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Assessing the efficacy of a novel galactooligosaccharide to promote fish health and robustness

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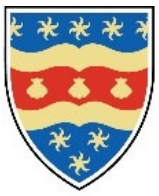

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Assessing the efficacy of a novel galactooligosaccharide to promote fish health and robustness

By

Lucy May Sykes

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

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Dedication

I dedicate this thesis to myself, as I have achieved so much over the course of this degree. Despite the last two years with no funding, trapped inside during an unprecedented and harrowing global pandemic, and working full-time for the last year of the PhD, I have managed to complete this research.

Author's Declaration

At no time during the registration for the degree of *Doctor of Philosophy* has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

This research has not been conducted under a formal agreement with another higher education institution. This thesis has not been proof read by a third party.

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Conferences Attended and Presentations of Research

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Abstract

Assessing the efficacy of a novel galactooligosaccharide to promote fish health and robustness

Lucy May Sykes

Aquaculture has been steadily increasing global production; however, there remains a progressively greater demand for sustainable sources of protein, whilst the prevalence of disease represents a major constraint to industry growth. Feed additives are gaining traction as useful tools to help combat disease and promote health, and previous research has documented benefits to the mucosal surfaces and microbial communities within a range of teleost species.

Three experimental in vivo feeding trials were undertaken using a range of in-depth analyses, such as growth performance, haematology, immunology, intestinal histology, intestinal gene expression and intestinal bacterial 16S rRNA sequence libraries. Studies were conducted on three important aquaculture species: rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and Nile tilapia (*Oreochromis niloticus*).

All three experiments revealed that dietary B-GOS® applications modulated the intestinal bacterial communities and intestinal gene expression. Some of these modulations may have potential benefits at the localised level for the hosts. However, no significant improvements of growth performance metrics were observed at the whole organism level, where FCR, SGR, survival, weight gain, condition factor and body composition remained unaffected by treatment. Despite the lack of statistical significance, numerical improvements indicated that GOS at the inclusion rate of 4g kg^{-1} was the optimal concentration.

Results from the experimental *in vivo* trials conducted during this research have provided evidence that a novel GOS tested in three commercially important teleost species had scope to improve localised intestinal health but offered little benefit to growth performance of fish reared with highly nutritious diets and excellent rearing conditions. Further research should prioritise the 4g kg^{-1} B-GOS® inclusion level for further investigation in these fish species whilst under pathogenic or environmental challenge, as this concentration presented the most potential to improve fish health and growth performance.

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List of Abbreviations

AB-vG	Alcian Blue – van Gieson
AFOS	Animal Feed Optimisation Software
AMPs	Antimicrobial peptides
AOAC	Association of Official Agricultural Chemists
APCs	Antigen-presenting cells
AR	Ash residue
AVCs	Antiviral components
β-actin	Beta-actin
BCA	Bicinchoninic acid
B-GOS®	Bimuno®-galactooligosaccharide
BSA	Bovine serum albumin
BT	Blank titre
BW	Body weight
CASP3	Caspase-3
cDNA	Complimentary deoxyribonucleic acid
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CFU	Colony forming unit
C_T	Cycle threshold
CW	Crucible weight
DAMPs	Damage-associated molecular patterns
DMT	Deltamethrin
DNA	Deoxyribonucleic acid
DW	Dry weight
Elf-1α	Elongation factor 1 alpha
FAO	Food and Agriculture Organisation
FCR	Feed conversion ratio
FOS	Fructooligosaccharide
GALT	Gut-associated lymphoid tissues
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GHG	Greenhouse gas
GIT	Gastrointestinal tract
GMOS	Galactomannanoligosaccharide
GOS	Galactooligosaccharide
Hb	Haemoglobin
Hct	Haematocrit
HSP70	Heat shock protein 70
IELs	Intraepithelial lymphocytes
Ig	Immunoglobulin
IgM	Immunoglobulin M

IgT	Immunoglobulin T
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
IMO	Isomaltooligosaccharide
K-factor	Condition factor
LAB	Lactic acid bacteria
LEAP	Liver-exposed antimicrobial peptides
LEfSe	Linear Discriminant Analysis Effect Size
LPS	Lipopolysaccharides
LW	Lipid weight
LYZ	Lysozyme
MAMPs	Microbe-associated molecular patterns
MALT	Mucosa-associated lymphoid tissues
MCC	Microcentrifuge tube
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MHC	Major histocompatibility complex
MOS	Mannanoligosaccharide
mRNA	Messenger ribonucleic acid
MS222	Tricaine methanesulfonate
MYD88	Myeloid differentiation primary response 88
NGS	Next generation sequencing
NF-κB	Nuclear-factor kappa-B
NLRs	Nod-like receptors
NRC	National Research Council
OSW	Original sample weight
OTUs	Operational taxonomic units
PAMPs	Pathogen-associated molecular patterns
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PCV	Packed cell volume
PRRs	Pathogen recognition receptors
PWG	Percentage weight gain
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RAS	Recirculating aquaculture system
RBC	Red blood cells (erythrocytes)
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real time polymerase chain reaction

SBM	Soybean meal
SCFAs	Short-chain fatty acids
SGR	Specific growth rate
SPB	Sodium phosphate buffer
SPC	Soy protein concentrate
ST	Sample titre
TCRs	T-cell receptors
TGF-β	Transforming growth factor beta
Th1	T helper cell 1
TLRs	Toll-like receptors
TNFα	Tumour necrosis factor alpha
TOS	Trans-galactooligosaccharide
WBC	White blood cells (leucocytes)
WW	Wet weight
XOS	Xylooligosaccharide

General Introduction

1.1 Global Aquaculture

The global population is set to reach over 9 billion by 2050 (FAO 2018). Providing sufficient protein to meet this increased demand is a challenging task, therefore, greater attention is being focused on aquatic sources, in addition to traditional terrestrial methods of farming. Farmed fish species are increasingly considered sustainable alternatives to traditional terrestrial meat producing species, such as beef cattle, poultry and swine (Philis *et al*. 2019). Recent research by Koehn *et al*. (2022) has investigated the nutritional benefits of producing different foods relative to the environmental impacts via measuring greenhouse gas (GHG) emissions needed to meet the average nutritional requirements of these foods. Figure 1.1 demonstrates how the GHG footprints of 35 foods sourced from either vegetables and fungi, livestock, capture fisheries and aquaculture score according to Koehn *et al*. (2022) environment-nutritional index. The authors demonstrate that the lowest GHG footprint (the GHG needed to meet the average of 12 nutrient requirements) were in plants and small pelagic fish, with the aquaculture of carps, bivalves and salmon falling below the median footprint required for all the food types tested. Among the highest of the GHG footprints were pork, crustaceans, lamb and the highest was beef (Koehn *et al*. 2022), indicating that while these food groups are nutrient rich and a reliable source of protein, the environmental impact is significantly greater than other forms of nutrient production, such as plant and aquaculture systems. A recent study on global environmentally sustainable diets determined that vegetable availability is already insufficient to meet nutrient requirements necessary for healthy growth, and so other food production

methods must contribute to fill this gap (Mason-D'Croz *et al.* 2019). Capture fisheries may provide highly nutrient rich products compared to traditional terrestrial production systems, however due to poorer management and slow gains in governance, wild fishery stocks alone will not meet the growing demand for seafood (Koehn *et al.* 2022). In this regard, food production from aquaculture may provide a more environmentally friendly and nutritionally rich solution to further contribute to the global food demands.

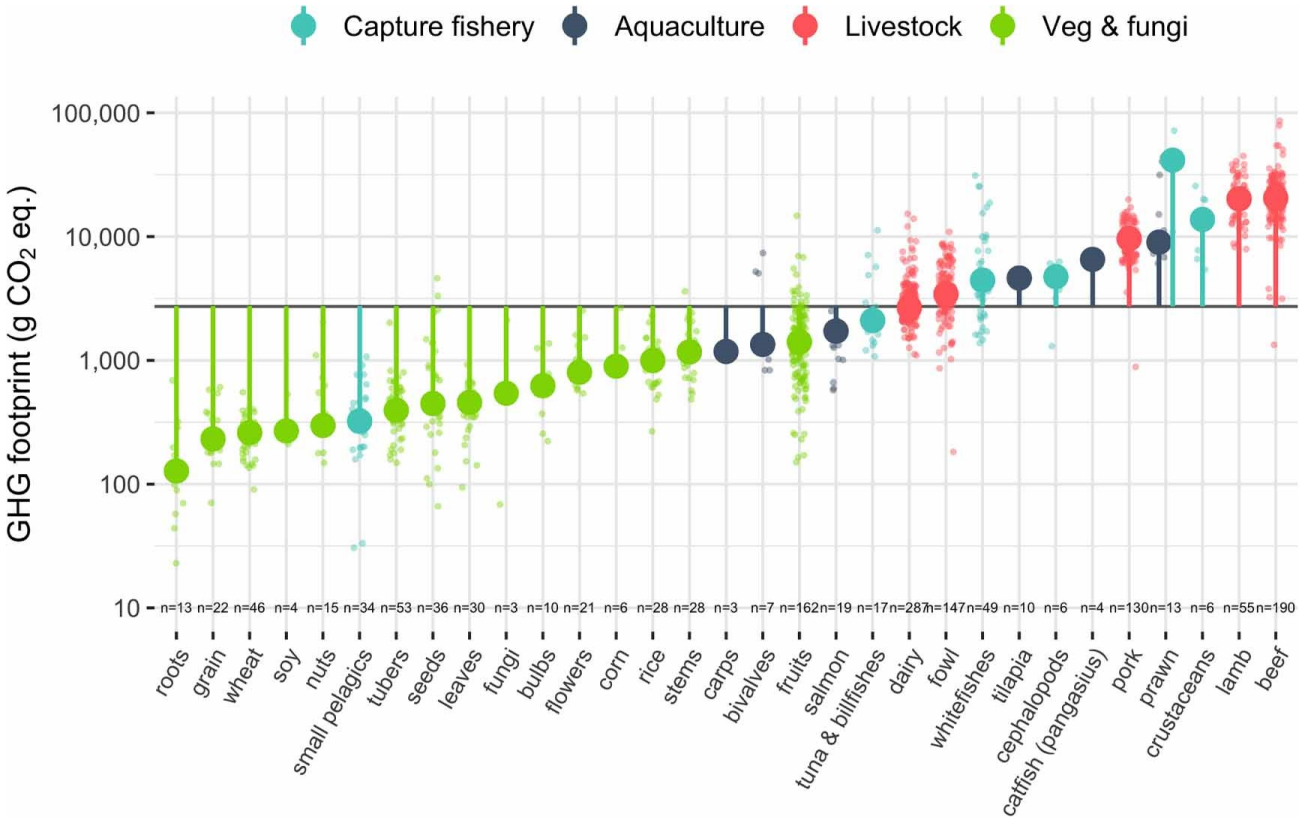


Figure 1.1 Greenhouse gas emissions relative to composite nutrient richness across major food groups on a logarithmic scale. Lower values indicate that lower GHG footprints are required to meet the nutrient requirement average across 12 nutrients. The grey horizontal line indicates the median for all observations across the food groups. Large points represent the median value across all species in each food group, whereas the vertical lines represent the distance from the median GHG footprint needed to meet 100% of the daily requirement across the nutrients across all food groups. Dots represent the individual species-level observations. Sample sizes above each food group on the *x*-axis represent the number of nutrient richness observations at species level within each food group. Colour coding represent broad production systems. Image from Koehn *et al.* (2022).

Over recent decades, the aquaculture industry has been growing by approximately 8% per year since the 1970s, and in 2018, the annual value of production was estimated at $250bn USD (FAO 2020). Of the 82 million tonnes of animal food produced in this market, finfish were a dominant contribution of 54.3 million metric tonnes to world aquaculture production (FAO 2020). The consumption of fish is in many countries the sole reliable source of protein, fats and omega 3's (Huss 1994; Ibrahem *et al*. 2010; FAO 2020), and restrictions to resources may mean aquaculture is an easy method of producing food to complement a mainly vegetable diet (FAO 2016).

This growing industry must focus on innovation through increasing production efficiency and biomass gain by improving the overall health of the cultured species (Secombes & Wang 2012). Such an undertaking will involve solving environmental challenges that are posed by parasites, eutrophication, and pathogens (Philis *et al*. 2019). As with any major food producing industry, disease remains a constant challenge and is the subject of continuous review due to the potential overwhelming mortality and morbidity rates that affect the aquaculture sector and environment (Magnadottir 2010; Hoseinifar *et al*. 2015; Mzula *et al*. 2021). In 2018, the China Fisheries Statistics Yearbook estimated that disease directly caused a production loss of 205,000 tonnes, equating to $401 million USD (FAO 2020). High and/or inappropriate stocking densities for the species grown, such as in tilapia or cold-water shrimp (Ziegler *et al*. 2016), increase stress, which impairs innate immunity. Such impairment may lead to atypical or diminished immune responses towards the GIT microbiota components and signals, thus allowing for the decrease of non-harmful or beneficial bacteria abundance and the prevalence of pathogenic microbes (Vargas-Albores *et al*. 2021).

Pathogenic microorganisms are more likely to proliferate and spread disease in heavily intensified and over-stocked production facilities, and in the past antibiotics were often relied upon to combat bacterial diseases (Ringø *et al*. 2014). However, there has been growing concern over the effectiveness of antibiotics and their effect on the environment. Previous antibiotic overuse as growth promoters was of concern, as antibiotic resistance in microorganisms in the local environment was linked to the impairment of the growth of aquatic species, and weakening of aquatic organisms' immune systems (Cabello 2006; Ringø *et al*. 2014; Pagano *et al*. 2016; Dawood *et al*. 2018). In addition to the impact on the environment, there are also concerns as to the overuse of unnecessary veterinary interventions to bolster farmed fish health and the addition of chemicals that may cause detriment to the fish themselves (Dawood *et al*. 2018). As of 2006, the EU ratified into law the banning of antibiotics as growth promoters, and there has since been a focus on alternative means of health promotion in finfish aquaculture. Vaccination presents an alternative approach to controlling infectious diseases; however, its efficacy is limited by the age of the species reared, as juvenile teleosts are not fully immunocompetent, and the vaccine may be ineffective (Pérez-Sánchez *et al*. 2018). Research has focused primarily on other sustainable methods of improving fish health and achieving global food security, such as the use of feed additives (Dawood *et al*. 2018).

Feed additives are designed in a way to supplement the basic nutritional requirements of the target species, and to enhance the growth, health and immune response within the host (Encarnação 2016). These additives have been presented as commercially viable and readily available products that many aquaculturists regularly use them to supplement fish diets to improve their health and growth (Fuchs *et al*. 2015; Dawood *et*

al. 2018). The nature of these groups of feed additives are diverse, and their applications are specified by their design, for example exogenous enzymes such as β-glucanase or xylanase may be used to improve digestibility, or immune stimulants such as β-glucans may improve the immune responses of important organs such as the intestine or skin (Castillo & Gatlin 2015; Encarnação 2016).

As fish production has provided more than 3.3 billion people globally with 20% of their average per capita intake of animal proteins, it is important to study these additives in commercially valuable and globally important fish species (FAO 2020). In 2018, the global production of salmonids (mainly Atlantic salmon, *Salmo salar,* and rainbow trout, *Oncorhynchus mykiss*) was over 3.2 million tonnes, and the strong demand means that salmonid production has become one of the largest fish commodities by value (FAO 2020). In other tropical regions, tilapia (predominantly the Nile tilapia, *Oreochromis niloticus*) have contributed over 4.5 million tonnes to aquaculture production and have ranked third, by volume, for finfish species (FAO 2020). With the ever-growing demand for seafood in mind, it is important to determine how the immune responses of commercially valuable teleost species function, and how their health and growth performance are affected by the addition of dietary additives.

1.2 Teleost Microbiome Host Interactions

The health of finfish species is linked to the complex and diverse host-microbial communities and the genetic information of these microorganisms (microbiomes) within and on the host (Merrifield & Rodiles 2015; Vargas-Albores *et al*. 2021), as this ecological community acts as one of the first major defence systems for fish (Ringø *et al.* 2014). The most notable regions are the mucosa-associated lymphoid tissues (MALT)

(Dimitroglou *et al.* 2011a; Akhter *et al.* 2015), which consist of the skin, gills, nose and the gastrointestinal tract (GIT) (Merrifield & Rodiles 2015; Salinas 2015; Bjørgen & Koppang 2021; Pontefract 2021). Considerable research has shown that these regions are important for host health, and a large portion of this research is biased toward the GIT itself, which plays an important role in immunological responses and defence (Gatesoupe 1999; Gómez & Balcázar 2007; Merrifield *et al.* 2010a,b,c; Dimitroglou *et al.* 2009, 2011a,b; Merrifield & Ringø 2014; Merrifield & Rodiles 2015; Hoseinifar *et al.* 2016a; Pontefract 2021; Vargas-Albores *et al.* 2021). This region of the host organism is a major infection route for pathogens, as its optimal environment allows for the proliferation of various microbes (Gatesoupe 1999; Hoseinifar *et al.* 2016a).

The environment in which teleosts live is a suitable medium for microbes as it provides a surplus of organic material for increased growth, therefore complex communities colonise the digestive tract and the mucosal surfaces (De Schryver & Vadstein 2014; Marchesi 2014; Rawling *et al.* 2019). Transient microbes are brought into the GIT and other mucosal surfaces, as water constantly flows over the gills and mouth, therefore allowing microorganisms to settle on the mucosa and within the digesta (Gatesoupe 1999; Hoseinifar *et al.* 2016a; Vargas-Albores *et al.* 2021). As fish develop throughout their life span, the composition of the communities changes within each section of the GIT (Egerton *et al.* 2018). Microbes that are able to colonise the mucosal surfaces make up the core microbiota and the autochthonous (resident) community, while the free-living, transient microbes are more associated with the digesta of the GIT (allochthonous community), and the community composition in teleosts varies between each section of the GIT (Llewellyn *et al.* 2014; Egerton *et al.* 2018). These microbial communities contain opportunistic pathogens that may infect

immunocompromised hosts as secondary pathogens, or primary pathogens that are kept at low levels due to competition from the commensal microbes present in the microbiota, as well as the localised immunity of the host (Merrifield & Rodiles 2015). There is clear evidence that the presence of such microbial communities within the host can influence how the functionality of the mucosa develops throughout all life stages of the host (Merrifield & Rodiles 2015).

Efforts have been made to understand how the microbiome influences the general health of teleosts using either germ free models, as demonstrated by Schaeck *et al*. (2016), who introduced a germ free European seabass (*Dicentrarchus labrax*) model to study interactions within the host and microbiota in a marine environment; *in vivo* trials (Kelly & Salinas 2017), or with specific bacterial loads within the host (Merrifield & Rodiles 2015; Montalban-Arques *et al*. 2015, Figure 1.2). Rawls *et al*. (2006) have demonstrated that mice and zebrafish which underwent GIT microbiota transplants resulted in the selection pressure of each host to resemble the typical microbial lineages before transplantation had occurred, however, the relative abundance of these taxonomic lineages changed to more greatly resemble the GIT microbiota of the recipient host. Physiochemical conditions also influence the composition of the microbiota, with the literature demonstrating that rearing water temperature, salinity, pH, oxygen content, seasonal changes, and the presence of pollutants or antibiotic resistant genes all impact how the commensal microorganisms are regulated (Fu *et al*. 2017; Huyben *et al*. 2017; Vargas-Albores *et al*. 2021). These results indicate that phylogeny and environmental pressures play a big part in the composition of the core microbiota (Rawls *et al*. 2006; Merrifield *et al*. 2014; Vargas-Albores *et al*. 2021).

From the increasing body of research into the microbiomes of teleosts, the types of microorganisms that are found within and on teleosts can vary from *Archaea*, *Bacteria*, *Eukarya* and viruses (Merrifield & Rodiles 2015). These microorganisms are extremely important in controlling the host's physiology, with the majority comprising of *Bacteria*, as each microorganism can affect the enzymatic capacity of the host and determines the health of the fish (Montalban-Arques *et al*. 2015; Vargas-Albores *et al*. 2021). The microbiome of fish is highly diverse and exists in an equilibrium with commensal microorganisms and host cells interacting with each other in a symbiosis (Vargas-Albores *et al*. 2021).

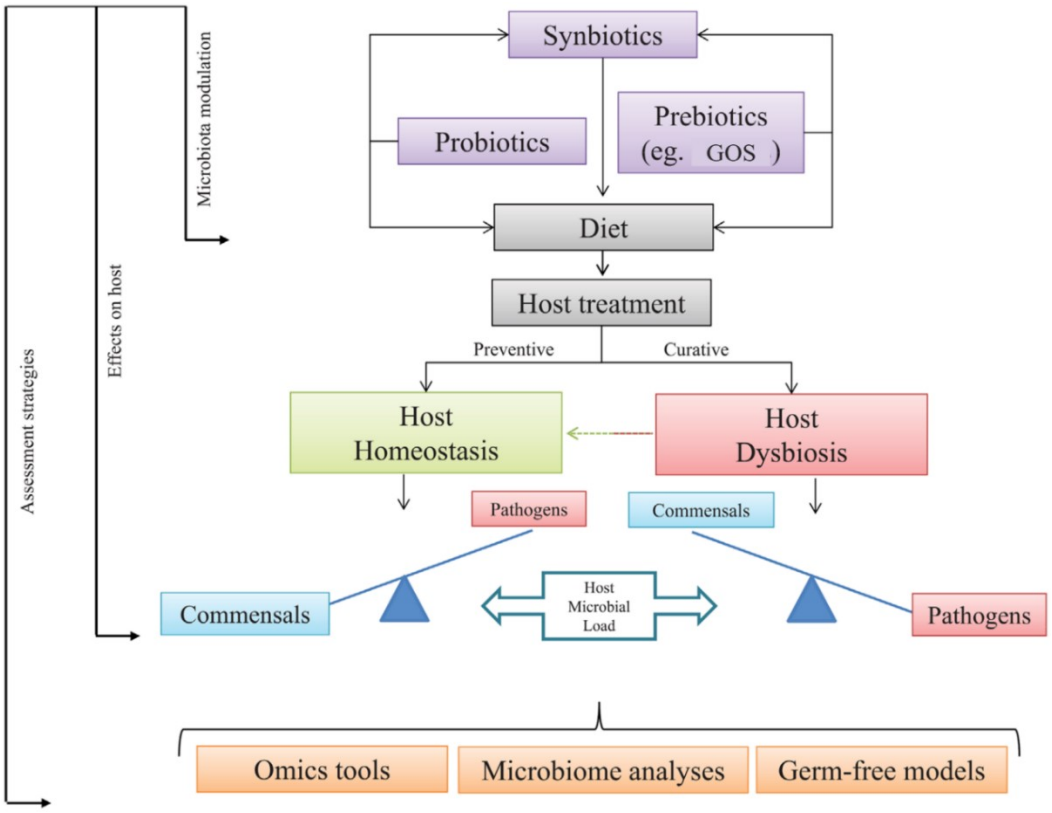


Figure 1.2 Diagram representing the interaction between host and microbial load. The addition of microbial sources in the form of feed additives to the diet may positively influence the host-microbial interactions within the gut. The host is treated via the diet and this will result in preventative or curative outcomes, leading to increased commensal communities within the gut. However, host dysbiosis and imbalance may increase pathogenic microorganism abundance. Assessment of these outcomes may be quantified using omics, germ-free models or microbiome analysis of the dissected samples. Image taken from Montalban-Arques *et al*. (2015).

If the commensal microbes that are found within the host are disturbed, be it by physical damage, stress or infection etc., then there is the possibility that the microbiota will enter dysbiosis, a state in which the microbiological balance is shifted negatively (Song *et al.* 2014; Montalban-Arques *et al.* 2015, Figure 1.2; Vargas-Albores *et al.* 2021). This change may allow commensal bacteria and other microorganisms that exist within the host at safe levels to multiply rapidly, and so cause pathological states within the host. Conversely, such changes may also reduce the number of healthy and beneficial commensals, and so too cause pathological states (Karlsson *et al.* 2013). To prevent this occurring, teleosts have a number of complex immunological interactions between different cells and organs that are able to protect the host from damage due to pathogenic infection.

1.3 Fish Immunoregulation

As within mammals, most teleost species utilise similar primary and secondary lymphoid organs, however, in fish, these are far less complex compared to mammals, and studies of antigen uptake and the mucosal barrier in the GIT are in their infancy (Løkka & Koppang 2016; Egerton *et al.* 2018; Smith *et al.* 2019). Teleost lymphoid organs are similar to those in mammals, such as the thymus, kidney, spleen and MALT, and produce different immune responses depending on the severity of risk to the host (Figure 1.3) (Rauta *et al.* 2012). For an overview of the more recently discovered immune organs within salmonids, i.e. the interbranchial lympohoid tissue, salmonid bursa and nasopharynx-associated lymphoid tissue please see Bjørgen & Koppang (2021).

The entire kidney within teleost fish is used for immunity; however, the head kidney or pronephros is primarily used for endocrine-immunity interactions, and is a major site

of antibody production, for example via B-cells, and cytokine-producing lymphoid cells such as T-cells (Whyte 2007; Geven & Klaren 2017; Bjørgen & Koppang 2021). The head kidney contains proliferating precursor B-cells and plasma cells, whilst the trunk kidney contains abundant B-cells, of which some are activated, and renal and immune tissues to produce urine and hormones, respectively (Bjørgen & Koppang 2021). The trunk kidney also hosts resident and circulating macrophages that are involved in filtration of the blood to remove aged blood cells and particulate matter, as well as the innate immune response via phagocytosis (Zwollo *et al*. 2008; Bjørgen & Koppang 2021).

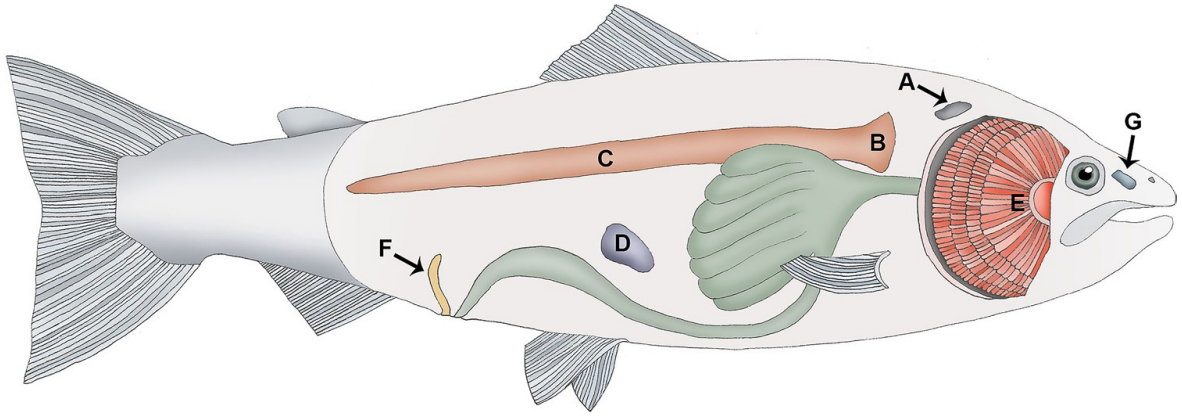


Figure 1.3 Image of immune organs in Atlantic salmon. **A)** Thymus, **B)** head kidney, **C)** trunk kidney, **D)** spleen, **E)** gills with the interbranchial lymphoid tissue, **F)** salmonid bursa and **G)** nasopharynx-associated lymphoid tissue. Image taken from Bjørgen & Koppang (2021).

Another important organ is the spleen, which as in mammals, plays an important role in filtration of the peripheral blood. The blood filters through endothelial cells and the basal lamina, the latter of which is surrounded by macrophages and reticulated cells and is known as white pulp (Bjørgen & Koppang 2021). Macrophages and T-cells are abundant in white pulp, and it is of similar structure and function to that in mammals (Koppang *et al*. 2010). The spleen is important in antigen presentation by antigen-presenting cells (APCs) and the initiation of the adaptive immune response (Chaves-Pozo *et al*. 2005; Rauta *et al*. 2012; Bjørgen & Koppang 2021).

The thymus plays an essential role in the production of lymphocytes, and the maturation of T-cells, as this organ consists of reticulated epithelial cells where T-cells are embedded (Bjørgen & Koppang 2021). Common T-cell markers include CD3, CD4 and CD8, which have been characterised in teleost fish, including salmonids such as rainbow trout (Bernard *et al*. 2006; Rauta *et al*. 2012). Developing T-cells migrate to the thymus, whereby they differentiate into two subtypes: CD4 helper T-cells which activate B-cells, and CD8 killer T-cells that are cytotoxic against infected host cells (Alberts *et al*. 2002; Bowden *et al*. 2005; Rauta *et al*. 2012).

The gut-associated lymphoid tissue (GALT) of teleosts, particularly the posterior segment, contains both innate and adaptive immune cells. Examples include macrophages, dendritic cells, T- and B- cells after they are produced in the head kidney and migrate to these tissues, and other lymphocytes similar to mammals such as natural killer cells (Secombes 1996; Rauta *et al*. 2012; Smith *et al*. 2019). The effects of the combination of all these immune responses are not yet fully understood in fish, and so research has been focused on understanding how these responses differ to terrestrial vertebrates, with major comparisons between fish and mammals (Smith *et al*. 2019). These different components within the associated mucosal tissues in teleost fish are described as mucosal function, innate (inborn) immunity and adaptive (acquired) immunity, and are described in the following sections.

1.3.1 Teleost Mucosal Function

Teleosts are able to prevent the introduction of harmful microbes through the presence of mucus that is concentrated on the epithelial surfaces; this mucus provides a mechanical, chemical and immunological barrier creating harsh environmental

conditions for many microbes (Chia *et al*. 2010; Secombes & Wang 2012; Merrifield & Rodiles 2015). The mucosal epithelium is the first interface between the host and the environment, which contains substantive populations of commensal, symbiotic and pathogenic microbes (Montalban-Arques *et al*. 2015; Løkka & Koppang 2016; Rawling *et al*. 2019). This physical barrier is important in maintaining homeostasis and the health of the fish, as most antigens and infectious agents will enter the host through the mucosal surfaces (Secombes & Wang 2012; Løkka & Koppang 2016).

Skin keratinocytes and mucosal epithelial cells of fish have specialised functions, with some of the most important being the production of anti-viral components (AVCs) (Raj *et al*. 2011), inducing humoral immunity through Ig production (Chia *et al*. 2010; Secombes & Wang 2012; Løkka & Koppang 2016), and antimicrobial peptides (AMPs). Immunoglobulin T (IgT) is a specialised mucosal antibody in teleosts that is analogous to mammalian IgA, which teleosts do not produce, and IgT has been identified in a number of teleost species, including salmonids, within the skin, gill and GIT mucus (Zhang *et al*. 2010; Merrifield & Rodiles 2015; Løkka & Koppang 2016; Mashoof & Criscitiello 2016). The production of IgT mRNA increases when pathogens are detected within the fish GIT (Pérez-Sánchez *et al*. 2011), and this antibody binds to pathogens within the mucus and prevents them from attaching to the mucosa, thus ensuring homeostasis by limiting mucosal interactions with too many, or hostile, microbes (Zhang *et al*. 2010; Merrifield & Rodiles 2015). The immunoglobulin M (IgM) is considered the most prevalent Ig in teleost plasma, and is found expressed on the surface of B-cells in either serum or mucus (Flajnik & Kasahara 2010; Mashoof & Criscitiello 2016). IgM contributes to innate and adaptive immunity in teleosts, with the effector functions of this Ig including cytotoxic activity, complement activation which

lyses pathogens, and agglutination for phagocytosis when destroying pathogens (Boshra *et al*. 2004; Mashoof & Criscitiello 2016). For a further comprehensive review of other Ig found in gnathostomes, please see Mashoof & Criscitiello (2016).

AMPs are also an important group of molecules that regulate mucosal tolerance, and include hepcidins, such as Th1-5, Th2-2, which have been observed in tilapia (*Oreochromis mossabicus,* Huang *et al*. 2007), or liver-expressed antimicrobial peptides, LEAP-2A and LEAP-2B, which have been observed in rainbow trout (Zhang *et al*. 2004). These AMPS and AVCs can be constitutively expressed or upregulated in response to pathogenic insult, the latter leading to direct interaction between Gram-positive or –negative bacteria, fungi, parasites or viruses that invade teleost host cells, which results in membrane disruption and the activation of other immune related cells (Chia *et al*. 2010; Secombes & Wang 2012; Montalban-Arques *et al*. 2015).

Within the mucus, there are also glycosylated mucin proteins, which are metabolised by mucus-degrading enzyme producing bacteria (Montalban-Arques *et al*. 2015). The oligosaccharides released during this process are used by the specific commensal microorganisms within the GIT, and these carbohydrate-based compounds have been reported to act as antiadhesives and prevent the binding of pathogens to the underlying epithelium in the GIT (Loke *et al*. 2007; Merrifield & Rodiles 2015). Balcázar *et al*. (2007) demonstrated that lactic acid bacteria (LAB) probiotic candidate could exclude the adhesion of fish pathogens *Aeromonas salmonicida* and *Vibrio anguillarum* by competitively binding to the mucus of rainbow trout, and the observed increased production of antagonistic compounds contributed to the reduction of their growth. Lazado *et al*. (2011) also demonstrated that host-derived immunostimulants displayed

adhesion specificity in Atlantic salmon intestinal epithelial cells, and research has presented that the mode of interference could be by exclusion, competition or displacement of pathogens (Caipang & Lazado 2015; Merrifield & Rodiles 2015). Evidently, there is a great deal of protection provided by the mucus layer within the fish GIT and on the skin, thus the mucins are important in maintaining and restricting the pathogenic bacteria, as well as affecting the abundance of certain bacterial subsets (Asselin & Gendron 2014).

1.3.2 The Innate Immune System in Teleosts

To combat disease, teleosts have evolved many different immunoregulatory responses which act in addition to the mucosal barrier functions. These responses are activated when the host experiences stress and changes within the exogenous and endogenous environment, for example by over-stocking in the rearing environment or through parasitic infection e.g. sea lice. One of the most important aspects of fish regulatory functions is the innate immune system (Secombes & Wang 2012). Innate immunity in addition to the mucosal barrier is one of the first lines of defence within the host from potential pathogens, primarily focussed on destroying any non-self cells, organisms or particles (e.g. viral). A key component of this is through recognition of pathogen cells and also inducing phagocytosis and further inflammation to counter pathogenic insult (Secombes & Wang 2012). Innate immune-competent APCs such as macrophages recognise damage or pathogen associated molecular patterns (DAMPs or PAMPs, respectively), with the latter encompassing molecular structures which are not present within the host's cells (Gaudino & Kumar 2019). APCs do this by expressing pattern-recognition receptors (PRRs) on their surfaces that bind to specific molecular patterns

and cause a response to a plethora of ligands including viral, bacterial and parasitic (Montalban-Arques *et al*. 2015; Gaudino & Kumar 2019).

PRRs, such as toll-like receptors (TLR) and nod-like receptors (NLR), are found across many cell types including macrophages and B-lymphocytes. The PRRs detect DAMPS and PAMPs, recognising foreign lipoproteins, lipopolysachharides, and peptidoglycan among other molecule types, for example damaged host cells, which stimulates a response (Rauta *et al*. 2012). TLRs have received the most attention of the PRRs studied, and their activation initiates important molecular cascades, whereby adaptor molecules such as myeloid differentiation primary response 88 (MYD88) and the transcription factor nuclear factor-κB (NF- κB) are activated (Merrifield & Rodiles 2015). This in turn promotes the production of antimicrobial molecules such as hepcidin, inflammatory cytokines (e.g. interleukin 1 beta (IL-1β) or tumour necrosis factor alpha (TNFα)), and interferons (Le Page *et al*. 2000; Rauta *et al*. 2012; Montalban-Arques *et al*. 2014, 2015; Merrifield & Rodiles 2015). The APCs bind to the appropriate PAMP or DAMP and internalise the target via phagocytosis, and degrade the molecules (Gaudino & Kumar 2019). In the absence of pathogenic insult, the excessive inflammatory response is dampened by regulatory molecules such as Tollip, and anti-inflammatory cytokines transforming growth factor beta (TGF-β) and interleukin 10 (IL-10) are expressed to maintain the mucosal tolerance of the immune system (Merrifield & Rodiles 2015).

Should these initial responses not be sufficient to ensure a pathogen is eliminated, further immune cells are utilised. In mammals, M-cells are found within the GIT epithelium and are specialised in antigen sampling as with other APCs, whereby particulate matter is recognised by receptors on the M-cell surface, and transported to

macrophages or lymphocytes within the lamina propria for further immune responses, such as phagocytosis and T-cell recognition (Neutra *et al*. 2001; Gaudino & Kumar 2019). Teleosts do not possess M-cells but specialised enterocytes within the hindgut, such as those found within Atlantic salmon, are able to absorb macromolecules and present antigens in a similar manner as M-cells (Fuglem *et al*. 2010; Løkka & Koppang 2016). Various antigens have been detected inside and on the surface of macrophages found in the intra-epithelium and lamina propria, providing evidence of antigen presentation from these cells (Løkka & Koppang 2016). Following antigen uptake, further immune activation occurs via cytokine and interferon signalling via effector cells. Examples of these cells are intestinal intraepithelial lymphocytes (IELs) found in the GALT, which are long-lived cells that move freely between the basement membrane and epithelial cells of the intestinal tract (McDonald *et al*. 2018). IEL populations contain $CD8^+$ T-cells which are an important part of the surveillance of antigens across the gut mucosal barrier, and act to clear pathogen-infected epithelial cells in this barrier (Salinas 2015). These cells acquire effector properties once antigen recognition occurs (McDonald *et al*. 2018), and more specific (adaptive) immune responses are prompted to initiate following signals from effector cells, and include augmenting specific T- and B-cell responses.

1.3.3 The Adaptive Immune System in Teleosts

Adaptive immunity components include immunoglobulins, T-cell receptors (TCRs) and major histocompatibility complex (MHC) class I and class II molecules (Flajnik 2018). MHC class I molecules are cell-surface recognition elements that signal the cell's physiological state to effector cells, such as T-cells or natural killer cells (Natarajan *et al*. 1999; Flajnik 2018; Gerdol *et al*. 2019). MHC class 11 molecules act in a similar manner

by residing on the surface of APCs (for example, macrophages and B-cells that secrete cytokines) and present self or non-self peptides to T-cells, such as $CD4^+$ T helper cells (Gerdol *et al.* 2019), and thus directly influence their differentiation (Gaudino & Kumar 2019). The production of these components allows for the clonal selection and augmentation of antigen specific B- and T-cells, as well as APCs, with a large proportion occurring in the spleen (Rauta *et al*. 2012; Flajnik 2018).

As within terrestrial vertebrates, it is incredibly important to have B-cell and T-cell activation and proliferation within the teleost host (Song *et al*. 2014). During antigen-dependent activation, B-cells mature into memory cells providing immunological memory upon contact with the specific antigen, or plasma cells that secrete antibodies specialised to the recognised antigen (Montalban-Arques *et al*. 2015; Gaudino & Kumar 2019). Once activated, T-cells that have matured into $CD8^+$ T cells express TCRs that recognise specific antigens and produce cytotoxic factors to destroy the cell (Gaudino & Kumar 2019). Other mature T-cells such as $CD4^+$ T helper cells produce signalling cytokines to recruit other cells to mount an immune response against infected host cells, for example by activating B-cells and other cytotoxic T-cells, and so pathogens are quickly destroyed by these activated cells (Alberts *et al*. 2002; Galindo-Villegas *et al*. 2012; Montalban-Arques *et al*. 2015; Gaudino & Kumar 2019). After activation and clonal expansion, a subpopulation of T-cells mature into memory cells (Gaudino & Kumar 2019). Should another infection occur, these specific B-cell and T-cell populations will proliferate rapidly in order to mount a rapid response upon exposures to specific pathogen cells (Galindo-Villegas *et al*. 2012; Gaudino & Kumar 2019).

Mucosal barrier function and subsequent immunity in teleosts can be greatly influenced by the GIT microbiota, as well as the microbiota of other mucosal organs. A great deal of research has demonstrated how the GIT microbiota can nutritionally benefit the host, and how a complex consortium of microbes are crucial for host development at a localised and systemic level (Merrifield & Rodiles 2015; Yukgehnaish *et al*. 2020). The microbiome within the GIT provides competition and antagonism against pathogens, and the way in which these responses are mediated and the importance of bacterial communities to the teleost host development and function has only received attention in recent years (Merrifield & Rodiles 2015). Previous research has demonstrated that the GIT microbiota can be manipulated to bolster the development and improve immune responses in teleosts, with a lot of attention focusing on feed additives.

1.4 Manipulating the Host-Microbe Interactions

Previous research has focused on the importance of commensal bacteria and the microbiota within humans and other mammals (Turnbaugh *et al.* 2008; Tremaroli & Bäckhed 2012), and a lot of what is known about host-microbe interactions is based on these higher vertebrates. As has been demonstrated previously in this chapter, teleost immune and mucosal function share many similar traits to that of mammals (Merrifield & Rodiles 2015). Additional research at the mucosa-microbiome level is required to understand how teleost hosts are affected by the microbiota and the benefits that arise from manipulating the host's microbiota through the environment and diet (Dimitroglou *et al*. 2009, 2011a,b; Merrifield *et al.* 2010a,b,c; Merrifield & Ringø 2014; Montalban-Arques *et al*. 2015; Hoseinifar *et al.* 2016a).

Evidence has been presented that the conversion of dietary components by the intestinal microbiota can induce beneficial effects on specific components of the teleost immune response (Vargas-Albores *et al*. 2021). The innate and adaptive immune responses act in tandem to provide protection for the host and coordinate using complex signalling hormones and molecules, enhancing the host's immune system and therefore overall health (Yukgehnaish *et al*. 2020). Despite the host's ability to regulate the GIT microbiota, various factors can prevent a synergistic balance between the host and its associated microbes. For example, fish fed nutrient-deficient diets that reduce the abundance of beneficial bacteria may be at increased risk of pathogenic infection, or the prevalence of pollutants such as microplastics or pesticides in polluted water can cause inflammation in intestinal tissues and a loss of microbial diversity (Jin *et al*. 2018; Wang *et al*. 2019; Yukgehnaish *et al*. 2020; Vargas-Albores *et al*. 2021). If these factors are not properly controlled, they may lead to an imbalance within the microbial community (dysbiosis) that may in turn lead to adverse effects on the host's health (Montalban-Arques *et al*. 2015; Yukgehnaish *et al*. 2020; Vargas-Albores *et al*. 2021). Dysbiosis can affect teleost performance, and consequently affect aquaculture production on a major scale through the increased likelihood of disease outbreaks from pathogenic infection on an already weakened immune system (Vargas-Albores *et al*. 2021).

The prevention of pathogenic infection within teleosts can be achieved by regulating some of the abiotic and biotic factors that are leading causes of pathology, such as ensuring good water quality and formulated diets that meet all the nutritional requirements of the species reared (Vargas-Albores *et al*. 2021). However, there are other factors that are not as easy to control, for example, increasing stocking densities

in sea-pens to ensure economic viability, and so the increased risk of pathogen proliferation is still of great concern. The possibility of improving the gut microbiota by adding exogenous sources of molecules that are utilised by favourable microorganisms is certainly a therapeutic possibility in most vertebrates, including fish (Zhang *et al*. 2011). These molecules may be represented and provided in teleost diets in the form of feed additives.

The diet is a major factor of health regulation within teleosts, as it can help to shape the representation of the microbiota within the GIT and other mucosal organs, provide the host with vital nutrients and bolster the immune response (van den Ingh *et al*. 1991; Merrifield *et al*. 2011: Merrifield & Rodiles 2015; Miyake *et al*. 2015; Yukgehnaish *et al*. 2020). The digestibility of feed and overall health status is affected by the diet, allowing for a healthy relationship between the microbiota and host to be established (Dimitroglou *et al*. 2011a; Butel 2014). The addition of dietary feed additives, whereby feed has added benefits for improving the health status/growth performance of animals which ingest them (Tacchi *et al*. 2011), can therefore be an effective alternative to using other expensive restorative treatments, such as chemotherapy or antibiotics (Buentello *et al*. 2010; Plant & La Patra 2011; Montalban-Arques *et al*. 2015; Carbone & Faggio 2016). It is estimated that feed costs equate to around 40-60% of fish farm operational expenditures (Jobling *et al*. 2010). Therefore, cost-effective strategies using feed additives as dietary supplements may ensure that the fish health requirements are met, as well as producing a high quality product with little additional cost to the producer (Tacchi *et al*. 2011).

There is incredible plasticity within the GIT microbiota of teleosts (Montalban-Arques *et al*. 2015), and this plasticity may ensure that commensal bacterial communities utilise feed additives, for example by breaking down fibres to short-chain fatty acids (SCFAs), but without altering the physiology and morphology of the GIT tract (Young & Schmidt 2004; McFarland 2014; Montalban-Arques *et al*. 2015). This is extremely useful when manipulating the communities present with feed supplemented with external live microorganisms or non-digestible substrates (Dimitroglou *et al*. 2011a).

1.4.1 Overcoming Challenges in Large-scale Aquaculture Production

1.4.1.1 Probiotics

Live microorganisms that confer benefits to the host microbial balance when administered in the rearing water, or as food ingredients or supplements are defined as probiotics (Merrifield *et al*. 2010c; Montalban-Arques *et al*. 2015). The most widely studied and tested microorganisms have come from the genera *Lactobacillus* (Montalban-Arques *et al*. 2015; Sarao & Arora 2015), however, there have been a number of other popular species from the genera *Bacillus*, *Enterococcus*, *Micrococcus*, *Shewanella*, *Streptococcus* and yeasts (Fernández *et al*. 2015; Akanmu 2018; Vargas-Albores *et al*. 2021). The inclusion of these probiotic strains has been studied in many endotherms, but also in many fish species. While probiotics suggest a practical alternative to antibiotics, it can be difficult to apply to large-scale operations (Merrifield *et al.* 2010c). Commercial aquaculture is on such a scale that administering pelleted probiotics, ensuring probiotic survival through pelleting/extrusion and ensuring viability of the microorganisms present within the GIT after feeding is a considerable undertaking.

The feed preparation with probiotics may present problems, as the pellets themselves may have low viability of bacteria after manufacture and during storage (Ringø *et al*. 2014; Gatlin 2015), or may not survive the feed extrusion process (Merrifield *et al*. 2010c). Of the viable pellets that are ingested, determining how viable the microorganisms are can be challenging on a large scale (Yousefian & Amiri 2009). Non-digestible additives may present an alternative option, as they are designed to directly stimulate the immune system and beneficial indigenous commensal microbiota, as opposed to introducing specific microorganisms (Ringø *et al*. 2014; Vargas-Albores *et al*. 2021).

1.4.1.2 Prebiotics

Non-digestible food/feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of specific health promoting bacteria that can improve host health in the GIT are described as prebiotics (Gibson & Roberfoid 1995; Manning & Gibson 2004; Merrifield & Rodiles 2015). Often, carbohydrates derived from plants or fruit matter are fermented and are used that promote GIT health, growth performance, immune response, and the suppression of pathogens (Gibson *et al.* 2003; Ringø *et al*. 2014). Previous research has reported that prebiotics promote the colonisation of beneficial bacteria within the GIT, and reduce pathogen abundance by competing for adhesion sites and improving mucus, SCFA and cytokine production (Pérez-Sánchez *et al*. 2018).

The success of the application of the prebiotic can depend on a number of factors, one such example being the bacterial metabolites formed from fermenting non-digestible additives by bacteria such as LAB within the intestine (Yukgehnaish *et al*. 2020). By-

products, such as SCFAs, are used by the host and commensal bacteria, and a considerable amount of energy supplied to the host is produced from microbial SCFA production (Ringø *et al*. 2014; Merrifield & Rodiles 2015). The enterocytes within the GIT of fish absorb these metabolites and utilise them for energy or for use elsewhere within the host via transport in the vascular system to promote healthy immune responses within the GIT and other mucosal surfaces (Gibson *et al*. 2004; Roberfroid *et al*. 2010; Merrifield & Rodiles 2015; Guerreiro *et al*. 2018a). Wider changes in other commensal microorganism's activities brought about by the presence of the prebiotic is also one element to measure prebiotic success against (Sako *et al.* 1999; Ringø *et al*. 2010; Akhter *et al.* 2015, see Figure 1.4; Yilmaz *et al*. 2022).

Common prebiotics studied in fish include inulin and oligofructose (Yousefian & Amiri 2009), mannanoligosaccharides (MOS), fructooligosaccharides (FOS), galactooligosaccharides (GOS), as well as other combinations such as yeasts (Ringø *et al*. 2014). These prebiotic groups are commonly used as immunomodulatory enhancers to augment the innate immune responses in fish, and to provide protection from pathogenic infection, as well as enhance growth performance in tested species (Dawood *et al*. 2020). Zhou *et al*. (2010) demonstrated how addition of FOS, tran-GOS, MOS and galactoglucomannans increased the serum lysozyme activity and microvilli and enterocyte lengths in juvenile red drum (*Sciaenops ocellatus*), with similar results shown by Hoseinifar *et al*. (2015) in rainbow trout fed GOS, *P. acidilactici* or synbiotics of both, with the latter demonstrating significantly increased protection from *Streptococcus iniae* challenge. Prebiotics have also been shown to significantly alter the microbiota within the GIT, and so the composition can be changed for the benefit of

the host by the addition of these types of additives (Gibson & Roberfroid 1995; Akhter *et al.* 2015; Peggs 2015; Standen *et al*. 2015, 2016).

Previous work has focused primarily on the effects of prebiotics on commercially valuable fish species, such as members of the Salmonidae (Merrifield *et al.* 2010c; Ringø *et al*. 2010; Merrifield *et al*. 2014; Ringø *et al.* 2014), Ascipernseridae (Reza *et al.* 2009; Hoseinifar *et al.* 2011; Ringø *et al.* 2014), Gadidae, Moronidae, Cyrinidae, and crustaceans such as shrimps, to name a few (Merrifield *et al*. 2014). There is an emphasis in research into the efficacy of prebiotic application in important fish species to focus on novel and well-established additives in a variety of fish species.

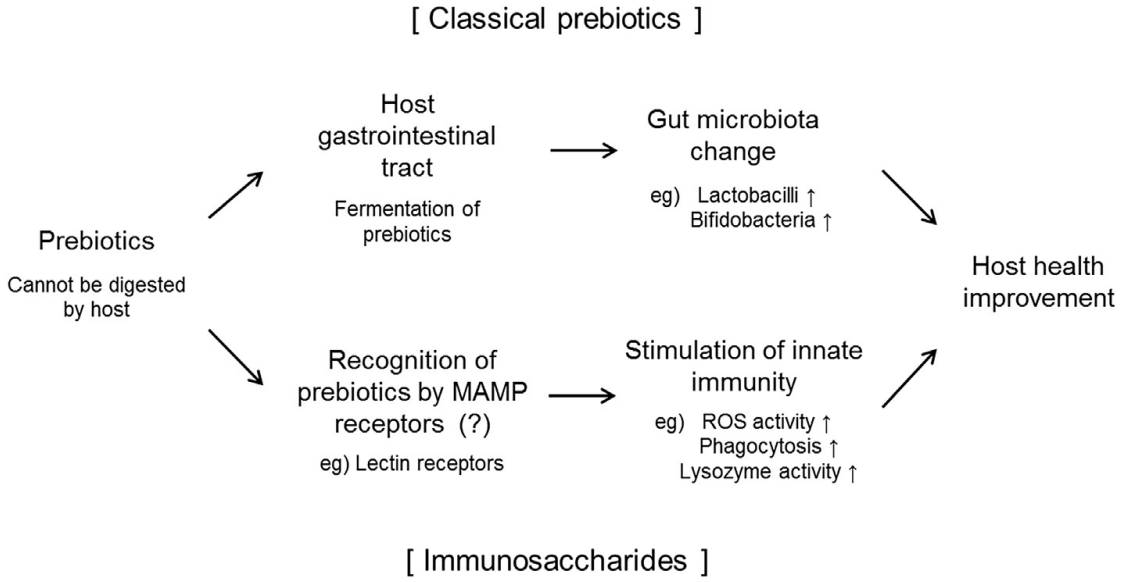


Figure 1.4 An overview of host benefits derived from prebiotic and immunosaccharide supplementation. Some compounds, such as MOS, may have both functional properties but dual function is not common to all prebiotics. MAMP, microbe-associated molecular pattern; ROS, reactive oxygen species. Image taken from Song *et al.* (2014).

Considerable research has focused on MOS or FOS as additives, as these are more commonly utilised in the aquaculture industry (Dawood *et al*. 2020). MOS is derived from carbohydrate molecules originating from the outer cell wall of brewer's yeast (*Saccharomyces cerevisiae*) (Broadway *et al*. 2015), while FOS is derived from inulin

degradation processes within plants and fruits (Campbell *et al*. 1997). Comparatively, the research conducted on teleost species using oligosaccharides derived from enzymatic activity acting on lactose, such as galactooligosaccharides, is minimal, and warrants further attention within teleost functional feed research, given the health benefits that have been demonstrated from the addition of this prebiotic in humans (Mao *et al*. 2015) and terrestrial livestock (Tzortzis *et al*. 2005b).

1.5 Galactooligosaccharides in Teleost Based Literature

Galactooligosaccharides are a group of prebiotics that are non-digestible carbohydrates produced using enzymatic hydrolysis of lactose using β-galactosidases, and chains of 2-20 galactose and glucose molecules arise from transgalactosylation reactions (Yang & Silva 1995; Sako *et al*. 1999; Ringø *et al*. 2014; Hoseinifar *et al*. 2015; Mao *et al*. 2015). These compounds are able to retain moisture well, are stable under high temperatures and resistant to enzymatic action within acidic environments such as the stomach and intestinal tract, making them suitable additives for fish feed processing methods (Macfarlane *et al*. 2008; Ringø *et al*. 2014). The benefits of supplementing GOS within the digestive tract of commercially important species result from the fermentation reactions from specific colonic bacteria, such as *Lactobacillus*, using GOS as a substrate. The presence of GOS as food supplements has led to significant increases in the levels of LAB, and levels of these bacteria may provide increased health benefits (Sako *et al*. 1999) as they metabolise by fermentation (Merrifield *et al*. 2014). Indigenous LAB that may be elevated due to the presence of a prebiotic, such as *Lactobacillus* populations, are known to boost immunological responses within the GIT (Ringø *et al*. 2014), as these taxa produce antimicrobial substances such as lactic acid, and retard pathogenic

colonisation on the fish GIT epithelial mucosal layer (Macfarlane *et al*. 2008). In addition, GOS have also been found to influence the production of microbial SCFAs, which in turn affects host lipid and carbohydrate metabolism, and growth and cellular differentiation of colonic epithelial cells (Macfarlane *et al*. 2008; Ringø *et al*. 2014; Gaudino & Kumar 2019).

Research in the last decades has focused on both warm-water and cold-water fish species, as these species are part of a multi-billion dollar aquaculture industry that is the fastest growing animal food sector across the globe (Dawood *et al*. 2020; FAO 2020). Studies have investigated different inclusion levels of the prebiotic GOS and its many variations over different periods with interesting results (Table 1.1). Hoseinifar *et al*. (2013) demonstrated how the administration of GOS at 0%, 1% and 2% positively affected the intestinal microbiota, stress resistance and performance of juvenile Caspian roach (*Rutilus rutilus*) fry. The authors observed significant improvements of final weight gain, specific growth rate (SGR), condition factor (K-Factor) and feed conversion ratio (FCR) in fry fed 2% GOS compared to other treatment groups (Hoseinifar *et al*. 2013). The addition of GOS also significantly elevated salt-water resistance in roach that were challenged, and significantly increased survival levels of these fish (Hoseinifar *et al*. 2013). There were no significant differences in the total autochthonous heterotrophic bacteria isolated from the intestinal microbiota from fish fed the experimental diets, but there were significantly elevated levels of total autochthonous LAB within the 1% and 2% diets compared to the control. The improvement of the weight gain, SGR, FCR and final weights may be attributed to elevated levels of digestive enzymes within the intestine of the fry (Hoseinifar *et al*. 2013), and the increase of LAB may have increased immunity and host health, as these bacteria are generally considered to be beneficial for

the host (Ringø & Gatesoupe 1998; Ringø *et al*. 2014). Similar instances of increased microbial diversity and increased intestinal absorptive area of the microvilli have also been observed within rainbow trout fed MOS diets at 0.2% inclusion rate (Dimitroglou *et al*. 2009), and in gilthead sea bream (*Sparus aurata*) fed MOS at 0.2% and 0.4% inclusion (Dimitroglou *et al*. 2010) compared to control fed fish.

Other studies have failed to report benefits with prebiotic applications. Growth performance parameters were not significantly affected by the inclusion of 1% dietary GOS in hybrid striped bass (*Morone chrysops* x *M. saxatilis*) when researched by Burr *et al*. (2010), and Atlantic salmon as investigated by Grisdale-Helland *et al*. (2008). Grisdale-Helland *et al*. (2008) investigated the effects of 1% prebiotics MOS, FOS and GOS on juvenile Atlantic salmon over a four-month trial, and observed no significant treatment effects on the intake, growth, and digestibility of the diet within fish fed prebiotics compared to the control group. Salmon fed the GOS diet had 11% and 7% significantly greater nitrogenous and energetic losses in the non-faecal nitrogen excretions, respectively (Grisdale-Helland *et al*. 2008). The body protein concentration was also reduced by 6% relative to the basal fish diet ($p<0.05$); likewise the protein retention was reduced in the fish fed the GOS diet compared to the basal diet fish by 9% ($p<0.05$) (Grisdale-Helland *et al*. 2008). The authors concluded that the GOS investigated was not the most effective prebiotic for the conditions of the trial, but there was potential for further improvement from prebiotics FOS and MOS. However, as there was no challenge trial or other stressors administered to the fish post prebiotic supplementation, caution should be advised when assuming that an additive is defunct when the rearing conditions are more than suitable for fish growth and survivability.

Table 1.1 An overview of GOS and other prebiotics investigated as feed additives in various commerically valuable species.

Prebiotic	Dose and Duration	Fish Species	Observations	Reference
GOS	0%, 1%, 2% for 7 weeks	Caspian roach juveniles (*Rutilus rutilus*)	↑ Final weight, weight gain, SGR, FCR in fish fed 2% diet ↑ Survival and salt-water resistance in GOS fed fish ↑ Total autochthonous LAB in fish fed 1% and 2% diets –– Total autochthonous heterotrophic bacteria in fish fed any diet	(Hoseinifar *et al*. 2013)
MOS	0%, 0.2% for 111 days	Rainbow trout juveniles (*Oncorhynchus mykiss*)	↑ Gut absorptive surface area, microvilli length/density, microbial diversity in fish fed 0.2% diet ↓ Levels of *Aeromonas*/*Vibrio* spp. in fish fed 0.2% diet	(Dimitroglou *et al*. 2009)
MOS	0%, 0.2%, 0.4% for 9 weeks	Gilthead sea bream (*Sparus aurata*)	–– Final weight, SGR, FCR, protein efficiency ratio ↑ Gut absorptive surface area, microvilli length/density, species richness and diversity of the gastrointestinal microbiota in fish fed 0.2% and 0.4% diets	(Dimitroglou *et al*. 2010)
GOS, MOS, GroBiotic®-A, inulin	0%, 1% each additive for 8 weeks	Hybrid striped bass (*Morone chrysops* × *Morone saxatilis*)	–– Weight gain, feed efficiency ratio, protein efficiency ratio, whole body ash/moisture/lipid The prebiotics altered the intestinal microbiota of fish fed these diets compared to the basal diet, and produced distinct microbial communities to each other	(Burr *et al*. 2010)
GOS, MOS, FOS	0%, 1% each additive for 16 weeks	Atlantic salmon juveniles (*Salmo salar*)	–– Feed intake, growth or digestibility ↑ Nitrogenous and energetic losses in non-faecal nitrogen excretions of fish fed GOS ↑ Feed efficiency and energy retention in fish fed FOS and MOS ↓ Body protein concentration and protein retention in fish fed GOS compared to basal diet	(Grisdale-Helland *et al*. 2008)

GOS	0%, 0.5%, 1%, 2% for 8 weeks	Zebrafish (*Danio reiro*)	–– Weight gain, SGR, FCR, survival rate, lysozyme activity, *IL-1β* gene expression ↑ Total protein level, total Ig, gene expression of *LYZ* and *TNFα* in fish fed 1% and 2% diets	(Yousefi *et al*. 2018)
GOS, *Pediococcus acidilactici*, symbiotic of both	0%, 1% GOS, 7.57 log CFU g^{-1} *P. acidilactici,* both, for 8 weeks	Rainbow trout juveniles	↑ Lysozyme activity, alternative complement, respiratory bust activity, skin mucus activity in fish fed all three treatments ↑ Highest innate immune response in fish fed symbiotic diet ↑ Survival after *Streptococcus iniae* challenge greatest in symbiotic, probiotic, then prebiotic fed fish compared to basal diet fed fish	(Hoseinifar *et al*. 2015)
Trans-GOS, FOS, MOS, Previda®	0%, 1% each additive for 8 weeks	Red drum junveniles (*Sciaenops ocellatus*)	↑ Weight gain in fish fed Previda® compared to basal and MOS fed fish –– Feed and protein efficiency, mucosal fold heights, enterocyte heights ↑ Microvilli heights in fish fed any additive compared to basal diet fed fish	(Zhou *et al*. 2010)
Trans-GOS, FOS, MOS, GroBiotic®-A	0%, and 1%, 2% each additive for 8 weeks	Red drum juveniles	↑ Length of intestinal folds, microvilli and enterocyte heights in the anterior intestine of fish fed Trans-GOS –– Enzyme activities such as lipase and acid/alkaline phsphatase, although aminopeptidase and α-amylase tended to increase in fish fed prebiotics	(Anguiano *et al*. 2013)
Short-chain FOS, XOS, GOS	0%, 1% of each additive for 12 weeks	White sea bream (*Diplodus sargus*)	–– Growth, feed efficiency, protein efficiency ratio ↑ Trypsin activity in fish fed GOS compared to basal and FOS regimes α-amylase levels lower in fish fed GOS and FOS ↓ Microbial richness at trial conclusion compared to 15 days –– Microbial abundance, e.g. LAB levels, between any dietary group	(Guerreiro *et al*. 2018b)

Trans-GOS, FOS, MOS, GroBiotic®-A	0%, 1% each additive for 4 weeks	Red drum juveniles	–– Weight gain, whole body composition ↑ Feed efficiency, serum lysozyme and intracellular superoxide anion production in fish fed prebiotic diets ↑ Survival in fish fed prebiotic diets after challenge with *Amyloodinium ocellatum* compared to basal diet group	(Buentello *et al.* 2010)

Further investigating of GOS as an additive has been conducted by Yousefi *et al*. (2018), whose authors studied the effect of different inclusion levels of GOS on innate immunological gene expression, innate immune response, and growth performance within adult zebrafish (*Danio rerio*). The treatments included a basal diet, 0.5%, 1% and 2% GOS inclusion rates, with growth parameters SGR, weight gain, FCR or survival rate presenting no significant improvements between GOS fed fish and the control group (Yousefi *et al*. 2018). The authors determined that fish fed 1% and 2% GOS inclusion presented significantly increased total protein and total Ig compared to other dietary regimes, however, lysozyme remained unaffected by treatment group.

The significant upregulation of the target genes *LYZ* (lysozyme) and *TNFα* were reported in GOS fed fish compared to the control group, but *IL-1β* (interleukin 1 beta) was not affected by dietary GOS addition (Yousefi *et al*. 2018). The authors suggested that the elevation of certain immune parameters such as total Ig and protein could be due to the improvement of the zebrafish immune system in response to GOS addition by increase constituent antibody production. In addition, Yousefi *et al*. (2018) suggested that the upregulation of *TNFα* and *LYZ* in GOS fed fish, but the lack of increased lysozyme activity, was inconsistent with other studies. The conflicting information found between growth parameters measured may be due to the differences in administration of the prebiotics, the length of the trial conducted, dosage of the prebiotic (Yousefi *et al*. 2018), as well as being influenced by biological parameters, such as the developmental stage of species used.

Despite the disagreeing results regarding growth parameters, there have been studies showing increased responses to administered GOS in the immune responses of fish.

Previous literature has also reported an increase in some immune responses, such as lysozyme activity, in rainbow trout fed 1% GOS (Hoseinifar *et al*. 2015). Hoseinifar *et al*. (2015) investigated the application of a control diet, 1% GOS (prebiotic), 7.57 log CFU g^{-1} of *Pediococcus acidilactici* (probiotic), and the synbiotic of both fed to rainbow trout fingerlings for 8 weeks. All dietary regimes presented significantly increased immune parameters investigated, including lysozyme activity, alternative complement and respiratory burst activity compared to control fish (Hoseinifar *et al*. 2015). The greatest improvements were observed between the fish fed the synbiotic diets and the pre- and probiotics in immune parameters, the skin mucus bactericidal activity and the skin mucus protein levels. After a challenge with *Streptococcus iniae*, survival rates were significantly higher in fish fed the synbiotic diet, followed by the individual probiotic, then prebiotic diets, compared to the control group (Hoseinifar *et al*. 2015). The results provide evidence that the addition of a synbiotic, prebiotic and probiotic increased the resistance of trout fingerlings to streptococcosis, with the combination of the GOS and *P. acidilactici* improving the performance and health of supplemented rainbow trout (Hoseinifar *et al*. 2015).

Zhou *et al*. (2010) also reported significantly increased serum lysozyme activity of juvenile red drum fed 10g kg^{-1} of four prebiotics: inulin (FOS), trans-galactooligosaccharides (TOS), Bio-MOS® (MOS), and Previda™ (galactoglucomannans) compared to the control group. The growth performance metrics of the juvenile red drum were not significantly affected by prebiotic addition, apart from in the 1% Previda™ group. Fish fed Previda™ had significantly higher weight gain than the basal diet fish and Bio-MOS® (Zhou *et al*. 2010). However, there were no significant differences among the fish fed FOS, GOS and Previda™ in growth

performance metrics, e.g. FCR, protein efficiency ratio (PER) and body condition indices (Zhou *et al*. 2010). Histological appraisal determined that mucosal fold heights and enterocyte heights in the pyloric caeca, proximal, mid- and distal intestines were not significantly affected by the diets ($p>0.05$). The microvilli heights were significantly increased in fish fed any prebiotic regime compared to the control fed fish in the pyloric caeca, proximal intestine and mid-intestine (Zhou *et al*. 2010).

As one of the first studies to compare these prebiotics, Zhou *et al*. (2010) demonstrated that dietary supplementation of different prebiotics such as MOS, FOS, trans-GOS and galactoglucomannans may induce a positive effect on the growth of juvenile red drum. The authors did acknowledge that the effects of growth could be contentious within the literature, as other studies have reported no differences in the growth parameters of the same species, and the same prebiotics used (Grisdale-Helland *et al*. 2008; Burr *et al*. 2010; Zhou *et al*. 2010; Yousefi *et al*. 2018). However, caution is advised as studies use different experimental designs, such as differing regions of the intestine to measure morphological and immune related parameters, trial lengths, doses and species.

The prebiotic supplements used by Zhou *et al*. (2010) significantly increased the heights of the microvilli in fish fed these additives compared to the control diet fish (as previously observed in cobia and rainbow trout fed MOS diets; Salze *et al*. 2008 and Yilmaz *et al*. 2007, respectively), suggesting that there could be increased potential for nutrient uptake. Similar effects were also observed by Anguiano *et al*. (2013), whereby the authors investigated four different prebiotics (FOS, Bio-MOS®, TOS and GroBiotic®-A (GBA)) at 1% and 2% compared to a control diet over 8 weeks, and their effects on the enzymatic properties and intestinal morphology of red drum. The intestinal structures

of the red drum were significantly improved by the inclusion of prebiotics, as the authors reported an increase in the length of the intestinal fold heights and heights of the microvilli and enterocytes in fish fed TOS ($p<0.05$) (Anguiano *et al*. 2013). As also observed by Zhou *et al*. (2010), the increased effects on the intestinal structures of the red drum suggested increased nutrient uptake during the length of the trial.

There is evidence from previous studies that the length of prebiotic administration affects the efficacy of the feed additive and its influence upon the intestinal microbiota and immune response of the chosen species. Guerreiro *et al*. (2018b) evaluated the growth performance and hepatic metabolism and digestive action of white sea bream (*Diplodus sargus*) juveniles fed 1% diets of FOS, GOS and xylooligosaccharides (XOS) for 12 weeks, with a sampling at 15 days. The growth, feed efficiency, and PER were unaffected by treatment diets after 3 months of feeding, similar to observations of other papers investigating FOS, GOS and MOS in Atlantic salmon (Grisdale-Helland *et al*. 2008), GOS, MOS and inulin in hybrid striped bass (Burr *et al*. 2010), and GOS in zebrafish (Yousefi *et al*. 2018). Guerreiro *et al*. (2018b) also reported that trypsin activity was significantly higher in white bream fed GOS compared to bream fed the control and FOS diets. The α-amylase levels were lower in the fish fed GOS and FOS compared to the control, which is contrary to findings of Anguiano *et al*. (2013), who reported that trans-GOS tended to increase red drum α-amylase and aminopeptidase. However, all digestive enzymes studied by Guerreiro *et al*. (2018b) were lower by the end of the trial than at 15 days, as similarly seen by Anguiano *et al.* (2013), suggesting that the probiotic effects occur in the early stages of prebiotic administration.

These results are contrary to the study of Buentello *et al*. (2010), whose authors researched dietary 10g kg^{-1} FOS, MOS, TOS and GroBiotic®-A (partially autolysed brewer's yeast) supplementation in juvenile red drum growth performance, feed efficiency and non-specific immunity. There were no significant differences observed in weight gain between fish fed any treatment (Buentello *et al*. 2010), and this was suggested to be due to the limited trial length of 4 weeks. In addition, authors Li & Gatlin (2004) suggested that the effects of prebiotic supplementation are only evident at 7 weeks or more. Conversely, there was a significant increase in feed efficiency and serum lysozyme and intracellular superoxide anion production ($p<0.05$) seen in fish supplemented with the prebiotic compared to the control diet, suggesting potentially enhanced disease protection (Buentello *et al*. 2010).

Comparing the intestinal bacterial communities present in the samples studied by Guerreiro *et al*. (2018b) revealed a lowered microbial richness at the end of the trial compared to the sampling at 15 days. Whilst a large proportion of prebiotics studied have been reported to improve the health, diversity and richness of the GIT microbial composition (Sullam *et al*. 2012; Baldo *et al*. 2015; Gajardo *et al*. 2016; Lyons *et al*. 2016; Villasante *et al*. 2019), this study did not find any changes within white sea bream fed either diet (Guerreiro *et al*. 2018b). LAB levels were reported to be increased, and four examples of *Lactobacillus* spp. were cultured, however, the abundance levels were not significantly increased between fish fed any dietary regime (Guerreiro *et al*. 2018b). These results are in conflict with the results of Hoseinifar *et al*. (2013), whose authors reported that increased abundance of LAB may have benefits for prebiotic fed fish, including increased immunity and host health, supporting the idea that the effects of GOS and other additives tested within teleosts varies between studies.

1.6 Conclusion

There is evidence from previous studies that suggests the efficacy of GOS administered to different commercial teleost species is dependent on a number of different factors. Results between studies have differed, as improvements reported on the health of the GIT by prebiotic supplementation in finfish can heavily depend on the diet, age, species, culture conditions or environment, and the length of feeding (Burr *et al.* 2005; Merrifield *et al.* 2010c; Ringø *et al.* 2010; Sweetman *et al*. 2010; Hoseinifar *et al*. 2013, 2015; Merrifield & Rodiles 2015; Eryalçin *et al.* 2017; Yousefi *et al*. 2018). One potential factor that may explain the lack of any significant benefits from prebiotic supplementation in some studies may be due to the proportion of plant feedstuffs within formulated diets, compared to diets produced with fish derived products.

Plant derived proteins that are used as a supplement or replacement to fishmeal typically contain oligosaccharides that may induce their own prebiotic effect within the GIT of teleosts by providing a source of carbon for metabolism (Burr *et al*. 2010; Dimitroglou *et al*. 2010). However, some plant feedstuffs, such as soybean meal (SBM), contain a number of anti-nutritional factors that may produce negative morphological changes in the GIT mucosal surfaces, resulting in a pathology known as enteritis (Merrifield *et al*. 2011; Adeoye *et al*. 2016a,b). There is the possibility that the effects of GOS fed to fish investigated by Guerreiro *et al*. (2018b) may be masked by an increased ratio of plant proteins to fishmeal, which may also explain the results of Buentello *et al*. (2010). The contrasting results with the immune response and gene expression seen by Zhou *et al*. (2010), Yousefi *et al*. (2018) and Hoseinifar *et al*. (2015) suggest that there may be possible immunomodulatory effects within the fish fed GOS, at varying levels.

However, the overall conclusion that these studies suggest is that the species, life stage and experimental design are factors that will affect the results across studies, and may be the cause for conflicting results in the literature.

Despite the conflicting results between studies, most reports have included some degree of positive effects of GOS supplemented feeds on the species studied. There have been demonstrated increases in growth performance parameters, as seen in Hoseinifar *et al*. (2013), while other studies have shown positive impacts on immunoregulatory responses (Buentello *et al*. 2010; Zhou *et al*. 2010; Hoseinifar *et al*. 2015; Yousefi *et al*. 2018). There are also enhancements observed in the intestinal structures of the fish species studied. Research has demonstrated increased microvilli fold length across different sections of the GIT following GOS supplementation, suggesting increased nutrient uptake and absorption (Zhou *et al*. 2010; Anguiano *et al*. 2013), as well as increased abundance and activity of microorganisms within the microbiome (Anguiano *et al*. 2013; Hoseinifar *et al*. 2013; Guerreiro *et al*. 2018b).

The supplementation of feed additives to the diets of cultured species may positively affect the microbiota of the GIT and other important mucosal lymphoid tissues of the host by improving commensal proliferation of beneficial bacteria and increasing the immune functions that help protect the host from pathogenic interactions. There is great scope for novel additives to be tested in a similar manner as described in the literature to determine the efficacy of novel prebiotics for widespread use within the wider aquaculture industry.

1.7 Research Aims and Enquiry

The present knowledge of dietary additive utilisation in the aquaculture industry has been extensively researched within teleosts, however, to date, there is a bias in prebiotic testing in aquaculture towards products that are derived from yeast, such as MOS, or plants sources, such as FOS, with fewer studies concentrating on GOS addition (Mugwanya *et al*. 2021). Within the literature presented, GOS has been reported to elicit health benefits in some teleost species, whilst also conferring no improvements in others. Multiple factors may lead to these observations, such as the type of GOS and experimental conditions for each trial, such as concentration of additive, the species investigated, or if fish are subjected to a/biotic challenge, which lead to difficulties in comparing the efficacy of GOS additives. In-depth appraisal of multiple aspects of growth performance and the immune response of investigated teleost species fed GOS is not as widely explored as probiotic additives, particularly between cold and warm-water species under the same testing conditions.

The aim of this PhD is to determine if the health and growth of rainbow trout, Atlantic salmon and Nile tilapia are improved by the addition of a novel dietary additive developed by Clasado Biosciences Ltd. called Bimuno®. This additive is a novel source of GOS that to the author's knowledge had not been tested in finfish prior to the current experimentations, and no data for this product in fish was available in published and publically accessible literature. This proprietary prebiotic is derived from the activity of galactosyltransferases from *Bifidobacterium bifidum* NCIMB 41171 in the presence of lactose (a natural component in bovine milk), and is composed of chains of galactose and glucose structural components (Tzortzis *et al*. 2005a,b). Previous research has

reported that this product has potential benefits humans and swine by improving digestive health and wellbeing, modulating the immune system and diversifying the microbiota by encouraging the growth of beneficial bacteria, such as bifidobacteria (Tzortzis *et al.*, 2005a, b; Mao *et al*. 2015). To the author's knowledge, there has been no research published using this novel GOS within commercially important aquatic species, and so it is of great interest to understand how fish respond to this particular GOS compared to other feed additives, especially GOS, and address how this prebiotic modulates the immune response in commercially important teleosts. Hence forth, this product shall be referred to as B-GOS® or GOS, unless otherwise stated.

The objectives of this research programme are as follows:

1. To ascertain if there are modulatory effects of GOS on the gut microbiome of three commercially important fish species.
2. To assess potential effects of GOS on the intestinal and systemic health of three commercially important fish species.
3. To determine an optimal GOS inclusion level for three commercially important fish species.

To meet these objectives and assess the effectiveness of B-GOS®, a series of three *in vivo* feeding trials were performed using rainbow trout (Chapter 3), Atlantic salmon (Chapter 4) and Nile tilapia (Chapter 6). The additive itself was administered to each species via the feed and each trial persisted over a growth period that saw at least a two-fold increase in weight. The conditions for each species were met within the aquaria facilities of the University of Plymouth, and the length of each trial was determined based upon the species and the age of the fish used, as well as other suitable growth

conditions, such as water temperature. The results from a multitude of analytical techniques have been generated from samples of fish tissues/organs collected at each trial's conclusion, with the specific details of these assays outlined in the Materials and Methods (Chapter 2) section of this thesis. The data generated from these experimental *in vivo* trials have been used to form novel endpoints in aspects of salmonid and cichlid growth performance and immune response, such as humoral immunity, more in depth histological appraisal, and intestinal microbiome analysis upon feeding with B-GOS®.

This body of work has been part funded by Clasado BioSciences Ltd., a company that specialises in additives for terrestrial animal feeds and human health. This company has provided partial funding and the test additive to be used in fish diets to determine if this additive will benefit the health and growth performance of commercial fish species. A further grant was obtained from the Seale-Hayne Educational Trust.

Materials and Methods

2.1 General Overview

In this series of studies, differing levels of B-GOS® were supplemented into experimental diets fed to rainbow trout (*O. mykiss*), Atlantic salmon (*S. salar*) and Nile tilapia (*O. niloticus*) to determine their effect, if any, on fish growth and health. A total of three fish feeding trials were designed, implemented, managed and concluded within the Aquaculture and Fish Health, Production and Nutrition research facilities within the University of Plymouth, UK. All experiments complied with the Animal Scientific Procedures Act 1986. Individual ethical approval was sought and granted for each trial, as outlined in the experimental systems sections. *In vivo* fish feeding trials are an extremely useful model to determine the optimum input of a feed additive product to the diet of commercial fish species as all rearing conditions can be controlled and managed in a secure and clean facility, thus allowing others factors to be removed from the experimental design. All feeds with additive concentrations of B-GOS® were formulated and produced in-house by the PhD candidate within the facilities of the University of Plymouth, and feed was administered to the experimental species by hand by the candidate four to six times a day, depending on the age and species. Fish were randomly sampled at each trial's conclusion at n=3 fish per tank and euthanised via Schedule 1 methods. Samples of fish skin, gut, blood, mucus and other relevant organs were aseptically removed from the carcasses and stored in MCC tubes at -20°/-80°C for further analysis. Other randomly sampled fish (n=6 per diet) were euthanised via Schedule 1 methods and were used for proximate compositional analyses.

2.2 Experimental Systems

The PhD candidate was responsible for the set-up, upkeep and running of the trials for each species of fish tested. The experimental designs of each trial, holding facilities, animal husbandry and specific licences are described in the respective experimental chapters for rainbow trout, Atlantic salmon and Nile tilapia. Please refer to these chapters for the specifics for each trial. All experimental analyses performed at the trials conclusions are presented under the following protocols, and all work involving the rearing of fish was conducted under the UK Home Office Institute licence of the University of Plymouth, under the UK Animals Scientific Procedures Act of 1986. Reagents and chemicals were sourced from Fisher Scientific, BIO-RAD, Qiagen, Sigma Aldrich, Thermo Scientific or Life Technologies.

2.3 Sample Collection

For all salmonid trials, fish were selected at random by netting, and individual fish were euthanised by a blow to the head and destruction of the brain. For the Nile tilapia, the fish were too small to be euthanised in this manner, and so fish were euthanised by overdose of MS222 (tricaine methanesulfonate, 400 mg L^{-1}) for 15 minutes, after which the brain was destroyed. For logistical and practical reasons, the samples obtained for each analysis were not always taken from the same fish; where required, samples (n=9 fish per diet, where available) were collected according to the following protocol, and outlined in Figures 2.1 and 2.2:

- **Morphometric measurements:** Each tank was batched weighed at the conclusion on the trial and the growth performance metrics calculated from these measurements (n=3 per diet), except for K-factor. Body weights and fork

lengths were recorded for individually sampled fish to calculate Fulton's condition factor (K-factor) measurements. The K-factor is a non-lethal morphometric index used to estimate body condition of a fish, and is calculated by measuring the body weight and fork length of a fish (Robinson *et al*. 2008). This method assumes that heavier fish of a given length are in better condition (Sutton *et al*. 2000).

- **Haematological samples:** Blood samples were taken from the caudal vessel of each fish, from both the trout and salmon trial, using a 25-gauge needle and 1 mL syringe, after Rawling *et al*. (2014). Blood was stored at 4°C overnight until further analysis. Blood from tilapia sampled at the end of the trial was not taken, as these fish were too small to extract enough blood for all further analyses.
- **Skin epidermal mucous collection:** Mucous was collected from the left flank of the body using a spatula directly after Schedule 1 termination. The mucous was collected and weighed in a pre-weighed MCC tube (±10mg) and frozen at -20°C.
- **Histological tissue samples:** Approximately 1 cm^2 of skin and 250 mg segment of posterior intestinal tissue was sampled via dissection and preserved in 10% buffered formalin pots for 1 week until transferred to 70% ethanol for storage until further processing, after Standen *et al*. (2015, 2016).
- **Gene expression samples:** Approximately 1 cm^2 of skin and 200mg segment of posterior intestinal tissue was sampled and preserved in 1 mL of RNAlater® (Thermo Fisher Scientific) for 24 hours at 4°C and thereafter stored at -80°C for long-term storage until further analysis.

- **Intestinal microbiome samples:** After tank weighing and before dissecting, the fish were fed their respective diets and sampled after 1 hour. Fish were sampled and wiped clean on the left flank in the presence of a Bunsen flame using 75% ethanol, and sterilised scalpels were used to dissect the body cavity. The posterior intestine was sectioned at the top and bottom of the tract, and the contents were squeezed into MCC tubes. The gut contents were labelled as digesta and the gut sections themselves were labelled as mucosa. All samples were immediately frozen at -20°C until further processing.

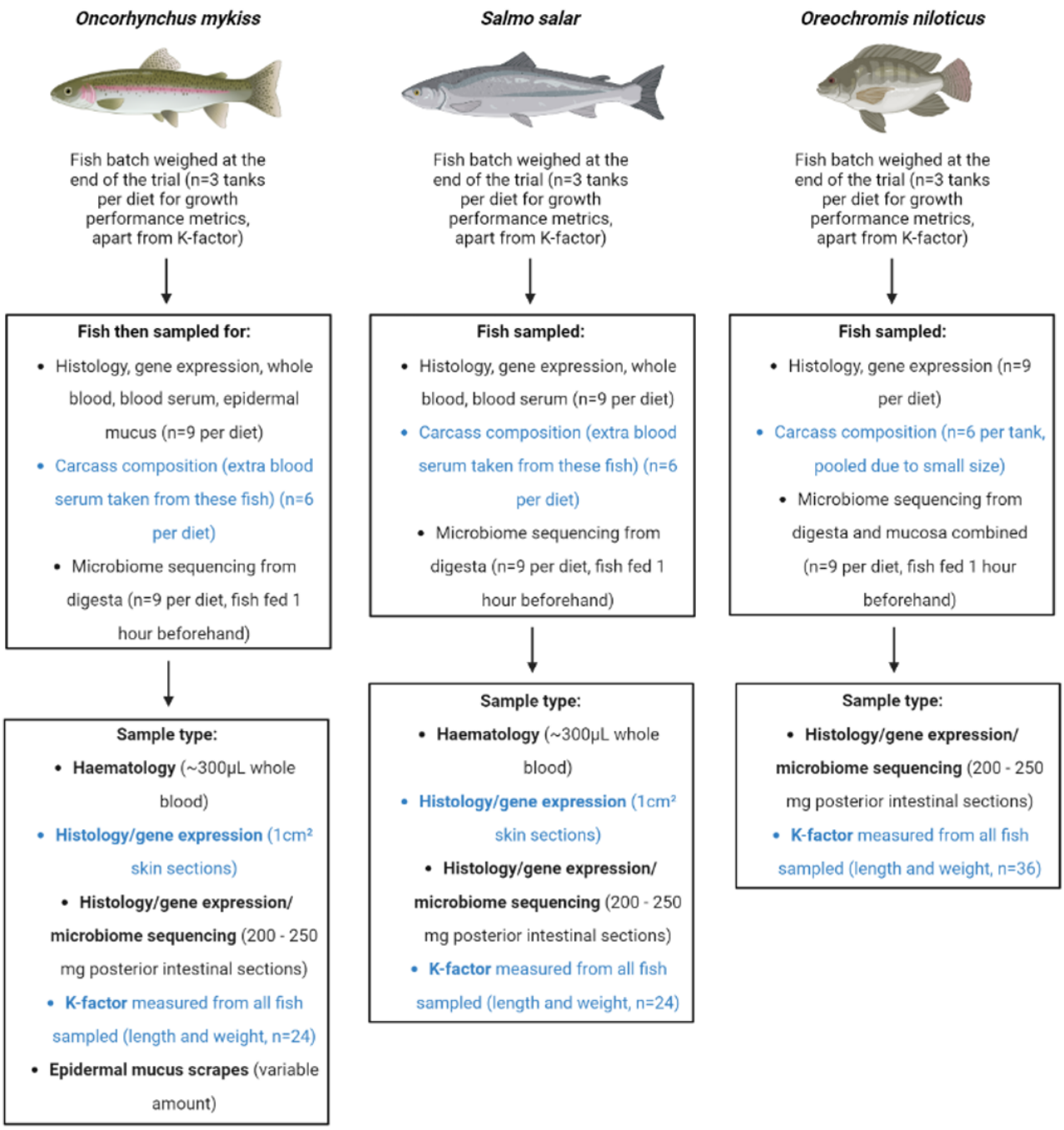


Figure 2.1 Sample collection methodology for each species trialled. For logistical reasons, whole blood and epidermal mucus scrapes could not be sampled from the tilapia, as these fish were too small. Image made in BioRender.com.

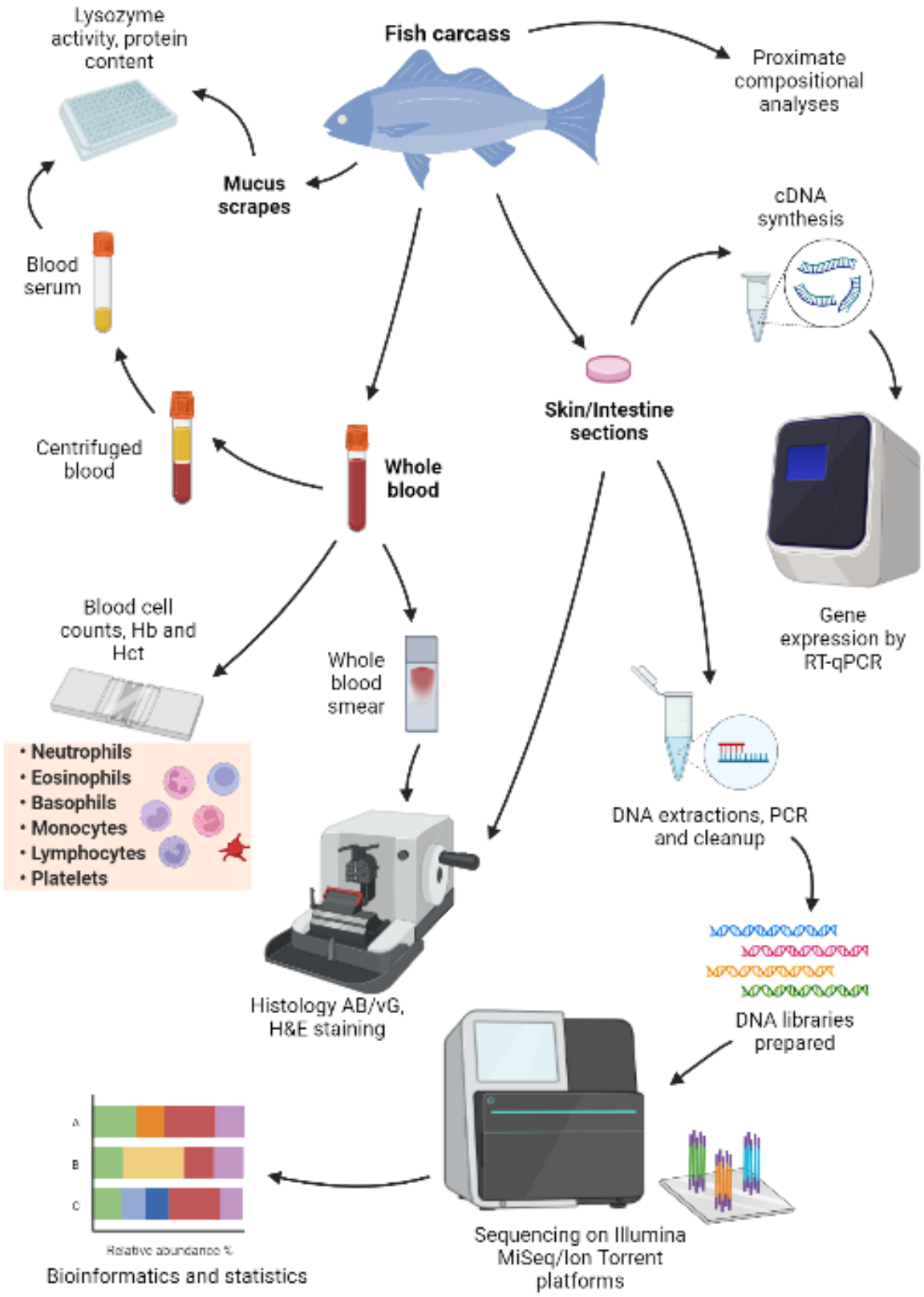


Figure 2.2 Analyses conducted from tissues and organs dissected as outlined in Figure 2.1 for each species trialled. For logistical reasons, whole blood and epidermal mucus scrapes could not be sampled from the tilapia at the conclusion of the trial, as these fish were too small. Image made in BioRender.com.

2.4 Diet Formulation and Growth Performance

The specific diet formulations and tables of chemical compositions for each trial are summarised in the methodology sections of each trial chapter. Diets were formulated for each species using Animal Feed Optimisation Software (AFOS) and were designed to meet the known nutritional requirements for each species (NRC 2011), without replicating commercial diet compositions. For each trial, diets were produced in batches by mixing dry ingredients in a Hobart food mixer (Hobart Food Equipment, Australia, model HL-1400-10STDA) to ensure a homogenous mix of ingredients before the slow addition of oil and warm water. These ingredients were mixed to form a consistency that is suitable for cold press extrusion using a PTM P6 extruder and producing appropriate size pellets for each species. The basal (Control) diets were void of the prebiotic B-GOS® and the incremental levels of the prebiotic were added to each diet at the expense of another ingredient; cornstarch for rainbow trout, sunflower meal for Atlantic salmon, and wheat for Nile tilapia.

Diets were dried for 48 hours in an air convection oven set to 45-50°C, and then placed within airtight containers and stored at 4°C to reduce the risk of bacterial growth. Dietary proximate composition analyses were performed as described in Methodology section 2.7 and under AOAC procedures (2016). Diets prepared for the Nile tilapia were ground to a crumb and sieved to produce 250, 500 and 750 micron grains to ensure that the fish could accept the diet.

Growth performance parameters provide an important toolkit for those who work in the aquaculture industry to determine the production and profitability of culture facilities. The measurements of growth, nutrient uptake and utilisation via feed

efficiency, fish condition and survivability are key factors in the assessment of overall performance, and these parameters for each experimental trial were determined before sampling after batch-weighing each tank on the final day of the trial (n=3 tanks per treatment, except for K-factor whereby individually sampled fish were measured) using the following calculations after Standen *et al*. (2016):

- Feed Conversion Ratio (FCR) = Amount fed (g)/ Weight gained (g)
- Specific Growth Rate (SGR) = ((lnW2 − lnW1)/T) x 100;

  lnW1 and lnW2 are the initial and final natural logarithmic weights, respectively, and T is the number of days in the feeding period.
- Percentage Weight Gain (PWG) = $\frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}}$ x 100
- Survival (%) = $\left(\frac{\text{Number of fish at the end of the trial}}{\text{Number of fish at the beginning of the trial}}\right)$ x 100
- Condition Factor (K − Factor) = Body weight(g)/(Body length (cm))^3 x 100

At the end of each trial, two fish per tank were used to determine carcass composition (thus n=6 fish per diet), however as the tilapia were small than 5 g each, any remaining carcasses were pooled by dietary treatment and used for these analyses. Proximate composition analyses were conducted on these samples as described in section 2.9.

2.5 Haematology

Blood samples used for calculating the haemoglobin (Hb) concentration were prepared from 4 µL of whole blood diluted in 1 mL of Drabkin's cyanide-ferricyanide solution, in a modified protocol from Svobodova & Vykusova (1991). Sample absorbance was read at A_{540nm} (Jenway 7305 Spectrophotometer, UK) against a blank cuvette of Drabkin's solution and 4 µL of distilled water. The Hb concentration was calculated using a

standard curve generated from haemoglobin porcine lyophilized powder (Sigma-Aldrich, UK), and multiplying by the dilution factor (Svobodova & Vykusova 1991). One fish sample was removed from the Hb analysis from the 8g kg^{-1} experimental diet in the salmon trial, as further statistical testing using Excel 2016 Quartile and OR functions determined that this sample was an outlier.

Haematocrit (Hct) samples were prepared using heparin treated haematocrit tubes (BRAND™, Fisher Scientific) and blood that was drawn into the tubes by capillary action. The sample tubes were spun at 12,500 xg for 5 minutes after a modified protocol from Svobodova & Vykusova (1991), and the results are expressed as Packed Cell Volume (PCV).

Total erythrocyte (RBC) and leucocyte (WBC) counts were made from 20 µL of whole blood diluted in 980 µL Dacies solution. Cells were counted in a Neubauer haemocytometer following standard procedures (Dacie & Lewis 1984; Svobodova & Vykusova 1991; Rao & Deshpande 2006). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from the previous parameters stated. All haematological counts were calculated using the equations below:

- $\text{PCV (L/L)} = \text{Percentage Hct} \times 0.01$
- $\text{RBC (cells} \times 10^6/\text{mm}^3) = (\frac{\text{Average total count}}{\text{Volume}} \times \text{Dilution factor})/1 \times 10^6$
- $\text{WBC (cells} \times 10^4/\text{mm}^3) = (\frac{\text{Average total count}}{\text{Volume}} \times \text{Dilution factor})/1 \times 10^4$
- $\text{MCV (fl)} = (\text{PCV} \times 1000)/\text{RBC}$
- $\text{MCH (pg)} = \text{Hb (g/L)}/\text{RBC}$
- $\text{MCHC (g/L)} = \text{Hb (g/L)}/(\text{PCV} \times 1000)$

Leucocyte counts were quantified using 5 µL of blood prepared on microscope slides using standard smear techniques (Berillis *et al*. 2016; Figure 2.2). The blood smears were allowed to air dry, fixed for 5 minutes with 100% ethanol, and stained in one-part May Grunwald (CellPath, Wales) to one-part Sorensens buffer pH 6.8 for 5 minutes. The slides were rinsed in buffer pH 6.8, stained in one-part Giemsa (CellPath, Wales) to nine-part buffer pH 6.8 for 10 minutes, and mounted with using DPX (Sigma-Aldrich). The cells were analysed using the Leica DMD108 microscope and digital software (magnification x200), and lymphocytes, monocytes and basophilic and neutrophilic granulocytes were differentiated using the shape of the nucleus, size and internal structure according to Rowley (1990) and Berillis *et al.* (2016) (Figures 2.3 and 2.4). A minimum of 200 cells were counted per fish, and the cells were expressed as a percentage of total leucocytes, after Rawling *et al*. (2012). Outliers were determined by statistical analysis in Excel 2016 using the Quartile and OR functions to determine the upper and lower limits based on interquartile ranges (Grech 2018), and remove data points that were TRUE as outliers. These outliers were removed from leucocyte and erythrocyte counts.

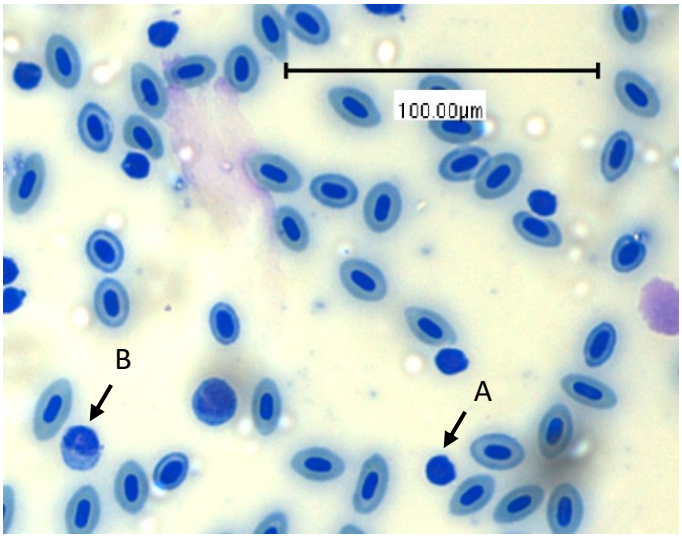


Figure 2.3 MGG stained blood smear from trout sample S2T5C. **A)** lymphocyte and **B)** basophilic granulocyte. Scale bar = 100 µm.

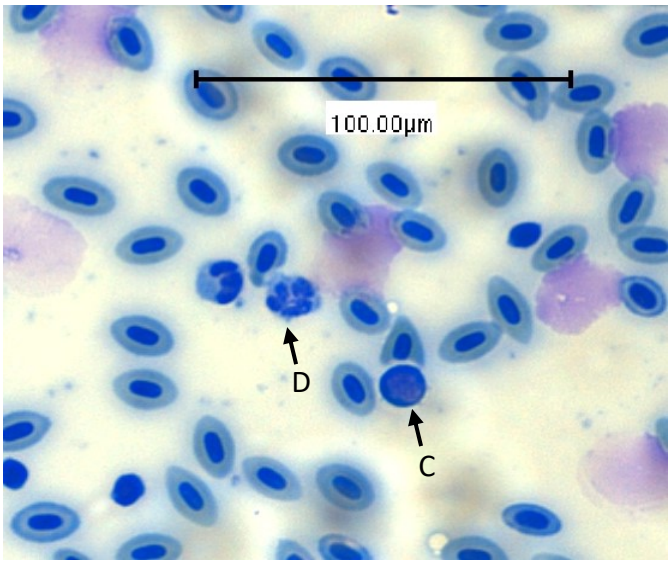


Figure 2.4 MGG stained blood smear from trout sample S2T5C. **C)** monocyte and **D)** neutrophilic granulocyte. Scale bar = 100 µm.

2.6 Determination of Lysozyme Activity

Lysozyme activity was measured from samples of the mucus and blood serum in fish following protocols described by Rawling *et al.* (2014). This enzyme has antibacterial properties and works by causing lysis of Gram-positive bacteria due to the breakdown of peptidoglycan within the cell walls. This assay uses *Micrococcus lysodeikticus* according to Ellis (1990).

Epidermal mucus scrapes were performed on fish sampled at the trial end points as described in section 2.3 (n=9 fish per diet). Blood serum was extracted from blood collected as described in section 2.3 (n=15 fish per diet, with the extra blood serum samples (an additional n=6 per diet) taken from fish that were used for carcass composition), and aliquoted into MCC tubes for storage overnight at 4°C. The blood samples were centrifuged at 5,000 x g for 5 min the following day, and the serum aliquoted into fresh tubes before being stored at -80°C (Figure 2.2). All samples were thawed on ice before the determination of lysozyme activity.

A 0.04 M, pH 5.8 (for trout) or pH 6.3 (for salmon) sodium phosphate buffer (SPB) was prepared by mixing $NaH_2PO_4.2H_2O$ and $Na_2HPO_4.2H_2O$ with distilled water and warmed to 25°C as a medium for *M. lysodeikticus*. A suspension of the bacteria in this solution was prepared at 0.2 mg mL^{-1}. Samples were prepared in duplicate on 96-well flat-bottomed plates (Costar® Corning Inc. USA) in a 1:20 dilution with the SPB. The reduction in absorbance at 540 nm (25°C) was measured between 1 min and 10 min on the VersaMax™ ELISA Spectrophotometer microplate reader and analysed using SoftMax® Pro Software (Molecular Devices LLC) against a standard curve. A unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance at 0.001 min^{-1}. To normalise the lysozyme activity of the serum samples, the lysozyme/min/mL measurements for each individual fish were divided by the protein content in mg/mL. Outliers were determined by statistical analysis in Excel 2016 using the Quartile and OR functions described in section 2.5 (Grech 2018), and removed from further statistical testing.

2.7 Determination of Protein Concentration

Total protein content was determined from samples of blood serum and epidermal mucus of rainbow trout using the BCA protein assay, and following protocols described by Rawling *et al*. (2014). This procedure involves bicinchoninic acid (BCA) and the reduction of Cu^{2+} to Cu^{1+} ions by protein in alkaline medium, combined with colourimetric detection of the Cu^{+1} cation using BCA.

The Pierce BCA Protein Assay kit (Thermo Scientific) was used to determine the protein content of the same blood serum and mucus extracted as in section 2.5 (n=9 per diet for each tissue type). Bovine serum albumin (BSA) was used from a range of 0.0 – 2.0 mg/mL to generate a standard curve. Three replicates of each sample were run on a microplate according to the manufacturer's instructions. The samples were used from the same blood serum and epidermal mucus scrapes as described in the lysozyme activity assay. Prior to this assay, aliquots of the serum samples were diluted 1:50 and the mucus diluted 1:10 before freezing at -80°C.

Samples were prepared in a ratio of 1:20 for the sample to the kit's working reagent, and each plate was incubated at 37°C for 30 min. After this incubation, the absorbance was measured at 562 nm using the VersaMax™ ELISA Spectrophotometer microplate reader at room temperature and analysed using SoftMax® Pro Software (Molecular Devices LLC). The results were standardised by subtracting the average blank absorbance measurements from the samples and standards, and a quadratic line of best fit was generated along the standard curve to calculate the protein concentrations. These results were then normalised to take into account the dilution factor.

2.8 Histology

The histological samples prepared as in section 2.3 were further dehydrated using the Leica TP 1020 processor and then embedded in paraffin wax according to standard histological procedures. Samples were sectioned at 5 µm thickness (Leica RM2235 microtome), and then dried at 37°C onto microscope slides. The sections were stained using Haematoxylin Alcian Blue and van Gieson (AB/vG) to measure the muscularis widths, mucosal fold heights, lamina propria widths and goblet cell counts (for both intestine and skin samples). The slides were mounted with cover slips using DPX and allowed to dry. Images were captured using the Leica DMD108 microscope and digital software (Figure 2.2).

Image analysis was conducted using Image J software (National Institutes of Health, Bethesda, Maryland, USA) and the Fiji processing package (Fiji Is Just Image J, GPL v2). Images were taken at x40 magnification for measuring the muscularis thickness (example in Figure 2.5), and further images were taken at x100 magnification for measuring lamina propria width, mucosal fold length (Figure 2.5 for salmonids and Figure 2.7 for tilapia), and goblet cells (Figures 2.6 and 2.8).

Muscularis widths were measured from the outermost point of the longitudinal muscular layer to the innermost side of the inner circular muscular layer, in triplicate per fish (as shown in Figure 2.5, A). Mucosal fold heights were measured from eight complete folds per fish, with measurements starting from the submucosa (base of the fold) to the apex (Figure 2.5, B). Lamina propria widths were measured from eight folds per fish from the bottom, middle and top points per fold (Figure 2.5, C). These same measurements were also taken from juvenile tilapia intestinal cross sections (Figure

2.7). Goblet cell counts were calculated within a length of 200 µm at the apex of four folds per fish (as shown in Figure 2.6, A), and within a length of 200 µm from the end of one scale across the mucosal layer (as shown in Figure 2.8).

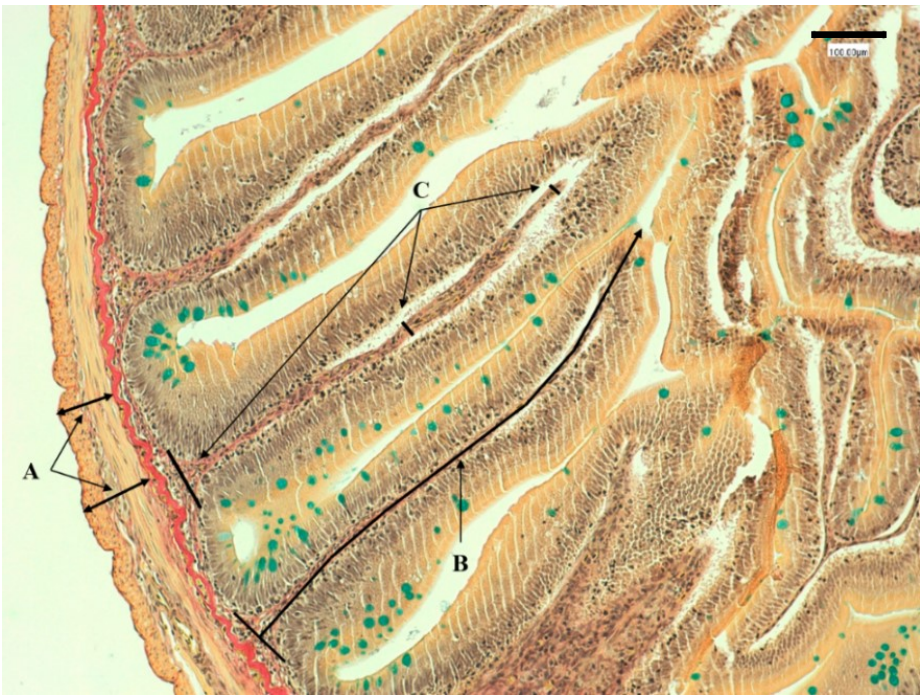


Figure 2.5 AB/vG stained image from a juvenile rainbow trout demonstrating the apical region of the mucosal folds used for **A**) muscularis thickness, **B**) mucosal fold height, **C**) lamina propria width. Scale bar = 100 µm.

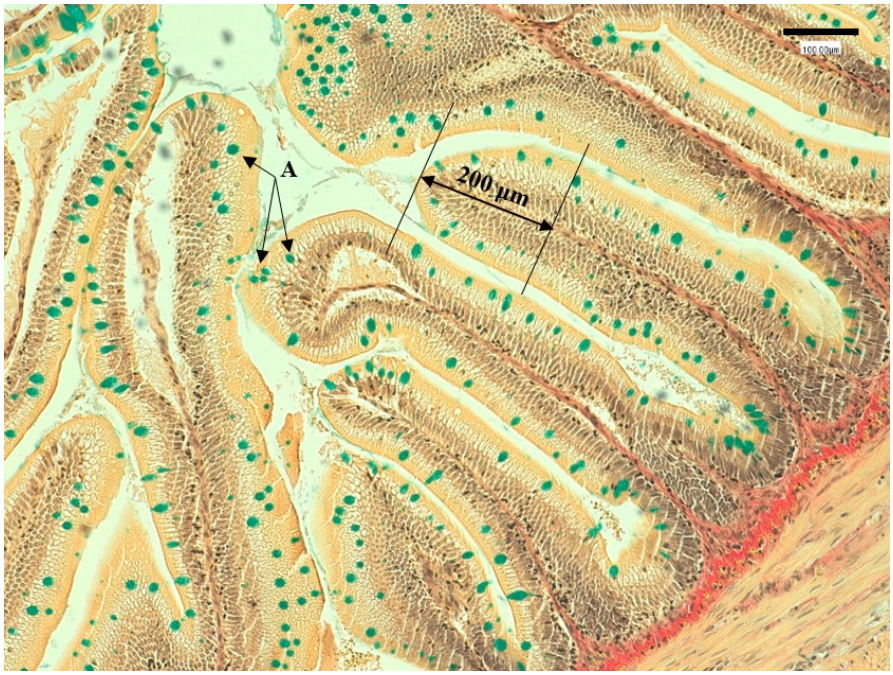


Figure 2.6 AB/vG stained image from a juvenile rainbow trout demonstrating the apical region of the mucosal folds used for goblet cell counts. **A**) Both acid and basic mucin cells (green) Scale bar = 100 µm.

Figure 2.7 AB/vG stained image from tilapia demonstrating the whole intestinal cross section of the mucosal folds and muscularis used for all histology measurements. Both acid and basic mucin cells (green) were used for goblet cell counts. Scale bar = 100 µm.

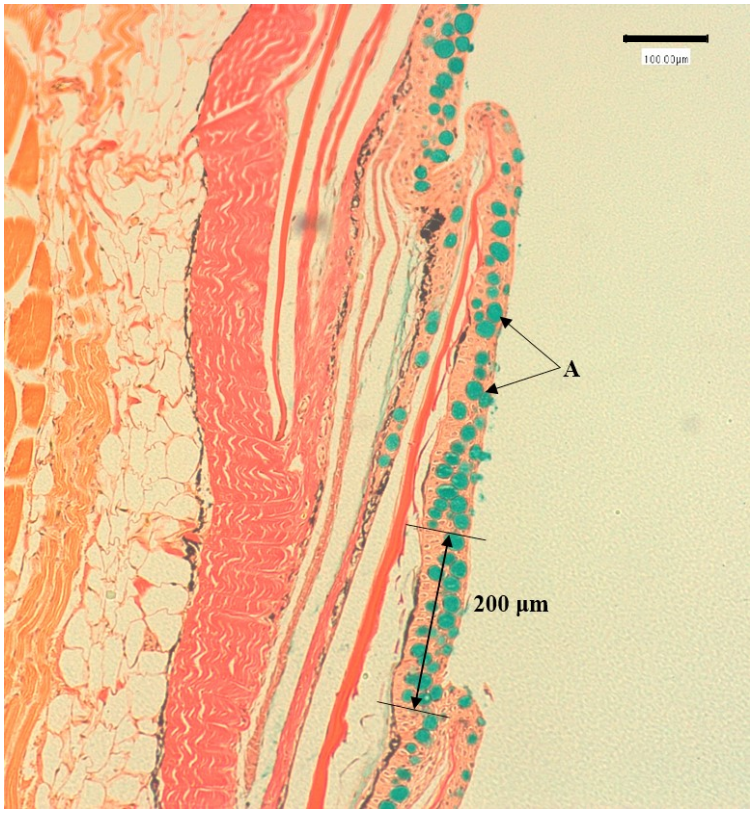


Figure 2.8 AB/vG stained skin image from a juvenile Atlantic salmon demonstrating the region of the mucosal layer used for goblet cell counts. **A**) Both acid and basic mucin cells (green). Scale bar = 100 µm.

2.9 Proximate Composition Analyses

The proximate compositions of all experimental diets were determined using adapted methodologies from standard AOAC (2000, 2016) procedures (Howitz 2000; Horowitz & Latimer 2000) described below, in triplicate. Carcass composition analyses of sampled fish (n=6 per diet for trout and salmon) were conducted in an identical manner to the proximate analyses of the feed formulations, according to standard AOAC (2016) procedures. For the Nile tilapia carcass compositions, due to the size of the fish, n=6 fish per tank were pooled for the proximate analysis, as individual fish would not yield sufficient dried matter for further analyses. Proximate compositional analyses are utilised to determine that all experimental treatments were comparable.

2.9.1 Moisture Content

Approximately 5.0 g of sample was weighed into a metal crucible in triplicate and air dried in a fan-assisted oven set to 105°C overnight and subsequently measured once an hour until a consistent weight had been achieved. Percentage moisture was calculated as:

$$Moisture\ content = ((WW - DW)/WW) \times 100$$

Where the WW is wet weight of sample (g), and the DW is the dry weight (g).

2.9.2 Ash Content

The ash content was determined by adding approximately 500 mg of sample to a pre-weighed porcelain crucible and placed within a muffle furnace (Carbolite, Sheffield, England) at 580°C to a constant weight. The ash content as a percentage was calculated as:

$$Ash\ content = ((AR - CW)/OSW) \times 100$$

Where AR is ash residue (g), CW is the crucible weight (g) and OSW is the original sample weight (g).

2.9.3 Crude Lipid Content

The Soxhlet extraction method was used to determine the lipid content of each sample. Samples were weighed into a cellulose thimble (2.5 ± 0.5 g), plugged with cotton wool and then placed into an insert resting in glass beakers containing bumping granules. Petroleum ether (140 mL per beaker) was added to the glass beaker and each sample was placed within the Soxtherm unit (Gerhardt Soxtherm, Bonn, Germany) under the manufacturer's instructions for lipid extraction. The beakers were removed from the

unit upon completion and excess solvent was allowed to evaporate overnight in a vented fume hood. The beakers were weighed to determine the extracted lipid content, and the percentage lipid was calculated as follows:

$$Lipid\ content = (LW/OSW) \times 100$$

Where LW is lipid weight after extraction (g) and OSW is original sample weight (g).

2.9.4 Crude Protein Content

The crude protein content was measured from samples using the Kjeldahl method after acid digestion and multiplying the result by 6.25 on the assumption that animal proteins will contain 1% nitrogen (AOAC 2016). Each sample was weighed to 150 (± 50) mg into sections of nitrogen free paper and placed within Kjeldahl digestion tubes. Standards were also used to correct for the nitrogen efficiency (two tubes containing acetanilide, nitrogen content 10.36%) and to validate the reaction (casein, bovine). A catalyst tablet (3g K_2SO_4, 105 mg $CuSO_4.5H_2O$ and 105mg TiO_2, BDH Ltd., UK) and 10ml of concentrated (98%) H2SO4 (Sp.Gr. 1.84, BDH Ltd., UK) were added to each tube, which were placed on a Gerhardt Kjeldatherm KB8 S digestion block. The tubes were heated to 105°C for 15 minutes, 225°C for 60 minutes and 380°C for 45 minutes. The day following digestion, each sample was distilled using a Vapodest-50 automatic distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany) following manufacturer's instructions for determining crude protein. The crude protein content as a percentage was calculated as:

$$Protein\ content = (((ST - BT) \times 0.2 \times 1.4007 \times 6.25)/OSW) \times 100$$

Where ST is sample titre (mL), BT is blank titre (mL), OSW is the original sample weight (mg), 0.2 is acid molarity and 1.4007 is the molecular weight of nitrogen.

2.10 Gene Expression

Three fish per tank were sampled for gene expression analyses at the end point (n=9 per diet), following protocols described in section 2.3. Total RNA was extracted using TRIzol™ (Invitrogen, Thermo Fisher Scientific) extraction method, as per the manufacturer's instructions. One mL of TRI reagent (Ambion, Thermo Fisher Scientific) was added to each sample within Lysing Matrix D tubes (MP Biomedicals) and homogenised using the FastPrep-24™ 5G Instrument (MP Biomedicals) for 40 seconds. After the sample was thoroughly dissociated, 200 µL of chloroform was added and the samples were shaken for 15 sec and allowed to stand at room temperature for 10 min. After this, the samples were centrifuged at 12,000 x g for 15 min at 2-8°C. The upper aqueous phase was pipetted into separate 1.5 mL Eppendorf tubes for the precipitation step.

To each sample, 500 µL of isopropanol (2-propanol) was added and allowed to stand at room temperature for 10 min, before being centrifuged at 14,000 x g for 15 min. The RNA pellet was cleaned via removal of the initial supernatant, and adding 1 mL of 70% ethanol to the sample and centrifuged at 10,000 x g for 10 min for a total of two times. The ethanol was removed and the sample allowed to stand at room temperature for 10 min while the residual ethanol evaporated. RNA was eluted in 30 µL of nuclease-free water, before being stored at -80°C.

The quality and quantity of the RNA was assessed and confirmed using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific) to verify the 260/280 nm and 260/230 nm absorbance ratios, and via 1% agarose gel electrophoresis. The sample concentrations were corrected to 500ng μL^{-1} before being stored at -80°C until further

use. The samples were cleaned of genomic DNA (gDNA) and complimentary DNA (cDNA) was reverse transcribed from 1 µg of gDNAsed RNA using the QuantiTect® Reverse Transcription kit, (Qiagen, UK), as per the manufacturer's instructions. The reaction was run for 42°C for 15 minutes and inactivated at 95°C for 3 minutes. The cDNA samples were diluted by 1:20 for the rainbow trout samples, and 1:10 for the salmon and tilapia samples. The cDNA samples were stored at -20°C until use in real-time qPCR.

The primer efficiencies were calculated for the reference genes, elongation factor 1 alpha (*Elf-1α*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and beta-actin (*β-actin*). The primer efficiencies were calculated for target genes: interleukin 1 beta (*IL-1β*), tumour necrosis factor alpha (*TNFα*), interleukin 10 (*IL-10*), transforming growth factor beta (*TGF-β*) and calreticulin (*Cal*) (see Chapter 3, section 3.2.3, Table 3.2; and Chapter 4, section 4.2.3, Table 4.2 for rainbow trout and Atlantic salmon primer sequences, respectively). In addition to these target genes, the primers caspase-3 (*CASP3*), myeloid differentiation primary response 88 (*MYD88*), proliferating cell nuclear antigen (*PCNA*) and heat shock protein 70 (*HSP70*) were assessed in Nile tilapia (See Chapter 5, section 5.2.3 Table 5.2 for primer sequences). All primer sequences were ordered from Eurofins.

Representative cDNA was pooled for each tissue type and PCR was performed using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad) according to manufacturer's instructions, in duplicate 20 µL reaction (primers 300 nM both forward and reverse, 2 µL of pooled cDNA) as the pre-amplification stage. The PCR thermal profile for each reaction was: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1

min. The product was checked for specificity on a 1.5% agarose gel against a 100 bp DNA Hyperladder (Bioline).

To purify the PCR product, the MinElute® PCR Purification Kit (Qiagen, 28004) was used according to manufacturer's instructions. Purified primer product from the columns was eluted in 10 µL DEPC treated water (Ambion), twice. The product was stored at -20°C until further use. Serial dilutions of 1:10 of the purified product was made, from 1 x 10^{-2} to 1 x 10^{-8}, whereby the standard curves for each primer were generated using each dilution, in triplicate, in a 7.5 µL qPCR reaction (2 µL of purified product), following the iTaq™ Universal SYBR® Green Supermix instructions. The PCR reaction conditions were the same as the pre-amplification stage.

Reference genes are commonly amplified simultaneously with the target genes to act as an internal reference so that RNA values can be normalised (Karge *et al*. 1998; Jorgensen *et al*. 2006; Bustin *et al*. 2009). The purpose of these genes is to reduce the likelihood of statistical error due to sampling when calculating differences in RNA in target tissues between target genes (Olsvik *et al*. 2005). Ideal reference genes will be expressed at a constant level among many tissues within a chosen organism, and should do so regardless of experimental treatment and life stages (Olsvik *et al*. 2005). The reference genes *β-actin*, *Elf-1α* and *GAPDH* were amplified with each sample to standardise the results by reducing the variation in mRNA and cDNA quality and quantity (Olsvik *et al*. 2005; Bustin *et al*. 2009). No amplification product or primer dimer formations were observed in the negative controls and control templates, respectively. The threshold cycle during PCR (C_T) is defined as the point at which the fluorescence rises appreciatively above the background fluorescence, and these values for each sample

were determined manually. The C_T values were used to calculate the primer efficiencies, using the slope of the standard curves generated for each primer, and expressed as the E-value (Ramussen 2001):

$$\text{PCR efficiency} = 10^{(-1/slope)}$$

The qPCR for each gene per tissue type was run under the following conditions: a 7.5 µL reaction volume in duplicate for each sample, using the iTaq Universal SYBR® Green Supermix kit, 250 nM forward and reverse primers for the trout samples (300 nM forward and reverse primers for the salmon and tilapia samples), and 2 µL cDNA skin and intestine samples as a 1:10 dilution (trout) or 1:20 dilution (salmon and tilapia). PCR conditions were run under the same reactions as the previous PCR stages. The C_T value duplicates were averaged for each sample, and used to generate delta (Δ) C_T values for each gene and each diet, as calculated using the equation below:

$$\Delta C\text{T for each sample} = Primer\ E.value^{(Mean\ of\ Control\ Diet\ CT - Individual\ Sample\ CT)}$$

The ΔC_T values for the two to three reference genes for each treatment (*Elf-1α, GAPDH* and *β-actin*) were confirmed by generating an expression stability measure, or 'M' value, in the programme geNorm (Vandesompele *et al*. 2002). The normalisation factors generated for each treatment were used to calculate normalised ΔC_T values for each treatment, for each target gene (according to a modified method in Riedel *et al*. 2014). Outlying results were removed from the analysis using the Quartile and OR functions in Excel 2016 as described in section 2.5 (Grech 2018); for the specific number of samples taken forward in analysis, please refer to the specific species chapter methodology and results sections. The fold change was calculated by dividing the treatment normalised ΔC_T values by the control normalised ΔC_T values, for each gene.

These fold changes were then log2 transformed to determine the up or down regulation of target genes in each prebiotic inclusion fed fish compared to the control group.

2.11 Intestinal Microbiome Analysis

The analysis of the microbial populations involved amplifying the V1-V2 hypervariable region of the highly conserved 16S rRNA gene. First, digesta samples (n=9 per diet for trout, and n=7 per diet for both salmon and tilapia) were aseptically removed from the posterior intestine of each fish sampled at the end of the trial and stored at -20°C, following protocols described by Standen *et al*. (2015, 2016). DNA extractions were then performed as outlined in the section below. For the specific methodology for preparing DNA from the trout samples using an older version of the DNA extraction kit, please see the Chapter 3, section 3.2.4.

Samples of digesta were thawed and 150-200 mg digesta was weighed into new MCC tubes for DNA extraction. Samples were lysed in 500 µL of 50mg mL^{-1} lysozyme (Sigma-Aldrich) in 1 x TE buffer. These samples were incubated for 30 min at 37°C, and then centrifuged at 13,000 x g for 5 min. All centrifuge steps were performed at room temperature and 13,000 x g, unless otherwise stated (GeneAmp® PCR System 9700, Applied Biosystems). The supernatant was aspirated and discarded, and the pellet was suspended in 750 µL PowerBead Solution. This solution was transferred to Dry Bead Tubes and 60 µL of C1 solution was added to the suspension. This solution was heated for 10 min at 65°C and then vortexed in a horizontal Vortex Adapter for 10 min at maximum speed.

After this, the samples were centrifuged for 1 min and 400 µL supernatant was transferred to a new tube. To this solution, 250 µL of C2 solution was added, the tubes

vortexed and then place on ice for 5 min. The tubes were further centrifuged and 600 µL of this supernatant was transferred to a new tube, whereby 200 µL of C3 solution was added by pipetting up and down. The samples were vortexed and the solution incubated on ice for a further 5 min. After centrifuging again for 1 min, 650 µL of this supernatant to a new tube and 1200 µL C4 solution was added before vortexing. All of this solution was transferred to the MB Spin Columns provided and centrifuged for 1 min per 600 µL to capture the DNA. To this column, 500 µL of C5 solution was added to the centre of the membrane, and the column centrifuged for another minute. The column was placed within a fresh Eppendorf tube and the DNA eluted with 30 µL of C6 solution provided by the kit. The DNA samples were frozen at -20°C for long-term storage, or kept at 4°C for further use. DNA samples were checked for quality and quantity using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific), before being taken forward in PCR.

PCR was performed using quasi-universal bacterial primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3'), and pooled 338R-I (5'-GCW GCC TCC CGT AGG AGT-3') and 338R-II (5'-GCW GCC ACC CGT AGG TGT-3') (Roeselers *et al*. 2011; Gajardo *et al*. 2016; do Vale Pereira *et al*. 2017). The PCR reaction was carried out in a 25 µL reaction, using 12.5 µL MyTaq™ Red 2x kit (Bioline) according to manufacturer's instructions, and primers were used at 0.5 µL of both forward (27F) and reverse (338R-I and 339R-II pooled) at 50 pM, as described in Gajardo *et al*. (2016). Digesta DNA template was added at 1 µL per reaction, and nuclease-free water made up the remainder of the reaction. The PCR reaction conditions were as follows: initial denaturation at 95°C for 7 min; followed by a 10 cycle touchdown strategy where the samples were heated to 94°C for 30 sec, annealing decreasing from 63°C to 53°C, 72°C for 30 sec; then 25 cycles of 94°C for 30

sec, 53°C for 30 sec, 72°C for 30 sec; finally followed by 72°C for 10 min. The PCR amplicons were immediately stored at 4-8°C for use in a 1.2% agarose gel electrophoresis to check the product length and for quality against the Bioline Hyperladder (50bp).

The samples were then purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, USA) according to manufacturer's instructions for 10 µL of sample. To each PCR reaction, 1.8 x sample volume of the AMPure XP beads were added and mixed by pipetting action. Each reaction was left for 5 min at room temperature and placed within a magnet rack for 2 min. The supernatant was aspirated and discarded, after which 200 µL of 70% ethanol was dispensed into each sample and allowed to incubate for 30 seconds before being removed and then the process repeated once more. Ethanol was removed and the samples allowed to dry for ~2 minutes. The beads were suspended in 40 µL nuclease-free water and mixed, and then incubated at room temperature for 2 min, before being placed on the magnet rack again for 2 min. Of the now purified product, 38 µL was transferred to a new MCC tube and these samples were quantified using the Qubit® 2.0 Fluorometer (Thermo Fisher) system. For the specific methodology outlining the preparation of sequencing libraries and the bioinformatics pipelines that follow, please see for the trout samples Chapter 3, section 3.2.4; for the salmon samples, Chapter 4, section 4.2.4; for the tilapia samples refer to Chapter 5, section 5.2.4.

Rarefaction plots were produced for alpha and beta diversity metrics, and taxonomic analyses were produced within the QIIME v2 environment (Quast *et al*. 2013; Yarza *et al*. 2014; Yilmaz *et al*. 2014; Bokulich *et al*. 2018; Bokulich *et al*. 2021). The operational taxonomic units (OTUs) observed, Good's coverage, Chao1, Shannon's diversity were presented from QIIME v2. Good's coverage ensures sufficient sequencing depth has

been achieved, and the Chao1 metric tests for the number of species in a given community, or the abundance, it is useful in giving more weight to low abundance species (Chao 1984; Kim *et al*. 2017). Therefore, any singletons or doubletons in the reads sequenced are used to estimate the number of missing species by calculating the estimated features present based on the observed features present. When the observed number of features is similar to the Chao1, this demonstrates that there are similar estimated number of species present, even the rare or missing. Generally, when species richness increases, so does the diversity (Kim *et al*. 2017). Shannon's diversity metric can provide more information about the community composition than the species richness or its evenness. The value of this metric will increase as the number of species and evenness in the distribution of individuals among the species increases (Lemos *et al*. 2011; Kim *et al*. 2017).

2.12 Statistical Analysis

All data are presented as mean ± standard deviation (SD), except for RT-qPCR data which are presented as mean ± standard error of the mean (SEM) as to be consistent and comparable with literature that investigates differences in RT-qPCR data. All statistical analyses were performed using R Studio version 1.461 based on the R software v4.0.4 (R Studio Inc., Boston, Massachusetts, USA), and additional software programme STAMP v2.1.3 and LEfSe Galaxy Version 1.0 via Galaxy Hub software were used to perform statistical tests and produce graphs for the high-throughput sequencing data. Outliers within each dataset were determined by using the Quartile and OR functions in Excel 2016 as described in section 2.5 (Grech 2018) and removed, before performing Shapiro-Wilk and Levene's tests to determine normality and equal variances on the

means of the data, respectively. Histograms of the data were also used to plot each dataset and further determine normality and inform which statistical analyses to perform. Statistical testing was used to determine if commercially relevant doses of a novel GOS product fed as part of a finfish diet would affect different aspects of health and performance compared to control diet fed fish. The doses were chosen according to manufacturer's instructions and were compared to control fed finfish to allign with existing oligosaccharide data within the literature, and so that the results would be of commercial interest within the industry. While there may be risks of pseudorepliation when fish are treated as independent data points, this was not a concern for the statistical testing during this thesis as the tests performed in each experimental chapter are in accordance with the statistical methods of other studies within this field. Normally distributed data were subjected to one-way ANOVA and Tukey *post hoc* analysis. Kruskal-Wallis and Kruskal-Wallis multiple comparison/Dunn test analyses were used to assess the differences between non-normally distributed data. All gene expression data statistical analyses were carried out using permutation tests in R Studio following Röhmel (1996). Permutation tests were used as they can account for a wide range of non-normal distributions, and create a reference of the F-statistic through random 1000 perumtations of the data which is then compared to the 'true distribution' of the data, resulting in a p-value based on the proportion of similairty between these values (Röhmel 1996; Borcard *et al*. 2011). In all cases, significant differences were accepted as $p<0.05$.

The effects of prebiotic GOS dietary inclusion upon the growth performance and intestinal health of rainbow trout (*Oncorhynchus mykiss)*

3.1 Introduction

The pressure to produce enough protein for an increasing global population has grown considerably, and the aquaculture industry is expanding in an effort to meet these demands. Fish have provided more than 3.3 billion people globally with 20% of their average per capita intake of animal proteins, and in some cases, this was as high as 50% (FAO 2020). The consumption of fish is in many countries the sole reliable source of protein, fats and omega 3's (Huss 1994; Ibrahem *et al*. 2010; FAO 2016), and restrictions to resources may mean aquaculture is an easy method of producing food to complement a mainly vegetable based diet when resources are restricted (FAO 2016). As stocking densities increase, fish are subjected to more intensive and prolonged environmental abiotic and biotic conditions, such as changes in pH, temperature fluctuations, over-crowding and handling during welfare checks (Oliva-Teles 2012). These factors increase stress levels, which impairs normal immune functions and overall performance, thus allowing for the proliferation of disease (Oliva-Teles 2012). Good management practices and additional measures such as administration of antibiotics and other antimicrobials have proven to be efficient in combating disease; however, there has been growing concern over how the former will affect the surrounding environment, for instance by promoting antibiotic resistance in microorganisms (Cabello 2006; Ringø *et al*. 2014; Dawood *et al*. 2018).

In recent decades, the addition of feed additives, such as prebiotics, to the diets of commercially valuable species has been recommended as a suitable alternative to other invasive therapeutic methods of disease control (Carbone & Faggio 2016). In view of this, the first *in vivo* feeding trial as part of this project was designed to determine if the

novel prebiotic B-GOS® could offer benefits to rainbow trout growth and health within a fish species appropriate diet. To the author's knowledge, no data are currently available regarding the effect of this prebiotic on rainbow trout growth, health or intestinal microbiome.

In 2018, global aquaculture production of salmonids (the main species comprising of *O. mykiss* and *S. salar*) was over 3.2 million tonnes, with a strong demand in developed and developing countries (FAO 2020). There has been rapid growth in markets for salmonids, with rainbow trout being a highly valued commodity in many Northern Pacific fisheries, as well as being a major farmed UK species (FAO 2020). As one of the most popular species to cultivate, this global success can be attributed to several factors. Several characteristics include; a tolerance to a diverse range of temperatures, thriving in a broad range of temperate water sources, rapid growth in a short space of time, and easily manipulated spawning times to allow for eggs to be available for year-round supply (Crawford & Muir 2008; Jobling *et al*. 2010; Stanković *et al*. 2015). As part of their life history includes a smolting stage from fresh water systems into salt water, this species is also ideal for countries that would prefer to farm rainbow trout in marine cages as opposed to fresh water flow-through systems that are commonly used in North America and parts of Europe and the UK (Crawford & Muir 2008; Jobling *et al*. 2010).

Rainbow trout are commonly farmed within England, and are in easy supply in the south-west of this country. Given their popularity and commercial success, rainbow trout was chosen as a candidate species to test a novel prebiotic that could potentially benefit the wider aquaculture market. The aim of the present study was to understand how a novel GOS additive may benefit rainbow trout growth performance and overall

health. This first experiment was designed to accommodate juvenile rainbow trout reared on a formulated control and prebiotic supplemented for 8 weeks until they had achieved at least a doubling of biomass. Samples of target organs were taken at the end of the trial, and the techniques used to analyses these samples, and the trial conditions, are outlined in Chapter 2 and in section 3.2 in the present chapter.

3.2 Methodology

The specific experimental design, growth performance metrics and diet formulation for this trial are described in the following sections, 3.2.1 – 3.2.2. As the process for the extraction of DNA to be used in NGS of the microbiome differs for this fish species compared to the methodology of the other two experimental trials conducted, the methods used for the samples taken for the present chapter are described in section 3.2.4. Please refer to Chapter 2 sections 2.5 – 2.11 for more details about other sample analyses from this trial.

3.2.1 Trout Experimental System

Juvenile female rainbow trout (*O. mykiss*) were purchased from Exmoor Fisheries, UK, and the fish were subjected to a 4-week conditioning period in which during the first 10 day period trout were treated twice daily with an F-M-G proprietary prophylactic treatment (NT Labs, UK). The fish were fed a commercial feed during this time as a maintenance diet. After the initial prophylactic treatment and conditioning period, fish averaging 53.2 ± 0.6 g were graded into 18 x 140 L aquaria in groups of 25 individuals for the start of the trial. Fish were batch weighed altogether from each tank by carefully netting all fish from individual tanks, allowing excess water to run off by holding the net out of the water for a few seconds, and fish were then carefully placed within a pre-

weighed and tared vessel of 10 L of tank water to avoid splashing. Starting weights were calculated to be within ±2.5% of the overall average tank biomass. The weights of each tank were recorded in this manner at the start of the trial, every two-week period, and at the conclusion of the trial.

Throughout the trial, water chemistry and quality parameters were maintained and adjusted with mechanical and biological filtration to meet the maintenance requirements for rainbow trout. UV sterilisation was implemented to ensure optimum water quality. Fresh water was supplied to and recirculated throughout the systems within the East Aquarium of the Davy Building, University of Plymouth. Ambient room temperature was maintained at 12.5 ± 0.5°C to maintain an average water temperature of 16.6 ± 0.4°C.

The pH was maintained at 6.4 ± 0.2 and was buffered using sodium bicarbonate ($NaHCO_3$), when necessary. Dissolved oxygen was maintained at 9.5 ± 0.2mg L^{-1} and was within the optimum ranges for rainbow trout. Air was supplied to the tanks via a low-pressure side channel blower (Rietschle, UK Ltd) and through air stones. A 12-hour light: 12-hour dark photoperiod was maintained throughout the trial using fluorescent bulbs, which was controlled by timers. The tank weights (total biomass) were measured every two weeks to calculate the rations for the fish for each day in that period. Feed was weighed into pots according to the feeding rate, and fish were fed four to five times a day by hand. The feeding rate varied between 1.5-2.3% bodyweight, depending on acceptance of the feed.

One sampling point was scheduled at the conclusion of the trial (8 weeks). Fish were taken at random during the sampling period, concussed by a blow to the head and once

determined to be unconscious, they were then euthanised by the destruction of the brain (following Schedule 1 procedures). This work was approved by the University of Plymouth Internal Ethical Review Committee on Animal Scientific Investigations (approval number ETHICS-07-2017).

3.2.2 Diet Formulation and Growth Performance Parameters

Six experimental iso-nitrogenous and iso-lipidic diets were formulated using Animal Feed Optimisation Software (AFOS) (Feedsoft Professional®, USA) to meet the known requirements of juvenile rainbow trout (NRC 2011), of which the compositions and proximate analyses are described in Table 3.1. From the basal diet (control), five formulations were produced using incremental levels of B-GOS® obtained from Clasado BioSciences Ltd. (USA) based on manufacturer's instructions and ranges of current finfish prebiotic oligosaccharides in the literature (for example, Yousefi *et al*. (2018)), and comprised of 2 - 10g kg^{-1} as shown in Table 3.1. The methodology for producing these diets is described in Chapter 2 section 2.4. Growth performance, feed efficiency and overall fish condition were determined using the

Table 3.1 Compositions of experimental diets for rainbow trout with levels of B-GOS® inclusion. Proximate composition analyses of each diet are included following adapted AOAC (2016) procedures, presenting the crude protein, crude lipid, ash and moisture content.

Ingredient (% Inclusion)	**Control**	**2g kg^{-1}**	**4g kg^{-1}**	**6g kg^{-1}**	**8g kg^{-1}**	**10g kg^{-1}**
Soy Protein Concentrate[a]	37.18	37.18	37.18	37.18	37.18	37.18
Soy Bean Meal[a]	12.45	12.45	12.45	12.45	12.45	12.45
Wheat Gluten flour[b]	11.44	11.44	11.44	11.44	11.44	11.44
Cornstarch[c]	9.49	9.29	9.09	8.89	8.69	8.49
Rapeseed Oil[d]	9.25	9.25	9.25	9.25	9.25	9.25
Fish Oil[a]	8.85	8.85	8.85	8.85	8.85	8.85
Fish Meal[a]	7.50	7.50	7.50	7.50	7.50	7.50
Vit/Min premix (PNP FISH 2%)[e]	1.97	1.97	1.97	1.97	1.97	1.97

Lysine[c]	0.90	0.90	0.90	0.90	0.90	0.90
CMC-binder[c,f]	0.59	0.59	0.59	0.59	0.59	0.59
Methionine[c]	0.38	0.38	0.38	0.38	0.38	0.38
B-GOS®	**-**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**
Crude Protein (%)	46.38 ± 0.74	45.92 ± 1.14	47.14 ± 1.03	46.75 ± 1.17	46.15 ± 1.04	42.11 ± 0.70
Crude Lipid (%)	19.26 ± 0.21	19.12 ± 0.72	17.82 ± 0.42	17.57 ± 0.74	17.79 ± 0.35	17.99 ± 1.86
Ash (%)	4.21 ± 0.39	5.18 ± 0.17	5.41 ± 0.21	4.70 ± 0.09	5.08 ± 0.32	4.50 ± 0.09
Moisture (%)	4.96 ± 0.05	4.54 ± 0.10	5.11 ± 0.08	5.06 ± 0.03	4.76 ± 0.08	5.06 ± 0.02

[a] Skretting, Norway
[b] Ethica, Plymouth
[c] Sigma-Aldrich, UK
[d] Tesco, UK
[e] Premier Nutrition, UK
[f] Carboxy methyl cellulose, Sigma-Aldritch

calculations for FCR, SGR, PWG, K-Factor as described in the Chapter 2 section 2.4.

The mean and standard deviation (SD) were recorded for the end-point FCR, SGR, final tank weights, PWG and K-Factor of each diet. The FCR was calculated using n=3 tanks per diet, and the SGR was based on the starting weights in October and the final weights of each tank in December 2017. The PWG was calculated from the mean tank weights at the beginning of the trial and the mean tank weights at the end; these values were then averaged across the diets. The K-Factor was calculated from the final lengths and weights of fish sampled at the end of the trial (n=24 fish per diet).

3.2.3 Gene Expression

The specific primers used for the gene expression analyses are described in Table 3.2. For the specific methodology of how the samples were prepared for gene expression analyses using qPCR, please see Chapter 2, section 2.10. The reference genes *Elf-1α* and *β-actin* were used to normalise the relative expression of target genes in geNorm. Outliers were identified using the Quartile and OR functions in Excel 2016 as described in section 2.5 and 2.10 (Grech 2018) and removed from further analysis to reduce

variation in C_T values obtained for the reference genes after qPCR, and so n=3 samples per diet were taken forward for further analysis.

Table 3.2 Primer sequences of target genes evaluated within the *O. mykiss* trial.

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Amplicon size (bp)	Tm (°C)	GenBank Accession Number
Elf-1α	TGCGGAGGCATTGACAAGAG	TCCAGCACCCAGGCATACTT	92	60.9	AF498320.1
β-actin	AGCCCTCCTTCCTCGGTATG	GGATGTCCACGTCACACTTCAT	81	60.6	AB196465.1
IL-1β	GGACATGCAGCAGGACTACA	GCTGGATGGTGAAGGTGGTA	83	59.4	AJ223954.1
TNFα	AGCCCTACTCTTTGCATGGT	GCACCAATGAGTATCTCCAGTT	87	58.5	AJ277604.2
IL-10	GCTGGACGAAGGGATTCTACA	GCACCGTGTCGAGATAGAACT	89	59.6	AB118099.1
TGF-β	CCCACTGGCTACTTTGCTAAC	TGCTTATACAGAGCCAGTACCT	95	58.6	XM_021618706.1
Cal	TGACACACCTGTACACTCTGAT	GCCTGACTCCACCTTCTCATT	80	59.2	AY372389.1

3.2.4 Intestinal Microbiome Analysis

As outlined in Chapter 2, section 2.11, the microbial populations of the intestine samples were analysed using amplification of the V1-V2 region of the 16S rRNA gene. Alterations to the methods described in section 2.11 are described below. These alterations to the methodology took place as the DNA extraction kit from QIAGEN was outdated and a newer version was developed in 2018.

Samples of digesta (n=9 per diet) from the posterior intestine of each fish were sampled at the end of the trial and stored as described in Chapter 2, sections 2.3 and 2.11. Digesta samples were thawed and between 150-200 mg digesta was weighed for DNA extraction. The extraction protocol involved a four-step process; lysis, inhibitor removal, protein removal and DNA clean-up. Samples were lysed in 500 µL of 50mg mL^{-1} lysozyme (Sigma-Aldrich) in 1 x TE buffer. These samples were incubated for 30 min at 37°C, before 700 µL of Buffer ASL (QIAmp Stool Mini Kit, Qiagen) was added to

each sample and centrifuged for 1 min. All centrifuge steps were performed at room temperature and 16,200 x g (GeneAmp® PCR System 9700, Applied Biosystems). The suspension was incubated for 5 min at 95°C, then vortexed and centrifuged again for 1 min.

The inhibitor was removed using half an Inhibitex tablet added to 800 µL of the supernatant. This was then vortexed and left to stand for 1 min at room temperature, before being centrifuged for 3 min. The resulting supernatant was centrifuged once again for 3 min to prepare for the protein removal. Up to 200 µL of the centrifuged supernatant was added to 15 µL Proteinase K, and 200 µL of Buffer AL. The samples were then vortexed and this resulting suspension was incubated for 30 min at 56°C. After this, 200 µL of absolute ethanol was added and the suspension vortexed. The samples were cleaned using the remaining QIAmp kit consumables and following the manufacturer's instructions. DNA was eluted in 50 µL of the Buffer AE provided by the kit, and stored at -20°C. DNA samples were checked for quality and quantity using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific), before being taken forward in PCR.

PCR was performed using universal bacterial primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3'), and pooled 338R-I (5'-GCW GCC TCC CGT AGG AGT-3') and 338R-II (5'-GCW GCC ACC CGT AGG TGT-3') (Roeselers *et al*. 2011; Gajardo *et al*. 2016; do Vale Pereira *et al*. 2017). The PCR reaction was carried out in a 25 µL reaction, using 12.5 µL MyTaq™ Red 2x kit (Bioline) according to manufacturer's instructions, and primers were used at 0.5 µL of both forward (27F) and reverse (338R-I and 339R-II pooled) at 50 pM, as described in Gajardo *et al*. (2016). Digesta DNA template was added at 1 µL

per reaction, and nuclease-free water made up the remainder of the reaction. The PCR reaction conditions were as follows: initial denaturation at 95°C for 7 min; followed by a 10 cycle touchdown strategy where the samples were heated to 94°C for 30 sec, annealing decreasing from 63°C to 53°C, 72°C for 30 sec; then 25 cycles of 94°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec; finally followed by 72°C for 10 min. The PCR amplicons were immediately stored at 4-8°C for use in a 1.2% agarose gel electrophoresis to check the product length and for quality against the Bioline Hyperladder (50bp).

The samples were then purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, USA) according to manufacturer's instructions for 10 µL of sample. To each PCR reaction, 1.8 x sample volume of the AMPure XP beads were added and mixed by pipetting action. Each reaction was left for 5 min at room temperature and placed within a magnet rack for 2 min. The supernatant was aspirated and discarded, after which 200 µL of 70% ethanol was dispensed into each sample and allowed to incubate for 30 seconds before being removed and then the process repeated once more. Ethanol was removed and the samples allowed to dry for ~2 minutes. The beads were suspended in 40 µL nuclease-free water and mixed, and then incubated at room temperature for 2 min, before being placed on the magnet rack again for 2 min. Of the purified product, 38 µL was transferred to a new Eppendorf tube and these samples were quantified using the Qubit® 2.0 Fluorometer (Thermo Fisher) system.

The trout samples (total of 54) were sequenced by University of Plymouth staff using Next Generation Sequencing (NGS) Ion Personal Genome Machine™ System (Life Technologies) at the Derriford Research Facility, University of Plymouth. Sequencing adapters and barcodes were ligated on to the amplicons using the Ion Plus Fragment

Library Kit (Life Technologies, CA, USA). The libraries were quantified using the Ion Library Quantitation Kit (Life Technologies, CA, USA) and concentrations were adjusted to 26 pM for all samples (Falcinelli *et al*. 2015; Gajardo *et al*. 2016). Amplicon 16S rRNA V1-V2 libraries were attached to Ion Sphere Particles (ISPs) using an Ion PGM Template OT2 HiQ view kit (Life Technologies, CA, USA) according to the manufacturer's instructions for 400bp sequencing. The torrent server analysed the raw data and trimmed poor quality sequences and adaptor/barcode sequences to produce FASTQ files. These files were exported and then examined using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline, and further documentation is available from the website https://docs.qiime2.org/2021.4/ (Caporaso *et al*. 2010; Edgar 2010; Bokulich *et al*. 2018; Bolyen *et al*. 2019).

For all the FASTQ files from this trial, the reads were filtered and analysed for quality using scripts specifically written to determine the intestinal microbiota of fish. The software used for this purpose is QIIME v2021.4 (hereafter QIIME v2), one of the most recent versions to be released from QIIME. The scripts were based on tutorials in the QIIME v2 website database (https://docs.qiime2.org/2021.4/tutorials/) to become familiar with this type of programming. The commands for these scripts were processed in the QIIME v2 environment using the Terminal.app in Mac OS X (MacBook, Apple). One specific difference between the v1 and v2 QIIME software is that the OTUs are called features, and the pipeline is based on using some add-ons that were originally produced in R-Statistical software.

The dataset is first imported into the QIIME v2 environment to be analysed in later steps in the pipeline using miniconda and python commands. Once imported, the

sequences were demultiplexed and denoised using the add-on 'DADA2' pipeline, which is able to perform these quality control checks on paired-end and single-end reads, the latter of which represents the data presented here. This process includes quality control, chimeric sequence and primer removal and further trimming and filtering. The minimum bases per read to retain during filtering was set to 325 bp to keep the highest possible percentage of reads during quality control. One sample was found to have statistically low quality reads and was excluded from further QIIME analysis.

Once the reads had been demultiplexed, phylogenetic trees were generated using the 'q2-phylogeny' plugin, and alpha and beta diversity analyses were performed. These included rarefied tables to produce Shannon's diversity index, Observed Feature tables and Chao1 for alpha diversity. The sampling depth for was chosen based on the lowest scoring reads for a sample out of the total, and so the depth of 10,000 was chosen for the trout reads based on this criterion.

Rarefaction plots were produced for alpha and beta diversity metrics, and taxonomic analyses were produced within the QIIME v2 environment. A pre-trained classifier was imported into the QIIME v2 environment using the 'q2-feature-classifier' plugin so that taxonomy can be assigned to the sequence reads. The 'SILVA 138 99% OTU full length sequences' Naïve Bayesian classifier was chosen as this reference dataset is more recent and robust for assigning taxonomy classification than the Greengenes reference file (Quast *et al*. 2013; Yarza *et al*. 2014; Yilmaz *et al*. 2014; Bokulich *et al*. 2018; Bokulich *et al*. 2021). The feature table was filtered to remove *Streptophyta*, *Cyanobacteria* and singletons from the analysis, as these sequences are considered diet associated and do not represent the populations of bacteria found within the trout gut (Baldo *et al*. 2015;

Gajardo *et al*. 2016). The characterisation of the microbiome of trout fed incremental levels of GOS against the control diet was presented as bar charts at the genus levels, or as close to this taxonomic level as possible, and the raw data was processed for statistical testing.

The feature/OTU tables generated from the QIIME analyses can be taken and used with other software to compare and characterise significant differences in the OTUs among the treatments. STAMP v2.1.3 (Parks & Beiko 2010; Parks *et al*. 2014) and Linear Discriminant Analysis (LDA) Effect Size (LEfSe) Galaxy Version 1.0 (Segata *et al*. 2011) via Galaxy Hub software (Afgan *et al.* 2018) analyses were used on the data generated from QIIME v2 to show the relationships and abundances of each taxonomic level between treatments. These data are converted into .tsv format to be used in the STAMP and LEfSe software. A metadata table was also generated to group the samples within STAMP by diet and this programme can be used to generate heatmaps showing relative abundance of distinct feature OTUs, statistical testing and post-hoc analyses. The feature raw data produced in QIIME v2 is processed in LEfSe and this programme can determine which bacterial genera are biomarkers of interest and plots the difference between the diets as abundance histograms, with the relevant class and any subclass information (Afgan *et al*. 2018).

LEfSe analysis uses an algorithm that identifies genomic features that characterise differences between treatments, called classes. The biological relevance is also explained alongside the statistical significance, and so researchers can identify "differentially abundant features that are also consistent with biologically meaningful categories" (Segata *et al*. 2011). The LEfSe analysis was performed using an alpha value of 0.05 for

both the Kruskal-Wallis ran sum test and pairwise Wilcoxon test. The threshold was set to 2.0 for the LDA, and the approach used an all-against-all multiclass analysis, with pairwise comparisons between the subclasses being performed only among the subclasses with the same name (Segata *et al*. 2011). For more information on the specific default analyses performed by the programme, please refer to Segata *et al*. (2011).

3.3 Results

3.3.1 Growth Performance

The fish readily accepted the diets and survival was over 99% during the length of the trial. No significant differences of trout growth performance parameters were observed between the dietary groups for final weights, FCR, SGR, PWG or K-Factor (Table 3.3). Although there was an increase in final tank weights from fish fed Control to 6g kg^{-1}, there was no significant difference detected between these diets.

3.3.2 Haematology

No significant differences of trout haematology parameters were observed between fish fed the different dietary treatments: Hb, Packed Cell Volume (PCV or Hct), RBC, WBC, MCV, MCH and MCHC (Table 3.4). In addition, no significant differences were observed between fish fed the dietary regimes for lymphocyte, basophilic granulocyte, monocyte and neutrophilic granulocyte cell counts (Table 3.5).

Table 3.3 Mean growth performance parameters (± SD) of rainbow trout fed incremental levels of B-GOS® over a period of 8 weeks.

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Initial Weight (g)	53.15 ± 1.16	53.81 ± 0.51	52.85 ± 0.68	53.87 ± 0.62	52.53 ± 0.53	52.83 ± 0.30	**0.142**	$\chi^2_{(5,18)}$=8.28
Final Weight (g)	142.50 ± 5.80	146.87 ± 4.87	149.63 ± 2.87	150.86 ± 10.22	139.40 ± 8.33	136.37 ± 4.25	**0.145**	$\chi^2_{(5,18)}$=8.21
FCR	1.17 ± 0.04	1.15 ± 0.03	1.10 ± 0.03	1.13 ± 0.05	1.18 ± 0.10	1.23 ± 0.05	**0.193**	$F_{5,12}$=1.78
SGR	1.76 ± 0.07	1.79 ± 0.07	1.86 ± 0.04	1.84 ± 0.11	1.74 ± 0.09	1.69 ± 0.05	**0.132**	$F_{5,12}$=2.13
PWG (%)	168.14 ± 9.84	172.96 ± 10.44	183.13 ± 5.85	179.98 ± 16.73	165.27 ± 13.14	158.13 ± 6.98	**0.132**	$F_{5,12}$=2.13
K-Factor	1.42 ± 0.08	1.44 ± 0.09	1.42 ± 0.10	1.46 ± 0.11	1.43 ± 0.14	1.38 ± 0.10	**0.083**	$\chi^2_{(5,18)}$=9.75

Table 3.4 Mean haemoglobin (Hb), haematocrit or packed cell volume (PCV or Hct), erythrocytes (RBC), leukocytes (WBC), Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) (± SD) of rainbow trout fed incremental levels of B-GOS® over a period of 8 weeks (n=9 fish per diet).

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Hb (g L^{-1})	82.23 ± 16.50	85.06 ± 14.98	84.43 ± 11.34	80.99 ± 14.38	88.88 ± 11.43	81.49 ± 8.54	**0.212**	$\chi^2_{(5,54)}$=7.04
PCV (L L^{-1})	0.27 ± 0.06	0.27 ± 0.08	0.23 ± 0.13	0.26 ± 0.08	0.27 ± 0.15	0.27 ± 0.09	**0.963**	$F_{5,48}$=0.194
RBC (x10^6/mm^3)	0.75 ± 0.23	0.66 ± 0.17	0.59 ± 0.17	0.71 ± 0.16	0.71 ± 0.20	0.69 ± 0.16	**0.559**	$F_{5,48}$=0.794

WBC ($x10^4/mm^3$)	0.15 ± 0.03	0.18 ± 0.06	0.17 ± 0.05	0.19 ± 0.05	0.16 ± 0.06	0.15 ± 0.04	**0.108**	**$\chi^2_{(5,54)}=9.03$**
MCV (fL)	426.34 ± 136.99	429.53 ± 126.91	454.43 ± 117.66	411.50 ± 154.52	417.38 ± 231.24	419.21 ± 121.19	**0.998**	**$F_{5,48}=0.05$**
MCH (pg)	91.14 ± 19.39	106.53 ± 19.62	106.91 ± 15.02	93.18 ± 14.30	102.81 ± 11.51	98.14 ± 22.35	**0.306**	**$\chi^2_{(5,54)}=6.01$**
MCHC (g/L)	0.24 ± 0.08	0.25 ± 0.08	0.25 ± 0.07	0.25 ± 0.10	0.17 ± 0.03	0.22 ± 0.08	**0.578**	**$\chi^2_{(5,54)}=3.80$**

Table 3.5 Mean percentages of lymphocytes, basophilic granulocytes, monocytes and neutrophilic granulocytes (± SD) of rainbow trout fed incremental levels of B-GOS® over a period of 8 weeks (n=9 fish per diet).

Diet	**Control**	**2g kg^{-1}**	**4g kg^{-1}**	**6g kg^{-1}**	**8g kg^{-1}**	**10g kg^{-1}**	**p-value**	**Test Statistic**
Lymphocytes (%)	89.28 ± 2.66	88.67 ± 3.69	84.94 ± 4.80	88.56 ± 1.96	88.39 ± 4.99	89.61 ± 3.85	**0.142**	**$F_{5,48}=1.75$**
Basophilic Granulocytes (%)	5.17 ± 1.66	5.39 ± 0.86	7.06 ± 1.74	5.33 ± 1.06	5.44 ± 3.20	5.56 ± 2.30	**0.355**	**$F_{5,48}=1.13$**
Monocytes (%)	2.67 ± 0.75	2.72 ± 1.87	4.06 ± 2.04	2.61 ± 1.22	3.06 ± 1.53	1.83 ± 0.97	**0.071**	**$F_{5,48}=2.19$**
Neutrophilic Granulocytes (%)	2.89 ± 0.82	3.22 ± 1.60	3.94 ± 1.86	3.50 ± 1.46	3.11 ± 1.54	3.0 ± 1.27	**0.674**	**$F_{5,48}=0.63$**

3.3.3 Lysozyme Activity

There were no significant differences in lysozyme activity observed between fish fed the dietary treatments within the epidermal mucus scrapes for the non-standardised mucus lysozyme activity, the standardised mucus lysozyme activity (in U/mg), and the serum lysozyme activity. While the fish fed the 2 g kg^{-1} diet did show the greatest lysozyme activity within the serum and the mucus, this metric was not significantly elevated compared to fish fed the control and other experimental diets (see Table 3.6).

3.3.4 Protein Concentration

There were no significant differences observed between fish fed either dietary regime and the control diet for protein content within the epidermal mucus scrapes, and for the serum. These results are shown in Table 3.7.

3.3.5 Normalised Lysozyme with Protein Content

To normalise the lysozyme activity of the serum and mucus samples, the lysozyme/min/mL measurements for each individual fish were divided by the protein content in mg/mL, with the results presented in Table 3.8. While there were no significant differences observed between fish fed GOS diets and the control diet for the mucus or serum lysozyme activity normalised with protein, the lower concentration of B-GOS® at 2g kg^{-1} has the highest normalised lysozyme activity for both mucus and serum.

Table 3.6 Mean lysozyme activity (± SD) within the epidermal mucus and blood serum of rainbow trout fed incremental levels of B-GOS® over a period of 8 weeks (n=9 fish per diet for mucus, n=15 fish per diet for blood serum). The mucus lysozyme activity (lysozyme/min/mL) was normalised using the mucus weight (in mg).

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Mucus Lysozyme Activity (lysozyme/min/mL)	345.82 ± 94.20	390.31 ± 94.18	286.13 ± 81.18	305.58 ± 79.84	296.10 ± 104.05	325.58 ± 67.24	**0.157**	**$F_{5,46}$=1.69**
Mucus Lysozyme Activity (U/mg)	14.73 ± 4.38	15.87 ± 5.00	13.80 ± 4.37	15.75 ± 9.28	13.87 ± 7.26	15.18 ± 7.39	**0.880**	**$\chi^2(5,52)$=1.77**
Serum Lysozyme Activity (lysozyme/min/mL)	138.59 ± 46.24	165.27 ± 55.48	128.94 ± 42.07	153.48 ± 45.52	149.01 ± 55.25	133.27 ± 50.68	**0.338**	**$F_{5,77}$=1.06**

Table 3.7 Mean protein content (± SD) within the epidermal mucus and blood serum of rainbow trout fed incremental levels of B-GOS® over a period of 8 weeks (n=9 fish per diet for mucus, n=9 fish per diet for serum).

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Mucus Protein Content (mg/mL)	7.74 ± 1.58	6.67 ± 2.62	8.29 ± 2.22	8.54 ± 2.43	9.77 ± 1.81	8.92 ± 2.30	**0.122**	**$\chi^2(5,52)$=8.70**
Serum Protein Content (mg/mL)	57.26 ± 8.11	50.19 ± 6.49	50.73 ± 6.70	52.95 ± 8.89	58.56 ± 6.50	58.44 ± 6.98	**0.105**	**$\chi^2(5,52)$=9.21**

Table 3.8 Mean lysozyme activity normalised with protein content (± SD) within the epidermal mucus and blood serum of rainbow trout fed incremental levels of B-GOS® over a period of 8 weeks (n=9 fish per diet for mucus, n=9 fish per diet for blood serum).

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Mucus Lysozyme Activity	47.76 ± 20.12	56.15 ± 22.75	35.26 ± 14.00	39.18 ± 12.30	32.64 ± 17.03	39.25 ± 14.11	**0.174**	**$\chi^2_{(5,52)}=7.69$**
Serum Lysozyme Activity	2.63 ± 1.22	3.45 ± 1.20	2.44 ± 0.92	2.89 ± 0.95	2.36 ± 1.05	2.31 ± 0.93	**0.209**	**$F_{5,46}=1.50$**

3.3.6 Histology

There were no significant differences detected in the muscularis thickness, mucosal fold heights, lamina propria widths or goblet cell counts of the intestine or skin in fish fed any experimental diet compared to the control group (Table 3.9). Representative images of fish from each dietary treatment are presented in Figure 3.1 and 3.2.

3.3.7 Body Composition

Proximate compositional analyses were utilised to determine that all experimental treatments were comparable. There were no significant differences between fish fed each dietary regime for the carcass moisture content, ash content, crude protein content or lipid content; see Table 3.10.

Table 3.9 Mean histological analyses (± SD) of rainbow trout fed incremental levels of B-GOS® over a period of 8 weeks (n=9 fish per diet).

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Muscularis Thickness (µm)	131.13 ± 32.20	134.40 ± 38.41	139.53 ± 24.87	119.39 ± 33.65	137.91 ± 33.98	114.83 ± 32.91	**0.492**	$F_{5,48}$=**0.90**
Mucosal Fold Height (µm)	606.67 ± 168.76	573.82 ± 123.87	594.99 ± 131.19	629.96 ± 142.53	569.10 ± 169.76	576.52 ± 134.18	**0.946**	$F_{5,48}$=**0.23**
Intestine Goblet Cell Counts	9.00 ± 3.66	11.06 ± 4.17	8.34 ± 2.86	11.08 ± 4.19	8.83 ± 3.61	9.00 ± 4.51	**0.531**	$F_{5,48}$=**0.84**
Lamina Propria Width (µm)	30.53 ± 5.36	31.70 ± 4.61	30.75 ± 5.18	31.81 ± 5.86	29.68 ± 6.00	32.91 ± 3.87	**0.815**	$\chi^2(5,54)$=**2.24**
Skin Goblet Cell Counts	17.61 ± 3.69	16.78 ± 5.13	15.11 ± 1.69	17.19 ± 4.76	17.11 ± 6.12	15.31 ± 3.23	**0.755**	$F_{5,48}$=**0.53**

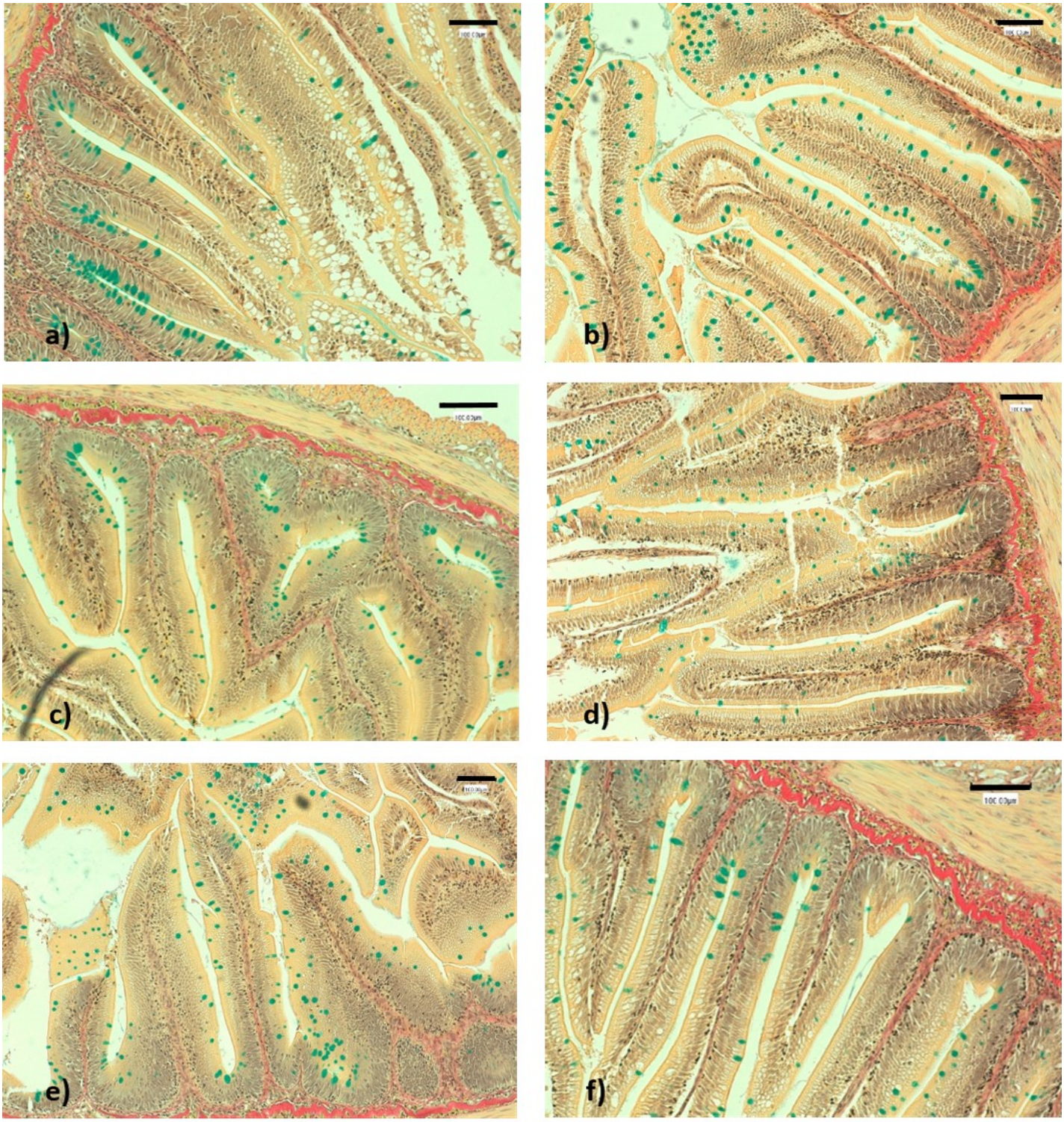


Figure 3.1 Representative intestinal images of individual rainbow trout from each diet, stained with AB/vG: **a)** Control, **b)** 2g kg^{-1}, **c)** 4g kg^{-1}, **d)** 6g kg^{-1}, **e)** 8g kg^{-1} and **f)** 10g kg^{-1}. Scale bar = 100 µm.

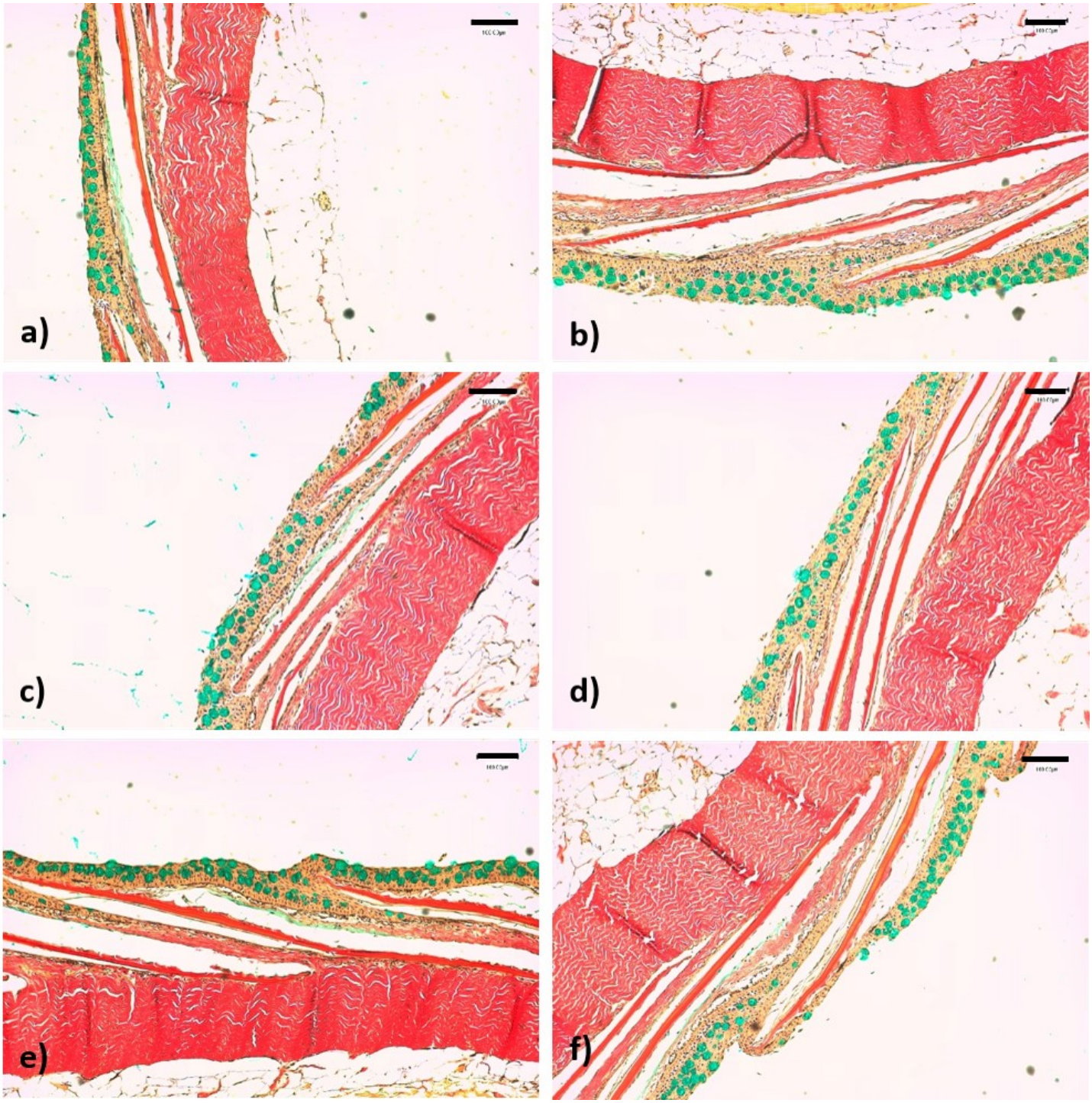


Figure 3.2 Representative skin images of individual rainbow trout from each diet, stained with AB/vG: **a)** Control, **b)** 2g kg^{-1}, **c)** 4g kg^{-1}, **d)** 6g kg^{-1}, **e)** 8g kg^{-1} and **f)** 10g kg^{-1}. Scale bar = 100 µm.

Table 3.10 Mean carcass compositional analyses (± SD) of rainbow trout before the start of the trial (n=6 fish) and rainbow trout fed incremental levels of B-GOS® over a period of 8 weeks (n=6 fish per diet). Ash content, Protein content and Lipid content are expressed as a percentage of the dry matter.

Diet	Pre-trial	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Moisture Content (%)	57.55 ± 0.68	71.96 ± 1.31	70.70 ± 1.46	72.20 ± 1.91	71.37 ± 0.97	71.45 ± 1.30	69.73 ± 2.33	**0.226**	**$\chi^2_{(5,36)}=6.94$**
Ash Content (%)	3.16 ± 0.40	1.34 ± 0.30	1.53 ± 0.17	1.48 ± 0.13	1.56 ± 0.18	1.39 ± 0.30	1.57 ± 0.29	**0.626**	**$\chi^2_{(5,36)}=3.48$**
Protein Content (%)	24.15 ± 0.55	16.13 ± 1.56	16.47 ± 1.33	15.96 ± 1.23	16.45 ± 0.67	16.41 ± 0.37	16.89 ± 1.35	**0.831**	**$F_{5,30}=0.42$**
Lipid Content (%)	13.27 ± 0.53	9.57 ± 1.12	10.68 ± 1.62	9.90 ± 1.06	9.91 ± 0.77	10.22 ± 1.21	10.91 ± 1.87	**0.734**	**$\chi^2_{(5,36)}=2.85$**

3.3.8 Gene Expression

The expression of five target genes in the skin and posterior intestine of rainbow trout after 8 weeks of dietary feeding on the experimental diets is represented as fold change relative to the control, and presented in Figures 3.3 and 3.4, respectively. Outliers that were previously removed as described in section 3.2.3 were not taken forward in this analysis, and so n=3 samples per diet were analysed.

3.3.8.1 Skin

Permutation tests were performed instead of ANOVA or Kruskal-Wallis models as described in section 2.12 to determine if significant differences between the expression levels of fish fed each diet were present for each target gene, for the target tissues. The significant outputs for these permutation tests are presented as different letters above the bars in Figure 3.3 for the skin, and Figure 3.4 for the posterior intestine. These tests for target genes measured in the skin of rainbow trout demonstrated that fish fed the 4g kg^{-1} diet had significantly downregulated expression of *Cal* compared to the control ($p=0.042$), 2g kg^{-1} ($p=0.049$), 6g kg^{-1} ($p=0.046$) and 10g kg^{-1} fed fish. The gene expression of *IL-10* was significantly downregulated in fish fed the 2g kg^{-1} diet compared to the control fed fish ($p=0.041$), and also compared to fish fed 4g kg^{-1} ($p=0.038$), 6g kg^{-1} ($p=0.026$) and 10g kg^{-1} ($p=0.048$). *IL-10* expression was significantly upregulated in fish fed the 4g kg^{-1} diet compared to the control fed fish ($p=0.042$), and also compared to fish fed 6g kg^{-1} ($p=0.043$) and 8g kg^{-1} ($p=0.042$) diets.

For fish fed 4g kg^{-1} and 10g kg^{-1} B-GOS® diets, the gene expression of *IL-1β* was significantly upregulated compared to control fed fish ($p=0.049$, $p=0.050$, respectively). The expression of *TGF-β* was significantly downregulated in fish fed the 2g kg^{-1} diet

compared to the control (p=0.045), 4g kg^{-1} (p=0.035) and 8g kg^{-1} (p=0.050) diets. Conversely, fish fed the 4g kg^{-1} diet presented upregulated expression of *TGF-β* in the skin compared to the control (p=0.044), 6g kg^{-1} (p=0.050), 8g kg^{-1} (p=0.039) and 10g kg^{-1} (p=0.039) fed fish.

TNFα gene expression was upregulated in fish fed 4g kg^{-1} (p=0.028) and 8g kg^{-1} (p=0.026) compared to the control, but downregulated in fish fed 2g kg^{-1} (p=0.050) and 6g kg^{-1} (p=0.050) compared to control fed fish. Fish fed the 2g kg^{-1} diet expressed significant downregulation of *TNFα* in the skin compared to fish fed 4g kg^{-1}, 8g kg^{-1} and 10g kg^{-1} fed fish (p=0.027, p=0.025, p=0.049, respectively). The expression of the same gene was significantly upregulated in 4g kg^{-1} fed fish compared to the 6g kg^{-1} diet (p=0.027) and 10g kg^{-1} diet (p=0.039), and this same trend was seen in 8g kg^{-1} fed fish compared to the 10g kg^{-1} diet (p=0.035). The expression of *TNFα* in fish fed 6g kg^{-1} diet was significantly downregulated compared to expression levels in fish fed the 8g kg^{-1} and 10g kg^{-1} diets (p=0.025 and p=0.050, respectively).

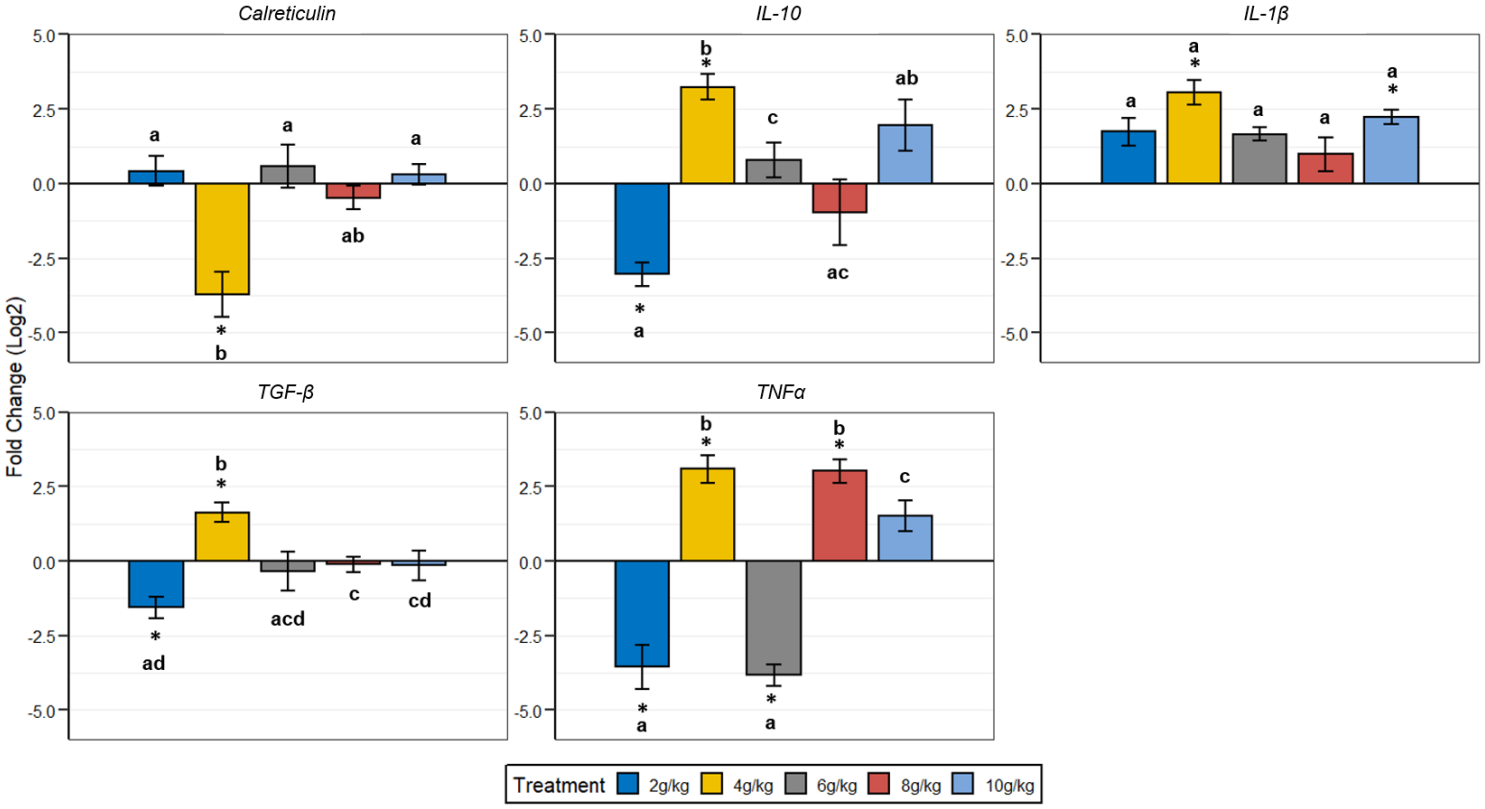


Figure 3.3 Gene expression data presented as fold change (log_2) of 5 target genes relative to the control group in the skin of rainbow trout fed six dietary treatments containing incremental levels of B-GOS® over a period of 8 weeks (n=3 fish per diet, per gene). Significant differences are denoted by differing letters between treatments, an asterisk between the treatment and control. Data presented as mean ± SEM.

3.3.8.2 Intestine

Permutation tests as described in section 3.3.8.2 for target genes measured in the intestine of rainbow trout demonstrated that fish fed the 2g kg^{-1} diet expressed significantly downregulated *IL-10* compared to the expression in all other diets excluding the control ($p<0.05$). The expression of *IL-1β* was significantly downregulated in 4g kg^{-1} and 10g kg^{-1} fed fish compared to control ($p=0.026$, $p=0.019$, respectively). Gene expression in 6g kg^{-1} fed fish for this same target get was significantly upregulated in this diet compared to the control ($p=0.049$).

The expression of *IL-1β* was also significantly upregulated in the 2g kg^{-1} fed fish compared to 4g kg^{-1} fed fish (p=0.038) and 10g kg^{-1} fed fish (p=0.028). A similar effect was also demonstrated in fish fed the 6g kg^{-1} and 8g kg^{-1} fed fish compared to fish fed 10g kg^{-1} diet (p=0.043, p=0.036, respectively). *IL-1β* expression was significantly downregulated in fish fed the 4g kg^{-1} diet compared to the expression levels in fish fed the 6g kg^{-1} and 8g kg^{-1} fed fish (p=0.044, p=0.039, respectively).

TNFα expression was upregulated in fish fed all experimental diets except the 8g kg^{-1} inclusion rate compared to the control fed fish ($p<0.05$). Fish fed the 2g kg^{-1} diet expressed significantly upregulated *TNFα* compared to fish fed 4g kg^{-1} (p=0.026), 6g kg^{-1} (p=0.031) and 8g kg^{-1} (p=0.026) diets. The expression of the same gene was significantly upregulated in 6g kg^{-1} and 10g kg^{-1} fed fish compared to the 8g kg^{-1} diet fed fish (p=0.047, p=0.050, respectively), but not compared to each other.

There were no significant differences in *Cal* and *TGF-β* gene expression in the intestine of fish fed any experimental diet relative to the control fed fish, or each other, for this trial.

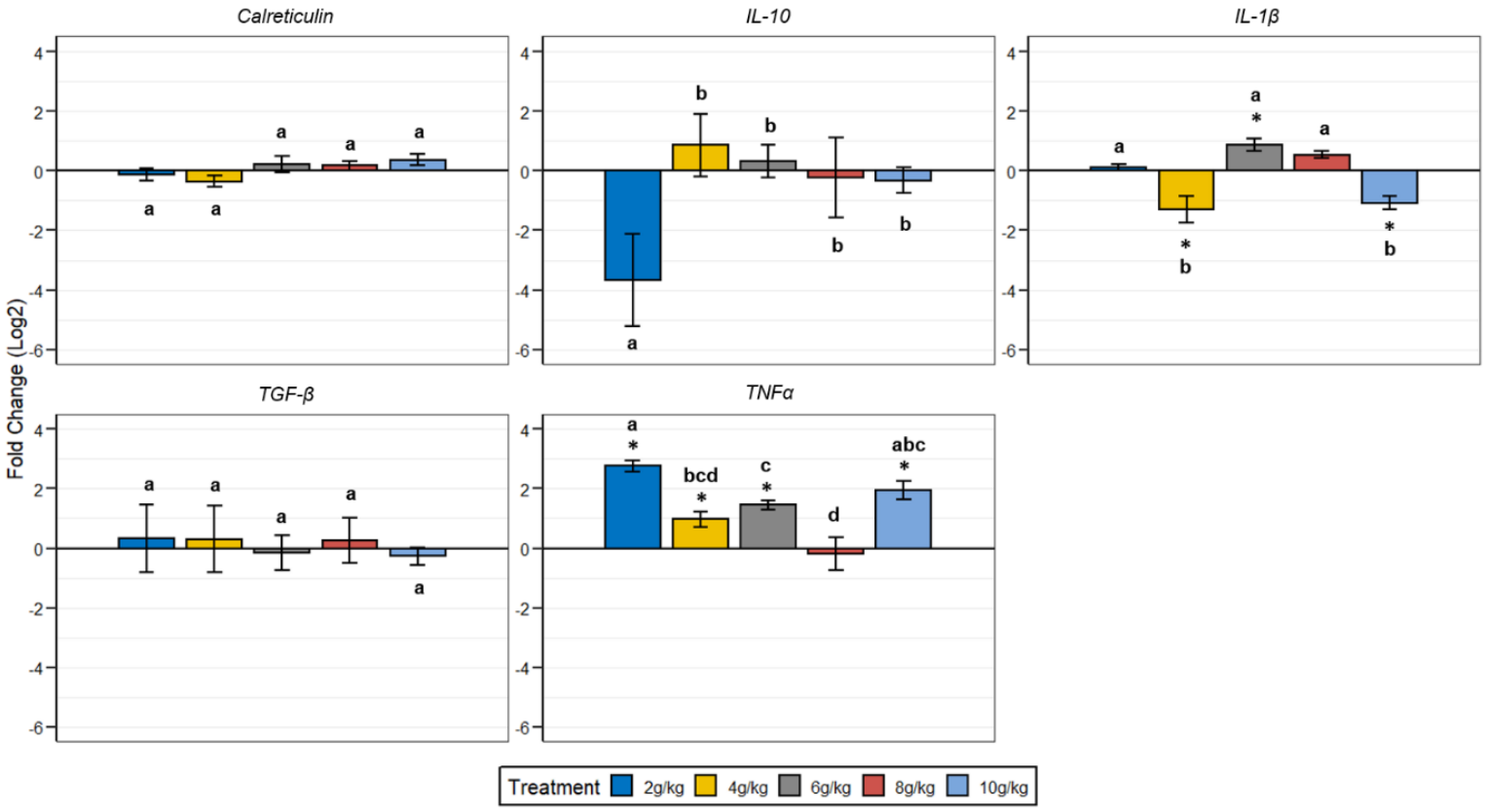


Figure 3.4 Gene expression data presented as fold change (log_2) of 5 target genes relative to the control group in the posterior intestine of rainbow trout fed six dietary treatments containing incremental levels of B-GOS® over a period of 8 weeks (n=4 fish per diet, per gene). Significant differences are denoted by differing letters between treatments, an asterisk between the treatment and control. Data presented as mean ± SEM.

3.3.9 Intestinal Microbiome Analysis

The high-throughput 16S rRNA gene sequencing analysis of the intestinal microbiomes of rainbow trout fed control vs prebiotic supplemented diets yielded a total of 4,797,140 single-end sequence reads from 53 samples, as one sample was filtered out of the analysis as an outlier, and the average number of reads per fish replicate being 90,512.08 ± 27,361 after demultiplexing. After 'DADA2' quality control and filtering, the average number of reads across all diets was 17,109.74 ± 6,373 and these sequences were taken forward to taxonomy assignment using the pre-trained classifier 'SILVA 138 99% OTU full-length sequences' as a reference dataset. The Good's coverage estimates for each diet were >0.999, indicating that the sequence coverage was sufficient for these

analyses. The number of OTUs observed within the intestine of rainbow trout did not significantly differ between fish fed control or prebiotic supplemented diets, however the control fed fish did have a slightly greater number of unique OTUs present (Table 3.11, Figure 3.5). The species richness diversity (Chao1) and Shannon diversity index did not differ significantly between treatment groups (Table 3.11).

The percentage relative abundance of some distinct genera (where relative abundance was reported at a threshold of over 0.25% of total reads) sequenced from the digesta of trout fed either the control or experimental diets trial are shown in Table 3.12 and Figure 3.6. Any unique genera detected that were not above the threshold level were grouped into the category 'Others', and this category also contained any unidentified sequences from the QIIME v2 analysis. Aside from the 'Others' category, *Firmicutes* was the dominant phyla in the control and prebiotic supplemented diets, accounting >52% of reads present across all diets. In addition to *Firmicutes*, the phyla *Actinobacteria*, *Proteobacteria*, *Patescibacteria*, *Spirochaetota*, *Bacteriodota* and *Verrucomicrobiota* were also detected across all treatments; however, the relative abundances of these phyla were not significantly different between dietary regimes. The relative abundance of above threshold reads belonged to 11 genera. The STAMP v2.1.3 (Parks & Beiko 2010; Parks *et al*. 2014) and LEfSe Galaxy Version 1.0 (Segata *et al*. 2011) via Galaxy Hub software (Afgan *et al.* 2018) software detected two distinct bacteria genera that were of significant interest. Of the genera sequenced in this trial, *Aerococcus* and *Macrococcus* relative abundances were determined to be significantly different between fish fed the experimental diets of this trial but not the control (Table 3.12). These data are represented in the relative abundance of features graph (Figure 3.6), and as a heatmap showing relative abundance and relatedness of the samples in each diet (Figure 3.7).

Table 3.11 OTUs observed after QIIME v2 analysis and alpha diversity/richness metrics of intestinal microbiota composition in rainbow trout fed incremental levels of B-GOS®, over a period of 8 weeks (n=9 fish per diet).

Alpha Diversity	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
OTUs observed	49.70 ± 26.86	39.27 ± 10.94	41.68 ± 7.56	42.10 ± 6.64	46.90 ± 14.81	33.82 ± 11.82	**0.503**	$\chi^2(5,54)=4.33$
Good's coverage	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	**0.931**	$\chi^2(5,54)=1.34$
Chao1 diversity	49.98 ± 27.07	39.31 ± 10.88	41.84 ± 7.75	42.38 ± 6.39	47.32 ± 14.99	34.01 ± 11.68	**0.275**	$\chi^2(5,54)=4.34$
Shannon's diversity index	3.80 ± 0.98	3.25 ± 1.29	3.79 ± 0.38	3.95 ± 1.07	3.79 ± 1.03	2.85 ± 1.33	**0.282**	$\chi^2(5,54)=6.26$

Table 3.12. Relative abundance (mean ± SD) of bacterial sequences at the genus level (or lowest taxonomic level) present within the intestinal digesta of rainbow trout fed incremental levels of B-GOS®, over a period of 8 weeks (n=9 fish per diet). Different letters between data denote significant difference between different letters on the same row, $p<0.05$.

Bacterial genus relative abundance (%)	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Others	46.57 ± 17.89	43.24 ± 17.90	44.38 ± 5.30	43.81 ± 9.97	51.54 ± 11.2	42.71 ± 20.31	**0.820**	$F_{5,48}=0.44$
Corynebacterium	2.41 ± 2.33	1.13 ± 1.39	1.21 ± 1.18	4.20 ± 3.86	3.33 ± 4.78	1.54 ± 2.63	**0.136**	$\chi^2(5,54)=8.39$
Bacillus	4.18 ± 7.30	4.00 ± 5.57	7.50 ± 6.73	4.91 ± 5.14	6.86 ± 8.22	2.01 ± 4.08	**0.273**	$\chi^2(5,54)=6.35$
Macrococcus	8.54 ± 8.73^{abc}	2.12 ± 1.83^{ac}	14.25 ± 7.33^{b}	1.84 ± 1.39^{a}	2.93 ± 2.95^{ac}	1.67 ± 2.29^{c}	**0.003**	$\chi^2(5,54)=18.23$
Staphylococcus	0.64 ± 0.82	1.08 ± 1.52	0.50 ± 0.53	1.75 ± 2.21	1.33 ± 1.39	0.56 ± 1.07	**0.331**	$\chi^2(5,54)=5.75$
Aerococcus	0.07 ± 0.12^{ae}	0.40 ± 0.64^{abde}	3.80 ± 2.91^{bcd}	8.68 ± 8.44^{c}	2.34 ± 2.64^{dce}	0.15 ± 0.13^{e}	**<0.001**	$\chi^2(5,54)=32.89$
o. *Enterobacterales*	0.42 ± 1.05	0.02 ± 0.02	0.00 ± 0.00	0.01 ± 0.02	2.85 ± 8.52	0.01 ± 0.02	**0.185**	$\chi^2(5,54)=7.51$
Enterococcus	31.78 ± 25.77	26.58 ± 23.43	14.51 ± 14.45	24.06 ± 14.56	24.70 ± 18.07	24.15 ± 21.53	**0.777**	$\chi^2(5,54)=2.50$
Aeromonas	0.00 ± 0.00	0.97 ± 2.55	0.00 ± 0.00	0.00 ± 0.00	0.49 ± 1.46	0.03 ± 0.08	**0.387**	$\chi^2(5,54)=5.25$
Candidatus Arthromitus	3.48 ± 7.80	18.42 ± 26.28	13.10 ± 17.75	9.95 ± 24.75	1.75 ± 2.06	24.26 ± 36.14	**0.067**	$\chi^2(5,54)=10.32$
f. *Ruminococcaceae*	1.90 ± 4.26	2.05 ± 4.64	0.75 ± 1.54	0.80 ± 1.64	1.90 ± 5.69	2.90 ± 4.98	**0.696**	$\chi^2(5,54)=3.03$

The 'Others' category was assigned in a similar way to the phyla present, and makes up the largest proportion of the relative abundance reads for this trial; this category was not significantly more or less abundant between diets (p=0.820, Table 3.12). *Enterococcus* was the next most abundant genus present across all diets, but no significant differences between dietary regimes was observed. In addition, no significant differences were observed between dietary treatments for the relative abundance of genera *Candidatus* Arthromitus, *Bacillus*, *Corynebacterium*, family *Ruminococcaceae*, *Staphylococcus*, order *Enterobacterales* and *Aeromonas* (Table 3.12).

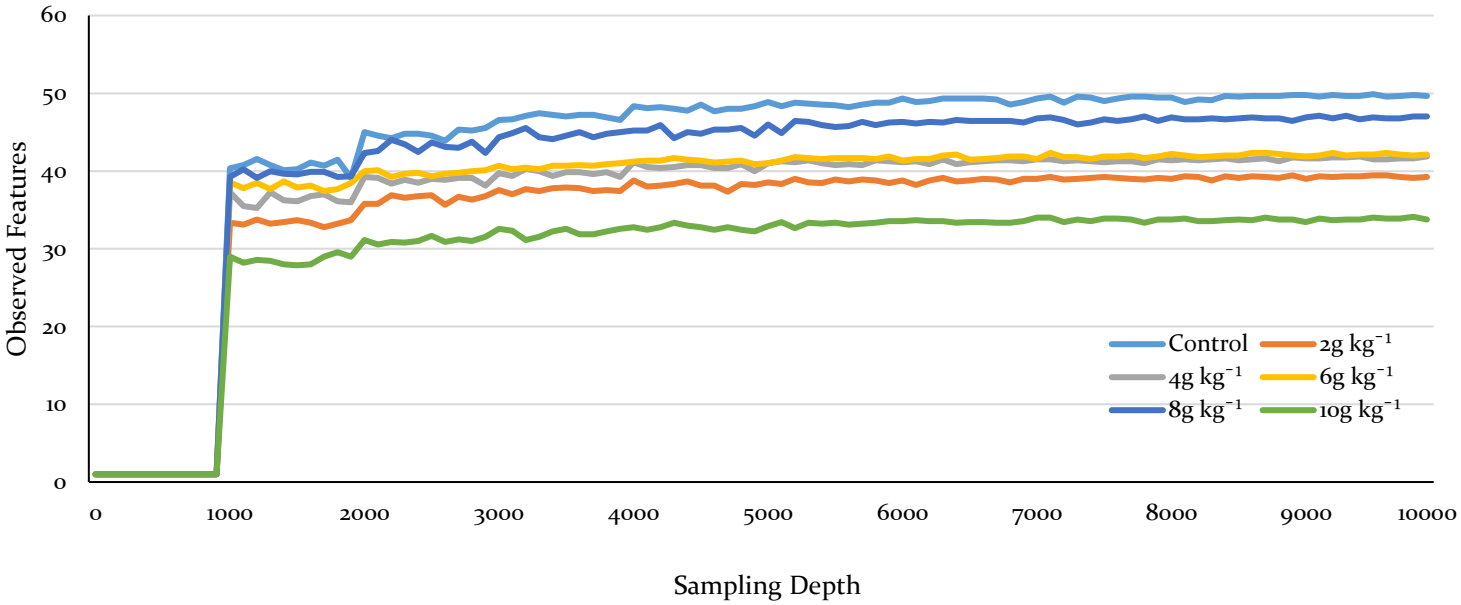


Figure 3.5. Comparison of observed OTU features as rarefaction curves within the digesta of rainbow trout (n=9, except for the 4g kg^{-1} diet where n=8), fed control diet or diets supplemented with incremental levels of B-GOS® over a period of 8 weeks.

The LEfSe histograms for the distinct bacteria genera detected are shown in Figure 3.8, where the relative abundance of each sample in each diet is plotted for each genus. Post-hoc analyses were competed within the STAMP software that are presented in Figure 3.9. These analyses display the differences detected between the mean proportions (%)

of the bacteria present within the diets, and then report the difference in mean proportions as a percentage with a p-value. The post-hoc results show differences between dietary regimes for genera *Aerococcus* and *Macrococcus* (Figure 3.9).

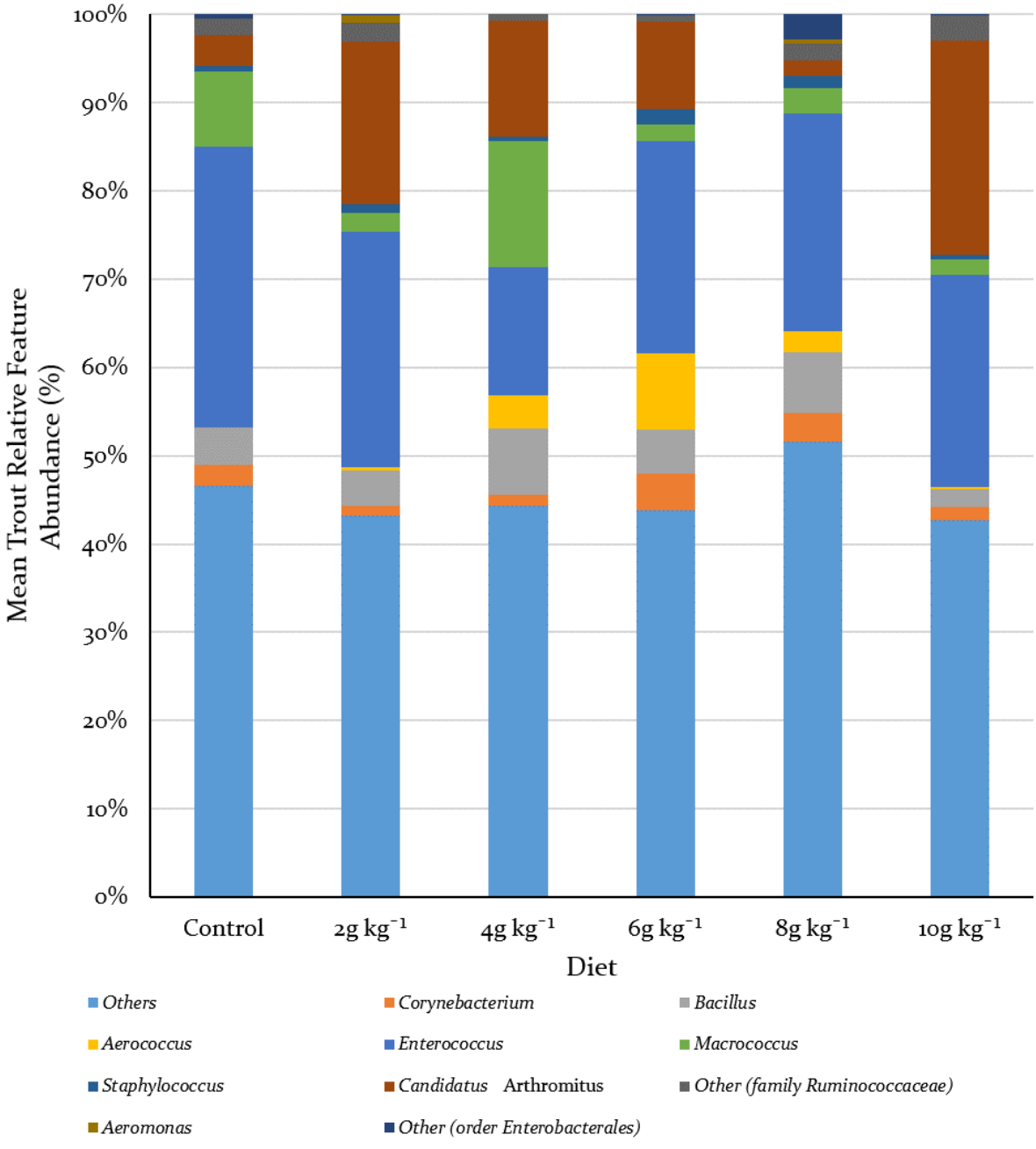


Figure 3.6. Percentage relative OTU abundance (%) of bacterial sequences at the genus level or lowest taxonomic level present within the digesta of rainbow trout (n=9, except for the 4g kg^{-1} diet where n=8), fed incremental levels of B-GOS® over a period of 8 weeks.

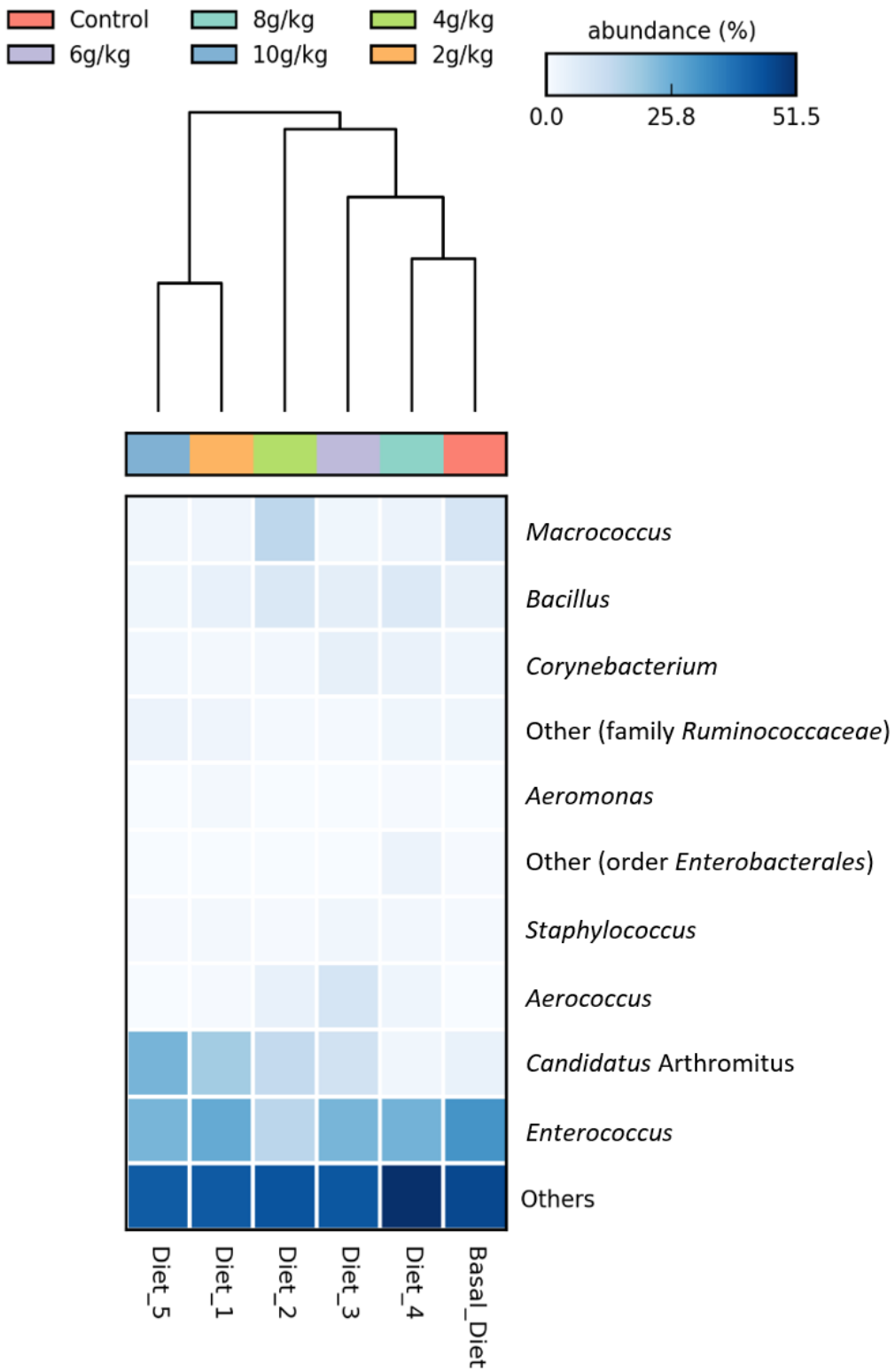


Figure 3.7 Mean abundance levels (%) of each genus present (or closest taxonomic level) within the microbiota of the intestinal digesta samples (n=9 fish per diet, except for n=8 in fish fed 4g kg^{-1}) for rainbow trout fed incremental levels of B-GOS® over a period of 8 weeks. The treatments are represented as: Control = Basal_Diet; 2g kg^{-1} = Diet_1; 4g kg^{-1} = Diet_2; 6g kg^{-1} = Diet_3; 8g kg^{-1} = Diet_4 and 10g kg^{-1} = Diet_5.

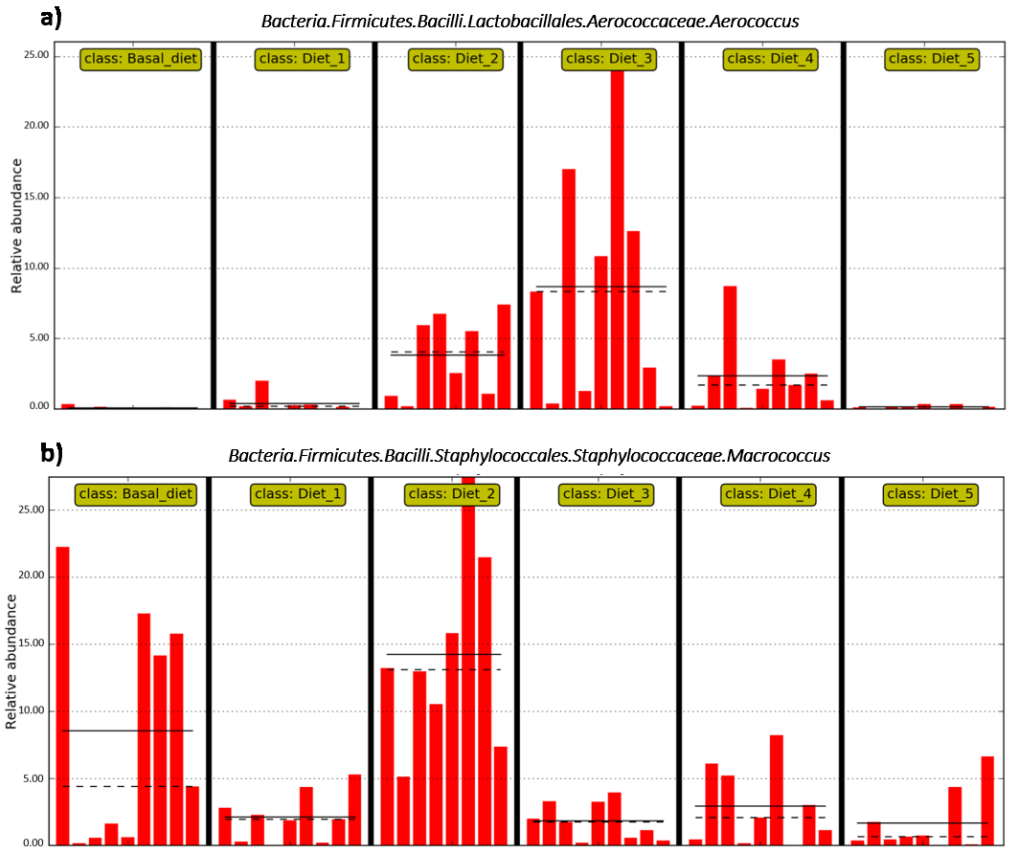


Figure 3.8 Differential features histogram plots of the biomarkers OTUs detected by LEfSe showing the relative abundance of each genus by diet. The diets are called class, as generated by the LEfSe analysis. The bacterial genera detected are shown as **a)** genus *Aerococcus*, and **b)** genus *Macrococcus*. The dotted line (- - -) represents the medians of each diet and the straight line (—) represents the means of each diet. The treatments are represented as: Control = Basal_Diet; 2g kg^{-1} = Diet_1; 4g kg^{-1} = Diet_2; 6g kg^{-1} = Diet_3; 8g kg^{-1} = Diet_4 and 10g kg^{-1} = Diet_5.

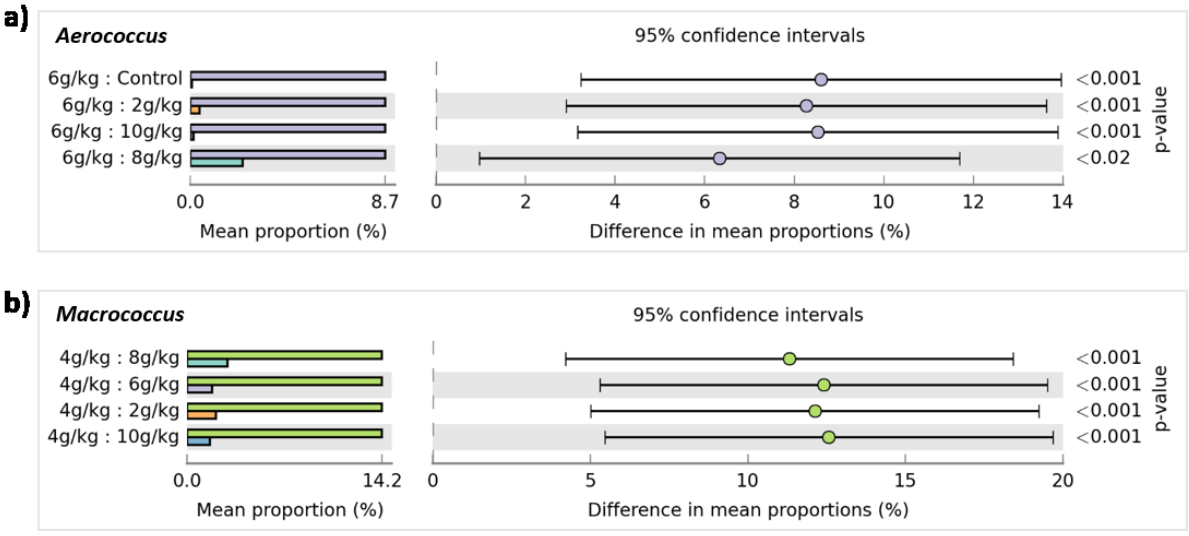


Figure 3.9 Post-hoc plots for the relative abundances of each bacterial genera that STAMP detected as being significantly different between fish fed the experimental diets. The mean proportions of the sequences for each diet are shown on the left, the differences in mean proportions for each statistical interaction are on the right, and the p-value is given indicating if the mean proportion is equal for a given interaction. Statistical differences were accepted at $p<0.05$. The genera detected are shown as **a)** genus *Aerococcus*, and **b)** genus *Macrococcus*.

The LEfSe software plotted a cladogram that represents the data based on taxonomical hierarchy and relatedness that are induced by the dataset label names (Figure 3.10). The middle unconnected point of the cladogram represents the Domain, and each point radiating out from the centre represents each incremental level of taxonomy, for instance the L2 (phyla) taxa are on the second level from the centre point. Based on the results from the LEfSe and STAMP analyses, the two genera *Aeroccocus* and *Macrococcus* are presented in Figure 11 as the sixth level from the centre point, with the order and family of these distinct genera also highlighted on their respective clades.

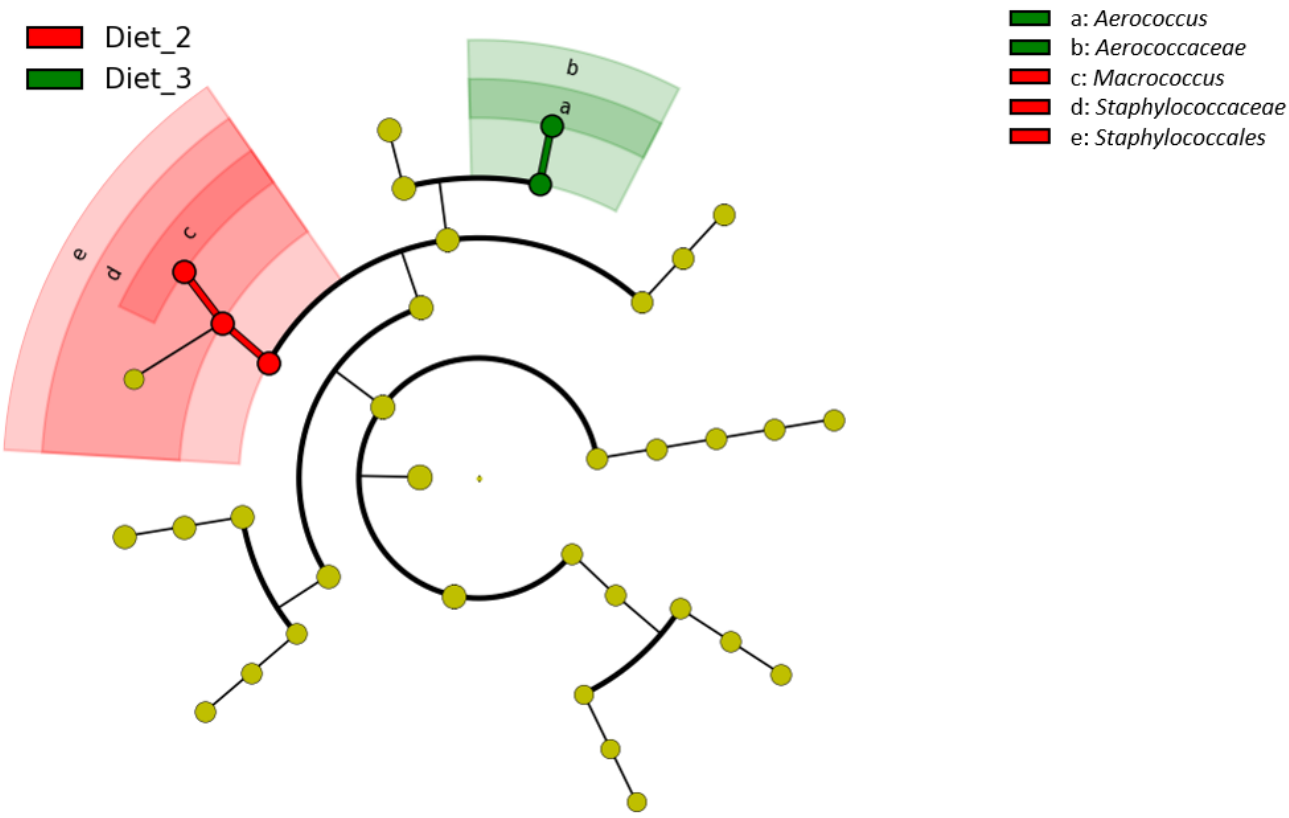


Figure 3.10 Circular cladogram reporting the identified trout OTUs from the LEfSe output and are distributed according to phylogenetic characteristics between treatments; for example, the phyla are represented by the circles on the second level of the cladogram from the central point. The order, family and genus that are significantly different between each compartment of the cladogram are coloured differently to the yellow taxon levels that indicate OTUs with similar abundances, and are listed on the right side of the figure. The diets are represented as: Control = Basal_Diet; 2g kg^{-1} = Diet_1; 4g kg^{-1} = Diet_2; 6g kg^{-1} = Diet_3; 8g kg^{-1} = Diet_4 and 10g kg^{-1} = Diet_5.

3.4 Discussion

The aim of the present study was to investigate the potential effects of different inclusion rates of a novel GOS product upon growth performance and immune response parameters in commercially valuable teleost species. The results will be used to further inform and build upon the knowledge that relates to alterations within fish mucosal tissues in response to a novel prebiotic. The species chosen for this study was rainbow trout, and is one of the most commercially valuable fish species in the world, with a high demand in North America and the UK, contributing towards over 3.2 million tonnes of salmonids produced from aquaculture in 2018 (FAO 2020).

The first *in vivo* feeding trial focused upon analysing the growth, immune response and overall performance of juvenile rainbow trout fed a novel prebiotic by utilising a variety of analytical techniques, including basic growth indices, histological appraisal of target organs, the expression of a selection of immunology related genes and the composition of the intestinal microbiota. Based on multiple metrics measured during the present chapter, the results of this study suggest that dietary inclusion of B-GOS® did not significantly affect the growth and immune functions of rainbow trout at the whole organism level under the conditions investigated. The results from the present study have contributed to the understanding of how growth performance and health of a commercially important salmonid species are influenced by the addition of a novel GOS prebiotic.

3.4.1 Growth performance

During the 8 week feeding trial, the rainbow trout readily accepted the control and experimental diets throughout, and exhibited an almost 3-fold increase in growth across

all diets, with acceptable ranges of FCR, SGR and PWG, indicating good growth and overall performance. The results of the present study suggest that there may be some numerical (but not significant) improvement in commercial fish species fed this novel prebiotic at 4 – 6g kg^{-1} B-GOS® inclusion compared to control fed fish. However, despite these improvements in growth across these metrics, the growth performance of fish fed any experimental B-GOS® diets was not significantly improved compared to fish fed the control diet. The rearing conditions of the trial were excellent and consistent, and these factors, in addition to the high quality feed administered, appeared to contribute to the overall good performance of the fish. It is therefore hard to conclude that the trout were gaining the full benefit of the prebiotic, as the growth performance levels of the control baseline of the fish were likely already good, or close to a maximum.

Previous studies have demonstrated that GOS prebiotics may improve overall growth performance in juvenile red drum (Zhou *et al*. 2010), snakehead (*Channa striata*) fingerlings (Talpur *et al*. 2014), common carp juveniles (Hoseinifar *et al*. 2017a) and Caspian roach fry (Hoseinifar *et al*. 2013); however, not all studies have reported improvements in these parameters for fish fed GOS. In agreement with the results in the present chapter, studies of GOS inclusion within zebrafish (Yousefi *et al*. 2018) and hybrid striped bass (Burr *et al*. 2010) suggest that the prebiotic did not improve fish growth performance under the conditions tested. Yousefi *et al*. (2018) reported that 0.5%, 1% and 2% GOS inclusion levels supplemented to zebrafish failed to produce significant improvements in growth performance metrics, such as SGR, weight gain, FCR and survival rate when compared to fish fed a basal diet after 8 weeks. Similar results have also been demonstrated after FOS, MOS and GOS supplementation in juvenile Atlantic salmon after 4 months by Grisdale-Hellend *et al*. (2008). Denji *et al*.

(2015) theorised that supplementation with higher doses of prebiotic in rainbow trout juveniles may lead to an accumulation of unfermented prebiotic that the intestinal microbiota is not able to effectively metabolise. This hypothesis may suggest why the trout fed higher doses of B-GOS® in the present chapter, 8 – 10g kg^{-1}, showed little improvement in growth performance and other immune response parameters measured.

It is vital that publications provide as much detail as possible to understand what differences in trial design might lead to contentious results. It is worth noting that snakehead fingerlings (*Channa striata)* investigated by Talpur *et al*. (2014) fed a control diet had an FCR of approximately 1.62 compared to the significantly lower FCR values in fish fed probiotic (*Lactobacillus acidophilus,* 1 x 10^{10} CFU) and prebiotic (1% yeast, 0.1% β-glucans, 0.2% MOS, and 1% GOS). There was a significant reduction of FCR in fish fed *L. acidophilus* (FCR of 1.22), yeast (1.29) and β-glucans (1.30) supplemented diets compared to the control and MOS and GOS formulated diets (FCR of 1.46 and 1.43, respectively) (Talpur *et al.* 2014). These data suggest that there may have been nutritionally beneficial effects within fish growth performance after supplementation of the probiotic, yeast and β-glucans experimental additives against a potentially sub-optimal basal diet, and allowed for increased nutrient uptake and enhanced growth. It is harder to make these comparisons within the literature if the performance metrics used to test for growth in experimental fish are not ubiquitous across publications, and so great care should be taken to report as many metrics as possible to help determine why differences in results in similar fish species occur. Thus, it may be suitable to conclude that the effectiveness of GOS prebiotics can be dependent on the differences

in prebiotic administration, length of trial, age and species of fish used (Merrifield & Rodiles 2015; Eryalçin *et al*. 2017; Yousefi *et al*. 2018).

In addition to the aforementioned rearing condition parameters, animals reared in conditions that are not designed to induce stress via pathogenic or environmental challenge may not be significantly improved by prebiotic addition if their growth is already consistent (Ziółkowska *et al*. 2020). Prebiotic supplementation may provide some measure of immune modulation in the face of challenging rearing conditions, as reported Salze *et al*. (2008) whose authors investigated how larval cobia (*Rachycentron canadum*) supplemented with diets of 0.2% dry weight MOS performed during a salinity challenge at six, seven, 13 and 14 days post-hatching. The authors reported that larval cobia fed supplemented MOS diets presented significantly greater survival rates at six days post-hatching compared to control diet fed fish, as well as significantly enhanced microvilli heights within the fish fed MOS diet (Salze *et al*. 2008). A similar example is also reported by Khodadadi *et al*. (2018), whereby the authors investigated how a novel MOS within rainbow trout performed against experimental yersiniosis, known for causing enteric redmouth disease. The authors concluded that prebiotic addition aided in healing gill and liver injuries that were sustained 30 and 60 days after intraperitoneal injection with bacterial culture. Fish fed with 0.1% and 0.5% MOS presented significantly improved growth during the challenge, suggesting that prebiotic addition can modulate the immune system and improve growth performance in spite of pathogenic insult (Khodadadi *et al*. 2018).

In response to the lack of significant differences in growth performance parameters under the rearing conditions studied in the present chapter, it is concluded that the

product B-GOS® investigated here does not significantly improve the growth of rainbow trout juveniles under the conditions or inclusion levels tested. As reported in previous studies that have conducted challenge trials whilst supplementing snakehead fingerlings and rainbow trout with MOS (Salze *et al*. 2008; Khodadadi *et al*. 2018), prebiotic inclusion may provide benefits to growth performance and stimulate the immune system to a degree and modulate the immune response to a potential disease or environmental challenge. Further research would benefit from testing B-GOS® with rainbow trout rearing under challenging conditions, for example by introducing husbandry stressors, sub-optimal diets and pathogen challenges.

3.4.2 Haematology

There is a great deal of research focused on the health and physiology of fish, and one such area comprises of haematological parameters to assess the effect of feed composition and nutrition in fish in relation to the environment (Svobodova *et al*. 2005, 2008; Rawling *et al*. 2012). In the present study, there were no significant differences observed between the GOS fed fish and control fed fish for the Hb, RBC and WBC, MCV, MCH and MCHC parameters. The ranges for these parameters are consistent and comparable to research conducted by McCarthy *et al*. (1973), Svobodova *et al*. (1991) and Svobodova & Vykusova (1991) where the authors investigated haematological parameter ranges in salmonid species. The results reported in the present chapter are in accordance with the findings demonstrated in Hoseinifar *et al*. (2017b), whose authors reported that rainbow trout fingerlings fed either GOS prebiotic, *P. acidilactici* probiotic, or a synbiotic of both presented no significant improvement in erythrocyte or total leucocyte levels, Hb, Hct, MCH or MCHC compared to control fed fish. In the present chapter, no significant differences of differential leucocyte abundances were

observed in fish fed B-GOS® dietary regimes compared to the control fed fish, which is a similar result as reported by Hoseinifar *et al*. (2017b).

Previous research has suggested that a significant elevation in leucocyte counts, for example lymphocytes, could indicate a cellular response to an endogenous or exogenous stress factor, such as pathogenic insult or environmental challenge (Liu *et al*. 2017b). As the results of the present chapter observed no significant increases in leucocyte abundance and other haematological parameters measured, it is suggested that B-GOS® addition at the concentrations tested here under good rearing conditions did not modulate the immune response in rainbow trout. Previous research by Munir *et al*. (2018) reported that snakehead fingerlings fed prebiotic (β-glucan, MOS and GOS) and probiotic (*Saccharomyces cerevisiae* and *L. acidophilus*) diets presented significant improvements of RBC and WBC levels, PCV, Hb concentration and serum protein levels and lysozyme activities compared to control fed fish. The authors also reported that these improvements were maintained 1 week post-infection from *A. hydrophila* injected into fish from each dietary regime, with probiotic and prebiotic fed fish presenting significantly greater survival and immune response in the form of increased WBC counts than control fed fish (Munir *et al*. 2018). With this in mind, future research should be conducted to determine if rainbow trout reared dietary B-GOS® under challenging conditions, such as introducing a pathogen to the rearing water or via intraperitoneal injection, may present improved performance and immune responses.

3.4.3 Lysozyme Activity and Protein Content

Lysozyme is one of the most important bactericidal enzymes within the innate immune response in fish, and constitutes a part of the first line of defence against opportunistic

pathogens (Soleimani *et al*. 2012; Akhter *et al*. 2015). These cationic proteins are produced by hepatic cells and are responsible for antimicrobial activity, bacteriolysis as part of the immune response (Magnadóttir 2010; Kiron 2012; Carbone & Faggio 2016), and can be found within many sites along the host, such as the epidermal mucus, blood plasma and lymphoid tissues (Alexander & Ingram 1992).

Previous studies have reported that teleost species supplemented with prebiotics presented significantly improved innate immune responses compared to fish fed control diets. Hoseinifar *et al*. (2015) reported significantly elevated serum lysozyme activity and skin mucus protein content after 8 weeks within juvenile rainbow trout fed GOS, *P. acidilactici* and a synbiotic diet of both when compared to control fed fish. A similar effect was reported by Zhou *et al*. (2010), whose authors investigated the effects of 1% GOS supplemented to juvenile red drum and observed significant increases in serum lysozyme activity within fish fed GOS compared to the control group. Soleimani *et al*. (2012) reported that Caspian fry fed 1, 2 and 3% FOS had significantly elevated levels of serum lysozyme compared to control fed fish after 7 weeks of feeding (Soleimani *et al*. 2012), and fish fed either experimental diet experienced significantly greater resistance to salinity challenge than fish fed the control diet.

Contrary to the aforementioned studies, the mucus and serum lysozyme activity reported in the present chapter was not significantly elevated in fish fed the different GOS dietary regimes compared to the control group. Despite the elevated levels of lysozyme activity within trout skin and mucus fed the 2g kg^{-1} diet, this was not significantly increased compared to the control group. This effect was also observed in the protein content of the epidermal mucus scrapes and when this lysozyme metric was

normalised with protein. It has been established that the lysozyme effect and overall activity can be influenced by the species of fish examined and the body site in which it is sampled (Dash *et al*. 2018), and so elevated immune responses in this immune parameter may be apparent when conditions are far from excellent.

As the rainbow trout in the present chapter were reared in good conditions without abiotic or biotic stressors introduced, it is possible that no elevated immune response was necessary, as there was nothing to mount a response against (Pontefract 2021). Previous research has determined that lysozyme activity may be influenced by a range of abiotic and biotic factors, such as season, sexual maturity, water temperature, nutrition, salinity and stress of infection (Saurabh & Sahoo 2008; Yousefi *et al*. 2018). It has been previously reported that post infection with sea lice (*Lepeophtheirus salmonis)*, rainbow trout and Atlantic salmon significantly increase mucus lysozyme activity in the earlier days of infection (Fast *et al*. 2002). A similar effect demonstrated by Leclercq *et al*. (2020) in Atlantic salmon fed a dietary MOS/β-glucan combination over 6 weeks produced a significant increase in lysozyme activity and mucus secretion compared to control fed fish when challenged by sea lice (*L. salmonis*). In addition, Hoseinifar *et al*. (2015) had observed that rainbow trout fed GOS, *P. acidilactici* and a synbiotic diet of both had significantly greater survival after a post-trial stress challenge with *S. iniae* than control fed fish. The authors suggested that the greater immunity built up from 8 weeks of synbiotic and pre/probiotic feeding had significantly improved the immune response once infection had occurred, and the increase in lysozyme activity may contribute to increased bactericidal activity to defend against pathogenic insult (Hoseinifar *et al.* 2015).

Other publications have also reported that a lack of significant difference between control fed fish and those administered with dietary prebiotic may not exhibit elevation in lysozyme activity within the blood serum or epidermal mucus, but may show significant differences in the expression of important immune system functions. Cavalcante *et al*. (2020) reported that the administration of probiotic (containing *Bifidobacterium* spp., *L. acidophilus* and *Enterococcus faecium*), prebiotics (MOS and chitosan) and synbiotic diets of these combinations to juvenile Nile tilapia did not significantly affect immunological parameters, such as plasma lysozyme, total protein and phagocytic activity of fish receiving either dietary regime. However, after intraperitoneal injection of *A. hydrophila*, fish fed the control group exhibited the highest mortality compared to treatment fed fish, and there appeared to be an upregulated immune response presented as elevated goblet cells, mast cells and preserved intestinal epithelium (Cavalcante *et al*. 2020). It is unclear from this study conducted by Cavalcante *et al*. (2020) if the immunological parameters they tested for prior to challenge were significantly different after the fish were infected with *A. hydrophila*. However, given that the fish which were supplemented with additive presented improvements in some morphological and immune responses, as well as having reduced mortality to *A. hydrophila* challenge, it is suggested that additive inclusion may also improve the lysozyme activity and protein content in fish fed the experimental diets (Cavalcante *et al*. 2020). Further testing for these immunological parameters post-challenge is a priority to ascertain if dietary inclusion of additives may lead to increased immune response and survival compared to fish that are not challenged.

Based on the results from the present chapter and the literature, the serum lysozyme activity and protein content in rainbow trout may be unaffected by dietary addition of B-GOS® due to the excellent conditions in which these rainbow trout were reared. It is suggested that further research should focus on rearing rainbow trout juveniles supplemented with dietary B-GOS® and introducing challenges to elucidate if this additive would improve performance whilst mimicking the conditions that might be experienced in an aquaculture facility.

3.4.5 Histology

Previous research has reported that prebiotic administration to teleost diets may positively affect the morphology of the GIT, and studies investigating dietary prebiotic effects have demonstrated improvements in nutrient uptake via greater absorptive surfaces; greater mucin cell production; and improved growth performance (Yilmaz *et al*. 2007; Salze *et al*. 2008; Zhou *et al*. 2010; Anguiano *et al*. 2013; Ziółkowska *et al*. 2020).

Zhou *et al*. (2010) reported that prebiotic supplemented diets Previda®, Bio-MOS®, FOS and GOS to juvenile red drum significantly enhanced microvilli heights within the pyloric caeca, proximal and mid intestine compared to control fed fish, however the distal intestine was not significantly affected. It was noted by the authors that the control diet fed fish presented the lowest microvilli heights compared to the other prebiotic supplemented diets in the same sections of the GIT studied (Zhou *et al*. 2010). Similar effects were observed in larval cobia (Salze *et al*. 2008), and rainbow trout (Yilmaz *et al*. 2007), whereby microvilli height was increased following supplementation of MOS to the diets of these fish compared to the control groups. In addition to these studies, Anguiano *et al*. (2013) reported significantly improved

intestinal fold and microvilli heights within the anterior intestine in red drum and the posterior intestine of hybrid striped bass fed four prebiotics (FOS, Bio-MOS®, TOS and GroBiotic®-A) compared to control fed fish. An increase in microvilli height is proposed to increase the uptake of nutrients across the GIT via increasing the absorptive area, and thus may improve the overall growth performance of fish species supplemented with these types of prebiotic (Yilmaz *et al*. 2007; Salze *et al*. 2008; Zhou *et al*. 2010; Anguiano *et al*. 2013).

In contrast to these studies, there were no significant differences detected in the muscularis thickness, mucosal fold heights, goblet cell counts and lamina propria widths between fish fed the control and B-GOS® supplemented diets in in the present chapter. Guerreiro *et al*. (2016b) reported similar results within gilthead sea bream anterior and posterior gut morphology, as the morphology of these GIT sections were not significantly improved by scFOS addition compared to control fed fish. In addition, Dimitriglou *et al*. (2010) reported that gilthead sea bream fed MOS did not present significantly improved anterior gut morphology compared to control fed fish when analysed using standard light microscopy techniques. However, when the ultrastructure of the anterior intestine was examined using electron microscopy, the authors observed significant differences present between sea bream fed MOS compared to the control group (Dimitriglou *et al*. 2010). While the results from the present chapter do not report any differences in the intestinal morphology of the GIT section studied here, this does not rule out the possibility that other aspects of intestinal mucous, such as the ultrastructure, may have been affected by B-GOS® addition. Future studies comparing light and electron microscopy may determine if this were the case.

Prebiotics have been reported to improve the morphology of the skin in teleosts and may positively affect the mucus membrane and innate immune response. Leclercq *et al*. (2020) demonstrated that dietary supplementation with yeast-based MOS to Atlantic salmon increased skin and distal intestine goblet cell proliferation of prebiotic fed fish, and an overall greater excretion of skin mucus in fish fed the prebiotic compared to the control group. The authors suggested that the increased mucus may prevent adhesion of chalimus sea lice to the skin of salmon by reinforcing the physical mucosal barrier, and this contributed to the prebiotic group experiencing lower susceptibility to this ectoparasite compared to the control group (Leclercq *et al*. 2020). In addition, Leclercq *et al*. (2020) reported a significant increase in skin lysozyme activity in prebiotic fed fish, suggesting that the addition of a MOS may increase anti-microbial defence and prevent lasting physical damage from sea lice settling on the skin surface.

The results from the present study do not support the findings of Leclercq *et al*. (2020), in that there were no significantly increased numbers of goblet cells present within the skin of rainbow trout fed any prebiotic inclusion against those fed the control diet. A lack of increase in goblet cell abundance in the skin and intestinal mucus of rainbow trout fed any B-GOS® inclusion rate compared to the control group suggests that this prebiotic does not influence mucus excretion levels at the time of sampling and under the rearing conditions tested. As the trout from the present chapter were reared in excellent conditions and with a nutritionally appropriate diet and exhibited good growth performance, there may be no scope for this dietary additive to be effective in the target organs investigated at the concentrations measured.

A contributing factor towards teleost responses to prebiotics within the intestinal structures is the addition of plant feedstuffs in fish diets that may unintentionally cause these diets to become nutritionally sub-optimal. Many aquafeed producers are focusing on the reduction of fish derived proteins and oil, and there had been an increasing focus on soybean meal (SBM) as a protein supplement as it has a high protein content and the amino acid profile is ideal (Merrifield *et al*. 2011; Adeoye *et al*. 2016a,b). In salmonids, the effects of SBM within the intestinal mucosa has been researched, and results have suggested the potential for deterioration in fish growth and the intestinal mucosa, and such changes may induce enteritis within the GIT (Merrifield *et al*. 2011). Changes that may occur are expressed as elevated goblet cell abundance within the microvilli and mucosal layers, widening of the lamina propria, shortening of the microvilli and lymphoid cell infiltration within the lamina propria (van den Ingh *et al*. 1991; Merrifield *et al*. 2011). In addition, Guerreiro *et al*. (2015) reported that European sea bass (*Dicentrarchus labrax*) fed plant-based diets presented deterioration of the distal intestine histomorphology after 15 days of feeding compared to a diet containing high percentage of fishmeal, regardless of whether sea bass were supplemented with prebiotics scFOS or XOS. Given that the composition of the diets formulated in the present study have a relatively high proportion of plant feedstuffs, including SBM, to fish derived proteins, any potential changes to intestinal morphological parameters were investigated analysed using light microscopy. No significant differences were detected in any histological measurement of rainbow trout fed any dietary regime, suggesting that the diet composition of SBM and plant feedstuffs to fish derived products did not significantly affect the trial conditions.

The results of this present chapter suggest that the B-GOS® prebiotic inclusion levels chosen for this experiment had no significant effect on the histomorphology of rainbow trout intestine studied at this age and within the section of intestine investigated. Other aspects of the intestinal mucosa, such as the biochemistry, viscosity of mucous and specific protein levels, may have been affected but could not be elucidated during this present study and so future studies using proteomics and biochemistry analyses should determine if this is the case using B-GOS® in rainbow trout.

3.4.6 Body Composition

No significant differences were observed between the carcass compositions of fish fed either dietary regime, suggesting that the B-GOS® did not significantly affect the body composition of rainbow trout.

3.4.7 Gene Expression

The relative expression of target genes within teleosts is a useful tool in evaluating how the innate and adaptive immune system response are affected by a number of different stimuli, for example different stocking densities, changes in water quality or pathogenic infection (Smith *et al*. 2019). Gene expression of key cytokine biomarkers has been studied within teleosts administered feed additives against control fish to determine if additives may enhance the immune response in studied species (Bustin *et al*. 2009; Smith *et al*. 2019). In the present chapter, the relative intestinal and skin gene expression levels of target genes *IL-1β*, *TNFα*, *IL-10*, *TGF-β* and *Cal* were studied in rainbow trout fed B-GOS® to determine if essential immune system pathways were affected by prebiotic supplementation. These target genes were chosen as they represent a broad spectrum of the different signalling pathways within the immune

system response, and the up or downregulation of these target genes can inform the response within the fish to any stimulant or additive.

The expression of *Cal* in this study was significantly downregulated in the skin of rainbow trout fed B-GOS® at 4g kg^{-1} inclusion rate compared to the control group and all other GOS groups apart from 8g kg^{-1}. *Cal* expression within the intestine of rainbow trout fed any dietary regime was not significantly affected by prebiotic addition. *Cal* is transcribed into a protein by the same name, which acts as a multi-functional chaperone protein, and is involved in processes such as calcium-binding and the degradation or folding of glycoproteins found in the endoplasmic reticulum (Kales *et al*. 2007; Micallef *et al*. 2017). This protein is involved in immune function and is crucial for early development in mice (Michalak *et al*. 2002), as well as important functions in wound healing in fish skin mucus, with the potential to protect against ectoparasites, such as sea lice (Micallef *et al*. 2017) making this target gene a potential biomarker for salmonid immune responses.

The results of the present study disagree with the work presented by Micallef *et al*. (2017), whose authors reported that 0.4% inclusion of yeast-cell wall extracts supplemented to Atlantic salmon significantly upregulated calreticulin-like gene expression in the skin compared to fish fed the control diet. Previous research has demonstrated that calreticulin has a possible role in the innate immune response in fish by binding to the complement and lectin pathways, and upon binding, calreticulin induces a change in the folding of the proteins, and so leads to the elimination of apoptotic cells (Cavill *et al*. 2004; Micallef *et al*. 2017). In addition to its role in the complement and lectin pathways, calreticulin has also been found to be over expressed

in the distal intestine proteome of Atlantic salmon after 24 hours from induction of inflammation (Vasanth *et al*. 2015). In addition, Vasanth *et al*. (2015) reported an increased abundance of goblet cells in the intestine of these same fish, further suggesting its role in the innate immune system pathways by contributing to the reduction of inflammation (Vasanth *et al*. 2015; Micallef *et al*. 2017). In the present study, the significant downregulation of *Cal* in the skin of rainbow trout fed 4g kg^{-1} B-GOS® may suggest that the activation of these immune pathways was not necessary due to the good rearing conditions and lack of pathogenic challenge, as evidenced by the good growth and performance of rainbow trout from this dietary group.

The expression of *IL-10* in the skin of rainbow trout fed 2g kg^{-1} diet was significantly downregulated compared to fish fed the control, 4g kg^{-1}, 6g kg^{-1} and 10g kg^{-1} B-GOS® supplemented diets. A similar effect was also observed in the expression of *IL-10* in the intestine of rainbow trout fed 2g kg^{-1} prebiotic compared to the other treatment groups apart from the control group. The expression of this target gene was also significantly upregulated in the skin of fish fed the 4g kg^{-1} diet compared to the fish fed control, 6g kg^{-1} and 8g kg^{-1} diets. *IL-10* is a gene that is transcribed into an anti-inflammatory cytokine, which is heavily involved in the immune response, primarily to downregulate the activation of macrophages, maintain the balance between rapid and intense inflammatory responses against pathogenic insult, and to enhance B-cell/T-cell survival, proliferation and antibody production (Zou & Secombes 2016; Rawling *et al*. 2019). When upregulation occurs, this cytokine may inhibit pro-inflammatory synthesis of other cytokines, such as *TNFα*, and have an essential role in modulating immune responses in fish (Bogdan *et al*. 1991; Zou & Secombes 2016). In the present chapter, upregulation of *IL-10* in the skin of fish fed 4g kg^{-1} GOS may suggest that an anti-

inflammatory response was induced, perhaps suggesting some readiness in the immune response. As the results from the present chapter suggest that there is some sensitivity in the gene expression between fish fed different diets, as trout skin *IL-10* expression exhibited 2.5-fold downregulation after 2g kg^{-1} GOS addition but 2.5-fold upregulation at 4g kg^{-1}, it is difficult to determine if this prebiotic is influencing the immune response pathways in a meaningful way.

A similar effect was also observed in the expression of *TGF-β,* whereby this gene was significantly downregulated in the skin of fish fed the 2g kg^{-1} diet compared to the control, 4g kg^{-1} and 8g kg^{-1} diets. There were no significant differences in *TGF-β* gene expression in the intestine of fish fed any experimental diet relative to the control fed fish, or each other, for this trial. In a similar manner to *IL-10*, fish fed the 4g kg^{-1} diet presented upregulated expression of *TGF-β* in the skin compared to the control, 6g kg^{-1}, 8g kg^{-1} and 10g kg^{-1} fed fish. *TGF-β* is a cytokine that functions to limit pro-inflammatory cells, and can facilitate cell growth, proliferation, differentiation and apoptosis (Yang *et al*. 2012). This cytokine is produced within from leucocytes and it is a vital part of the immune system pathway, as shown in mice embryos, as 50% of mice that have *TGF-β* removed died in utero (Clark & Coker 1998). This cytokine expression has been demonstrated to be upregulated in Atlantic salmon that had been challenged by sea lice (*L. salmonis*) and had damage induced in the skin from their contact, suggesting that this cytokine is involved in wound healing (Skugor *et al*. 2008). The significant upregulation of *IL-10* and *TGF-β* in the skin of rainbow trout fed 4g kg^{-1} B-GOS® may suggest that there is an activation towards the anti-inflammatory response, while the significant downregulation of these genes in fish fed the 2g kg^{-1} diet may suggest the opposite.

The target genes *IL-1β* and *TNFα* are both transcribed into pro-inflammatory cytokines of the same names that can be stimulated in response to immune stress within the host. *IL-1β* belongs to the interleukin 1 group and is one of the first effector cytokines to be activated in the immune response (Dinarello 2011; Zou & Secombes 2016; Rawling *et al*. 2019; Sakai *et al*. 2021). When activated, this cytokine is an important mediator for the inflammatory response in terms of increasing phagocytic and lysozyme activity in macrophages, mediating cell proliferation, differentiation and apoptosis (Montalban-Arques *et al*. 2014, 2015; Zou & Secombes; Sakai *et al.* 2021). *TNFα* is also an important regulator during the inflammatory response, and is activated by lymphoid cells, activated macrophages and endothelial cells. Previous research has reported that expression of *TNFα* is important for the regulation of leucocyte migration and proliferation as part of the immune response in teleosts (Sakai *et al*. 2021).

The results in the present chapter suggest that there may be a level of modulation of the pro-inflammatory response, as the expression of *IL-1β* was upregulated in the skin of trout fed 4g kg^{-1} and 10g kg^{-1} B-GOS® diets compared to the control group. For *TNFα* expression, a similar effect occurs as fish fed the 4g kg^{-1} diet displayed upregulated expression levels in the skin compared to the control, 2g kg^{-1} and 6g kg^{-1} fed fish, perhaps suggesting a potential regulation of pro-inflammatory responses. However, as skin *TNFα* gene expression levels were significantly downregulated in the fish fed 2g kg^{-1} and 6g kg^{-1} compared to the control group, while the 4g kg^{-1}, 8g kg^{-1} and 10g kg^{-1} fed fish exhibited elevated *TNFα* expression levels compared to the control fed fish. There appears to be no particular trend in the data for expression levels of TNFα in the skin of rainbow trout fed any GOS inclusion rate, and a level of sensitivity appears to be present, as an increase of 2g kg^{-1} GOS for each successive dietary treatment produced

an almost 5-fold difference in expression. It is difficult to determine how the expression of *TNFα* affects trout skin immune responses and so further research should be conducted to elucidate this.

For the gene expression of *IL-1β* in the intestine of rainbow trout, there was significant upregulation in 2g kg^{-1}, 6g kg^{-1} and 8g kg^{-1} fed fish compared to 4g kg^{-1} and 10g kg^{-1} fed fish, but not compared to the control group. These results are not reflected in the expression of *TNFα* in the intestine of rainbow trout, as *TNFα* expression was significantly upregulated in fish fed all dietary treatments (except the 8g kg^{-1} group) compared to the control group and the 8g kg^{-1} dietary inclusion. Of the expression levels observed for each dietary treatment, the fish fed 2g kg^{-1} GOS had the greatest fold change in intestinal expression of *TNFα*. Previous research by Dawood *et al*. (2020) reported that recent teleost studies focusing on cytokine gene expression of *IL-1β* and *TNFα* show the most pronounced upregulation after prebiotic treatment, however, other cytokine expression such as *IL8* or *IL-10* show alterations close to the control group or baseline. This appears to be in accordance with the intestinal samples measured in the present study from fish supplemented with B-GOS®; however, it is not the case with the skin samples.

These mixed significant results displaying no particular trend across the expression levels with increasing B-GOS® supplementation may be due to biological variation at the transcriptomic level being quite high between replicates. Replicates that were identified as outliers (as described in sections 2.10 and 3.2.3) were removed from further analysis to reduce the variation in C_T values obtained for the reference genes after qPCR. In addition, transcriptomic effects are especially transient in comparison to other omics

analyses, in particular the proteomic approach, thereby more turnover of proteins results in more changes detected at the protein level than gene expression level (Micallef *et al*. 2017). Despite this, the use of gene expression biomarkers is still cost-effective, practical and widely used within aquaculture research (Rawling *et al*. 2019), and can be useful comparisons for studies comparing transcriptomic expression versus protein expression. The presence and/or content of these target gene protein levels used for this present chapter could not be elucidated, and so future work should focus on how expression levels of these important immune-related genes compares to the levels of proteins in rainbow fish skin after supplementation with B-GOS®.

The fish reared in this present study were not challenged with pathogens or sub-optimal environmental conditions, and so testing this GOS prebiotic at the same inclusion levels and inducing stress through administration of a fish pathogen or poor water quality may influence the regulation of key immune response genes in rainbow trout. Further research is required to determine if the inclusion of B-GOS® to the diets of challenged fish may improve expression of target immune response genes.

3.4.8 Intestinal Microbiome Analysis

The intestinal microbiome is one of the most widely studied MALTs in teleosts, with attention focusing on how the microbial communities on and around the mucosal structures interacts with the host, and how these host-microbe interactions may be influenced by prebiotic addition (Gatesoupe 1999; Gómez & Balcázar 2007; Merrifield *et al.* 2010a,b,c; Egerton *et al*. 2018; Vargas-Albores *et al*. 2021). There is evidence that the microbial communities and their abundances within the host GIT can influence how the functionality of the mucosa develops throughout all host life stages (Merrifield &

Rodiles 2015), and other external factors, such as physiochemical conditions including water temperature and salinity, have also been reported to play a big part in the composition of the core microbiota (Rawls *et al*. 2006; Merrifield & Rodiles 2015; Vargas-Albores *et al*. 2021).

For the present study, the sequencing depth achieved through bioinformatics analysis was confirmed to be adequate as the Good's coverage for all treatments was >99%. The species richness (Chao1) metric was not significantly affected by the addition of B-GOS® to rainbow trout compared to a control group, and this was reflected in the Shannon diversity index. As the OTUs and Chao1 values were similar to each diet group, this indicates that there were similar species present with a similar diversity composition (Kim *et al*. 2017). Previous studies indicate that the rainbow trout GIT microbiota relative abundance is dominated by bacterial phyla *Firmicutes* and *Proteobacteria* (Sullam *et al*. 2012; Wong *et al*. 2013; Lyons *et al*. 2016), both of which were sequenced within the rainbow trout digesta from the present chapter. These phyla are also represented in other fish species, such as in Nile tilapia (Standen *et al*. 2015), and in both marine and freshwater species, carnivorous, omnivorous or herbivorous, suggesting that most species of gut bacteria found across many species are from these taxa (Sullam *et al*. 2012; Peggs 2015; Standen *et al*. 2015; Gajardo *et al*. 2016).

The presence of these taxa in the present study has been determined using similar or the same high-throughput 16S rRNA sequencing techniques as those used in other studies (Wong *et al*. 2013; Standen *et al*. 2015; Lyons *et al*. 2016). Previous studies support the idea of the phyla *Firmicutes* and *Proteobacteria* playing a part in the microbiota of fish (Ringø 2004; Kim *et al*. 2007). Further research has provided

evidence that a proportion of the microbiome in rainbow trout grown in both aquaria and farms also contains the taxa *Actinobacteria*, *Spirochaetes*, *Bacteriodetes and Verrucomicrobia* (Sullam *et al*. 2012; Lyons *et al*. 2016; Villasante *et al*. 2019), of which these taxa's relative abundances were observed in the present chapter.

One of the more relatively abundant group of genera identified from the 16S rRNA sequencing reads was 'Others', and this group contained reads that did not average over the percentage threshold of the total reads, but also contained a large number of reads that could not be identified by the QIIME v2 software. These reads may have arisen from the nature of the data, in that the reads from the rainbow trout digesta samples were single-end and there may have been information on genera identity lost due to the stringency of the filtering used in the QIIME v2 pipeline.

In terms of relative abundance sequencing data, the bacterial genera identified by STAMP and LEfSe as significantly different between fish fed control and one or more of the B-GOS® supplemented diets within the present chapter were *Aerococcus* and *Macrococcus*. These genera have been sequenced in teleost species in previous studies (Michel *et al*. 2007; Wang *et al*. 2013; Zhang *et al*. 2017), and relative abundance of reads assigned to bacterial genus *Aerococcus,* a collection of Gram-positive bacterial cocci, were identified to be significantly abundant in fish fed the 6g kg^{-1} B-GOS® diet compared to all other dietary regimes including the control group, except for 4g kg^{-1} fed fish. Michel *et al*. (2007) isolated and identified this genus using amplified 16S rRNA gene restriction analysis, and the authors reported that this genus may be present in healthy rainbow trout intestine and other fresh water fish samples. Given that the fish in the present chapter fed the 6g kg^{-1} prebiotic inclusion did have the most increased

weight gain at the end of the trial and had the biggest final weights, it would appear that the presence of this genus of bacteria does not limit or pose a health risk to these juvenile rainbow trout.

The relative abundance of reads assigned to the family *Staphylococcaceae* have previously been sequenced within rainbow trout juveniles primarily fed a grain-based diet and tested under low rearing densities compared to fish fed a control diet and under control rearing densities (Wong *et al*. 2013). *Staphylococcaceae* contains the genus *Macrococcus*, which is comprised of non-motile, Gram-positive bacterial species previously identified from sequence data in Atlantic salmon fed a diet with 5% supplemented chitin (Askarian *et al*. 2012). Within the results of this chapter, the relative abundance of reads assigned to *Macrococcu*s were significantly more abundant in trout fed the 4g kg^{-1} diet compared to all other B-GOS® inclusion rates, except the control group. This genus has previously been sequenced in common carp and is suggested to comprise part of the core microbiota in control fish before receiving dietary supplementation of cinnamon essential oil (Zhang *et al*. 2017), and also as part of the microbiome in sea bream (Parlapani *et al*. 2015). These studies suggest that this genus may be part of the core microbiome in rainbow trout, as *Macrococcus* was also present within the intestinal digesta of control fed fish of the current chapter and was most relatively abundant in fish fed 4g kg^{-1} prebiotic, which presented the biggest final weights compared to other B-GOS® fed fish. Further research is required to determine how this genus may influence the health and overall immune response in salmonids to ascertain if this genus and species may benefit rainbow trout growth and performance.

Given that the relative abundances of the significant genera identified here are quite low compared to the 'Other' group and the *Enterococcus* present, caution is advised when comparing these relative abundances. As the sequencing method used here relied on the relative abundances of reads assigned to each taxonomic group, we cannot say for sure the absolute abundances of all bacteria present, especially if some are uncultivable or left unknown. Furthermore, caution is advised when comparing abundances, especially if the 16S rRNA copy numbers differ between bacterial species, and so bacterial sequence reads may be skewed or unreliable in a given sample (Fogel *et al*. 1999; Peggs 2015).

The results of this chapter suggest that rainbow trout fed the 2 – 6g kg^{-1} B-GOS® diets appeared to exhibit marginal improvements in performance, albeit not significant, in terms of growth compared to the control fed fish, and there appears to be increased relative abundance of some genera of the GIT microbiota at the localised level in fish fed these treatment groups. A lack of significant and overwhelming increase in any measured bacterial genera may tie into the previous results sections of this chapter that have highlighted the excellent conditions in which the rainbow trout were reared, and so dietary prebiotic addition to enhance what may already be a diverse microbial community may not have been necessary for an effective immune response. Future work investigating the novel prebiotic B-GOS® should examine the hypothesis that challenges to the trout will be mitigated by the inclusion of this prebiotic, and the dosage to be tested should be between 2 – 6g kg^{-1} inclusion rate, as this range has the potential for improvement in juvenile rainbow trout intestinal microbiota.

3.5 Conclusions

There is minimal significant improvement to the health and growth of rainbow trout after addition of the dietary prebiotic B-GOS® when reared in excellent conditions, and with optimally designed, nutritious diets. The numerous and in-depth metrics utilised in this chapter to measure immune response and performance parameters suggest that there is little scope for this GOS to stimulate further improvement above the basal levels. However, marginal increased final growth, decreased FCR, and slight improvements in the diversity of beneficial bacteria within the microbiota, such as elevated relative abundance of *Enterococcus* spp. within the GIT, suggest that a potentially suitable prebiotic inclusion level lies between 2 - 6g kg^{-1} B-GOS® for rainbow trout.

The combined results from the haematological, immunology, histomorphology, and gene expression parameters also support the range of 2 – 6g kg^{-1} as having the potential for future investigation, as fish fed these ranges presented the best numerical improvement in the parameters tested, despite a lack of significant difference between fish fed these ranges and the control fed fish. Further work in this species should investigate this prebiotic under challenging conditions, as such challenges have been reported within the scientific literature to allow for greater utilisation of the prebiotic, and may enhance overall growth and health by improving the diversity of the microbiota and providing a carbon source for beneficial bacteria to proliferate. Previous research has also demonstrated that the benefits of prebiotic addition in one species does not necessarily translate to another, and so further research must focus on how this novel B-GOS® prebiotic may influence the immune defences of another

commercially important species, such as Atlantic salmon, and this shall form the basis of the next research chapter.

The effects of prebiotic GOS dietary inclusion upon the growth performance and intestinal health of Atlantic salmon (*Salmo salar*)

4.1 Introduction

Similar teleost species may show different growth performance and health-related responses to the same feed additive, as other factors such as age of the fish, length of the experiment and diet formulation can affect the efficacy of a prebiotic (Merrifield *et al*. 2010c; Merrifield & Rodiles 2015; Yousefi *et al*. 2018; Mugwanya *et al*. 2021). While there are insights as to how this novel additive affects rainbow trout (Chapter 3), to the author's knowledge there are no data currently available regarding the effect of this prebiotic on Atlantic salmon growth, health or microbiome.

As with rainbow trout, there has been rapid growth in markets for other popular salmonids, with Atlantic salmon accounting for the largest production in export revenue for this group of species (FAO 2020). In 2018, Atlantic salmon accounted for 2.44 million tonnes of fish produced globally, with countries such as Norway and Chile generating multi-billions of dollars from this industry (FAO 2020). Salmonids are a huge trading commodity in terms of value and have made up an incredibly important proportion of the market for internationally traded fish products since 2013 (FAO 2020). Atlantic salmon are also widely preferred by the consumer and are highly sought after in developing and developed nations around the globe.

As this species has a high fecundity and can breed over more than one spawning season, this fish is a great commodity in terms of broodstock management and the production of larvae for widespread supply (Jobling *et al*. 2010). The control of photoperiods and temperature of the water make this species, as with other salmonids, incredibly useful to raise throughout the year, and can ensure high rates of growth (Jobling *et al*. 2010). Being anadromous, Atlantic salmon are very similar to rainbow trout in that they are

grown in freshwater raceway or RAS systems until a smolt size (~100 g), before growing on in sea cages until marketable size. This makes this species suitable to grow in Norway, North America, Canada, Chile, and the UK (Jobling *et al*. 2010; Houston & Macqueen 2019), and Atlantic salmon are obtainable from northern parts of the UK, predominantly reared in Scottish waters.

The major aim of the present chapter was to understand how a novel GOS additive may benefit Atlantic salmon growth performance and health. As with the previous experimental system, this salmonid was chosen as another commercially important species to investigate how the novel prebiotic B-GOS® that could potentially benefit the wider aquaculture market. This second experiment was designed to accommodate an 8 week feeding trial using juvenile Atlantic salmon, and grow them on formulated control and prebiotic supplemented diets until they achieved at least a doubling of biomass. Samples of target organs were taken at the end of the trial and in-depth methods used throughout this are outlined in Chapter 2 and the trial conditions in section 4.2 in this chapter.

4.2 Methodology

The specific experimental design, growth performance metrics and diet formulation for this trial are described in the following sections. Please refer to Chapter 2 sections 2.5 – 2.11 for more details about other sample analyses from this trial.

4.2.1 Salmon Experimental System

Juvenile female Atlantic salmon (*S. salar*) were sourced from Landcatch Natural Selection Ltd., Scotland, weighing approximately 20 g each. The fish were subjected to a 20-day acclimation and conditioning period in which during the first 10 day period

salmon were treated twice a day with an F-M-G proprietary prophylactic treatment (NT Labs, UK). Throughout this time, the fish were fed a commercial feed as a maintenance diet, and after the initial conditioning period, fish averaging 20.6 ± 0.2 g were graded into 18 x 140 L aquaria in groups of 32 individuals at the start of the trial. As described in Chapter 3, section 3.2.1, fish were batch weighed altogether from each tank by netting individuals and moving the net side-to-side to remove as much water as possible from the fish before they were placed in a pre-weighed and tared vessel of 10 L of tank water. Starting weights were calculated to be within ±2.5% of the overall average tank biomass. The weights of each tank were recorded in this manner at the start of the trial, every two-week period, and at the conclusion of the trial.

As with the trout trial, this feeding trial took place within the East Aquarium facility of the University of Plymouth and used the same experimental design. The water chemistry and quality parameters were maintained and adjusted with mechanical and biological filtration to maintain suitable requirements for Atlantic salmon. Fresh water was UV sterilised to ensure optimum water quality and the room temperature was maintained at 12.5 ± 0.5°C to maintain an average water temperature of 16.9 ± 0.1 °C.

The pH was maintained at 7.1 ± 0.5 and buffered using powdered sodium bicarbonate $NaHCO_3$, when necessary, and dissolved oxygen was maintained at 9.6 ± 0.1 mg L^{-1}. The photoperiod and air supply were the same as described in Chapter 3 section 3.2.1. The tank weights (total biomass) were measured every two weeks to calculate the rations for the fish for each day in that period. Feed was weighed into pots according to the feeding rate, and fish were fed between four to six rations a day by hand of 1.0 - 2.0% bodyweight, depending on acceptance of the feed.

One sampling point was scheduled at the end of the trial (60 days). Fish were taken at random during the sampling period, whereby they were concussed by a blow to the head, and euthanised by destruction of the brain (following Schedule 1 procedures). This work was approved by the University of Plymouth Internal Ethical Review Committee on Animal Scientific Investigations (approval number ETHICS-28-2019).

4.2.2 Diet Formulation and Growth Performance Parameters

Six experimental iso-nitrogenous and iso-lipidic diets were formulated using AFOS (Feedsoft Professional®, USA) to meet the known requirements of juvenile Atlantic salmon (NRC 2011). One basal diet (control) and five feed formulations including B-GOS® based on manufacturer's instructions ranging from 2 – 10g kg^{-1} were formulated, as shown in Table 4.1. The methodology for producing these diets is described in Chapter 2, section 2.4. Growth performance, feed efficiency and overall fish condition were determined using the calculations for FCR, SGR, PWG, and K-Factor as described in Chapter 2, section 2.4.

The mean and standard deviation (SD) were recorded for the FCR, SGR, final tank weights, Percentage Weight Gain (PWG) and Survival (%) of each diet. The FCR was calculated using n=3 tanks per diet, and the SGR was based on the starting weights on 26th September and the final weights of each tank on the 26th November 2019. The PWG was calculated from the mean tank weights at the beginning of the trial and the mean tank weights at the end; these values were then averaged across the diets. The K-Factor was calculated from the final lengths and weights of fish sampled at the end of the trial (n=24 fish per diet).

4.2.3 Gene Expression

The specific primers used for the gene expression analyses are described in Table 4.2. For the specific methodology of how the samples were prepared for gene expression analyses using qPCR, please see Methodology Chapter, section 2.10. Outliers were identified using the Quartile and OR functions in Excel 2016 as described in section 2.5 and 2.10 (Grech 2018) and removed from further analysis to reduce variation in C_T values obtained for the reference genes after qPCR, and so n=4 samples per diet were taken forward for further analysis.

Table 4.1 Compositions of experimental diets for Atlantic salmon with levels of B-GOS® inclusion. Proximate composition analyses of each diet are included following adapted AOAC (2016) procedures, presenting the crude protein, crude lipid, ash and moisture content.

Ingredient (% Inclusion)	**Control**	**2g kg^{-1}**	**4g kg^{-1}**	**6g kg^{-1}**	**8g kg^{-1}**	**10g kg^{-1}**
Sunflower Meal[a]	21.36	21.16	20.96	20.76	20.56	20.36
Corn Gluten Meal[a]	20.00	20.00	20.00	20.00	20.00	20.00
Soy Protein Concentrate[a]	20.00	20.00	20.00	20.00	20.00	20.00
Fish Meal[a]	8.00	8.00	8.00	8.00	8.00	8.00
Fish Oil[a]	8.00	8.00	8.00	8.00	8.00	8.00
Rapeseed Oil[b]	7.83	7.83	7.83	7.83	7.83	7.83
Soy Bean Meal[a]	7.00	7.00	7.00	7.00	7.00	7.00
Wheat Gluten Meal[a]	5.21	5.21	5.21	5.21	5.21	5.21
Vitamin/Mineral premix[c]	1.00	1.00	1.00	1.00	1.00	1.00
Lysine HCL[d]	0.70	0.70	0.70	0.70	0.70	0.70
CMC Binder[d,e]	0.50	0.50	0.50	0.50	0.50	0.50
DL Methionine[d]	0.40	0.40	0.40	0.40	0.40	0.40
B-GOS®	**-**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**
Crude Protein (%)	48.39 ± 1.34	47.95 ± 0.41	47.82 ± 0.41	47.93 ± 0.74	47.08 ± 0.25	47.31 ± 0.08
Crude Lipid (%)	21.00 ± 0.49	20.96 ± 0.11	20.92 ± 0.06	19.98 ± 0.05	19.83 ± 0.13	20.60 ± 0.63
Ash (%)	4.99 ± 0.10	4.78 ± 0.34	4.92 ± 0.19	5.21 ± 0.33	5.24 ± 0.14	4.99 ± 0.18
Moisture (%)	4.20 ± 0.24	4.19 ± 0.12	3.75 ± 0.23	3.87 ± 0.05	4.26 ± 0.07	3.81 ± 0.09

[a] Skretting, Norway

[b] Sainsbury's, Plymouth

[c] Premier Nutrition, UK

[d] Sigma-Aldrich, UK

[e] Carboxy methyl cellulose

Table 4.2 Primer sequences of target genes evaluated within the *S. salar* trial.

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Amplicon size (bp)	Tm (°C)	GenBank Accession Number
GAPDH	GCACCCATCGCCAAGGTTAT	AGTCTTCTGTGTGGCTGTGA	90	59.8	XM_014141819.1
β-actin	ACGGCATCGTCACCAACTG	CTCCTCTGGTGCCACTCTCA	83	60.8	NM_001123525.1
Elf-1α	GGCTGATTGTGCTGTGCTTAT	CACGAGTCTGCCCGTTCTTT	80	59.9	AF321836.1
IL-1β	AGGAGGGAAGCAGGGTTCA	CATCAGGACCCAGCACTTGT	81	60.1	AY617117.1
TNFα	GCACCGAAGACAACAAGGTTTA	GCTGAACACTGCTCCCACATA	131	59.9	EF079662.1
IL-10	ACGAAGGCATTCTACACCACTT	CACCGTGTCGAGGTAGAACT	83	59.5	EF165028.1
TGF-β	AAGGACCTGGGCTGGAAATG	CCTGGGAGTACTTGTTCTCTGT	106	59.5	EU082211.1
Cal	AGGCAAGAACCACCTCATCAA	GTGCCTGACTCCACCTTCTC	132	59.8	BT058985

4.2.4 Intestinal Microbiome Analysis

The posterior intestinal samples used for extracting DNA were obtained as per the sampling methods in Chapter 2 section 2.3. The salmon (total of 42) PCR amplicons were prepared for sequencing as described in Chapter 2, section 2.11, by using the Qubit® 2.0 Fluorometer (Thermo Fisher) system to determine sample concentrations. Samples were analysed for quality and integrity, before being prepared into amplicon libraries by ligating adapters and index sequences and hybridised overnight. PCR was performed and further clean-up steps were performed using AMPure XP bead washing (Beckman Coulter, USA). The 16S rRNA V1-V3 libraries were quantified and then further pooled and analysed for quality and concentration before sequencing on the MiSeq PEx300 Illumina platform (50K reads either direction) (Omega Bioservices, USA). The FASTQ files were downloaded from the Illumina BaseSpace online Sequencing Hub and further

analysed in the QIIME v2 pipeline (Caporaso *et al*. 2010; Edgar 2010; Bokulich *et al*. 2018; Bolyen *et al*. 2019). The QIIME v2 software was used as described in Chapter 3, section 3.2.4. The data was imported into the QIIME v2 environment to be analysed later down the pipeline using miniconda and python command lines. Once imported, the sequences were demultiplexed and denoised using the add-on 'DADA2' pipeline, which works to join paired-end forward and reverse reads together after each sample had passed the filtering criteria. The minimum bases per read to retain during this filtering was set to 295 bp to keep the highest possible percentage of reads during quality control.

Phylogenetic trees were generated using the 'q2-phylogeny' plugin, and alpha and beta diversity analyses were performed as described in section 3.2.4. The sampling depth for each species was chosen based on the lowest scoring reads for a sample in either species group, and so the depth of 6,000 was chosen for the salmon reads based on this criterion. Rarefaction plots were produced for alpha and beta diversity metrics, and taxonomic analyses were produced within the QIIME v2 environment using the same pre-trained classifier described in section 3.2.4. The feature table generated in QIIME v2 was filtered to remove *Streptophyta* and singletons from the analysis, as described in the Chapter 3, section 3.2.4 (Baldo *et al*. 2015; Gajardo *et al*. 2016).

Bar charts were generated from the feature tables that contained the assigned relative abundance sequence reads at the genus level, or as close to this taxonomic level as possible, to characterise the microbiome of Atlantic salmon fed incremental levels of GOS against fish fed a control diet. The software STAMP v2.1.3 and programme LEfSe Galaxy Version 1.0 were used to determine if there were significant differences in the

OTUs among the treatments (Parks & Beiko 2010; Segata *et al*. 2011; Parks *et al*. 2014; Afgan *et al.* 2018). Please refer to Chapter 3, section 3.2.4 for more information about these programmes.

4.3 Results

4.3.1 Growth Performance

No significant differences of salmon growth performance parameters were observed between fish fed the different dietary regimes for initial weights, final weights, FCR, SGR, PWG and K-Factor (Table 4.3).

4.3.2 Haematology

No significant differences were observed in Hb, RBC, WBC and MCH between fish fed the different dietary regimes (Table 4.4). In addition, no significant differences were observed for lymphocyte, basophilic granulocyte, monocyte and neutrophilic granulocyte cell counts between each dietary regime (Table 4.5).

4.3.3 Lysozyme Activity and Protein Concentration

There was a significant increase of serum lysozyme activity detected within fish fed the 2g kg^{-1} diet compared to fish fed the control diet. This significant difference was also apparent when the serum lysozyme activity was normalised against serum protein content. The opposite trend was also observed for the serum protein content, with levels lower in fish fed the 2g kg^{-1} and 8g kg^{-1} GOS diets compared to the control fed fish (Table 4.6).

Table 4.3 Mean growth performance parameters (± SD) of juvenile salmon fed incremental levels of B-GOS® over a period of 8 weeks (60 days).

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Initial Weight (g)	20.60 ± 0.18	20.56 ± 0.06	20.69 ± 0.29	20.71 ± 0.29	20.75 ± 0.13	20.52 ± 0.38	**0.805**	$\chi^2_{(5,18)}=2.31$
Final Weight (g)	42.75 ± 0.47	42.46 ± 1.61	43.29 ± 0.60	41.55 ± 0.65	42.40 ± 0.96	42.90 ± 1.08	**0.545**	$F_{5,12}=0.84$
FCR	0.99 ± 0.03	1.00 ± 0.06	0.97 ± 0.01	1.04 ± 0.03	1.01 ± 0.04	0.98 ± 0.02	**0.332**	$\chi^2_{(5,18)}=5.75$
SGR	1.22 ± 0.03	1.21 ± 0.06	1.23 ± 0.01	1.16 ± 0.03	1.19 ± 0.04	1.23 ± 0.01	**0.268**	$\chi^2_{(5,18)}=6.41$
PWG (%)	107.50 ± 3.89	106.47 ± 7.35	109.27 ± 0.98	99.33 ± 3.41	104.32 ± 4.73	109.02 ± 1.82	**0.253**	$\chi^2_{(5,18)}=6.59$
K-Factor	1.28 ± 0.08	1.26 ± 0.06	1.27 ± 0.06	1.27 ± 0.06	1.29 ± 0.14	1.26 ± 0.06	**0.894**	$\chi^2_{(5,18)}=1.66$

Table 4.4 Mean haemoglobin (Hb), erythrocytes (RBC), leucocytes (WBC), mean corpuscular haemoglobin (MCH) (± SD) of Atlantic salmon fed incremental levels of B-GOS® over a period of 8 weeks (n=9 fish per diet).

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Hb (g L^{-1})	85.31 ± 15.97	80.78 ± 7.44	89.60 ± 14.95	90.44 ± 14.08	82.24 ± 14.70	92.07 ± 16.55	**0.472**	$F_{5,48}=0.93$
RBC ($x10^6/mm^3$)	0.87 ± 0.13	0.81 ± 0.31	0.74 ± 0.21	0.85 ± 0.18	0.76 ± 0.36	0.81 ± 0.21	**0.759**	$F_{5,48}=0.52$
WBC ($x10^4/mm^3$)	1.98 ± 0.32	2.03 ± 0.48	2.12 ± 0.21	1.97 ± 0.24	2.33 ± 0.53	2.08 ± 0.48	**0.619**	$F_{5,48}=0.71$
MCH (pg)	100.65 ± 28.40	100.37 ± 27.96	120.26 ± 38.21	100.47 ± 16.94	112.32 ± 21.42	121.80 ± 40.06	**0.462**	$F_{5,48}=0.95$

Table 4.5 Mean percentages of lymphocytes, basophilic granulocytes, monocytes and neutrophilic granulocytes (± SD) of Atlantic salmon fed incremental levels of B-GOS® over a period of 8 weeks (n=9 fish per diet).

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Lymphocytes (%)	93.28 ± 2.33	92.83 ± 1.97	92.39 ± 2.52	94.22 ± 1.73	94.06 ± 2.90	90.89 ± 3.66	**0.095**	**$F_{5,48}$=2.01**
Basophilic Granulocytes (%)	0.44 ± 0.39	0.39 ± 0.55	0.56 ± 0.39	0.22 ± 0.26	0.28 ± 0.36	0.44 ± 0.46	**0.521**	**$\chi^2(5,54)$=4.20**
Monocytes (%)	2.06 ± 0.85	2.00 ± 0.71	3.11 ± 0.93	2.17 ± 0.97	1.78 ± 1.15	3.33 ± 1.64	**0.114**	**$\chi^2(5,54)$=9.12**
Neutrophilic Granulocytes (%)	4.22 ± 1.75	4.78 ± 1.77	3.94 ± 1.72	3.39 ± 1.08	3.89 ± 2.18	5.33 ± 2.45	**0.350**	**$\chi^2(5,54)$=5.57**

Table 4.6 Mean lysozyme activity and protein content (± SD) within the blood serum of Atlantic salmon fed incremental levels of B-GOS® over a period of 8 weeks (n=7 fish per diet for blood serum). The serum lysozyme activity (lysozyme/min/mL) was normalised using the serum protein concentration (in mg/mL). Outliers were removed from the analysis. Different letters between data denote significant difference between different letters on the same row, $p<0.05$.

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Serum Lysozyme Activity	152.38 ± 30.83^{a}	209.33 ± 45.46^{b}	165.58 ± 15.01^{ab}	194.25 ± 22.76^{ab}	170.43 ± 37.06^{ab}	195.62 ± 28.92^{ab}	**0.013**	**$F_{5,36}$=3.40**
Serum Protein Content	45.21 ± 5.70^{a}	37.69 ± 3.47^{b}	41.09 ± 3.03^{ab}	42.14 ± 4.78^{ab}	36.92 ± 3.58^{b}	41.74 ± 3.35^{ab}	**0.002**	**$F_{5,36}$=4.49**
Normalised Serum Lysozyme Activity	3.41 ± 0.71^{a}	6.02 ± 1.50^{b}	3.98 ± 0.43^{ab}	4.62 ± 0.42^{ab}	4.70 ± 1.32^{ab}	4.84 ± 0.63^{ab}	**0.002**	**$\chi^2(5,42)$=19.20**

4.3.4 Histology

There were no significant differences in the intestinal muscularis thickness, mucosal fold heights, lamina propria or intestinal goblet cell counts between fish fed the different dietary treatments. In addition, no significant differences were detected in goblet cell counts within the mucosal layer of the skin of fish fed any diet (Table 4.7). Representative images of fish from each dietary treatments are presented in Figures 4.1 and 4.2.

4.3.5 Body Composition

Proximate compositional analyses were utilised to determine that all experimental treatments were comparable. There were no significant differences between fish fed each diet for the carcass moisture content, ash content, crude protein content or lipid content; see Table 4.8.

Table 4.7 Mean histological analyses (± SD) of Atlantic salmon fed incremental levels of B-GOS® over a period of 60 days (n=9 fish per diet).

Diet	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Muscularis Thickness (µm)	42.05 ± 9.80	43.23 ± 10.38	49.79 ± 16.39	46.88 ± 19.21	41.90 ± 8.28	43.20 ± 7.97	**0.899**	$\chi^2_{(5,54)}=1.62$
Mucosal Fold Height (µm)	434.43 ± 78.97	454.12 ± 67.27	437.63 ± 121.08	424.36 ± 74.37	441.49 ± 53.42	461.24 ± 60.20	**0.932**	$F_{5,48}=0.26$
Lamina Propria Width (µm)	22.24 ± 2.46	23.13 ± 3.23	25.53 ± 3.38	23.66 ± 2.33	25.23 ± 4.02	24.92 ± 3.42	**0.202**	$F_{5,48}=1.52$
Intestine Goblet Cell Counts	12.11 ± 1.84	10.75 ± 1.92	12.28 ± 3.13	11.82 ± 1.84	12.17 ± 1.44	10.80 ± 2.77	**0.477**	$F_{5,48}=0.92$
Skin Goblet Cell Counts	18.81 ± 1.65	17.67 ± 1.89	17.21 ± 4.17	18.83 ± 2.82	16.92 ± 3.05	18.40 ± 2.16	**0.551**	$F_{5,48}=0.81$

Table 4.8 Mean carcass compositional analyses (± SD) of Atlantic salmon before the start of the trial (n=6 fish) and salmon fed incremental levels of B-GOS® over a period of 8 weeks (n=6 fish per diet). Ash content, Protein content and Lipid content are expressed as a percentage of the dry matter. One sample in the 2g kg^{-1} diet was removed as an outlier.

Diet	Pre-trial	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Moisture Content (%)	66.65 ± 4.57	66.62 ± 0.69	66.30 ± 0.63	66.59 ± 0.79	67.49 ± 0.70	66.83 ± 0.83	67.29 ± 0.96	**0.119**	$F_{5,30}=1.93$
Ash Content (%)	7.37 ± 0.67	5.47 ± 0.49	5.22 ± 0.52	5.23 ± 0.16	5.46 ± 0.49	5.26 ± 0.42	5.55 ± 0.43	**0.669**	$F_{5,30}=0.64$
Protein Content (%)	61.16 ± 3.27	58.19 ±1.60	58.77 ± 0.94	58.82 ± 0.67	58.45 ± 1.71	57.83 ± 1.24	59.33 ± 1.47	**0.477**	$F_{5,30}=0.93$
Lipid Content (%)	29.77 ± 1.82	36.20 ± 1.77	37.37 ± 1.18	36.55 ± 1.19	35.33 ± 1.44	35.91 ± 2.02	35.05 ± 1.97	**0.241**	$F_{5,30}=0.24$

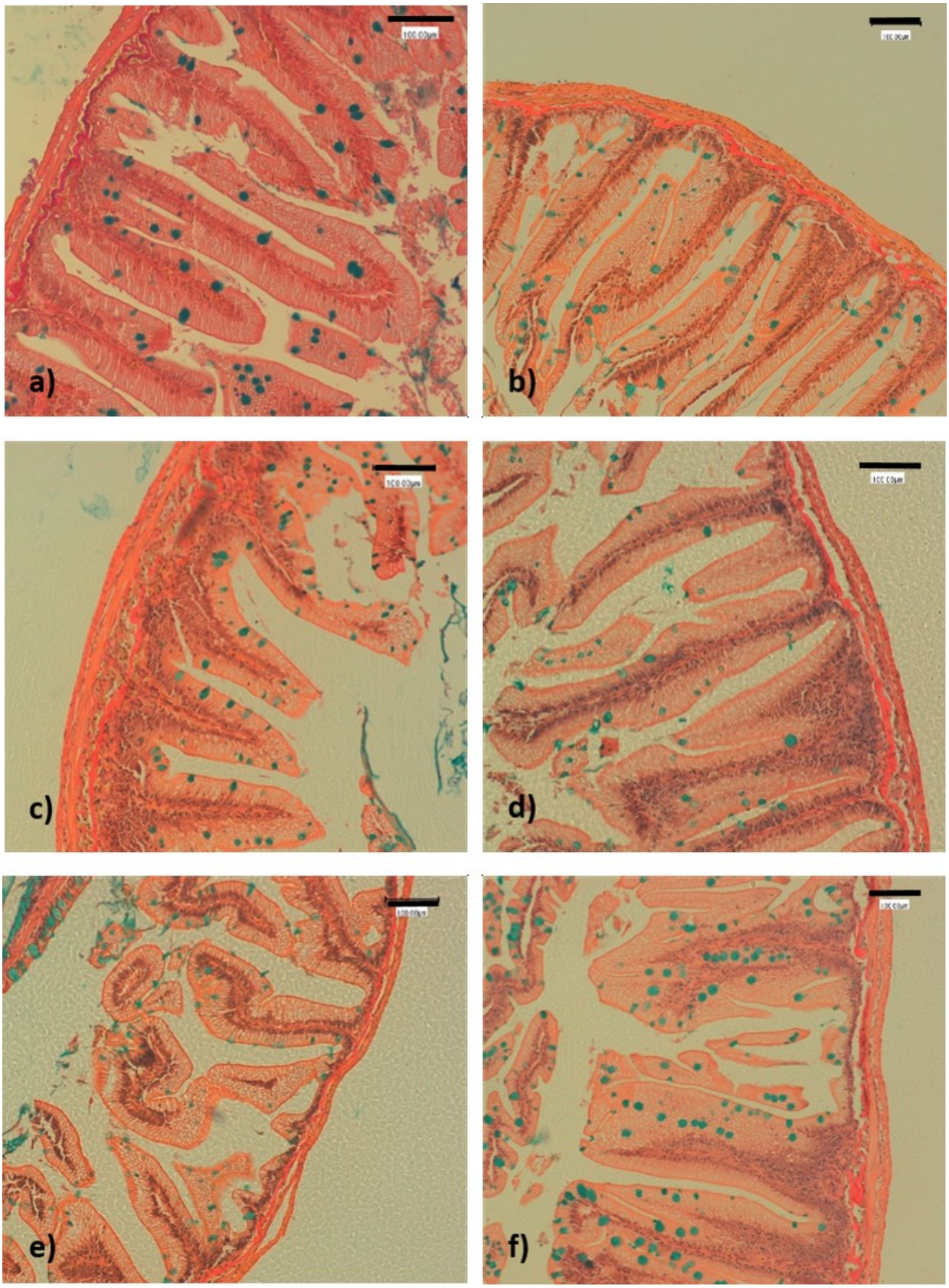


Figure 4.1 Representative intestinal images of individual Atlantic salmon sampled from each diet, stained with AB/vG: **a)** Control, **b)** 2g kg^{-1}, **c)** 4g kg^{-1}, **d)** 6g kg^{-1}, **e)** 8g kg^{-1} and **f)** 10g kg^{-1}. Scale bar = 100 µm.

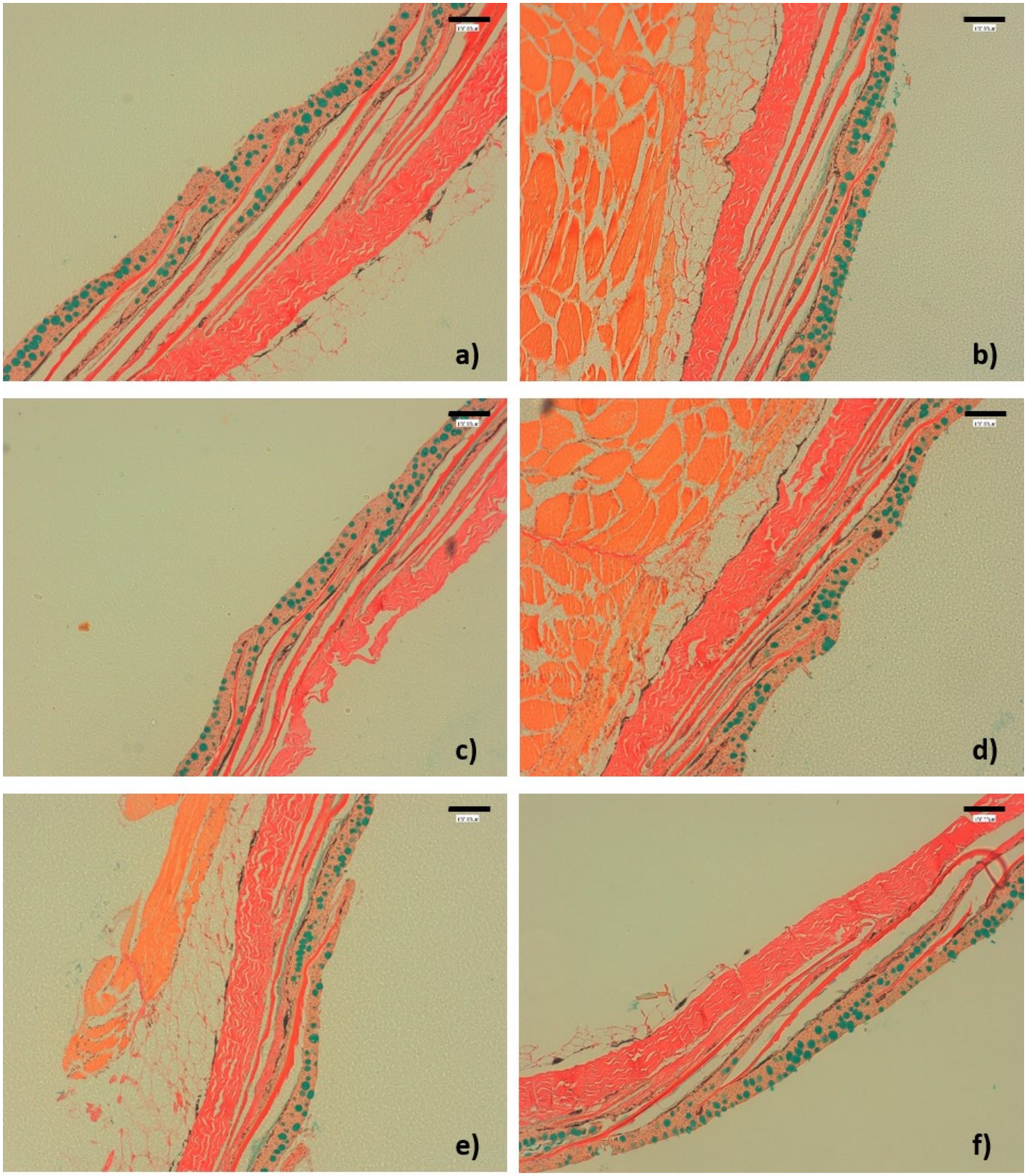


Figure 4.2 Representative skin images of individual Atlantic salmon sampled from each diet, stained with AB/vG: **a)** Control, **b)** 2g kg^{-1}, **c)** 4g kg^{-1}, **d)** 6g kg^{-1}, **e)** 8g kg^{-1} and **f)** 10g kg^{-1}. Scale bar = 100 µm.

4.3.6 Gene Expression

The expression of five target genes in the posterior intestine of Atlantic salmon after 8 weeks of dietary feeding on the experimental diets is represented as fold change relative to the control, and presented in Figure 4.3. Outliers that were previously removed as described in section 4.2.3 were not taken forward in this analysis and so n=4 samples per diet were analysed.

4.3.6.1 Intestine

Permutation tests were performed instead of ANOVA or Kruskal-Wallis models, as described in section 2.12, to determine if significant differences between the expression levels of fish fed each diet were present for each target gene, for the target tissue. The significant outputs for these permutation tests are presented as different letters above the bars in Figure 4.3. These tests for target genes measured in the intestine of Atlantic salmon demonstrated that fish fed the 2g kg^{-1}, 4g kg^{-1} and 8g kg^{-1} diets had significantly upregulated expression of *Cal* compared to the control (p=0.024, p=0.045, p=0.011, respectively). In a similar trend for the same target gene, the fish fed 2g kg^{-1}, 4g kg^{-1} and 8g kg^{-1} diets had significantly upregulated expression compared to fish fed the 6g kg^{-1} diet (p=0.021, p=0.036, p=0.009, respectively).

The gene expression of *IL-10* was significantly downregulated in fish fed the 6g kg^{-1}, 8g kg^{-1} and 10g kg^{-1} diets compared to the control fed fish (p=0.013, p=0.032, p=0.018, respectively). Fish fed the 2g kg^{-1} diet had significantly upregulated expression of *IL-10* compared to all other experimental diets except the control fed fish ($p<0.05$). The expression of 6g kg^{-1}, 8g kg^{-1} and 10g kg^{-1} was significantly downregulated compared to the 4g kg^{-1} diet fed fish (p=0.013, p=0.034, p=0.019, respectively); the 6g kg^{-1} diet

group also demonstrated significant downregulation of this target gene compared to fish fed 8g kg^{-1} and 10g kg^{-1} diets (p=0.039, p=0.036, respectively).

The gene expression of *IL-1β* was significantly upregulated in the 8g kg^{-1} diet compared to the control fed fish (p=0.017), and this target gene was also significantly elevated in the 2g kg^{-1} and 8g kg^{-1} fed fish compared to those fed 10 g kg^{-1} diet (p=0.050, p=0.019). The expression of *TGF-β* was significantly downregulated in fish fed the 6g kg^{-1} and 10g kg^{-1} diets compared to the control (p=0.026, p=0.033, respectively), and this effect was also present in fish fed the 2g kg^{-1} diet compared to those fed 4g kg^{-1} inclusion rate (p=0.042). Atlantic salmon fed the 4g kg^{-1} diet displayed significantly upregulated *TGF-β* expression compared to those fed the 6g kg^{-1} (p=0.033) and 10 g kg^{-1} diets (p=0.038), and this was also demonstrated in the 6g kg^{-1} diet fed fish compared to the 8g kg^{-1} group (p=0.046). *TNFα* expression was significantly upregulated in fish fed 2g kg^{-1} (p=0.039) and 4g kg^{-1} (p=0.025) compared to the control, and this trend was demonstrated by these same diets each against the 6g kg^{-1}, 8g kg^{-1} and 10g kg^{-1} diets ($p<0.05$).

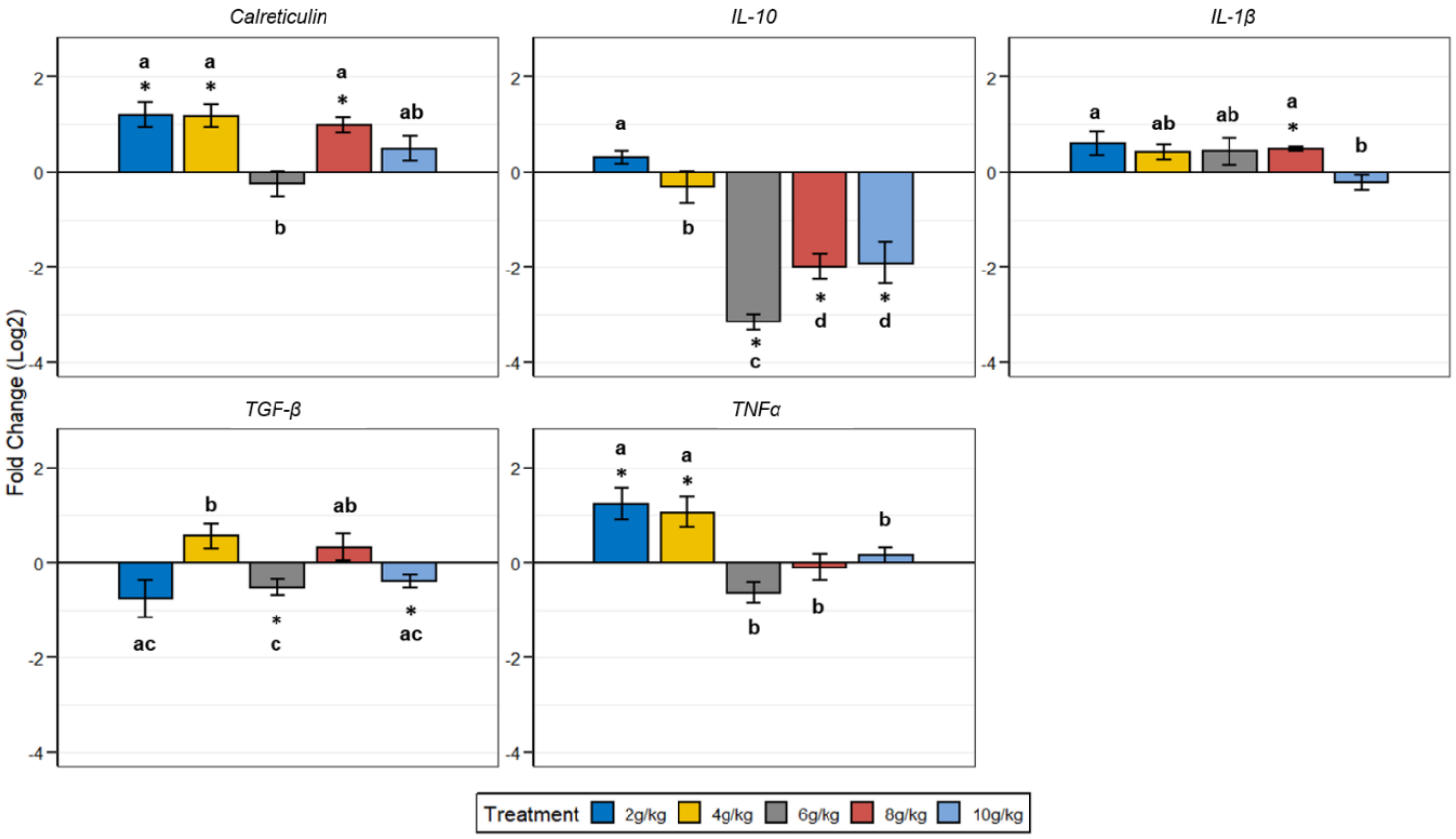


Figure 4.3 Gene expression data presented as fold change (log_2) of 5 target genes relative to the control group in the intestine of Atlantic salmon fed six dietary treatments containing incremental levels of B-GOS® over a period of 8 weeks (n=4 fish per diet, per gene). Significant differences are denoted by differing letters between treatments, an asterisk between the treatment and control. Data presented as mean ± SEM.

4.3.7 Intestinal Microbiome Analysis

The high-throughput 16S rRNA gene sequencing analysis of the microbiomes of Atlantic salmon fed control vs prebiotic supplemented diets yielded a total of 4,762,065 paired-end sequence reads from 42 samples, and the average number of reads per replicate fish being 113,382.50 ± 48,163.32 after demultiplexing. After 'DADA2' quality control and filtering, the average number of reads across all diets was 19,347.40 ± 24,684.74 and these sequences were taken forward to taxonomy assignment using the pre-trained classifier 'SILVA 138 99% OTU full-length sequences' as a reference dataset. The Good's coverage estimates for each diet were >0.999, indicating that the sequencing coverage

was sufficient. The number of OTUs observed within the intestine of salmon did significantly differ between fish fed the 4g kg^{-1} diet (13.57 ± 3.46 %) and the 6g kg^{-1} diet (41.14 ± 40.14 %; p = 0.044), (Table 4.9, Figure 4.4). The species richness diversity (Chao1) and Shannon diversity index did not differ significantly between treatments (Table 4.10).

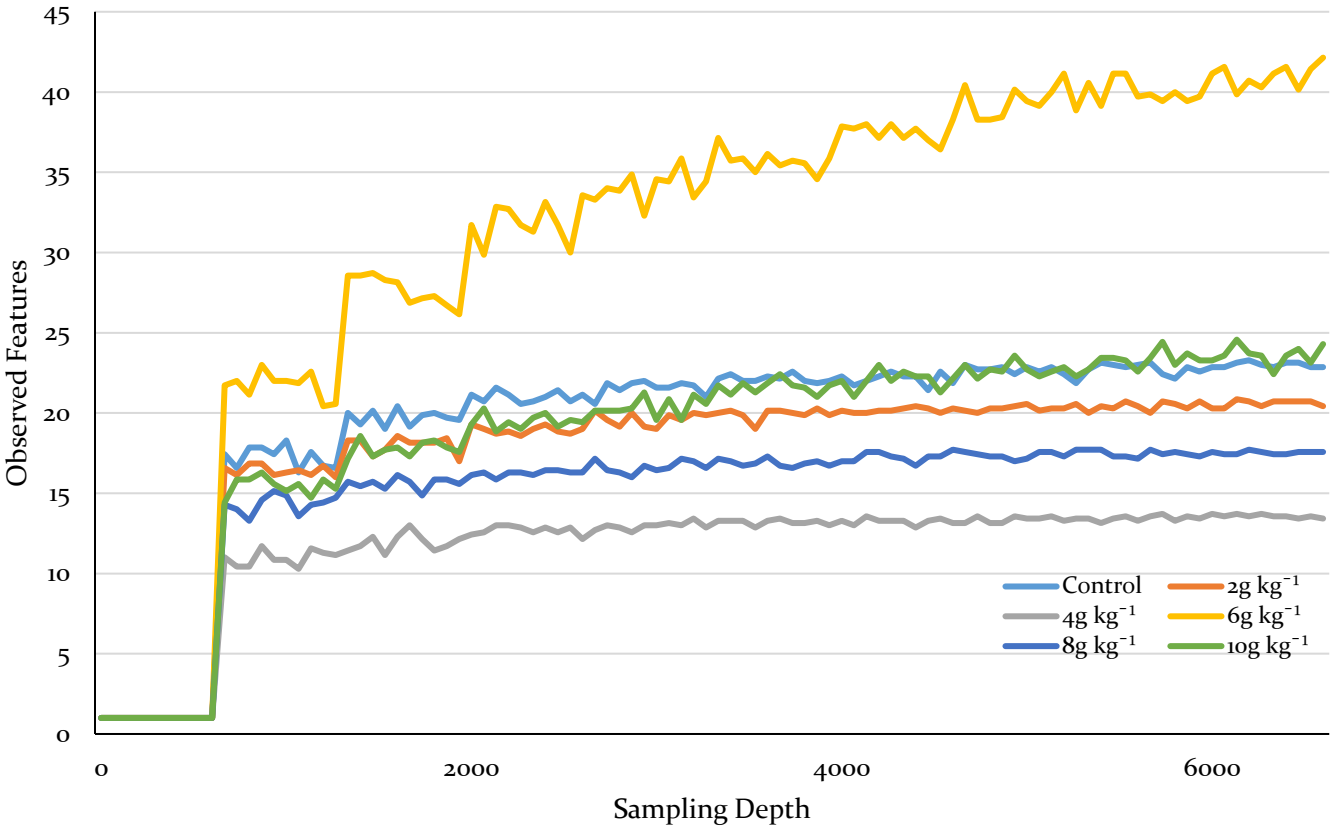


Figure 4.4 Comparison of observed OTU features as rarefaction curves within the digesta of Atlantic salmon (n=7 fish per diet), fed control diet or diets supplemented with incremental levels of B-GOS® over a period of 8 weeks.

Table 4.9 OTUs observed after QIIME 2 analysis and diversity/richness metrics of intestinal microbiota composition in Atlantic salmon fed incremental levels of B-GOS®, over a period of 8 weeks (n=7 fish per diet). Different letters between data denote significant difference between different letters on the same row, p<0.05.

Alpha Diversity	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
OTUs observed	23.14 ± 7.60^{ab}	20.71 ± 10.31^{ab}	13.57 ± 3.46^{a}	41.14 ± 40.14^{b}	17.43 ± 3.10^{ab}	24.29 ± 21.66^{ab}	**0.044**	**χ^2(5,42)=11.40**
Good's coverage	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	**0.343**	**χ^2(5,42)=5.64**
Chaol diversity	23.38 ± 23.38	20.74 ± 10.37	13.55 ± 3.55	48.55 ± 52.36	17.96 ± 3.52	25.69 ± 25.33	**0.053**	**χ^2(5,42)=10.95**
Shannon's diversity index	3.17 ± 0.36	3.04 ± 0.48	2.82 ± 0.07	3.14 ± 0.34	3.00 ± 0.18	2.99 ± 0.33	**0.395**	**χ^2(5,42)=1.07**

Table 4.10 Relative abundance (mean ± SD) of bacterial sequences at the genus level (or lowest taxonomic level) present within the intestinal digesta of Atlantic salmon fed incremental levels of B-GOS®, over a period of 8 weeks (n=7 fish per diet). Different letters between data denote significant difference between different letters on the same row, p<0.05.

Bacterial genus relative abundance (%)	Control	2g kg^{-1}	4g kg^{-1}	6g kg^{-1}	8g kg^{-1}	10g kg^{-1}	p-value	Test Statistic
Others	0.41 ± 0.40	0.28 ± 0.41	0.35 ± 0.70	1.55 ± 3.30	0.08 ± 0.11	0.34 ± 0.56	**0.285**	**χ^2(5,42)=6.23**
Corynebacterium	0.20 ± 0.37	0.07 ± 0.09	0.00 ± 0.00	0.04 ± 0.08	0.00 ± 0.00	0.02 ± 0.06	**0.123**	**χ^2(5,42)=8.68**
Frigoribacterium	0.01 ± 0.03	0.01 ± 0.03	0.00 ± 0.01	0.08 ± 0.17	0.00 ± 0.00	0.02 ± 0.03	**0.443**	**χ^2(5,42)=4.78**
Microbacterium	0.00 ± 0.01	0.03 ± 0.05	0.00 ± 0.00	0.02 ± 0.03	0.01 ± 0.03	0.05 ± 0.11	**0.476**	**χ^2(5,42)=4.53**
c. Bacilli	0.00 ± 0.00	0.06 ± 0.14	0.00 ± 0.00	0.07 ± 0.20	0.00 ± 0.00	0.00 ± 0.01	**0.258**	**χ^2(5,42)=6.53**
Bacillus	1.88 ± 2.99	2.24 ± 2.82	0.00 ± 0.01	0.13 ± 0.25	0.40 ± 0.61	4.27 ± 10.90	**0.265**	**χ^2(5,42)=6.45**
Rummeliibacillus	0.02 ± 0.05^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.93 ± 1.47^{ab}	1.40 ± 1.70^{b}	0.31 ± 0.60^{ab}	**0.002**	**χ^2(5,42)=18.50**
Aerococcus	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.53 ± 0.61^{b}	0.55 ± 1.42^{ab}	0.00 ± 0.00^{a}	**0.001**	**χ^2(5,42)=25.48**
Enterococcus	5.48 ± 11.28	1.11 ± 1.82	0.00 ± 0.00	0.79 ± 1.54	1.08 ± 2.00	0.29 ± 0.61	**0.240**	**χ^2(5,42)=6.74**
Lactobacillus	89.31 ± 16.79^{ab}	84.34 ± 17.90^{a}	98.98 ± 0.71^{b}	94.43 ± 5.04^{ab}	94.46 ± 5.65^{ab}	94.03 ± 12.28^{ab}	**0.038**	**χ^2(5,42)=11.79**
Weissella	1.76 ± 2.89	1.02 ± 1.82	0.12 ± 0.18	0.42 ± 0.30	0.15 ± 0.10	0.16 ± 0.13	**0.135**	**χ^2(5,42)=8.71**

Streptococcus	0.37 ± 0.21	0.37 ± 0.33	0.37 ± 0.19	0.59 ± 0.19	0.44 ± 0.29	0.37 ± 0.24	**0.485**	**$F_{5,36}$=0.91**
Macrococcus	0.09 ± 0.19	0.01 ± 0.03	0.00 ± 0.00	0.04 ± 0.11	0.12 ± 0.32	0.02 ± 0.06	**0.737**	**$\chi^2(5,42)$=2.76**
Staphylococcus	0.46 ± 0.44	2.91 ± 4.85	0.17 ± 0.17	0.38 ± 0.24	1.30 ± 3.01	0.12 ± 0.20	**0.064**	**$\chi^2(5,42)$=10.44**
Aeromonas	0.00 ± 0.44	7.56 ± 20.00	0.00 ± 0.17	0.00 ± 0.24	0.00 ± 3.01	0.00 ± 0.20	**0.676**	**$\chi^2(5,42)$=3.16**

The percentage relative abundance of some distinct genera (where relative abundance was reported at a threshold of over 0.01% of total reads) sequenced from the digesta of Atlantic salmon fed either the control or experimental diets trial are shown in Table 4.10 and Figure 4.5. Any unique genera detected that were not above the threshold level were grouped into the category 'Others'. The phylum *Firmicutes* accounted for >90% of the assigned taxonomy reads in the control and prebiotic dietary groups, however the relative abundances were not significantly different between dietary groups. The relative abundances of phyla *Cyanobacteria*, *Proteobacteria* and *Actinobacteriota* were also detected across all treatments, however were also not significantly different between dietary regimes.

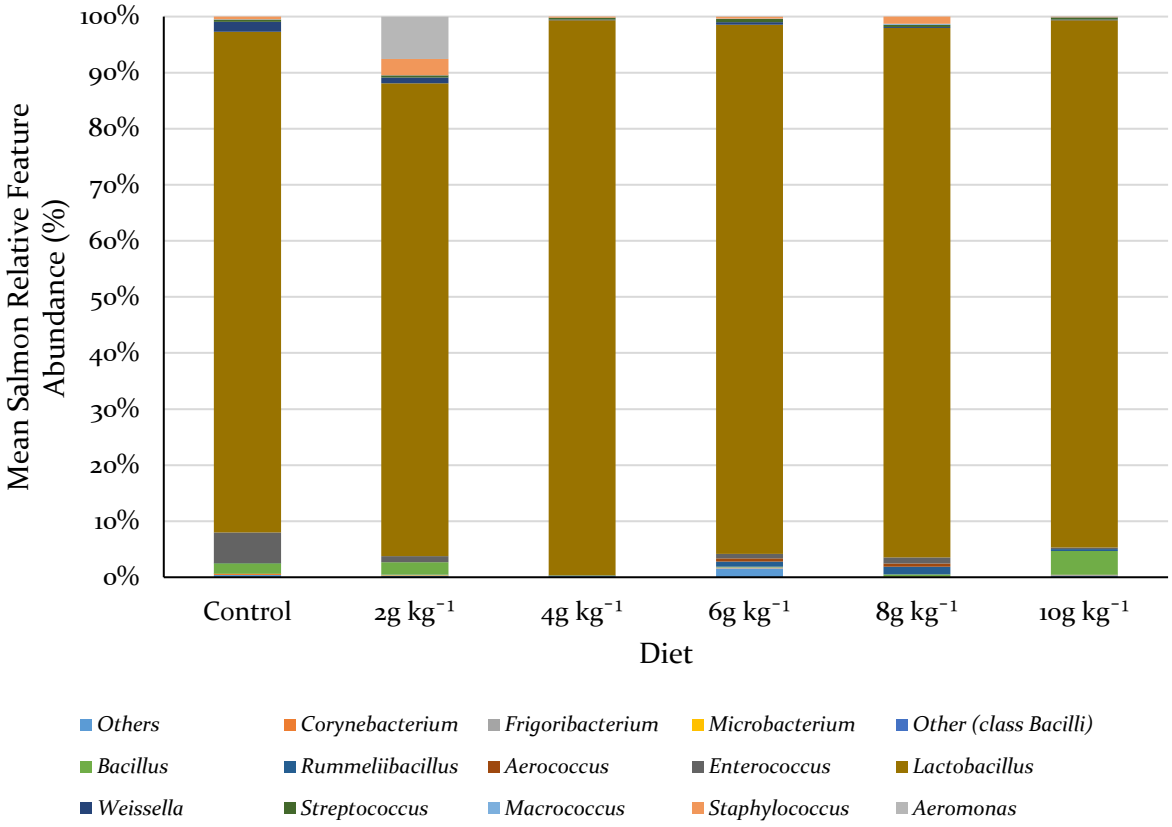


Figure 4.5 Percentage relative OTU abundance (%) of bacterial sequences at the genus level or lowest taxonomic level present within the digesta of Atlantic salmon (n=7 fish per diet), fed incremental levels of B-GOS® over a period of 8 weeks.

The relative abundance of above threshold reads belonged to 15 genera. As described in Chapter 3, section 3.3.9, STAMP v2.1.3 (Parks & Beiko 2010; Parks *et al*. 2014) and LEfSe Galaxy Version 1.0 (Segata *et al*. 2011) via Galaxy Hub software (Afgan *et al.* 2018) were used to detect any distinct genera. Of the genera sequenced in this trial, *Rummeliibacillus*, *Aerococcus* and *Lactobacillus* were determined to be significantly different within fish fed the experimental diets of this trial (Table 4.10). These data are represented in the relative abundance of features graph (Figure 4.5), and as a heatmap showing relative abundance and relatedness of the samples in each diet (Figure 4.6).

The 'Others' category was assigned in a similar way to the phyla present, and makes up a small proportion of the reads for this trial; this category was not significantly more or less abundant between diets (Table 4.10). *Enterococcus* and *Bacillus* were the next most abundant genera present across all diets, but no significant differences between dietary treatments was observed. This result was also observed for the relative abundances of *Weissella*, *Staphylococcus*, *Corynebacterium*, *Streptococcus*, *Macroccocus*, class *Bacilli*, *Frigoribacterium*, *Microbacterium* and *Aeromonas* (Table 4.10).

The LEfSe histogram for the most distinct bacterial genus detected is presented in Figure 4.7, where the relative abundance for the genus *Lactobacillus* is plotted as this taxa was found to be significantly distinct between the experimental diets. The statistical tests that were performed in R Studio complement the data used in STAMP, and further post-hoc analyses were completed within the STAMP software, which are represented in Figure 4.8. The post-hoc tests show the differences between diets for genera *Rummeliibacillus*, *Aerococcus* and *Lactobacillus* (Figure 4.8 a, b, c respectively).

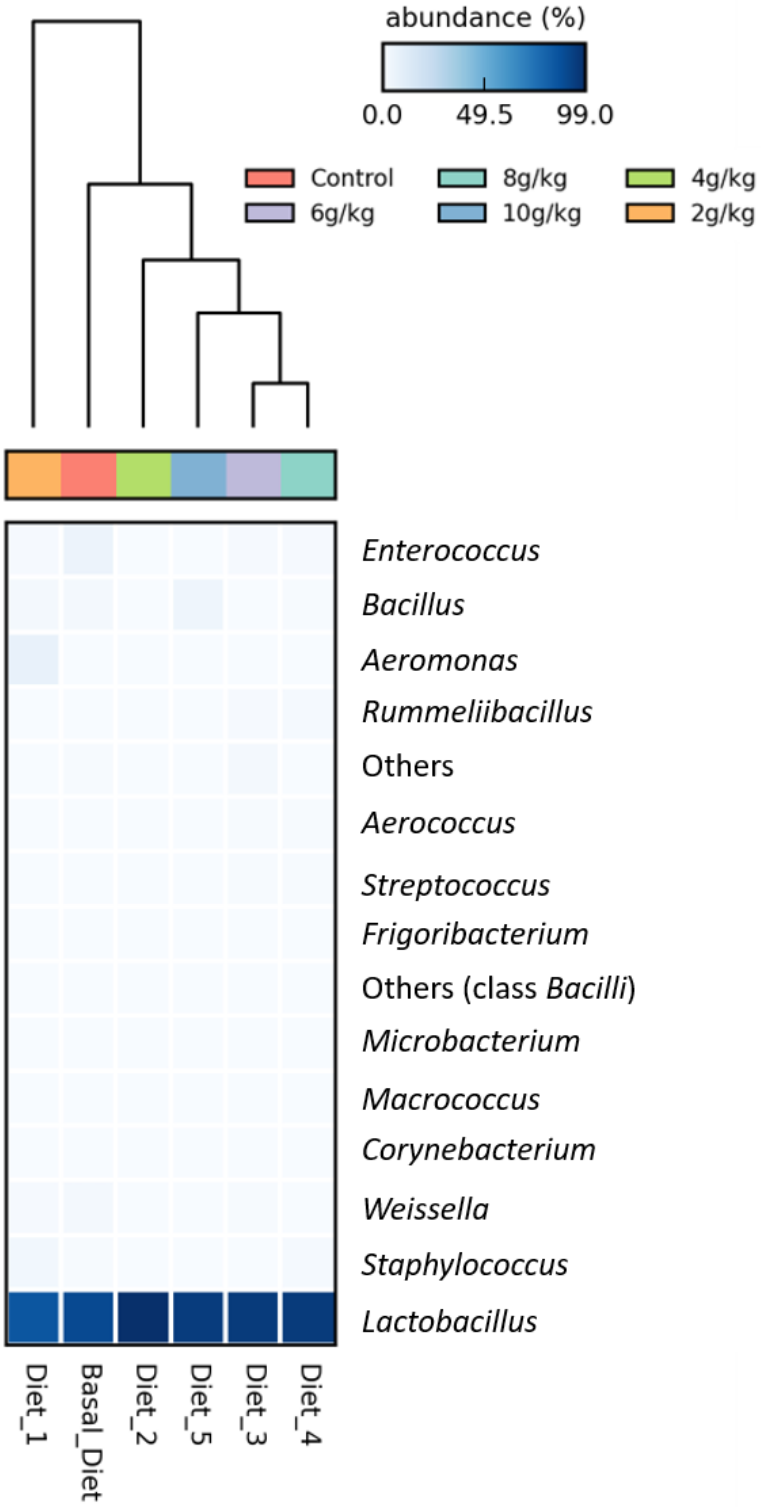


Figure 4.6 Mean abundance levels (%) of each genus present (or closest taxonomic level) within the microbiota of the intestinal digesta samples (n=7 fish per diet) for Atlantic salmon fed incremental levels of B-GOS® over a period of 8 weeks. The treatments are represented as: Control = Basal_Diet; 2g kg^{-1} = Diet_1; 4g kg^{-1} = Diet_2; 6g kg^{-1} = Diet_3; 8g kg^{-1} = Diet_4 and 10g kg^{-1} = Diet_5.

The LEfSe software generated a cladogram for the significant genus detected and this is represented in Figure 4.9. The middle unconnected point of the cladogram represents the Domain, and each point radiating out from the centre represents each incremental level of taxonomy, for instance, the L2 (phyla) taxa are on the second level from the centre point. Based on the results from the LEfSe, the genus shown as the sixth level from the centre point is *Lactobacillus* (Figure 4.9).

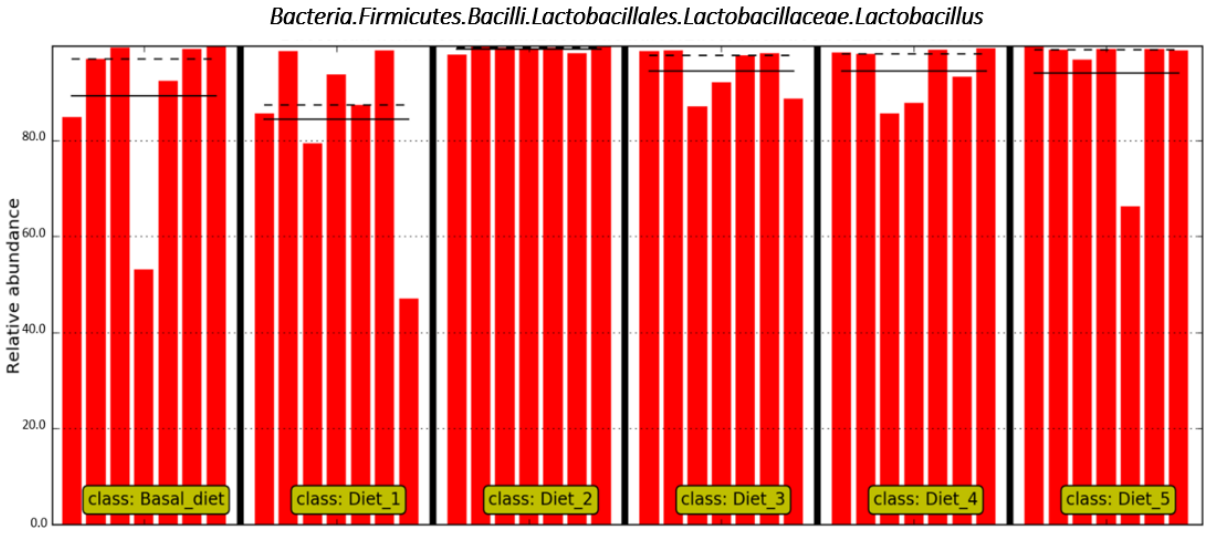


Figure 4.7 Differential features histogram plots of the biomarker OTUs detected by LEfSe showing the relative abundance (%) of each genus by diet. The diets are called class, as generated by the LEfSe analysis. The bacterial genus detected is shown as *Lactobacillus*. The dotted line (- - -) represents the medians of each diet and the straight line (—) represents the means of each diet. The treatments are represented as: Control = Basal_Diet; 2g kg^{-1} = Diet_1; 4g kg^{-1} = Diet_2; 6g kg^{-1} = Diet_3; 8g kg^{-1} = Diet_4 and 10g kg^{-1} = Diet_5.

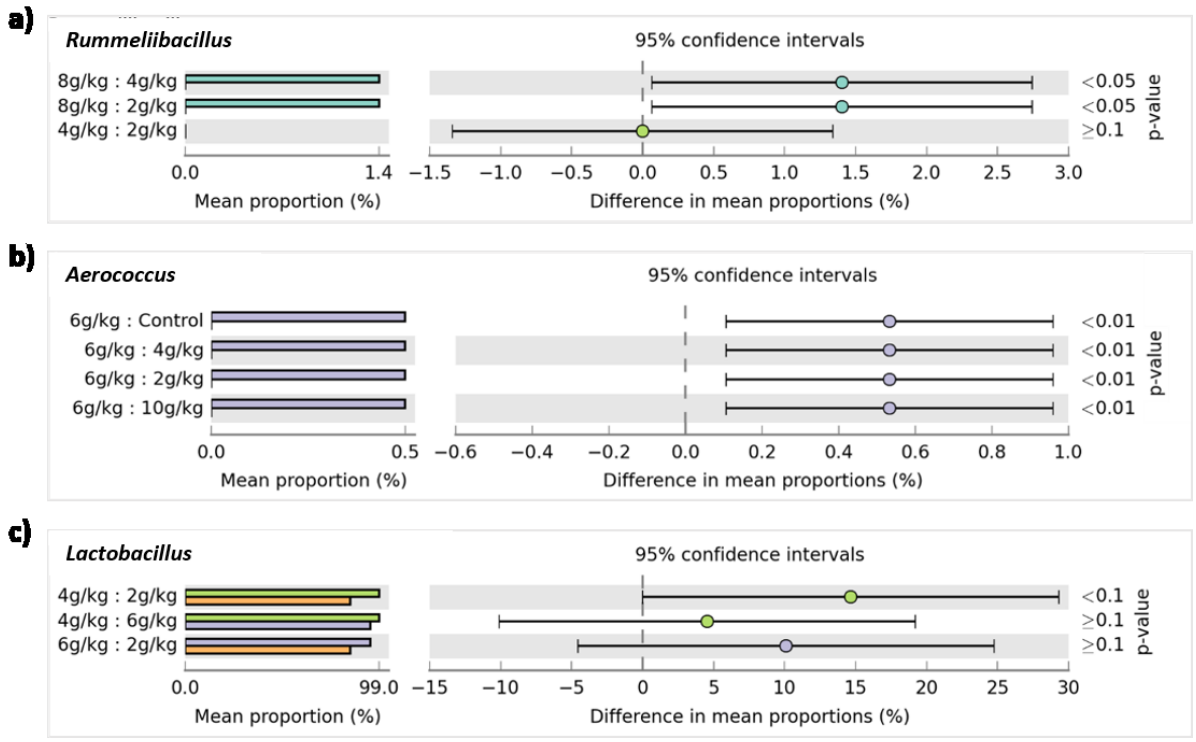


Figure 4.8 Post-hoc plots for the relative abundances of each bacterial genus that STAMP detected as being significantly different between fish fed the experimental diets. Statistical differences were accepted at p<0.05. The genera detected are shown as **a)** *Rummeliibacillus*, **b)** *Aerococcus* and **c)** *Lactobacillus*.

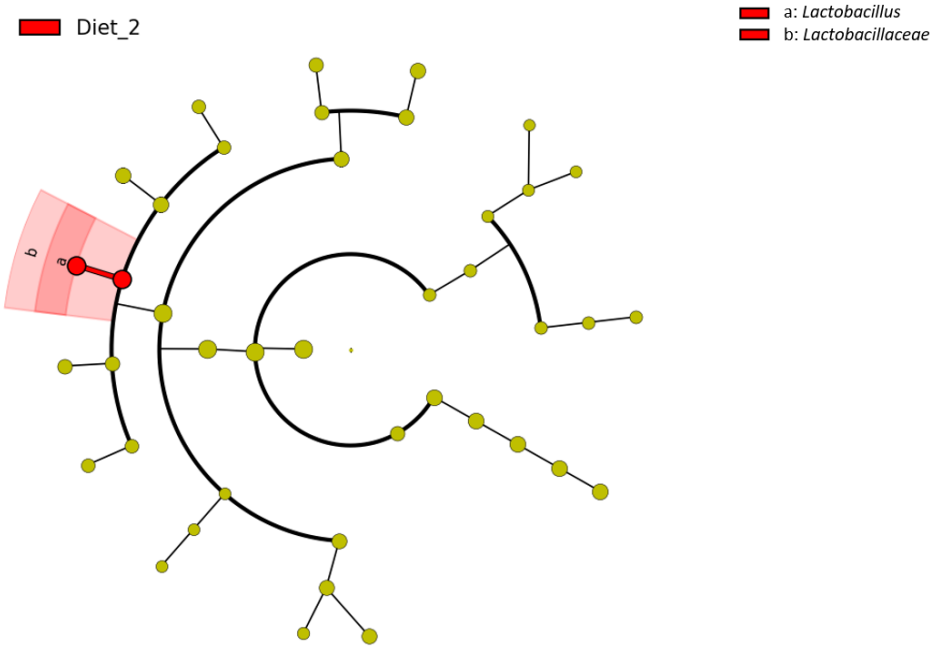


Figure 4.9 Circular cladogram reporting the identified OTU from the LEfSe output for salmon and are distributed according to phylogenetic characteristics between treatments. The family and genus that are significantly different between each compartment of the cladogram are coloured differently to the yellow taxon levels that indicate OTUs with similar abundances, and are listed on the right side of the figure. The diets are represented as: Control = Basal_Diet; 2g kg^{-1} = Diet_1; 4g kg^{-1} = Diet_2; 6g kg^{-1} = Diet_3; 8g kg^{-1} = Diet_4 and 10g kg^{-1} = Diet_5.

4.4 Discussion

The aim of the present chapter was to understand how a novel GOS additive may benefit Atlantic salmon growth performance and health. This study has contributed to the understanding of how performance and immune responses to dietary additives differ between closely related species, as in-depth analytical techniques such as intestinal expression of key biomarkers, changes to the histomorphology in the intestine and the abundance of microorganisms within the GIT microbiome have demonstrated this.

The aquaculture industry has endeavoured to meet rising international demands for protein, with Atlantic salmon accounting for a significant proportion of finfish production (FAO 2020). As this species has been comprehensively investigated in studies formulating higher percentage plant-based diets in comparison to their naturally piscivorous diet (Ytrestøyl *et al*. 2015), there is a substantial amount of literature focusing on the GIT microbiome (Gatesoupe 1999; Gómez & Balcázar 2007; Merrifield *et al.* 2010a,b,c; Ringø *et al.* 2014; Merrifield & Rodiles 2015; Vargas-Albores *et al*. 2021). More recent high-throughput sequencing techniques of the Atlantic salmon metagenome have further broadened our understanding of how commensal bacteria are affected by diet (Gajardo *et al*. 2016).

This second *in vivo* feeding trial was designed with similar objectives as with Chapter 3; to investigate the efficacy of the prebiotic B-GOS® supplemented to a closely related salmonid to rainbow trout to understand how fish growth and performance are affected. Based on multiple metrics measured in this study, such as histological appraisal and microbiome relative abundance sequencing, the results from this chapter suggest that the inclusion of dietary B-GOS® did not significantly affect salmon growth at the whole

organism level, however, some modulations in immune functions at a localised level in the GIT suggest some potential benefits to Atlantic salmon under the conditions investigated.

4.4.1 Growth performance

Despite relative improvements in multiple growth metrics, such as final weights, FCR, SGR and PWG, the growth performance of fish fed 2 or 4g kg^{-1} B-GOS® inclusion levels did not significantly outperform the control fed fish. It is hard to conclude if these fish were gaining the full benefit of the prebiotic given the rearing conditions were consistent and good for salmonid growth, and thus performance levels of the control baseline were already suitable. Similar to the rainbow trout investigated in Chapter 3, the FCR was lower than or at 1.00 in all experimental diets during the course of this trial, which indicates good growth and overall performance.

The SGR and weight gain also reflect this high growth rate, as the best rates numerically (but not significantly) for both SGR and FCR were exhibited in fish fed the 2 or 4g kg^{-1} diets. These levels of inclusion may provide some benefit to Atlantic salmon growth by providing suitable carbohydrate for the GIT microorganisms to ferment, and potentially increasing nutrient uptake within the intestine (Zhou *et al*. 2010; Merrifield & Rodiles 2015; Miyake *et al*. 2015; Yukgehnaish *et al*. 2020). However, the inclusion levels here failed to lead to fish outperforming the control, and so it is also worth considering that animals reared in environments that include no stress-inducing conditions, such as pathogens or challenging environmental parameters, may not be significantly affected by prebiotic supplements (Ziółkowska *et al*. 2020). As Salze *et al*. (2008) and Talpur *et al*. (2014) have demonstrated in larval cobia and fingerling snakehead, respectively, fish

trialled with pathogenic and salinity challenges whilst fed prebiotic supplemented diets, including yeast, β-glucans, MOS and GOS, had significantly higher survival rates than fish fed the control diets. These findings from the aforementioned studies suggest that GOS may stimulate the immune system to a degree and modulate the immune response to a potential disease even if treated fish growth performance parameters are not significantly improved in suitable rearing conditions. Further research would benefit from testing B-GOS® in Atlantic salmon reared under challenging conditions, such as suboptimal water quality, variable and inconsistent water chemistry, husbandry stressors, suboptimal diets and pathogen challenges.

It can be concluded that the product B-GOS® investigated in the present chapter does not significantly improve the growth of Atlantic salmon juveniles during the 8 week testing period, or with the inclusion levels tested under suitable rearing conditions. The results presented in this study indicate consistently good growth and performance of fish which is often enabled by the type of system in which they are reared. The aquaculture facilities utilised for this study are RAS, and these systems are known to offer more control over the feed input and water quality, thus improving and in some cases lowering the FCR in tested fish, as well as reducing the risk of disease than within open-net pen systems or raceways (Philis *et al*. 2019; Bergman *et al*. 2020).

Despite the lack of significant improvement in the growth performance for fish fed one particular B-GOS® inclusion rate, there may be some indication that the 4g kg^{-1} diet is able to benefit juvenile salmon health in marginal ways at the localised level that may be interesting for potential aquaculture enthusiasts. Guerreiro *et al*. (2016a) determined that short chain FOS application at two different rearing temperatures (15 and 20°C)

did not affect juvenile turbot (*Scopthalmus maximus*) fish performance, but enzymatic activities were higher in fish reared at 15°C, and fish grown at 20°C had higher intestinal bacterial diversity and richness. Leclercq *et al*. (2020) reported that MOS supplemented to Atlantic salmon juveniles did not significantly alter the body sizes and growth after a 65-day trial; however, fish presented an improved immune response within the intestinal morphology and mucosal surfaces in response to sea lice challenge. Based on the results of the present study, the prebiotic B-GOS® prebiotic has little effect when supplemented to salmon diets in suitable growing conditions, but it would be prudent to investigate how the immune response changes to any deviation from that, for instance by mimicking seasonal temperature fluctuations or potential pathogenic infection as what might be experienced in a farm.

4.4.2 Haematology

Blood and serum-based assays are useful in determining the primary innate immune responses, such as the presence of leucocytes, lymphocytes and lysozyme active in the blood and mucus (Kiron 2012). In the present study, there were no significant differences observed between the GOS fed fish and control fed fish for the Hb, RBC and WBC, and MCH parameters. The ranges for these parameters are consistent and comparable to research conducted on this species as per studies produced by McCarthy *et al*. (1973), Svobodova *et al*. (1991), and Svobodova & Vykusova (1991) which was focused on the haematology of salmonids.

Differential cell count ranges are influenced by endogenous and exogenous factors, but are used as simple diagnostic tools for assessing the health and conditions of fish (Dobšíková *et al*. 2013). The data presented in this chapter falls within the accepted

ranges for lymphocytes (76-97.5%), basophilic granulocytes (0-1%), monocytes (3-5%) and neutrophilic granulocytes (2-10%) for salmonids (Svetina *et al*. 2002; Dobšíková *et al*. 2013). No significant differences of leucocyte abundances were reported in fish fed GOS diets compared to the control fed fish, suggesting the immune system was not elevated in response to prebiotic addition.

An increase in the number of lymphocytes would indicate a cellular response to some form of stress, thus indicating the potential for pathogenic defence (Liu *et al*. 2017b). An increase in RBC and the concentration of haemoglobin may also indicate higher oxygen demand to the tissues and a need for greater oxygenation within the blood (Braun *et al*. 2010). When coupled with an increase in leucocyte cell counts, these results may indicate an increased immune response to adapt to higher energy demands when fish are stressed (Liu *et al*. 2017b). Given that the results in the present study show no significant differences in haematological parameters between fish fed the control or B-GOS® supplemented diets, as was also reported in Chapter 3, it can be determined that the addition of this prebiotic at the concentrations tested here did not modulate the immune response in this manner. As proposed with the rainbow fish trialled with B-GOS® in Chapter 3, further research should test whether this prebiotic may be fully utilised by the fish should they be reared in more challenging conditions by introducing either abiotic or biotic stressors, such as pathogen challenges.

4.4.3 Serum Lysozyme Activity and Protein Content

The innate immune response in fish is incredibly important in the regulation of health and preparing the host to combat infection, and lysozyme activity is one of many important measures of the level of this response (Hoseinifar *et al*. 2017c). Aftabgard *et*

al. (2019) investigated dietary supplementation of GOS and *Bacillus* spp. to Caspian salmon (*Salmo trutta caspius*) fingerlings, whereby salmon fed the synbiotic diet had significantly greater growth performance, survival, serum protein, lysozyme activity and serum alkaline phosphatase levels compared to control fed fish. Similar results were also reported by Talpur *et al*. (2014), who demonstrated elevated serum protein content, RBC, WBC, Hb% and PCV in fingerling snakehead fed 1% yeast, 0.1% β-glucan, 1% GOS, 0.2% MOS, and 1g kg^{-1} feed containing *L. acidophilus* (1 x 10^{10} CFU). The lysozyme activity of fish fed the pro- or prebiotics was significantly higher compared to fish fed the control group, and this effect was similar when fish were challenged with *A. hydrophila* (Talpur *et al*. 2014). Similarly Giri *et al*. (2015) demonstrated that *Chlorophytum borivilianum* polysaccharide administered to *Labeo rohita* carp fingerlings modulated the immune response by upregulating serum lysozyme activity, increasing phagocytosis and increasing the survival of carps after infection with *A. hydrophila* challenge. Coupled with the increase in other immune parameters measured, these authors suggested that these significant improvements in the immune response are from the addition of feed additives, and are further supported by the reduced mortality of all treatment fed fish compared to the control groups.

For the present study, the serum lysozyme activity was significantly elevated in fish fed 2g kg^{-1} B-GOS® compared to the control fed fish. These data suggest that the 2g kg^{-1} dietary inclusion of B-GOS® may elevate the immune response in Atlantic salmon juveniles fed with this prebiotic concentration by activating lysozyme pathways as part of an improved immune response. This finding is similar to Miandare *et al.* (2016), who determined that goldfish (*Carassius auratus gibelio*) fed 1% and 2% GOS for 6 weeks had significantly higher serum lysozyme activity than fish fed 0.5% GOS and the control

diet, leading to increased humoral and skin mucus immunity following dietary prebiotic administration.

Contrary to the literature, the serum protein content was highest in control fed fish during this study, and a significant decrease was detected in fish fed 2 and 8g kg^{-1} diets, but not against each other or other dietary treatments. There is little research in the literature as to how the serum protein levels decreasing with elevated serum lysozyme affects growth and immune response parameters in fish without further analysis of the specific proteins produced. There are a range of protective proteins found in the skin mucus and blood serum of fish that help to augment the innate immune response in fish, for example, immunoglobulins, cytokines, transferrin proteins and other complement proteins (Esteban 2012; Karimi *et al*. 2020). It would be prudent to further research potential useful proteins by utilising additional proteomic assays in addition to those used in this study from fish administered with B-GOS® in a range of 2 – 8g kg^{-1}.

4.4.4 Histology

The histology of the teleost GIT is widely studied and has been mapped extensively. Numerous studies have reported improved intestinal morphology in fish upon feeding with prebiotics, with the benefits including increased nutrient uptake, greater mucin cell production, enhanced immune responses due to increased cell abundance and an overall improved performance (Burr *et al*. 2008; Dimitriglou *et al*. 2010; Zhou *et al*. 2010; Anguiano *et al*. 2013; Leclercq *et al*. 2020; Ziółkowska *et al*. 2020). Despite marginal improvements in the muscularis thickness and mucosal fold heights within the intestine of fish fed 4 and 6g kg^{-1} diets, these data presented suggest that the prebiotic inclusion levels chosen for this experiment had no significant effect on the

morphology of the intestine in salmon at this age, and at the studied area of the intestine.

Results from prebiotic research within various commercial fish species are often in conflict with previously reported findings, in that some concentrations of an additive will produce positive effects, while the same concentrations in other studies produce no effect on intestinal morphology of the treated fish. Caution is advised when comparing studies that deal with differing inclusion levels, life stages, basal diets, rearing conditions, length of administration and differing species, as well as different organ sites (Yousefi *et al*. 2018). The section of the GIT sampled may also be a factor. Khojasteh *et al*. (2009) determined that the length of the villi within the GIT of rainbow trout decrease towards the end of the intestine, in particular with the posterior intestine, whilst the number of goblet cells increase. This is possibly due to the increased need for mucus to aid in faecal expulsion (Khojasteh *et al*. 2009), but more likely as an important barrier function in host defence, acting as a chemical and physical deterrent to potential pathogens (Standen *et al*. 2016). As reported by Dimitriglou *et al*. (2010) using standard light microscopy techniques and electron microscopy to evaluate the effects of a prebiotic on the intestinal structures of gilthead sea bream, the sections of the intestine chosen for analysis may have significantly altered morphology to other sections of the GIT. Only one section of the GIT was sampled as part of the research from the present chapter, so it is possible that changes that may be attributed to GOS supplemented may not have been detected if their effects were localised to other sections of the intestine.

The results presented in the current chapter do not support the findings of Khojasteh *et al*. (2009), in that there were no significantly increased numbers of goblet cells

present within the skin of Atlantic salmon fed any prebiotic inclusion against those fed the control diet. The lack of differentiation in numbers of goblet cells between the additive diets suggest that there is no significant impact upon the mucus excretion levels as part of the immune mucosal response at the time of sampling in fish fed either inclusion level. This does not however rule out the possibility that other aspects of the mucous, such as biochemistry, viscosity, protein levels or activity, could have been affected. Future studies using proteomics and biochemical analyses would elucidate if this were the case.

4.4.5 Body Composition

There were no significant differences observed between the carcass compositions of fish fed each diet, suggesting that the GOS did not significantly affect body composition.

4.4.6 Gene Expression

The innate and adaptive immune responses are important components in the defence from infection and is reactive to a number of different stimuli, such as changes in water quality, stocking density, and the detection of PAMPs from potential pathogens (Smith *et al*. 2019). Many cytokine genes have been characterised from a number of fish species in recent years to be used as biomarkers for evaluating immune response activities (Bustin *et al*. 2009; Abid *et al*. 2013). In the present study, the relative intestinal gene expression levels were analysed for the target genes *Cal, IL-10, IL-1β, TGF-β* and *TNFα* in Atlantic salmon to determine if any effects of prebiotics are seen in essential immune system pathways. The target genes were chosen to compliment the research completed in the third chapter of this body of work to provide a better understanding of how this

novel B-GOS® prebiotic may influence the immune response in a commercially valuable salmonid species.

The expression of target gene *Cal* was significantly upregulated in the intestine of fish fed the 2g kg^{-1}, 4g kg^{-1} and 8g kg^{-1} B-GOS® diets compared to the control and 6g kg^{-1} fed fish. Contrary to the findings in the third chapter concerning the gene expression of rainbow trout fed this prebiotic for 8 weeks, the findings here suggest that dietary addition of B-GOS® may modulate the immune response by upregulating *Cal* gene expression within the intestine to potentially ready the immune system in the event of pathogenic infection. Calreticulin is a multi-purpose chaperone protein and is involved in protein folding and mucin synthesis (Micallef *et al*. 2017). Vasanth *et al.* (2015) detected an increased abundance of goblet cells in the intestine of these Atlantic salmon 24 hours after inflammation was induced, further supporting its role in the innate immune system by helping to regulate inflammation. In relation to the present chapter, this may suggest that there is a state of elevated immune readiness within the intestine of the 2, 4 and 8g kg^{-1} GOS fed salmon in the present study. The significant elevation of *Cal* gene expression in the intestine of fish fed the 4g kg^{-1} GOS diet is similar to the observation of Micallef *et al*. (2017) who reported that a 0.4% inclusion rate of yeast-cell wall extracts in Atlantic salmon significantly upregulated skin calreticulin-like gene expression compared to control fed fish. As the growth performance and other immune parameters investigated in the present chapter have determined the overall good health of the salmon that have been reared in good conditions, the upregulation of *Cal* in the 2, 4 and 8g kg^{-1} GOS fed fish is promising as its role as a biomarker for immune responses.

The gene expression of *IL-10* was significantly downregulated in fish fed the 6g kg^{-1}, 8g kg^{-1} and 10g kg^{-1} diets compared to the control, 2 and 4g kg^{-1} fed fish, with the most significant downregulation displayed in fish fed the 6g kg^{-1} diet compared to fish fed all other diets. Fish fed the 2g kg^{-1} diet presented significantly upregulated expression of *IL-10* compared to all other experimental diets except the control fed fish. These findings are contrary to the data in Chapter 3 for rainbow trout fed B-GOS^{-1} at these inclusion rates, suggesting that there are still differences in immune responses to dietary prebiotics in very closely related fish species. As the regulation of this anti-inflammatory cytokine is controlled to maintain the balance between rapid and intense inflammation responses (Rawling *et al*. 2019), significant downregulation may suggest that an elevated immune response has not been triggered under the conditions tested.

Hoseinifar *et al*. (2017c) demonstrated that common carp (*C. carpio*) fed 2% GOS, FOS and inulin modulated the expression of immune-related genes *IL-1β, IL-10, TNFα* and *TGF-β*, and found that the degree of expression was affected by the prebiotic used, as well as organ (head kidney or intestine) sampled. The expression of *IL-10* was significantly downregulated in fish intestine and head kidney that had been dosed with GOS compared to all other diets (Hoseinifar *et al*. 2017c), which is in agreement with the results of the present chapter as the salmon fed the higher incremental doses of B-GOS® presented the most downregulation of this target gene. This trend within the *IL-10* expression in the intestine of salmon fed the higher doses (6 – 10g kg^{-1} B-GOS®) corresponds to the decreased expression of pro-inflammatory cytokines *TNFα* and *IL-1β*, as the gene expression of these target genes is close to the control, suggesting that the necessity for anti-inflammatory cytokines to inhibit inflammatory responses was not necessary throughout the duration of the trial.

In addition to the expression of *IL-10*, Hoseinifar *et al*. (2017c) determined that *IL-1β* expression in carp was not affected by prebiotic addition (GOS, FOS or inulin) in the intestine, but was significantly lower in the head kidney. In the present chapter, a combination of anti-inflammatory cytokines *IL-10* and *TGF-β* being up and down regulated may have reduced the production of the pro-inflammatory cytokines (*IL-1β* and *TNFα*), thus limiting their expression. Samples from the intestine of carp fed prebiotic diets as investigated by Hoseinifar *et al*. (2017c) have been reported to have significantly higher expression of *TNFα* in the intestine and head kidney of fish fed the prebiotics GOS or inulin compared to the control and FOS fed fish. The latter results from this paper are in agreement with the results from the present chapter regarding *TNFα* expression in Atlantic salmon intestine that were fed 2 – 4g kg^{-1} B-GOS®.

Previous literature has suggested that the gene expression of *IL-1β* and *TNFα* cytokines in teleost species show the most pronounced upregulation after prebiotic treatment, however the expression of other cytokines such as *IL8* or *IL-10* may also show alterations close to the control, or baseline (Dawood *et al*. 2020). The results of this present chapter are not in accordance with the intestinal samples measured from trout supplemented with B-GOS® in Chapter 3, and there appears to be no specific trend in the expression levels as B-GOS® supplementation increases. Biological variation was quite high between the replicates in this chapter, as also described in section 3.4.7, and so replicates that were identified as outliers (as described in sections 2.10 and 4.2.3) were removed from further analysis to reduce the variation in C_T values obtained for the reference genes after qPCR. The results from this present study suggest that there may be some modulation in the intestine of Atlantic salmon juveniles fed 2 – 4g kg^{-1} B-GOS®, as the gene expression of key immune response biomarkers such as *Cal* and *TNFα* was

significantly elevated, perhaps suggesting a state of immune readiness in response to supplementation of the GOS prebiotic.

The fish reared in this study were not challenged with pathogens or sub-optimal environmental parameters, as the scope of this trial could not include these testing conditions in the time available. However, testing this prebiotic at the inclusion levels that were investigated in the present study, as well as administering a known fish pathogen or changing the environmental rearing conditions, may produce upregulation of other key immune response genes, such as *TGF-β* or *HSP70*, in fish fed the prebiotic diets versus the control diets. Further research is required to determine if the inclusion of B-GOS® to the diets of challenged fish would improve the immune system by modulating the gene expression in key immune response cytokine genes.

4.4.7 Intestinal Microbiome Analysis

The microbiome is important for the function of health in fish, and it plays a huge part in the regulation of immune pathways and functions within healthy hosts, and fish that have experienced pathogenic infection or stress through dysbiosis. There have been previous studies demonstrating that the microbiota of fish can be influenced by the introduction of dietary feed additives, and there can be positive health benefits to the continued supplementation of these functional feeds (Dimitroglou *et al*. 2009; Wong *et al*. 2013; Baldo *et al*. 2015; Gajardo *et al*. 2016; Hoseinifar *et al*. 2017b).

The Good's coverage for the high-throughput sequencing demonstrated that the microbiome had been fully sequenced, as all diets showed >99% at each sequencing depth. The sequence reads obtained from 16S rRNA sequencing revealed that the number of features or OTUs present in fish fed the 4g kg^{-1} diet was significantly less

than fish fed the 6g kg^{-1} diet, but were not significantly different from fish fed the other diets. This may indicate that the fish fed 4g kg^{-1} GOS diet had a less diverse range of bacterial species present within the GIT that could be identified, at the time of sampling. While the number of identifiable features was higher in fish fed the 6g kg^{-1} diet compared to all other diets, and so the diversity may be considered as greater, caution should be taken when examining this result as the standard deviations are very high within this experimental group. Indeed, the dataset generally is quite distinct from the rest of the data and it is not clear why this is the case, or whether it is a genuine observation or due to an unidentified artefact. The number of species that are presented here in this chapter are those that were above the threshold, and so the number of unique sequences for other reads from these samples may be higher, however their relative abundances are too low.

In terms of relative sequence abundance, any reads that were unidentifiable or did not pass the threshold of 0.01% were grouped as the group 'Others'. The most dominant phyla was *Firmicutes* at 93.06% for 16S rRNA sequencing reads, followed by *Cyanobacteria* (5.32%), *Proteobacteria* (1.41%) and *Actinobacteria* (0.19%). These phyla have previously been reported as constituents of the intestinal microbiota of various fish species (Navarrete *et al*. 2009; Sullam *et al*. 2012; Peggs 2015; Gajardo *et al*. 2016; Lyons *et al*. 2016). The reads assigned to the *Cyanobacteria* phylum were concluded to be artefacts of sampling and are present due to the high plant-based diets, and so these reads were filtered and removed as in Chapter 3, section 3.2.4 (Baldo *et al*. 2015; Gajardo *et al*. 2016).

There were three genera identified from high-throughput sequencing that were of specific interest identified from the STAMP and LEfSe programmes: *Rummeliibacillus*, *Aerococcus* and *Lactobacillus*. Other genera, such as *Bacillus*, *Enterococcus* and *Weisella*, were also present at a lower level of abundance. Relative abundance reads which were assigned to *Rummeliibacillus* were significantly more abundant in fish fed the 8g kg^{-1} diet compared to fish fed the 2 and 4g kg^{-1} prebiotic diets, but not the control. This Gram-positive bacterial genus was once categorised as part of the *Bacillus* genus, however, has since been reclassified. Few studies have utilised species of this genus as feed additives or probiotics, however, Tan *et al*. (2019) isolated *Rummeliibacillus stabekisii* from the gut of Nile tilapia and investigated it as a probiotic in juvenile Nile tilapia. The results were positive, with fish fed the probiotic exhibiting increased weight gain, improved feed efficiency, reduce FCR and an increase in serum lysozyme activity (Tan *et al*. 2019). Disease resistance was also significantly increased in fish fed the probiotic when challenged with *A. hydrophila* and *S. iniae* (Tan *et al*. 2019). Data presented in the current chapter suggest that there may be possible benefits to the presence of *Rummeliibacillus* spp. within the GIT of Atlantic salmon, with individual fish presenting elevated levels of this genus also demonstrating improved lysozyme activity, weight gain and FCR. The presence of this genus in salmon fed this prebiotic should be investigated further in other fish species to determine if this is a species-specific genus that could potentially be part of the core GIT microbiome.

Relative abundance reads assigned to the genus *Aerococcus* were significantly more abundant in the 6g kg^{-1} fed fish compared to all other diets except the 8g kg^{-1} inclusion rate. This genus comprises of round clusters of Gram-positive species commonly found within human and animal microbiomes. Some species of this genus can become

pathogenic given the right environment, for instance in lobsters (Stewart 1975) or Nile tilapia (Elgohary *et al*. 2021). The presence of *Aerococcus* spp. within fish tested in this experiment did not present symptoms of pathogenic insult or detriment to overall health, as fish fed either of the experimental diets had improved growth performance via a doubling of biomass after the 8 week feeding trial. More research should be conducted to determine if this genus could contain any previously unknown beneficial bacteria within fish GIT that may become more relatively abundant upon prebiotic supplementation.

The relative abundance of reads assigned as *Lactobacillus* was significantly increased in the 4g kg^{-1} fed fish compared to the 2g kg^{-1} fed fish. While this genus was the most dominant taxon across fish fed all dietary treatments, the highest relative abundances were observed in the GIT of 4 – 10g kg^{-1} fed fish, suggesting that a significant proportion of the microbiota of fish fed these prebiotic inclusions were species of *Lactobacillus*. As this genus was the most dominant taxon within the fish fed 4g kg^{-1} B-GOS®, this would explain why this treatment regime had fewer OTUs identified and lower diversity of species and richness.

The genus *Lactobacillus* contains species that are rod-shaped, Gram-positive, non-spore-forming colonies, found in a significant portion of human and animal microbiota (Duar *et al*. 2017). Like many commensal bacteria, species within this genus provides mutualistic relationships with the host, likely providing protection against potential pathogens as well as metabolites of benefit to the host and other gut microbes (Martín *et al*. 2013). *Lactobacillus* spp. are a key component of the microbiota in fish, such as Nile tilapia (Standen *et al*. 2015), and this group is one of the most researched bacterial

genera, as species from this genus are popular probiotics in aquaculture, terrestrial animal farming and human nutrition (Rodiles *et al*. 2018). Mohammadian *et al*. (2020) observed that rainbow trout supplemented with *Lactobacillus bulgaricus* in microencapsulated alignate-chitosan produced significant improvements in health and immune function when the fish were exposed to lead nitrate. *Lactobacillus rhamnosus* has been predominantly used as a probiotic in rainbow trout to prevent furunculosis (Nikoskelainen *et al*. 2001), modulate blood profiles (Panigrahi *et al*. 2010), and increase growth and overall performance when exposed to *Yersinia ruckeri* (Hooshyar *et al*. 2020). Other *Lactobacillus* spp. and strains have been isolated from 12 marine fish species off the coast of Spain and were reported as exhibiting antibacterial activity against commonly found pathogens in aquaculture industries, such as *V. harveyii* and *V. splendidus* (Alonso *et al*. 2018).

There is evidence to suggest that the presence of *Lactobacillus* in the GIT may modulate the immune system in salmonids by protecting against pathogens, and metabolise non-digestible compounds, which alters the production of bacterial metabolites (Yukgehnaish *et al*. 2020). A high abundance of LAB such as *Lactobacillus* spp. in cultivated salmonids fed high plant-based diets has been speculated to be resulting from the presence of plant-derived fibres acting as prebiotics in the GIT (Gajardo *et al*. 2016). Based on this information, Atlantic salmon supplemented with 4g kg^{-1} B-GOS® exhibit increased *Lactobacillus* spp., suggesting that *Lactobacillus* could be considered potentially beneficial given their positive attributes demonstrated in many other studies. More information is needed to ascertain how these *Lactobacillus* spp. function in response to prebiotic supplementation such as GOS in other commercially valuable fish species.

4.5 Conclusions

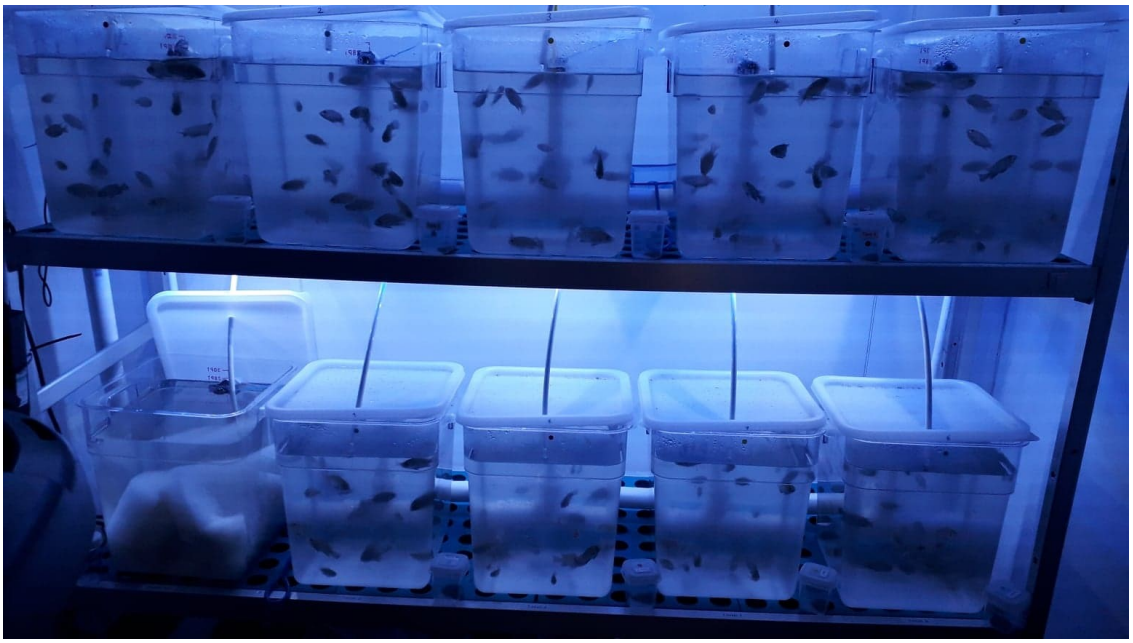
While there is marginal improvement to the localised health and growth of Atlantic salmon with the supplementation of the dietary prebiotic B-GOS®, these results were not significantly different to control fed fish. The addition of this prebiotic to an already highly nutritious and optimally formulated diet provided almost no opportunity for improvement in the health and growth of these fish as evidenced by the extensive immune metrics tested in this chapter. Despite the lack of significant improvements in most parameters tested from prebiotic addition, the combined results of improved final weight, FCR, histology morphology, and gene expression provide scope for a range of 2 – 4g kg^{-1} BGOS® to positively affect the immune response of Atlantic salmon within the intestine and skin at a localised level. The results presented here are in conjunction with the range of inclusion levels of this prebiotic determined in Chapter 3 to be the most suitable for rainbow trout. Administration of this chosen range of 2 – 4g kg^{-1} B-GOS® resulted in significantly increased lysozyme activity in the serum of Atlantic salmon compared to the control group, in addition to significantly more reads of *Lactobacillus* in the 4g kg^{-1} fed fish, suggesting that this range of additive may help to modulate the microbiota of the intestine and help to improve immune readiness within the salmon fed this inclusion level.

To further determine how this additive may improve the performance of Atlantic salmon, research should focus on how these responses would be affected by the addition of challenge trials in addition to B-GOS® supplementation at these concentrations. Challenges to fish relating to the abiotic and biotic factors within their rearing environment would provide a similar environment to the challenges faced in an

aquaculture facility, where there may be increased pressures that will increase the risk of pathogenic infection and overall poor health and performance. Determining if the addition of a challenge to the rearing conditions may present a greater utilisation of the prebiotic in salmon, and thus produce different proportions of bacterial reads within the microbiota.

The research conducted in Chapters 3 and 4 has illustrated that there is some scope for the marginal improvement of salmonid health and performance when supplemented with a novel GOS prebiotic. There are other important commercial species that fall into the category of warm-water aquaculture, which is an industry that is rapidly expanding in Africa and Asia, and recently has become popular in the UK. One of the most popular species in developing and developed countries is Nile tilapia, and makes up a large portion of the world's protein intake. As this fish species is readily available and can be grown in a range of different environmental conditions, Nile tilapia is a good candidate species to focus how this novel B-GOS® prebiotic may influence the immune defences and overall growth of a commercially important warm-water species. The results of this chapter and Chapter 3 have indicated a suitable range of B-GOS® prebiotic concentration as 4 and 6g kg^{-1}, and so these two inclusion levels were tested in juvenile Nile tilapia using similar immune parameters tested in the last two chapters, and this premise shall form the basis of the next research chapter.

The effects of prebiotic GOS dietary inclusion upon the growth performance and intestinal health of Nile tilapia (*Oreochromis niloticus*)



5.1 Introduction

Much research has focused on the salmonid family in terms of prebiotic application (Bakke-McKellep *et al*. 2007; Dimitroglou *et al*. 2009; Askarian *et al*. 2012; Gajardo *et al.* 2016; Aftabgard *et al*. 2019; Pontefract 2021); however, these species have specific temperature requirements to grow efficiently, preventing a large portion of the world from contributing to this industry (FAO 2020). Warm-water aquaculture dominates production in Asia and Africa, and teleost species with higher temperature thresholds are routinely grown in in-land and semi-intensive facilities in countries that might not naturally meet these requirements (Fitzsimmons & Watanabe 2010). Within warm-water aquaculture, the potential for pathogenic infection is an ever-growing concern and so additives that may increase fish species robustness and health in intensive farms are of great interest. Previous research within this body of work has provided insights into how this novel prebiotic B-GOS® affects salmonids (Chapters 3 and 4); however, to the author's knowledge there is no data currently available regarding the effect of this prebiotic on the immune responses, growth performance and microbiome in Nile tilapia.

Tilapia belong to the cichlids, a group of tropical fish species that are relatively easy to culture, as they thrive in a wide range of environmental conditions and are incredibly hardy (Grassi *et al*. 2016). Their availability is high in countries that would not have access to other globally cultured species, for example, salmonids or other whitefish that require colder water temperatures to achieve healthy growth (FAO 2020). In addition to inland monoculture production, this species is often integrated into hydroponics

systems, where aquaculture and agriculture are combined into one recirculating system (Hisano *et al*. 2019).

Commercial production of Nile tilapia is high in semi-arid and arid regions of the globe when coupled with agriculture, as this method of growing complex carbohydrates in the form of crop, and protein in the form of fish is less intensive on the environment if done in a controlled and highly regulated manner (Fitzsimmons & Watanabe 2010). Additional benefits of cultivating this species include its ability to produce huge quantities of eggs with high survival rates, rapid and superior growth, its omnivorous diet, tolerance to adverse environmental conditions and high quality of fillet (Fitzsimmons & Watanabe 2010; Grassi *et al*. 2016; Hisano *et al*. 2019; Mzula *et al*. 2021).

Tilapia species are a rapidly growing commodity in many developed and developing nations, with dominance coming from Chinese aquaculture facilities (Fitzsimmons & Watanabe 2010; FAO 2020). Nile tilapia contributed over 4.53 million tonnes to global aquaculture in 2018 and ranked third for finfish production for that year (FAO 2020). As fish in many parts of the world supplement part of a balanced diet, tilapia are an affordable import and represent an important source of nutrition and diversification in an otherwise narrow range of staple food available (FAO 2020).

The final experimental trial from the present chapter was designed to accommodate warm freshwater RAS in the University of Plymouth tropical aquaria. Due to limitations in space, the system was designed to house nine tanks and focus on a control diet and two B-GOS® prebiotic inclusion levels for juvenile Nile tilapia. The major aim of this study was to understand how a novel GOS additive may benefit Nile tilapia growth performance and health. The *in vivo* feed trials from Chapters 3 and 4 demonstrated

that the inclusion of the prebiotic B-GOS® did not significantly improve growth and performance in the whole organism of salmonids under the conditions tested. However, there were marginal numerical improvements to the health on a localised level to the intestine of fish fed a select number of inclusion levels. The optimal inclusion levels for B-GOS® prebiotic inclusion for rainbow trout (Chapter 3) were 2 – 6g kg^{-1} supplemented to the basal diet, and for Atlantic salmon (Chapter 4), it was 2 – 4g kg^{-1} B-GOS®. The improvements in overall size and performance of these juvenile salmonids were seen from the mid-range of GOS addition, primarily in fish fed the 4g kg^{-1} diet across both experimental trials, therefore this chapter focused on the inclusion levels at 4 and 6g kg^{-1} prebiotic against a basal control diet in the experimental design. The trial lasted 29 days, and the tilapia were reared using in-house formulated control and prebiotic supplemented diets until they achieved at least a doubling of biomass. Samples of target organs were sampled at the end of the trial in a similar way to Chapters 3 and 4, and the techniques used throughout this and the trial conditions are outlined in Chapter 2 sections 2.2-2.3, 2.7-2.11 and in section 5.2 in this chapter.

5.2 Methodology

The experimental design, growth performance metrics and diet formulation for this trial are described in the following sections. At the final sampling, the tilapia were measured at ~3.5 g each and so the blood and some mucosal tissues that were sampled for the rainbow trout and Atlantic salmon trials were unable to be taken or preserved from this trial due to the small size of the fish. Please refer to Chapter 2 sections 2.8 – 2.11 for more details about other sample analyses from this trial.

5.2.1 Tilapia Experimental System

Juvenile male Nile tilapia (*O. niloticus*, produced from YY supermales crossed with standard XX females) were sourced from FishGen Ltd., UK weighing approximately 0.2 g each. The fish were subjected to a 4-week acclimation and conditioning period in which the water temperature was raised from 24°C to 27°C. The fish were fed a commercial feed (Zebrafish Management Ltd.) during this time as a maintenance diet, and after the initial conditioning period, fish averaging 1.6 ± 0.01 g were graded into 9 x 15 L aquaria in groups of 30 individuals for the start of the trial. . As described in Chapter 3, section 3.2.1, fish were batch weighed altogether from each tank by netting individuals and moving the net side-to-side to remove as much water as possible from the fish before they were placed in a pre-weighed and tared vessel of 1 L of tank water. Starting weights were calculated to be within ±2.5% of the overall average tank biomass. The weights of each tank were recorded in this manner at the start of the trial, every one-week period, and at the conclusion of the trial.

Throughout the trial, water chemistry and quality parameters were maintained and adjusted with mechanical and biological filtration to maintain suitable requirements for tilapia. Fresh water was supplied to a reservoir tub within the Temperature Control Laboratory on the 7th Floor of the Davy Building, University of Plymouth, and then supplied to the recirculating system once acclimated to the ambient water temperature. The room temperature was maintained at 24.0 ± 0.5°C to maintain an average water temperature of 27.3 ± 0.3°C. The pH was maintained at 6.4 ± 0.1 and buffered using crushed coral/NaHCO, and dissolved oxygen was maintained at 7.4 ± 0.2 mg L^{-1}. A 12-hour light: 12-hour dark photoperiod was maintained throughout the trial using fluorescent bulbs, which was controlled by timers. The tank weights (total biomass)

were measured every week to calculate the rations for the fish for each day in that period. Feed was weighed into pots according to the feeding rate, and fish were fed by hand between four to six rations a day of 2.0-6.0% bodyweight, depending upon acceptance of the feed.

One sampling point was scheduled at the end of the trial (day 29). Fish were sampled at random and were anaesthetised using an overdose of MS222 (tricaine methanesulfonate) at 400mg L^{-1}. Once determined to be unconscious, whereby the opercula movement had ceased, sampled tilapia were then euthanised by the destruction of the brain (following Schedule 1 procedures). This work was approved by the University of Plymouth Internal Ethical Review Committee on Animal Scientific Investigations (approval number ETHICS-16-2019).

5.2.2 Diet Formulation and Growth Performance Parameters

Three experimental iso-nitrogenous and iso-lipidic diets were formulated using Animal Feed Optimisation Software (AFOS) (Feedsoft Professional®, USA) to meet the known requirements of juvenile Nile tilapia (NRC 2011), of which the compositions and proximate analyses are described in Table 5.1. One basal diet (control) and two feed formulations in the range of 4 – 6g kg^{-1} B-GOS® were formulated, as presented in Table 5.1. These ranges were chosen based on the results from the previous two fish feeding trials, and based on manufacturer's instructions. The methodology for producing these diets is described in Chapter 2 section 2.4, and includes the calculations for growth performance, feed efficiency and overall fish condition.

The mean and SD were recorded for the FCR, SGR, final tank weights, PWG and Survival (%) of each diet. The FCR was calculated using n=3 tanks per diet, and the SGR was

based on the starting weights on 18th February 2019 and the final weights of each tank on the 19th March 2019. The PWG was calculated from the mean tank weights at the beginning of the trial and the mean tank weights at the end. The K-Factor was calculated from the final lengths and weights of fish sampled at the end of the trial (n=36 fish per diet). Survival was calculated based on the number of fish that were present at the end of the trial and the beginning expressed as a percentage.

Table 5.1 Compositions of experimental diets for Nile tilapia with levels of B-GOS® inclusion. Proximate composition analyses of each diet are included following adapted AOAC (2016) procedures, presenting the crude protein, crude lipid, moisture and ash content.

Ingredient (% Inclusion)	**Control**	**4g kg^{-1}**	**6g kg^{-1}**
Sunflower Meal[a]	20.00	20.00	20.00
Soy Bean Meal[a]	20.00	20.00	20.00
Fababeans (ground)[a]	18.00	18.00	18.00
Corn Gluten Meal[a]	15.00	15.00	15.00
Soy Protein Concentrate[a]	10.17	10.17	10.17
Vegetable Oil[b]	6.64	6.64	6.64
Wheat[a]	4.99	4.59	4.39
Vit/Min premix (PNP FISH 2%)[c]	2.00	2.00	2.00
Gelatin[d]	2.00	2.00	2.00
CMC Binder[d,e]	0.50	0.50	0.50
DL Methionine[d]	0.40	0.40	0.40
Lysine HCL[d]	0.30	0.30	0.30
B-GOS®	-	**0.4**	**0.6**
Crude Protein (%)	42.81 ± 0.44	42.71 ± 0.45	43.32 ± 0.26
Crude Lipid (%)	7.34 ± 0.37	8.80 ± 0.65	7.72 ± 0.71
Moisture (%)	6.96 ± 0.39	7.16 ± 0.12	6.57 ± 0.08
Ash (%)	6.07 ± 0.15	5.87 ± 0.24	5.72 ± 0.70

[a] Skretting, Norway
[b] Tesco, Plymouth
[c] Premier Nutrition, UK
[d] Sigma-Aldrich, UK
[e] Carboxy methyl cellulose

5.2.3 Gene Expression

The specific primers used for the gene expression analyses are described in Table 5.2. For the specific methodology of how the samples were prepared for gene expression analyses using qPCR, please see Chapter 2, section 2.10. Outliers were identified using the Quartile and OR functions in Excel 2016 as described in section 2.5 and 2.10 (Grech 2018) and removed from further analysis to reduce variation in C_T values obtained for the reference genes after qPCR, and so n=6 samples per diet were taken forward for further analysis.

Table 5.2 Primer sequences of target genes evaluated within the *O. niloticus* trial.

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Amplicon size (bp)	Tm (°C)	GenBank Accession Number
GAPDH	CCGATGTGTCAGTGGTGGAT	GCCTTCTTGACGGCTTCCTT	82	59.4	JN381952.1
β-actin	TGACCTCACAGACTACCTCATG	TGATGTCACGCACGATTTCC	89	58.8	KJ126772.1
IL-1β	TGGTGACTCTCCTGGTCTGA	GCACAACTTTATCGGCTTCCA	86	58.7	XM_005457887.1
TNFα	CCAGAAGCACTAAAGGCGAAGA	CCTTGGCTTTGCTGCTGATC	82	59.9	AY428948.1
IL-10	CTGCTAGATCAGTCCGTCGAA	GCAGAACCGTGTCCAGGTAA	94	59.6	XM_003441366.2
TGF-β	GTTTGAACTTCGGCGGTACTG	TCCTGCTCATAGTCCCAGAGA	80	59.8	XM_003459454.2
CASP3	GGCTCTTCGTCTGCTTCTGT	GGGAAATCGAGGCGGTATCT	80	59.4	GQ421464.1
MYD88	AGCTCGAAGTAAACGCCTGAT	ACAAATGGTGAGGAAGCGTAAA	85	59.3	KJ130039.1
PCNA	CCCTGGTGGTGGAGTACAAG	AGAAGCCTCCTCATCGATCTTC	80	60.9	XM_003451046.2
HSP70	ACCCAGACCTTCACCACCTA	GTCCTTGGTCATGGCTCTCT	84	59.4	FJ213839.1

5.2.4 Intestinal Microbiome Analysis

The posterior intestinal samples used for extracting DNA were obtained as per the sampling methods in Chapter 2 section 2.3. The Nile tilapia (total of 21) PCR amplicons were prepared for sequencing as described in Chapter 2, section 2.11, and quantified using the Qubit® 2.0 Fluorometer (Thermo Fisher) system to determine sample

concentrations. These amplicons were sent on ice packs to for 16S rRNA sequencing of the V1-V3 hypervariable region using the MiSeq PEX300 Illumina platform (50K reads in either direction) (Omega Bioservices, USA). Please refer to Chapter 4, section 4.2.4 for more detailed explanations of the bioinformatics analysis using the QIIME v2 'DADA2' pipeline, as the tilapia sample FASTQ files were processed and analysed in the same manner as the Atlantic salmon samples. Phylogenetic trees were generated using 'q2-phylogency' plugin, and alpha and beta diversity metrics were produced as described in section 3.2.4.

The sampling depth for tilapia was chosen based on the lowest scoring reads for a given sample and so the depth of 14,000 was chosen for the tilapia reads based as per this criterion. Rarefaction plots were produced for alpha and beta diversity metrics, and taxonomic analyses were produced within the QIIME v2 environment using the same pre-trained classifier described in section 3.2.4. The feature, or OTU, table was filtered to remove *Streptophyta* and singletons from the analysis, as described in the Chapter 3, section 3.2.4.

Bar charts were generated using data obtained from the feature tables that contained the assigned relative abundance sequence reads at the genus level, or as close to this taxonomic level as possible, to characterise the microbiome of Nile tilapia fed two inclusion levels of B-GOS® against fish fed a control diet. The software STAMP v2.1.3 and programme LEfSe Galaxy Version 1.0 were used to determine if there were significant differences in the features identified from the intestine of fish fed each prebiotic diet (Parks & Beiko 2010; Segata *et al.* 2011; Parks *et al.* 2014; Afgan *et al.* 2018). Please refer to Chapter 3, section 3.2.4 for more information about these programmes.

5.3 Results

5.3.1 Growth Performance

No significant differences of Nile tilapia growth performance metrics were observed between fish fed experimental diets compared to fish fed the control diet: final weights, FCR, SGR, PWG, K-factor or Survival (Table 5.3). There was an increase in final tank weights from fish fed the 4g kg^{-1} inclusion of B-GOS® to the control; however, this increase was not significant.

Table 5.3 Mean growth performance parameters (± SD) of tilapia fed incremental levels of B-GOS® over a period of 4 weeks (29 days).

Diet	Control	4g kg^{-1}	6g kg^{-1}	p-value	Test Statistic
Initial Weight (g)	1.56 ± 0.004	1.57 ± 0.022	1.58 ± 0.010	**0.419**	$F_{2,6}$=**1.01**
Final Weight (g)	3.40 ± 0.17	3.62 ± 0.17	3.39 ± 0.16	**0.256**	$F_{2,6}$=**1.73**
FCR	2.15 ± 0.31	1.91 ± 0.18	2.09 ± 0.23	**0.506**	$F_{2,6}$=**0.77**
SGR	2.68 ± 0.18	2.88 ± 0.12	2.64 ± 0.16	**0.209**	$F_{2,6}$=**2.06**
PWG (%)	117.89 ± 11.26	130.55 ± 7.82	115.08 ± 9.78	**0.197**	$F_{2,6}$=**2.16**
K-Factor	1.79 ± 0.17	1.84 ± 0.12	1.88 ± 0.24	**0.220**	$\chi^2(2,9)$=**3.03**
Survival (%)	70.00 ± 3.33	70.00 ± 5.77	72.22 ± 1.92	**0.714**	$\chi^2(2,9)$=**0.67**

5.3.2 Histology

There were no significant differences observed in the intestinal muscularis thickness, mucosal fold heights and intestinal goblet cell counts between fish fed the different dietary treatments. There was a significant increase in lamina propria width between fish fed the 4g kg^{-1} B-GOS® diet compared to the control fed fish, but not the fish fed 6g kg^{-1} B-GOS® diet (Table 5.4). Representative images of fish from each dietary regime are presented in Figure 5.1.

Table 5.4 Mean histological analyses (± SD) of Nile tilapia fed incremental levels of B-GOS® over a period of 29 days (n=9 fish per diet). Different letters between data denote significant difference between different letters on the same row, $p<0.05$.

Diet	Control	4g kg^{-1}	6g kg^{-1}	p-value	Test Statistic
Muscularis Thickness (µm)	23.89 ± 6.46	26.53 ± 5.61	25.47 ± 5.79	**0.289**	$\chi^2(2,27)=2.48$
Mucosal Fold Height (µm)	145.60 ± 59.34	181.21 ± 49.11	160.89 ± 25.52	**0.114**	$\chi^2(2,27)=4.34$
Lamina Propria Width (µm)	16.51 ± 1.63^{a}	19.33 ± 3.09^{b}	18.10 ± 1.42^{ab}	**0.022**	$\chi^2(2,27)=7.63$
Intestine Goblet Cell Counts	10.22 ± 2.41	11.39 ± 2.63	11.76 ± 2.80	**0.442**	$F_{2,24}=0.85$

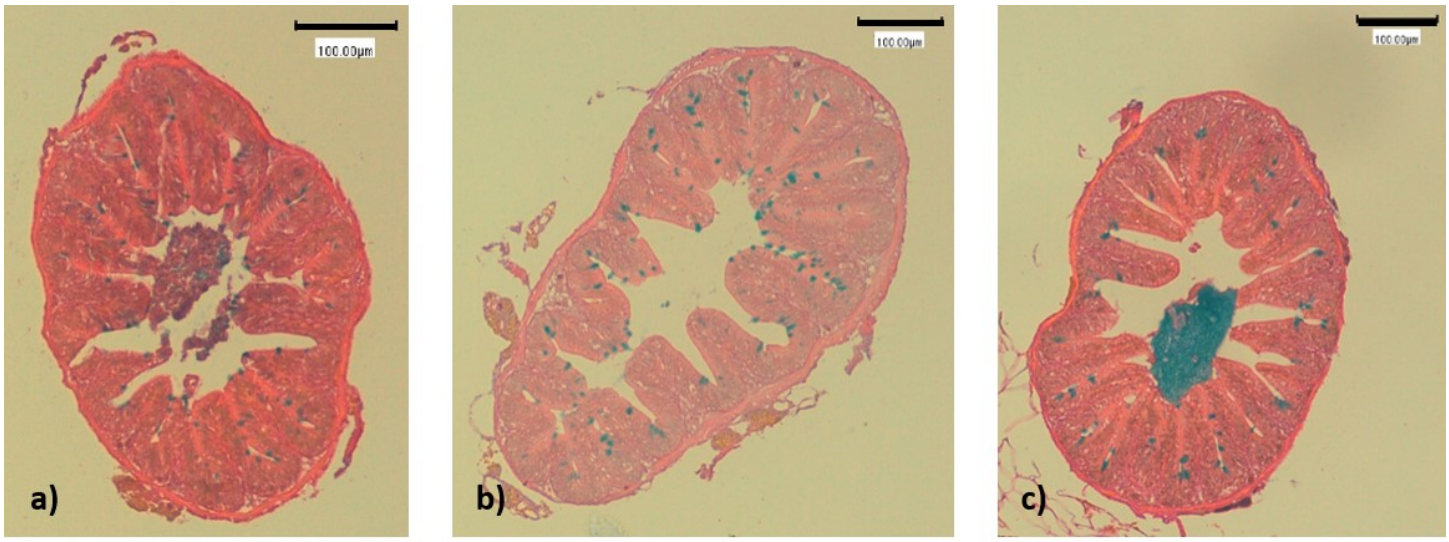


Figure 5.1 Representative intestinal images of individual Nile tilapia sampled from each diet, stained with AB/vG: **a)** Control, **b)** 4g kg^{-1} and c) 6g kg^{-1}. Scale bar = 100 µm.

5.3.3 Body Composition

Proximate compositional analyses were utilised to determine that all experimental treatments were comparable. There were no significant differences between fish fed any diet for carcass ash content, crude protein content or lipid content (Table 5.5). Fish fed the 4g kg^{-1} B-GOS® diet presented significantly lower moisture content than fish fed the control diet. To determine the moisture content, juvenile tilapia were grouped by tank, as opposed to individual analyses being conducted, as the weight of dried matter per fish was too small to use for all proximate compositional measurements. For this same

reason, it was not possible to obtain enough dried matter to evaluate the pre-trial proximate compositions of Nile tilapia.

Table 5.5 Mean carcass compositional analyses (± SD) of Nile tilapia fed incremental levels of B-GOS® over a period of 29 days (n=3 tanks per diet). Ash, Protein and Lipid content are expressed as a percentage of the dry matter. Different letters between data denote significant difference between different letters on the same row, $p<0.05$.

Diet	Control	4g kg^{-1}	6g kg^{-1}	p-value	Test Statistic
Moisture Content (%)	72.54 ± 0.14^{a}	71.48 ± 0.47^{b}	72.08 ± 0.22^{ab}	**0.010**	**$F_{2,6}$=10.73**
Ash Content (%)	8.61 ± 0.11	8.15 ± 0.18	8.21 ± 0.36	**0.113**	**$F_{2,6}$=3.21**
Protein Content (%)	56.14 ± 0.69	55.74 ± 0.95	56.01 ± 1.76	**0.921**	**$F_{2,6}$=0.08**
Lipid Content (%)	32.81 ± 1.59	34.98 ± 0.69	33.41 ± 2.06	**0.284**	**$F_{2,24}$=1.56**

5.3.4 Gene Expression

The expression of eight target genes in the mid intestine of Nile tilapia after 29 days of dietary feeding on the experimental diets is represented as fold change relative to the control group, and presented in Figure 5.2. Outliers that were previously removed as described in section 5.2.3 were not taken forward in this analysis, and so n=6 samples per diet were analysed.

5.3.4.1 Intestine

Permutation tests were performed instead of ANOVA or Kruskal-Wallis models as described in section 2.12 to determine if significant differences between the expression levels of fish fed each diet were present for each target gene, for the instestinal samples. The significant outputs for these permutation tests are presented as different letters above the bars in Figure 5.2 for the posterior intestine. These tests for target genes measured in the mid-intestine of Nile tilapia demonstrated that fish fed the 4g kg^{-1} and

6g kg^{-1} diets exhibited significantly elevated *IL-10* expression compared to the control diet (p=0.002 and p=0.034, respectively). *TNFα* expression was significantly upregulated in fish fed 4g kg^{-1} B-GOS® inclusion compared to the 6g kg^{-1} diet (p=0.023), however, no significant differences were observed in these fish compared to the control fed fish. The gene expression of *HSP70* was significantly upregulated in fish fed the 6g kg^{-1} diet compared to the control (p=0.007) and 4g kg^{-1} fed fish (p=0.013). Fish fed the 4g kg^{-1} diet had significantly decreased expression of *PCNA* compared to the control fed fish (p=0.050).

There were no significant differences between fish fed any prebiotic diet and the control, or between each treatment, for intestinal gene expression levels of target genes *IL-1β*, *TGF-β*, *CASP3* and *MYD88* (p>0.05).

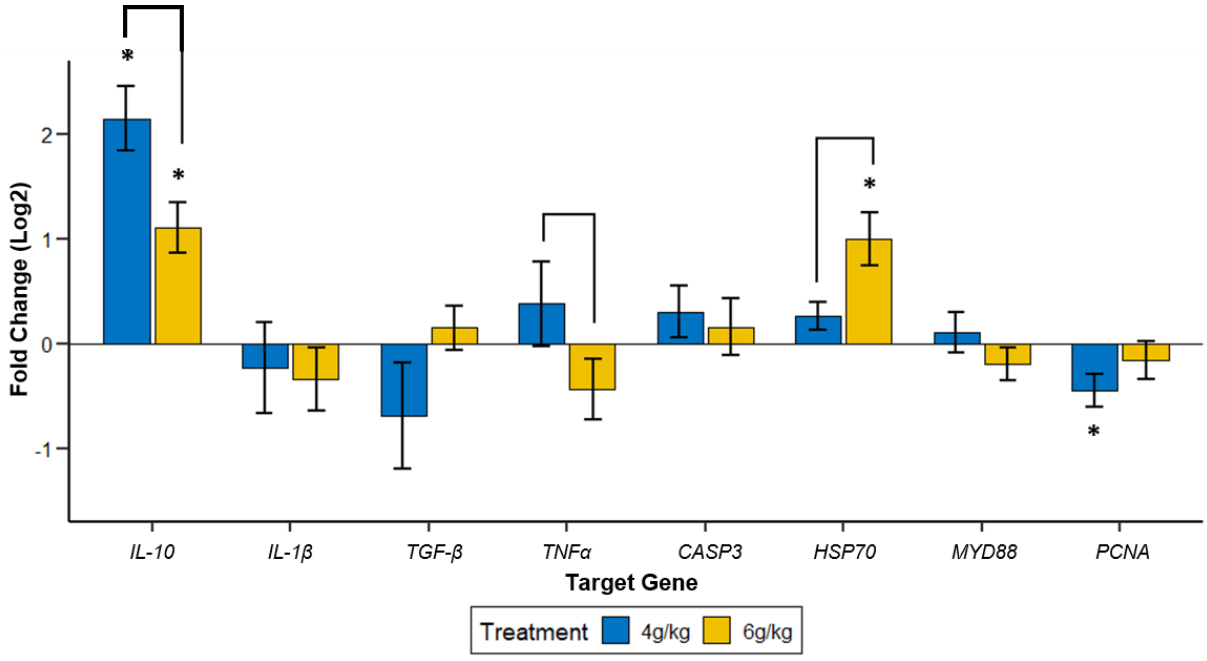


Figure 5.2 Gene expression data presented as fold change (log_2) of 8 target genes relative to the control group in the intestine of Nile tilapia fed three dietary treatments containing incremental levels of B-GOS® over a period of 29 days (n=6 fish per diet, per gene). Significant differences are denoted by an asterisk for the treatment to the control, and as a line between treatments. Data presented as mean ± SEM.

5.3.5 Intestinal Microbiome Analysis

The high-throughput 16S rRNA gene sequencing analysis of the microbiomes of Nile tilapia fed control vs prebiotic supplemented diets yielded a total of 3,327,000 paired-end sequence reads, and the average number of reads per replicate fish being 158,428.60 ± 23,861.51 after demultiplexing. After 'DADA2' quality control and filtering, the average number of reads across all diets was 22,157.95 ± 4,055.62 and these sequences were taken forward to taxonomy assignment using the pre-trained classifier 'SILVA 138 99% OTU full-length sequences' as a reference dataset. The Good's coverage estimates for each diet were >0.999, indicating that the sequencing coverage was sufficient for these analyses. The number of features detected within the intestine of Nile tilapia did not significantly differ between fish fed the control or prebiotic diets (Table 5.6, Figure 5.3). The species richness diversity (Chao1) and Shannon diversity index did not differ significantly between dietary treatments (Table 5.6).

The percentage relative abundance of some distinct genera (where relative abundance was reported at a threshold of over 0.25% of total reads) sequenced from the digesta of Nile tilapia fed either the control or experimental diets trial are shown in Table 5.7 and Figure 5.4. Any unique genera detected that were not above the threshold level were grouped into the category 'Others'. The relative abundance of many phyla and genera were detected for sequences assigned taxonomy from tilapia samples. The phyla *Proteobacteria* and *Fusobacteria* were two of the most dominant taxa in the control and prebiotic supplemented diets, albeit not significantly, accounting >25% of reads present across all diets. The phyla *Firmicutes* was also prevalent, followed by the phyla *Actinobacteriota*, *Dependentiae*, *Planctomycetota*, *Bacteriodota*, and *Cyanobacteria,*

however, the relative abundances of these phyla were not significantly different between dietary regimes.

Table 5.6 OTUs observed after QIIME v2 analysis and alpha diversity/richness metrics of intestinal microbiota composition in Nile tilapia fed incremental levels of B-GOS®, over a period of 29 days (n=7 fish per diet).

Alpha Diversity	Control	4g kg^{-1}	6g kg^{-1}	p-value	Test Statistic
OTUs observed	85.00 ± 19.69	77.43 ± 14.56	70.57 ± 12.71	**0.264**	**$F_{2,18}$=1.44**
Good's coverage	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	**0.267**	**χ^2(2,21)=2.64**
Chao1 diversity	85.75 ± 19.88	70.53 ± 12.66	77.56 ± 14.94	**0.236**	**$F_{2,18}$=1.56**
Shannon's diversity index	4.73 ± 0.44	4.82 ± 0.67	4.46 ± 0.58	**0.320**	**χ^2(2,21)=2.28**

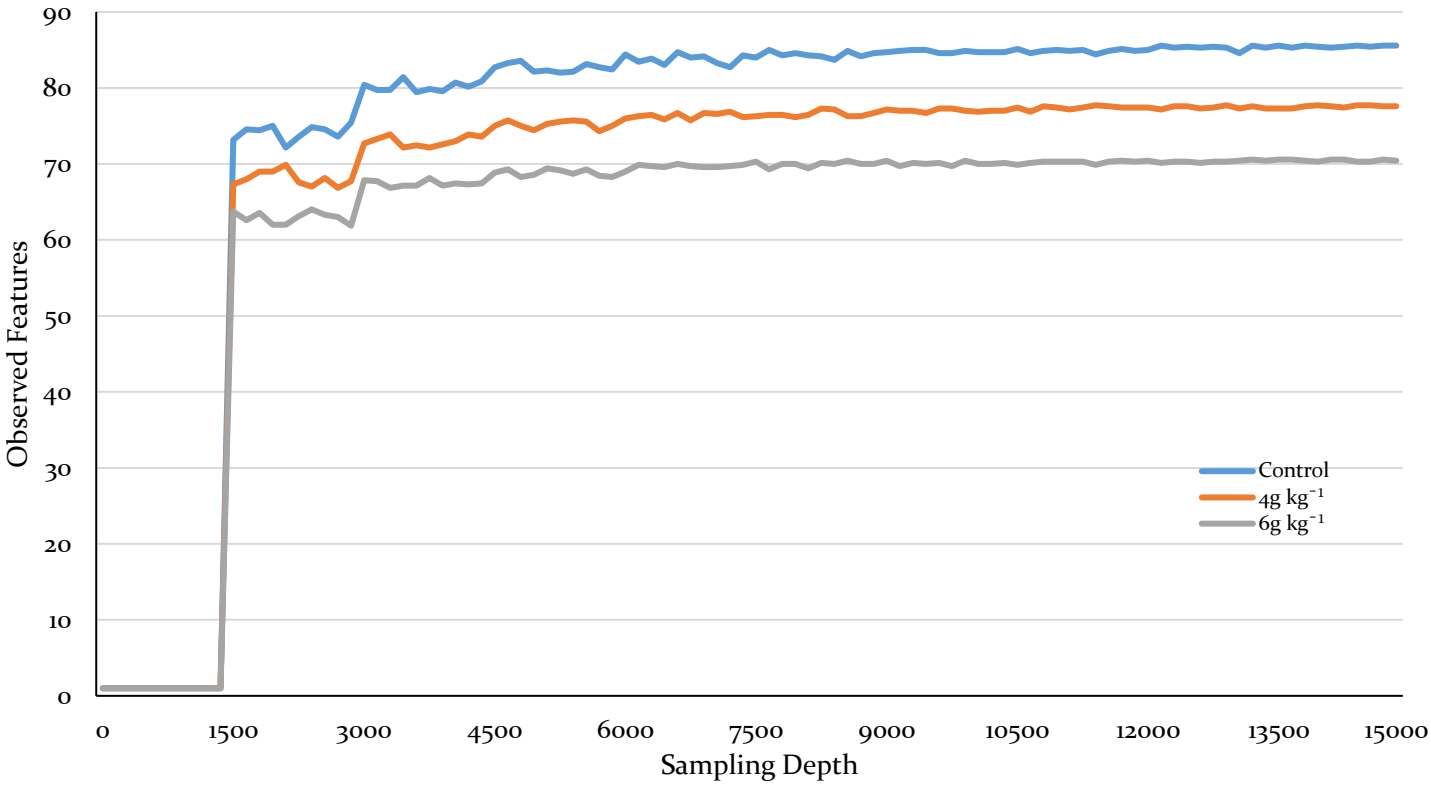


Figure 5.3 Comparison of observed OTU features as rarefaction curves within the digesta of Nile tilapia (n=7 fish per diet), fed control diet or diets supplemented with incremental levels of B-GOS® over a period of 29 days.

The relative abundance of above threshold reads identified belonged to 31 genera (Figure 5.4). As described in the Chapter 3, section 3.3.9, STAMP v2.1.3 (Parks & Beiko 2010; Parks *et al.* 2014) and LEfSe Galaxy Version 1.0 (Segata *et al.* 2011) via Galaxy Hub software (Afgan *et al.* 2018) were used to detect any distinct genera. Of the genera

sequenced in this trial, *Bacillus*, *Enterococcus* and *Weissella* relative abundances were significantly elevated in fish fed the 4g kg^{-1} experimental GOS diet compared to fish fed 6g kg^{-1}, but not the fish fed the control diet (Table 5.7 and Figure 5.4).

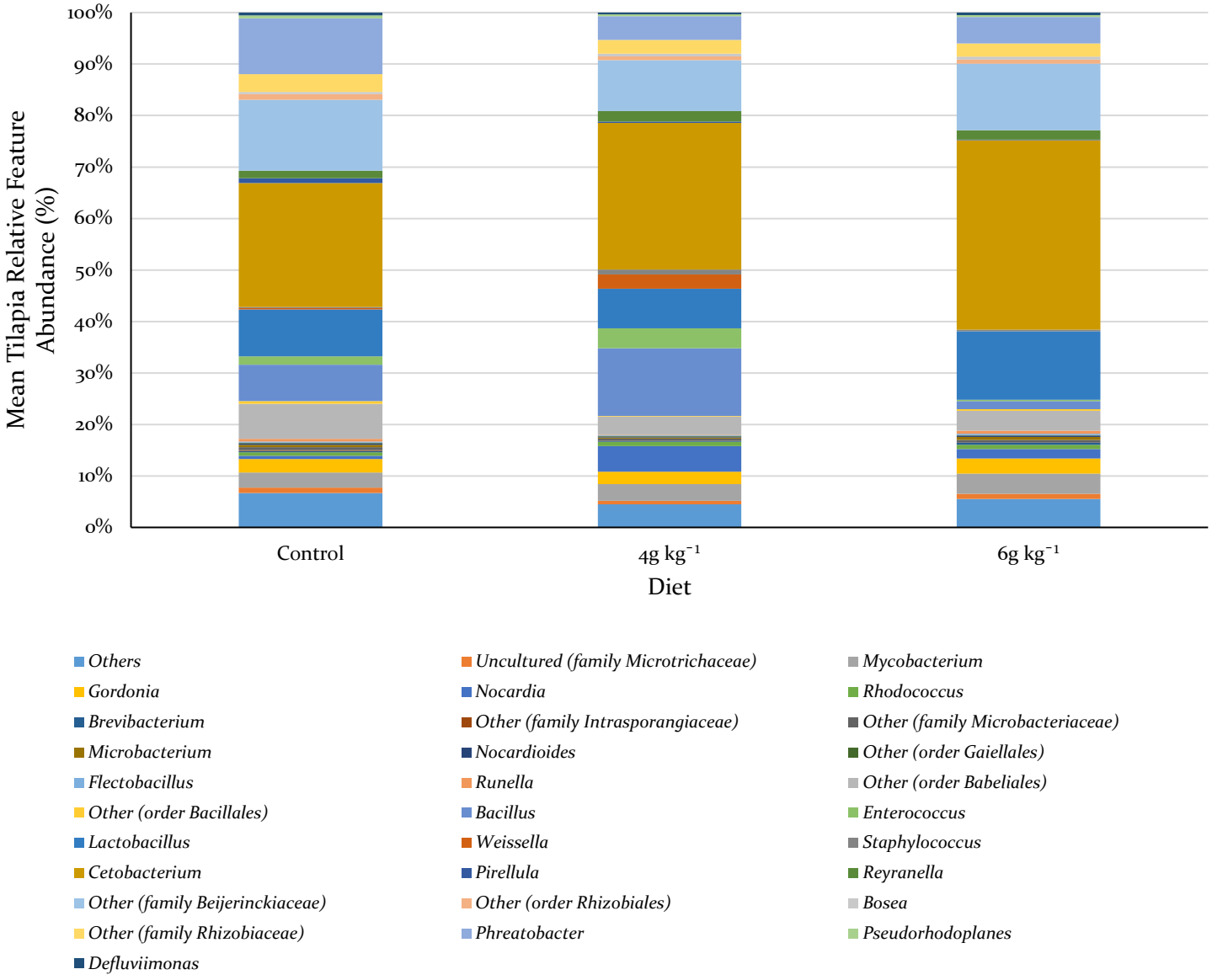


Figure 5.4 Percentage relative OTU abundance (%) of bacterial sequences at the genus level or lowest taxonomic level present within the digesta of Nile tilapia (n=7 fish per diet), fed incremental levels of B-GOS® over a period of 29 days.

Table 5.7 Relative abundance (mean ± SD) of bacterial sequences at the genus or lowest taxonomic level present within the intestinal digesta of Nile tilapia fed B-GOS® over a period of 29 days (n=7 fish per diet).

Bacterial genus relative abundance (%)	Control	4g kg^{-1}	6g kg^{-1}	p-value	Test Statistic
Others	6.71 ± 5.61	4.48 ± 2.93	5.56 ± 2.26	**0.713**	**$\chi^2(2,21)=0.68$**
Uncultured (family *Microtrichaceae*)	1.05 ± 0.67	0.69 ± 0.39	0.96 ± 0.78	**0.638**	**$\chi^2(2,21)=0.90$**
Mycobacterium	2.88 ± 1.44	3.24 ± 3.10	3.93 ± 2.77	**0.662**	**$\chi^2(2,21)=0.82$**
Gordonia	2.66 ± 2.05	2.39 ± 1.52	2.95 ± 2.23	**0.974**	**$\chi^2(2,21)=0.05$**
Nocardia	0.59 ± 0.56	4.98 ± 12.62	1.84 ± 4.01	**0.963**	**$\chi^2(2,21)=0.73$**
Rhodococcus	0.73 ± 0.42	0.88 ± 0.87	0.84 ± 0.41	**0.746**	**$\chi^2(2,21)=0.59$**
Brevibacterium	0.26 ± 0.24	0.17 ± 0.17	0.44 ± 0.50	**0.752**	**$\chi^2(2,21)=0.57$**
Other (family *Intrasporangiaceae*)	0.11 ± 0.12	0.07 ± 0.07	0.08 ± 0.09	**0.821**	**$\chi^2(2,21)=0.39$**
Other (family *Microbacteriaceae*)	0.61 ± 0.40	0.35 ± 0.19	0.42 ± 0.13	**0.350**	**$\chi^2(2,21)=2.10$**
Microbacterium	0.46 ± 0.43	0.24 ± 0.23	0.53 ± 0.54	**0.660**	**$\chi^2(2,21)=0.83$**
Nocardioides	0.27 ± 0.32	0.17 ± 0.13	0.30 ± 0.35	**0.912**	**$\chi^2(2,21)=0.18$**
Other (order *Gaiellales*)	0.17 ± 0.20	0.13 ± 0.18	0.09 ± 0.08	**0.791**	**$\chi^2(2,21)=0.47$**
Flectobacillus	0.20 ± 0.17	0.11 ± 0.21	0.28 ± 0.37	**0.458**	**$\chi^2(2,21)=1.56$**
Runella	0.51 ± 0.56	0.11 ± 0.21	0.56 ± 0.84	**0.159**	**$\chi^2(2,21)=3.68$**
Other (order *Babeliales*)	6.82 ± 4.91	3.59 ± 2.74	3.88 ± 3.02	**0.340**	**$\chi^2(2,21)=2.16$**
Other (order *Bacillales*)	0.51 ± 0.77	0.08 ± 0.12	0.31 ± 0.43	**0.751**	**$\chi^2(2,21)=0.57$**
Bacillus	7.06 ± 12.44^{ab}	13.14 ± 12.23^{a}	1.55 ± 0.91^{b}	**0.046**	**$\chi^2(2,21)=6.17$**
Enterococcus	1.63 ± 0.98^{ab}	3.89 ± 2.11^{a}	0.29 ± 0.39^{b}	**0.001**	**$\chi^2(2,21)=14.57$**
Lactobacillus	9.10 ± 4.45	7.66 ± 5.16	13.28 ± 12.52	**0.865**	**$\chi^2(2,21)=0.29$**
Weissella	0.29 ± 0.55^{ab}	2.79 ± 2.53^{a}	0.00 ± 0.00^{b}	**<0.001**	**$\chi^2(2,21)=16.09$**
Staphylococcus	0.23 ± 0.14	0.93 ± 1.71	0.35 ± 0.33	**0.558**	**$\chi^2(2,21)=1.17$**
Cetobacterium	24.07 ± 15.15	28.47 ± 24.05	36.80 ± 20.36	**0.428**	**$\chi^2(2,21)=1.70$**
Pirellula	0.94 ± 1.71	0.25 ± 0.32	0.09 ± 0.10	**0.226**	**$\chi^2(2,21)=2.98$**
Reyranella	1.43 ± 0.66	2.04 ± 1.70	1.82 ± 1.61	**0.825**	**$\chi^2(2,21)=0.39$**
Other (family *Beijerinckiaceae*)	13.80 ± 4.39	9.94 ± 3.61	12.92 ± 4.94	**0.246**	**$F_{2,18}=1.52$**
Other (order *Rhizobiales*)	1.12 ± 0.68	0.72 ± 0.64	0.78 ± 0.65	**0.522**	**$\chi^2(2,21)=1.30$**
Bosea	0.40 ± 0.26	0.51 ± 0.67	0.61 ± 0.44	**0.620**	**$\chi^2(2,21)=0.96$**
Other (family *Rhizobiaceae*)	3.43 ± 1.76	2.68 ± 1.00	2.56 ± 0.84	**0.399**	**$F_{2,18}=0.97$**
Phreatobacter	10.86 ± 10.93	4.59 ± 3.12	5.14 ± 6.38	**0.478**	**$\chi^2(2,21)=1.48$**
Pseudorhodoplanes	0.59 ± 0.44	0.40 ± 0.26	0.34 ± 0.26	**0.611**	**$\chi^2(2,21)=0.99$**
Defluviimonas	0.54 ± 0.63	0.32 ± 0.40	0.51 ± 0.97	**0.625**	**$\chi^2(2,21)=0.94$**

The 'Other' category comprises a small proportion of the reads for the present study; this category was not significantly more or less abundant between treatment groups (p=0.713, Table 5.7). *Cetobacterium*, *Phreatobacter*, and sequences identified to family *Beijerinckiaceae* were the next most abundant genera present across all diets, but no significant differences between dietary regimes was observed. All other genera relative abundance data were not significantly different between fish fed different dietary treatments. Sequencing data identified by STAMP and LEfSe software as of significant interest were identified as genera *Bacillus*, *Enterococcus* and *Weissella*, and these genera are presented as a heatmap showing relative abundance and relatedness of the samples in each diet (Figure 5.5).

The LEfSe histogram for the most distinct bacteria genus detected is shown in Figure 5.6, where the relative abundance for the genera *Bacillus*, *Enterococcus* and *Weissella* are plotted. The statistical tests that were performed in RStudio complement the data used in LEfSe and STAMP, and further post-hoc analyses were completed within the STAMP software, which are represented in Figure 5.7. The LEfSe software generated a cladogram for the significant genera detected and this is represented in Figure 5.8. The middle unconnected point of the cladogram represents the Domain, and based on the results from the LEfSe, family are shown at the fifth level from the centre point, and the genera at the sixth level (Figure 5.8).

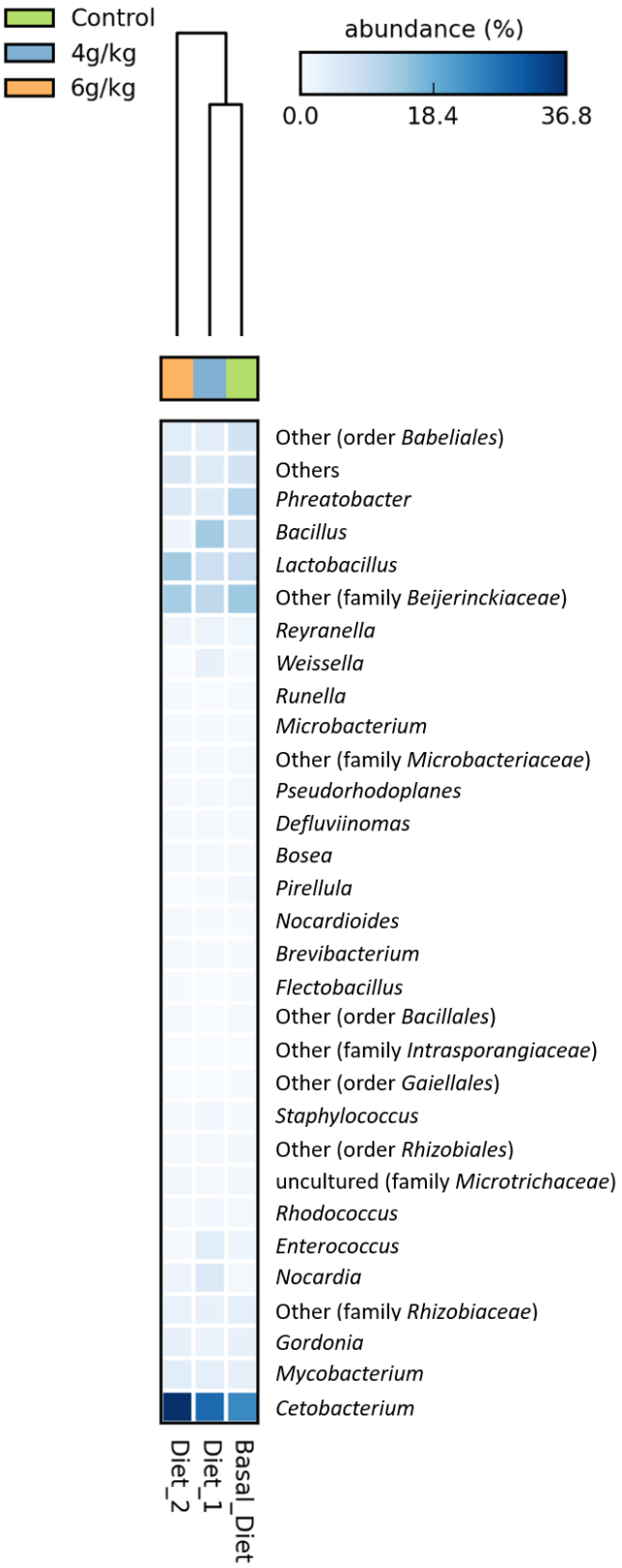


Figure 5.5 Mean abundance levels (%) of each genus present (or closest taxonomic level) within the microbiota of the intestinal digesta samples (n=7 fish per diet) for Nile tilapia fed incremental levels of B-GOS® over a period of 29 days. The treatments are represented as: Control = Basal_Diet; 4g kg^{-1} = Diet_1; 6g kg^{-1} = Diet_2.

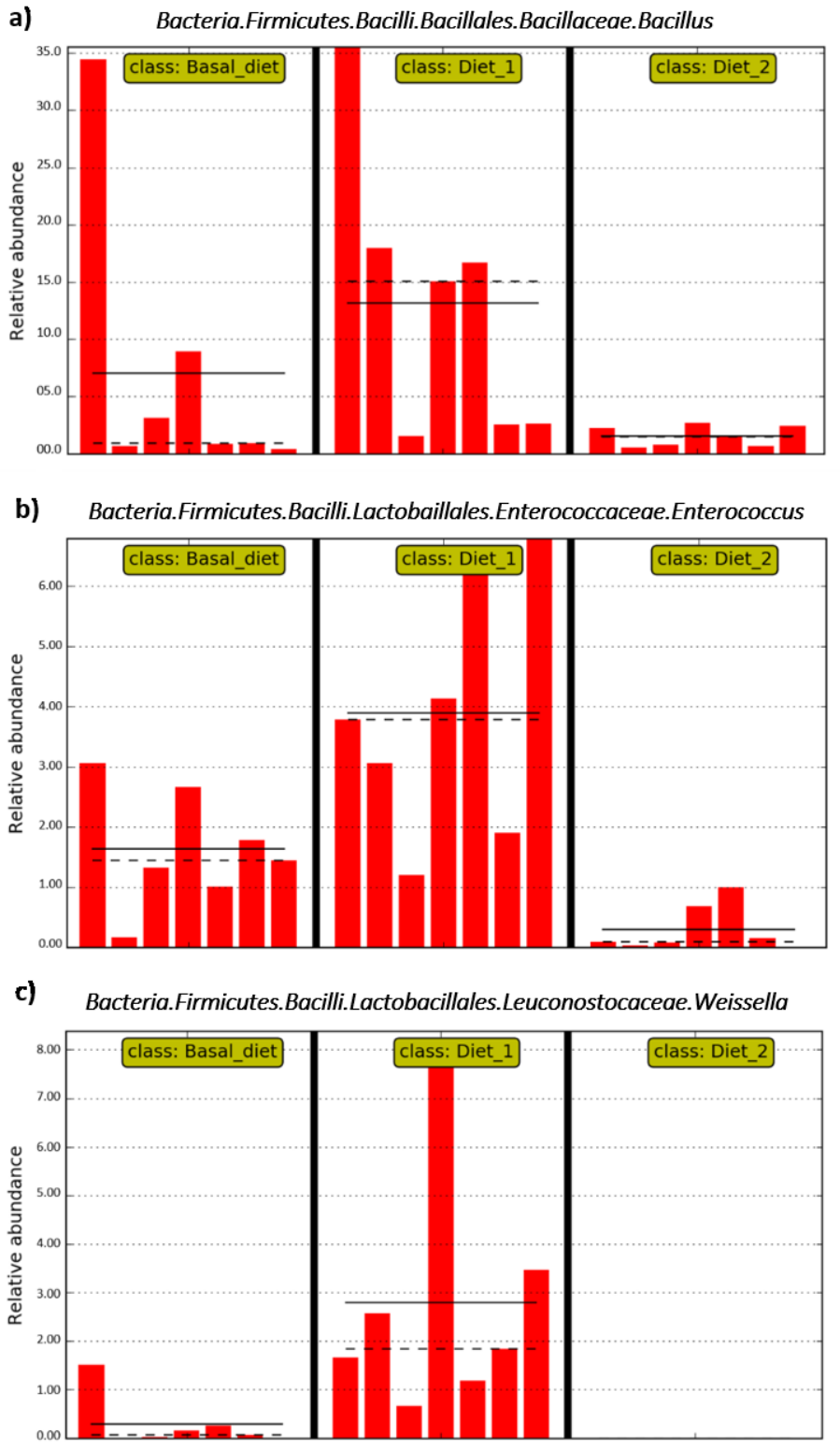


Figure 5.6 Differential features histogram plots of the biomarker OTUs detected by LEfSe showing the relative abundance (%) of each genus by diet. The bacterial genera detected are shown as **a)** *Bacillus*, **b)** *Enterococcus* and **c)** *Weissella*. The dotted line (- - -) represents the medians of each diet and the straight line (—) represents the means of each diet. The treatments are represented as: Control = Basal_Diet; 4g kg^{-1} = Diet_1; 6g kg^{-1} = Diet_2.

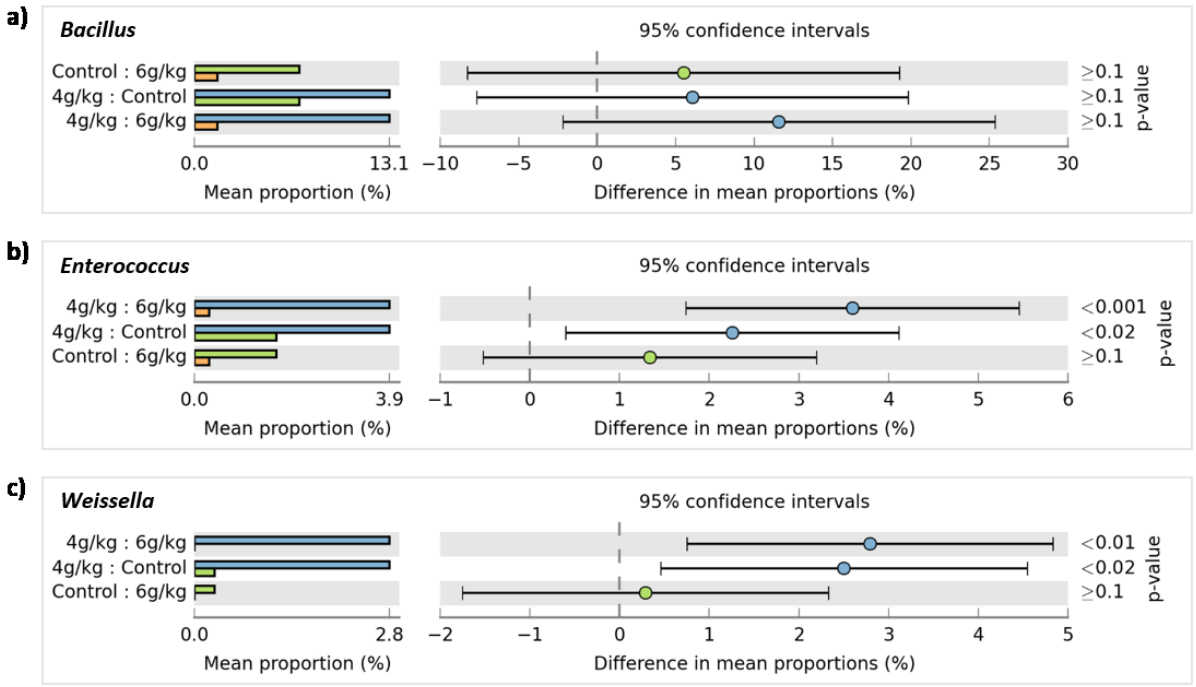


Figure 5.7 Post-hoc plots for the relative abundances of each bacterial genera that STAMP detected as being significantly different between Nile tilapia fed the experimental diets. Statistical differences were accepted at p<0.05. The genera detected are shown as **a)** *Bacillus*, **b)** *Enterococcus* and **c)** *Weissella*.

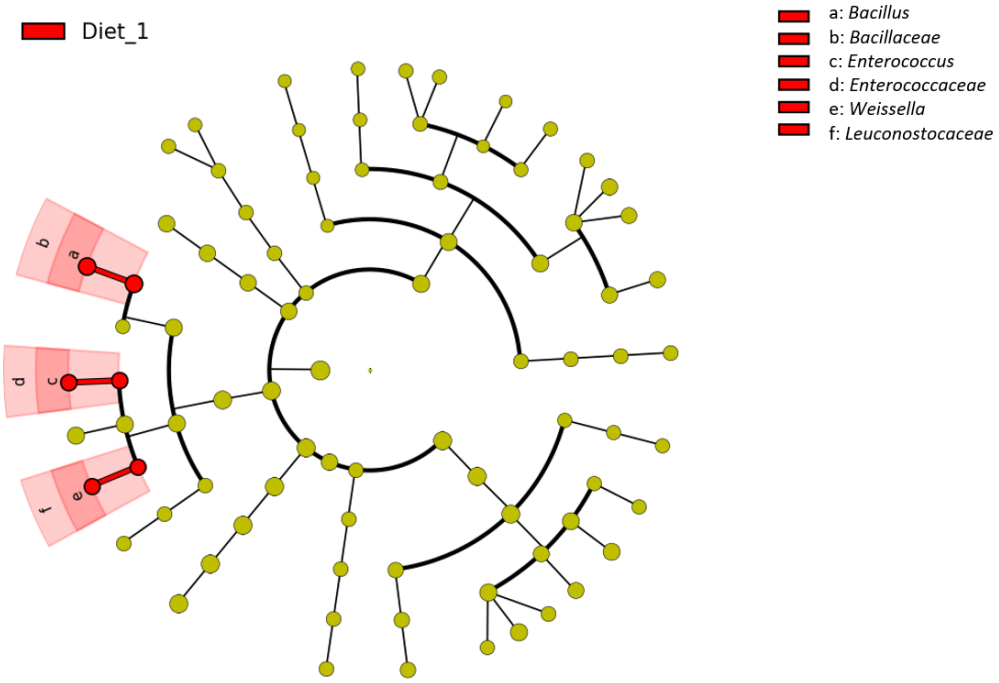


Figure 5.8 Circular cladogram reporting the identified OTUs from the LEfSe output and are distributed according to phylogenetic characteristics between treatments. The family and genus that are significantly different between each compartment of the cladogram are coloured differently to the yellow taxon levels that indicate OTUs with similar abundances, and are listed on the right side of the figure. The diets are represented as: Control = Basal_Diet; 4g kg^{-1} = Diet_1; 6g kg^{-1} = Diet_2.

5.4 Discussion

This study has contributed to the understanding of how growth performance and immune responses of a commercially valuable warm-water species are affected by a novel dietary additive. Finfish supply to the global markets is increasing, and one of the most rapidly growing sectors in aquaculture is cichlid production, in particular tilapia (FAO 2018, 2020). Nile tilapia have been consistently a popular and highly sought commodity, with over 4.5 million tonnes contributed to the global aquaculture industry in 2018 (FAO 2020). Given that this species is a staple part of a balanced diet in many developing and developed nations, it is vital that this sector continues to grow to meet demand in an environmentally and socially viable way (Adeoye *et al*. 2016a,b).

The effects that prebiotics elicit upon the growth and immune responses in Nile tilapia at an extremely vulnerable life stage has been researched relatively recently in comparison to other feed additives, such as probiotic inclusion, and there are comparatively fewer studies focussing on novel GOS products (Mugwanya *et al*. 2021). The third *in vivo* feeding trial was designed with similar objectives to Chapters 3 and 4, to investigate the efficacy of B-GOS® supplementation in Nile tilapia, a commercially important warm-water teleost species, to understand how growth performance and the immune response is influenced. In-depth analytical methods, such as measuring changes within the histomorphology of the intestine, gene expression of key biomarkers, and the relative abundance of microorganisms within the GIT microbiota, have demonstrated how Nile tilapia growth and health remain unaffected by GOS prebiotic addition under the conditions tested, despite some minimal but not significant improvements in the metrics tested.

5.4.1 Growth performance

The results of the present study suggest that there may be marginal, albeit not significant, improvements to growth performance in Nile tilapia fed the novel prebiotic B-GOS® at the concentration of 4g kg^{-1}. Of the parameters studied, including final weights, FCR, weight gain, SGR, K-Factor and survival, the most improvement was observed in fish fed the 4g kg^{-1} inclusion. These findings are in line with the results presented in Chapters 3 and 4, and previously reported in Atlantic salmon (Grisdale-Hellend *et al*. 2008) and Nile tilapia supplemented with feed additives including pre and probiotics (Standen *et al*. 2015). Similar results have also been demonstrated by Ziółkowska *et al*. (2020) who reviewed carp (*Cyprinus carpio*) and concluded that prebiotic inclusion at 1% or 2% GOS did not affect growth parameters, but haematological parameters such as phosphorous and histological morphology were improved by GOS addition. Pietrzak *et al*. (2020) also determined that carp fed 2% GOS did not present significantly increased growth compared to control fish, however, the immunomodulatory effects on the skin mucosa, in the form of gene expression in lysozyme, cytokines and protein production, were improved. While there is evidence to suggest that other health-related parameters may be significantly improved by dietary addition of prebiotics despite the growth remaining unaffected, the literature presents conflicting results.

Aryati *et al*. (2021) reported that growth performance, histomorphology and intestinal microbiota of Nile tilapia fed a diet containing a prebiotic honey supplement was significantly improved in fish fed the higher concentrations of supplement over 30 days compared to control fed fish. As the honey prebiotic was determined to contain multiple oligosaccharides, including FOS and GOS, the use of multiple prebiotics was suggested

to facilitate improved growth performance within fish over a short-term period (Aryati *et al*. 2021). Additionally, dietary supplementation of synbiotics has been investigated by Addo *et al*. (2017) in Nile tilapia fed a probiotic *B. subtilus* strain and the prebiotic Previda®, and the authors reported that the fish fed the combination of pro and prebiotic exhibited the highest survival rates compared to the control group when challenged with *A. hydrophila* against the control group. The combination of prebiotics and probiotics may benefit fish species over shorter time periods, and when fish species tested are at particularly vulnerable life stages. The addition of B-GOS® in Nile tilapia did not significantly improve growth performance in the present chapter, however further investigations are warranted to determine if additional dietary additives used in conjunction to B-GOS® may present improved performance metrics in tilapia.

Another factor to consider is the life history of cichlids. Nile tilapia are omnivorous and exhibit cannibalism that intensifies as they age, especially in heterogeneous size distributions within the rearing tanks that result in greater social aggression, it is especially important to reduce the potential for fry or fingerling loss at the early life stages (Pantastico *et al*. 1988; Fessehaye *et al*. 2004). This factor may explain why the FCR was higher during this trial than in the previous chapters, as cannibalism may be a cause or consequence of size heterogeneity since the smallest fish are eaten and so the largest ones survive (Fessehaye *et al*. 2004), thus influencing the final FCR result at the end of the trial. Despite the increased FCR compared to the previous growth performance results in Chapters 3 and 4, the 4g kg^{-1} prebiotic inclusion did provide some improvement in the overall final weight and feed utilisation in fish fed this diet, as this group exhibited the lowest FCR compared to the control fed fish. There may be marginal numerical improvements in fish growth of this size in Nile tilapia fed B-GOS®

over 29 days, however, as the tilapia were reared during one of their most vulnerable life stages, it would be useful to determine if the prebiotic tested here could be beneficial for fish that grow on from this size and for longer trial periods, or in combination with other additives.

5.4.2 Histology

The intestinal histological analyses revealed normal and healthy morphology of the mid-intestine of Nile tilapia fed either dietary regime. There were no significant improvements of muscularis thickness, mucosal fold heights and intestinal mean goblet cell counts between fish fed each dietary regime, suggesting that the inclusion of B-GOS® does not enhance these histomorphological features of the mid-intestine in Nile tilapia under the conditions tested.

However, there was a significant increase in the lamina propria thickness of tilapia fed the 4g kg^{-1} prebiotic inclusion compared to the control and the 6g kg^{-1} inclusion fed fish. Similar thickening of the lamina propria has also been demonstrated in the posterior section of the intestine in juvenile tiger grouper (*Epinephelus fuscoguttatus*) in a study conducted by Firdaus-Nawi *et al*. (2013). As the GIT is portioned along its entirety with different functions in each section, it is prudent to examine a representative section for analysis. The authors reported that the posterior intestine of tiger grouper is linked to immune function when examined using light microscopy, as the authors observed a significantly higher number of lymphoid and goblet cells, and significantly thicker lamina propria, thus suggesting that this section of the gut has a clear role in immunity (Firdaus-Nawi *et al*. 2013). The mid-section of the intestine was reported to be the transitional phase between the two structures, with the anterior

section being used for absorption and the posterior section used for immune responses (Firdaus-Nawi *et al*. 2013). Given that the mid-section of the tilapia intestine was sampled for the present study, it may be possible that other changes attributed to GOS supplemented diets could not be discovered at this time. These changes may be present in other sections of the intestine, or as part of the ultrastructure that may only be observed using electron microscopy (Dimitriglou *et al*. 2010; Zhou *et al*. 2010).

It is difficult to determine an exact cause for the increased lamina propria thickness in the results of this chapter. Previous evidence has observed that IgM positive cells, T cells, APCs and mast cells may be located in the lamina propria of salmon, and are upregulated when an immune response is triggered (Rombout *et al*. 2014; Bjørgen *et al*. 2020). However, the widening of the lamina propria may also be presented as the result of an enteric state elicited by a high plant-based diet (van den Ingh *et al*. 1991; Merrifield *et al*. 2011; Bjørgen *et al*. 2020). The diet compositions formulated in this study have a high proportion of plant feedstuffs, which includes SBM and sunflower meal, with little input from fish derived proteins. As the tilapia reared in this trial were at a vulnerable life stage, any potentially harmful changes to morphological parameters were investigated and analysed using light microscopy. As no detrimental effects to the morphology of the tilapia GIT were apparent, the results of the present study suggest that the diet composition of SBM and plant stuffs did not alter the growth of the fish or impact the trial conditions and were suitable for tilapia.

The increased length of the mucosal folds within teleosts may indicate a greater uptake and utilisation of nutrients within the intestine (Bjørgen *et al*. 2020), and so allow for increased growth and immune function (Anguiano *et al*. 2013). The marginal, albeit not

significant, improvements to the muscularis thickness and mucosal fold heights within the 4g kg^{-1} diet fed fish, in addition to an overall increased final weight compared to the other treatment groups, suggest that this inclusion level may have the potential to improve the intestinal morphology of tilapia fed B-GOS® at an early life stage. These findings from this chapter are also in accordance with Chapters 3 and 4 for salmonids fed this 4g kg^{-1} B-GOS® inclusion level. Future studies using additional sections of the intestine and other analytical techniques that focus on other aspects of the mucous, such as protein levels or activity, should be considered to determine if other aspects of the immune response are affected by GOS addition.

5.4.3 Body Composition

No significant differences were observed between the carcass ash content, crude lipid content and protein content compositions of fish fed the dietary regimes, suggesting that GOS addition did not significantly affect tilapia body composition. The exception to this finding was the moisture content of the 4g kg^{-1} fed fish carcasses was significantly decreased compared to the control fed fish. As the carcass composition analyses required a certain amount of material to be performed, individuals were pooled by tank to produce enough dry matter for further lipid, protein and ash content measurements. The reason for this significant result is not clear.

5.4.4 Gene Expression

As discussed in Chapter 1, the immune system in fish is comprised of innate and adaptive responses that interact through complex subsystem signalling pathways to detect and mount a defensive response against pathogenic infection within the host (Yukgehnaish *et al*. 2020). Prebiotics may also act as immunostimulants in tilapia spp.

through the stimulation of the host immune system by increasing cytokine production to modulate the inflammatory response during normal or stressful conditions (Mugwanya *et al*. 2021). The expression of certain genes relating to the health of fish can be measured to ascertain how the diet can influence these cytokines and determine if a prebiotic may influence the immune response. The target genes that were chosen to compliment the previous research outlined in Chapters 3 and 4 were *IL-10*, *IL-1β*, *TNFα*, *TGF-β, HSP70, CASP3, PCNA* and *MYD88*. These genes have been categorised in Nile tilapia as biomarkers for evaluating immune responses in previous literature (Mugwanya *et al*. 2021), but there is comparatively little information regarding the addition of prebiotics and how they may influence the expression of immune-related genes in tilapia. Therefore, it was prudent to investigate the expression of these biomarkers in this fish species, and how they are influenced, if at all, by the addition of B-GOS® to the diet.

The upregulation of anti-inflammatory cytokine *IL-10* in tilapia fed 4 and 6g kg^{-1} B-GOS® suggests a dampening of the inflammatory response compared to control fed fish. As discussed in previous chapters, this pleiotropic cytokine *IL-10* is readily expressed in host tissues, and is capable of inhibiting the synthesis of pro-inflammatory cytokines, reactive oxygen and nitrogen radicals, and downregulates the expression of Th1 cytokines (Bogdan *et al*. 1991; Opp *et al*. 1995; Zou & Secombes 2016; Wu *et al*. 2021). Authors Wu *et al*. (2021) have previously observed that Nile tilapia *IL-10* is constitutively expressed in a number of different tissues and organs, such as the gills, kidney and spleen. The expression of *IL-10* was significantly elevated in tilapia that had been challenged with *S. galactiae* and lipopolysaccharide (LPS), and elevated IgM antibody production levels were detected, further providing evidence that upregulated *IL-10* may

promote pathogenic resistance in Nile tilapia (Wu *et al*. 2021). The increased expression of *IL-10* within the present chapter, particularly in fish fed 4g kg^{-1} diet, suggests that anti-inflammatory responses may potentially be elevated by GOS addition and may provide balance between intense immune responses to potential infection and pathological injury (Rawling *et al.* 2019).

The relative expression of *IL-1β* in tilapia was reduced (albeit not significantly) in fish fed B-GOS® at any concentration compared to the control, suggesting that the pro-inflammatory response was not activated by the addition of this prebiotic under the conditions investigated. This effector cytokine is one of the first cytokines to be activated during intracellular stress (Rawling *et al*. 2019), and so would further activate lysozyme activity in macrophages, cell proliferation and apoptosis (Montalban-Arques *et al*. 2014, 2015; Zou & Secombes). The results of the present chapter are similar to those of Hoseinifar *et al*. (2017c), whose authors observed that the expression of *IL-1β* in the kidney of common carp was significantly downregulated in fish fed 2% GOS, FOS and inulin compared to the control group. A similar significant downregulation in the intestinal expression of *IL-1β* was also determined by Modanloo *et al*. (2017) in common carp fed 1% GOS versus a control group. The results of the present study suggest that B-GOS® likely has little effect on the pro-inflammatory response via *IL-1β* regulation, perhaps due to the significant upregulation of *IL-10* in the same tissue site, or perhaps due to the excellent rearing conditions in which the tilapia were raised that has provided little scope for the prebiotic to be fully utilised by the host.

Significant upregulation in the expression of *TNFα* in tilapia fed 4g kg^{-1} B-GOS compared to 6g kg^{-1} inclusion suggests that there is a potential elevation in pro-

inflammatory responses that may be induced by this cytokine, such as apoptosis, necrosis and cell survival, as well as increases in Th1 and Th2 cytokine transcription (Wang & Secombes 2013). Qin *et al*. (2014) reported that hybrid tilapia (*O. niloticus* ♀ x *O. aureus* ♂) fed incremental inclusion levels of chito-oligosaccharides exhibited lower gene expression levels of *TNFα*, protein *HSP70*, and increased gene expression levels of the anti-inflammatory cytokine *TGF-β* (Qin *et al*. 2014). These results are similar to the results of the 6g kg^{-1} GOS fed fish in this chapter, but not the fish fed 4g kg^{-1}. The upregulation of this gene within the intestine of Nile tilapia fed the 4g kg^{-1} B-GOS® may suggest a slight elevation in the pro-inflammatory response, as *TNFα* is secreted by activated macrophages and can be associated with the regulation of immune cells during pathogenic infection (Paul *et al*. 2012).

In a study conducted by Abu-Elala *et al*. (2018), the addition Immunowall® (yeast cell wall and MOS compounds) to Nile tilapia promoted the significant up-regulation of the *TNFα* cytokine and enhanced survival and disease resistance against *Lactococcus garvieae* and *A. hydrophila* infection. It is difficult to conclude if the addition of the prebiotic in the present chapter at 4g kg^{-1} is enhancing the immune response by elevating the expression of *TNFα,* especially as fish fed the 6g kg^{-1} diet expressed a downregulation in relation to the control group of the same gene. Further research should focus on how an abiotic or biotic challenge may influence the expression of this cytokine, as demonstrated by Abu-Elala *et al*. (2018) to determine if 4g kg^{-1} is the suitable level of inclusion of B-GOS® to administer to tilapia.

While not significant, there was a 1-fold level of downregulation in the expression of *TGF-β* within the intestine of Nile tilapia fed 4g kg^{-1} B-GOS® diet. *TGF-β* has been

reported to be involved in wound healing in the skin of teleosts (Skugor *et al*. 2008), as well as cellular proliferation and differentiation during anti-inflammatory responses (Yang *et al*. 2012). Previous research has demonstrated significantly increased expression of *TGF-β* in the intestine of hybrid tilapia fed probiotic *B. subtillis* C-3102 (He *et al*. 2013), and upregulation expression of immune-related genes *TNFα, IL-1β, IL-10* and *TGF-β* in Nile tilapia in response to probiotic AquaStar® (*Bacillus* spp., *Enterococcus* spp. and *Lactobacillus* spp.) addition (Standen *et al*. 2016). The results of the present chapter contradict this previous research, as the downregulation of *TGF-β* may suggest a possible inactivation of the anti-inflammatory response in tilapia fed this prebiotic, whilst the fish fed 6g kg^{-1} dose were unaffected by B-GOS® addition. As the increase in *IL-10* expression, another anti-inflammatory cytokine, was significantly upregulated in tilapia fed B-GOS® there may be no scope for the activation of *TGF-β* in these tilapia fed GOS at these doses.

The expression of *CASP3* was slightly, but not significantly, elevated in tilapia fed both prebiotic concentrations in relation to the control group, suggesting that the inclusion of B-GOS® does little to stimulate the expression of this cytokine. *CASP3* plays an important role in activating other caspases and catalysing protein degradation during cell apoptosis (Nesic *et al*. 2001; Serradell *et al*. 2020), and its elevated expression has been used as a biomarker for the presence of apoptosis in teleost leucocytes (Reyes-Becerril *et al*. 2018). Serradell *et al*. (2020) previously reported that GMOS and phytogenic functional feeds (PHYTO, garlic and labiatae essential oils) have little effect on basal *CASP3* and *IL-1β* expression in European seabass after feeding for 9 weeks in suitable rearing conditions for seabass, suggesting that there was no scope for these prebiotics to be fully utilised by the fish.

Conversely, once stress tested with confinement and/or *V. anguillarum* (1 x 10^5 CFU per fish), seabass fed all treatment diets expressed significantly elevated *CASP3* and *IL-1β* expression two hours post-challenge, with the control group exhibiting the greatest fold change compared to the prebiotic diets (Serradell *et al*. 2020). These expression levels of *CASP3* then decreased to pre-challenge levels 24 hours after infection, however, *IL-1β* was significantly elevated in the fish fed prebiotic diets relative to the control group at this time period. The presence of prebiotics may have mitigated apoptotic mechanisms in response to stress in seabass by presenting lower expression of *CASP3* to protect leucocytes from degradation, while also enhancing the immunity by increasing pro-inflammatory responses via upregulating *IL-1β* to respond to pathogenic challenges (Serradell *et al*. 2020). In the present study, the lack of significant upregulation in *CASP3* suggests that there may have been little apoptotic activity occurring within tilapia fed either B-GOS® diet. Further research testing this prebiotic in tilapia reared under challenging conditions should be conducted to determine if the GOS concentrations investigated here act to mitigate increased apoptotic mechanisms during stress from pathogenic or environmental challenges.

The lack of significant *MYD88* expression suggests that the addition of any concentration of B-GOS® would leave little scope for improvement in tilapia immune response by activation of this gene. Myeloid differentiation primary response 88 is an adapter protein encoded by *MYD88* gene expression, and plays a pivotal role in the activation of PRRs, such as TLRs (Deguine & Barton 2014). These PRRs detect DAMPS and PAMPS that are produced by pathogens upon entry into the host, as *MYD88* acts to connect these receptor proteins receiving signals from outside the cell with proteins located inside the cell. *MYD88* activation may also activate further expression of

nuclear-factor kappa-B (*NF-κB*), a protein that controls DNA production and cell survival, and can increase expression of pro-inflammatory cytokines in response to pathogens (Deguine & Barton 2014). Trung & Lee (2020) reported that *MYD88* obtained from Nile tilapia was readily expressed in the head kidney and spleen, and is essential in the innate immune system response. The authors observed that *MYD88* expression was elevated when tilapia were challenged by *S. agalactiae*, and activated the expression of *NF-κB*, which in turn induced the activation of pro-inflammatory cytokines *IL-1β* and *IL-12b*, further demonstrating *MYD88*'s role in innate immunity (Trung & Lee 2020). As the results from the present chapter have demonstrated a lack of significant up/downregulation of *MYD88* in B-GOS® supplemented fish from the control, it is unlikely that further pro-inflammatory responses are activated in tilapia above what are constitutively expressed. This may be due to the lack of a challenge present in the form of pathogenic infection, and so there is little need for fully utilising the prebiotic in already suitable rearing conditions.

The significantly upregulated gene expression of *HSP70* in fish fed the 6g kg^{-1} diet compared to other dietary treatments suggests that these fish may have been responding to potential stress. Heat shock proteins are highly conserved within most terrestrial and aquatic species, with members of the *HSP70* family playing important roles as molecular chaperones, involved in protein folding, cell cycle regulation and apoptotic mechanisms (Hendrick & Hartl 1993; Ming *et al*. 2010) under normal conditions. When an organism is stressed, for example by pathogen infection or overcrowding, the synthesis of *HSP70* mRNA gene is upregulated to allow cells to cope with acute stressor insults, especially those which affect protein signalling mechanisms (Molina *et al*. 2000; Boone & Vijayan 2002; Sørensen *et al*. 2003; Ming *et al*. 2010). The

production of inducible *HSP70* in fish depends on the strength and duration of exposure to a stressor, with the elevation in expression levels considered short-term (Molina *et al*. 2000). This principle was observed by Ming *et al*. (2010) who reported that Wuchang bream (*Megalobrama amblycephala* Y.) infected with *A. hydrophila* demonstrated a maximum elevation of two *HSP70* mRNA expression levels at 6 hours post-infection, which then gradually decreased to control levels after 24 hours.

Comparatively, there are fewer studies investigating how prebiotics affect fish *HSP70* expression compared to probiotic addition (Yilmaz *et al*. 2022), however, there are studies investigating synbiotics supplementation. Previous research by Gewaily *et al*. (2021) demonstrated that a synbiotic combination of β-glucan and *L. plantarum* may protect Nile tilapia challenged by deltamethrin (DMT) pesticide from increased apoptosis and acute stress to cellular processes. This was supported by *HSP70* and *CASP3* both expressing downregulated mRNA levels compared to fish stressed with DMT without synbiotic feeding (Gewaily *et al*. 2021). Other cytokines involved in the pro-inflammatory response, such as *IL-8* and *IL-1β*, were significantly elevated in tilapia treated with DMT and synbiotics compared to only-DMT treated fish (Gewaily *et al*. 2021). These results indicate that there was a synbiotic effect exhibited in fish fed this diet that prevented the immune-suppressing effects of DMT previously seen within fish, such as a reduction in physiological, immunological and pro-inflammatory responses (Dawood *et al*. 2022).

In the present chapter, the upregulation of *HSP70* in tilapia fed 6g kg^{-1} B-GOS® may indicate some stress that the fish were experiencing, and this gene expression may have been activated to mitigate potential cellular damage that may be induced. The fish were

reared in a benign rearing environment, and so no stressors were introduced to the tilapia as part of the trial. As there were mortalities across all treatment groups, the weights of these fish were recorded and included in the biomass calculations for growth performance. Mortalities are common in juvenile tilapia due to instinctive cannibalism displayed between different size ranges (Pantastico *et al*. 1988; Fessehaye *et al*. 2004, 2006; Abdel-Hakim *et al*. 2009; Hezron *et al*. 2019), and so perhaps increased aggression had induced an inadvertent social stressor within the tanks, leading to increased *HSP70* expression. Caution should be advised however, regarding this conclusion. The gene *HSP70* also acts as a molecular chaperone involved in protein folding, and stress responses were not measured in this study. In addition, the survival rates of each tank were not significantly affected by any dietary treatment within this chapter, and there has not been other genomic work to compliment the gene expression performed in this chapter to support the conclusion that the tilapia were under stress. Further research should focus on how *HSP70* and others related to it in Nile tilapia may influence the immune stress response by the addition of these ranges of B-GOS® under more challenging conditions, for example introducing pathogens found in warm-water aquaculture (e.g. *A. hydrophila*) or by inducing extremes in rearing water temperature.

The significant downregulation of *PCNA* in fish fed the 4g kg^{-1} B-GOS® diet suggests that tilapia may not have exhibited cellular responses in which DNA would be repaired, or to increase cellular division. Proliferating cell nuclear antigen is a protein encoded by *PCNA*, and is found in the nucleus. This gene plays an essential role in metabolising nucleic acids as a component of DNA replication and repair machinery, and as a processivity factor for DNA polymerases δ and ε (Kelman 1997; Essers *et al*. 2005). Probiotic research has reported upregulated gene expression of PCNA in Nile tilapia

supplemented with probiotics such as *Bacillus* spp., *Enterococcus* spp., *Lactobacillus* spp. and *Pediococcus* spp. (Standen *et al*. 2015, 2016). Peggs (2015) determined that European seabass fed a prebiotic (Previda®), probiotic (*B. subtilis*) or a synbiotic diet of both expressed downregulation of the gene *PCNA* relative to a control group. Similar results were also reported by Rawling *et al*. (2019), whose authors demonstrated that feeding European seabass single or multi-strain yeast fractions expressed significant downregulation of *PCNA* relative to control fish, in agreement with the results of the present chapter. Upregulation of this gene may suggest that there is a high cellular turnover within the intestine (Peggs 2015), and so the results of the present chapter suggest that B-GOS® does not induce cellular turnover by increased *PCNA* expression in Nile tilapia.

The results from the present chapter suggest that there is minimal improvement in the expression of key immune-related genes within the mid-intestine of tilapia fed B-GOS®. The expression levels of *IL-10, TNFα, HSP70* and *PCNA* within fish fed 4g kg^{-1} suggest that this inclusion rate may induce an improved immune response by modulating the pro- and anti-inflammatory responses within the intestine, and potentially reducing stress responses within this target organ. Despite marginal improvements within the expression levels of these key immune biomarkers, other immune parameters tested in this chapter suggest that there is little enhancement in the histology and overall performance of tilapia supplemented with B-GOS® at 4 - 6g kg^{-1} inclusion level. Further research should be conducted utilising abiotic/biotic challenges, such as pathogenic infection or extremes in rearing water temperature, to determine if the inclusion of B-GOS® to the diets of challenged fish would influence the immune system by modulating the gene expression in key immune response genes.

5.4.5 Intestinal Microbiome Analysis

Tilapia fry and juveniles offer a suitable life stage to study the colonisation of the intestinal microbiota, and thus are suitable to study how the microbiome can be altered by the addition of prebiotic supplementation (Giatsis *et al*. 2014; Haygood & Jha 2018). Previous research has indicated that the intestine of tilapia can be modulated by feed additives as a preventative measure to fight potential pathogens, and to induce a state of immune readiness in the host (Li *et al*. 2015; Standen *et al*. 2015; Adeoye *et al*. 2016; Xia *et al*. 2020; Yukgehnaish *et al*. 2020). The sequencing depth achieved through the bioinformatics analysis was confirmed to be adequate, as the Good's coverage for all treatments was >99%. The resulting sequencing libraries from the high-throughput 16S rRNA sequencing revealed that there were no significant increases or decreases in the number of features/OTUs, Good's coverage, Shannon or Chao1 indices between any of the experimental diets. The species richness (Chao1) within the fish fed each experimental diet was not significantly different from each other or the control fed fish, and this was likewise reflected in the Shannon diversity index. As the OTUs and Chao1 values were similar to each dietary group, this indicates that there were similar species present with a similar diversity composition (Kim *et al*. 2017).

In terms of the sequencing relative abundance, any reads that were unidentifiable, or did not pass the threshold (>0.25%), were grouped as 'Others', and this group averaged 0.92% of the total number of reads sequenced. The most dominant phyla was *Fusobacteria*, averaging 29.65%, then *Proteobacteria* (28.30%), followed by *Firmicutes* (21.59%), *Actinobacteria* (12.20%), *Dependentiae* (4.73%), *Planctomycetota* (1.18%), *Bacteriodota* (0.69%), *Cyanobacteria* (0.67%). The core microbiome of tilapia spp. is suggested to be dominated by the presence of *Fusobacteria, Proteobacteria, Firmicutes*

and *Actinobacteria,* with other phyla and genera differing depending on age, location and species of tilapia (He *et al*. 2013; Baldo *et al*. 2015; Standen *et al*. 2015; Haygood & Jha 2018; Yukgehnaish *et al*. 2020). The reads assigned to the *Cyanobacteria* phylum were concluded to be artefacts of sampling and are present due to the high plant derived diets, and so these reads were filtered and removed as in Chapter 3, section 3.2.4.

There were three genera identified from high-throughput sequencing, STAMP and LEfSe that exhibited significantly higher relative abundances in fish fed 4g kg^{-1} B-GOS® compared to fish fed 6g kg^{-1}, but not the control group: *Bacillus*, *Weisella* and *Enterococcus*. Other genera, such as *Cetobacterium*, were also present at substantial abundances (see Table 5.7). This is unsurprising, as *Cetobacterium* has been isolated from tilapia and other warm-water species and is largely considered part of the core microbiome in cichlids (Li *et al*. 2015; Standen *et al*. 2015; Adeoye *et al*. 2016b; Xia *et al*. 2020). However, the relative abundance of this genus within Nile tilapia was not significantly affected by dietary treatment.

Bacillus is a genus that comprises of Gram-positive, rod-shaped bacteria, with a large number of species and strains available as probiotics. A popular probiotic is *B. subtilus* and associated strains that have been utilised in various fish species as part of feeding trials, such as in rainbow trout (Merrifield *et al*. 2010a), European seabass (Peggs 2015), Nile tilapia (Standen *et al*. 2015), and hybrid tilapia (*O. niloticus* x *O. aureus*; He *et al*. 2013) to name a few. Within the present chapter, the relative abundance sequence reads representing *Bacillus* are significantly more abundant in the 4g kg^{-1} diet fed fish than the 6g kg^{-1} group, however, this difference is not significant from the control group. It is suggested that the presence of *Bacillus* spp. within the GIT of fish are able to modulate

the intestinal microbiota and produce useful metabolites that are utilised by the host, such as turins or cyclic lipoproteins (Sugita *et al*. 1998). There is further evidence to suggest that *Bacillus* spp. used in dietary trials may reduce the populations of some putative pathogens commonly found in juvenile tilapia, such as *Pseudomonas* spp. and *Aeromonas* spp. (Del'Duca *et al*. 2013), and also improve the immune response by increasing leucocyte phagocytic activity (Aly *et al*. 2008). The results of the present chapter suggest that increased *Bacillus* spp. abundance may contribute towards greater nutritional utilisation of metabolites produced by this bacterium, and may potentially reduce pathogenic abundance. Future research should focus on the presence of *Bacillus* as part of the core microbiome in tilapia fed GOS prebiotics, as there are fewer studies focusing on the addition of prebiotics to the diet of this fish species than there are probiotics.

The latest NGS techniques have been utilised in intestinal microbiome studies, and more recent reports suggest that the core microbiome in fish may be less likely to have a dominant component of *Carnobacterium* species, previously thought to have comprised up to 15% of the microbiota (Merrifield *et al*. 2014). Indeed, it appears that indigenous GIT species are also *Lactobacillus* spp., *Streptococcus* spp., *Lactococcus* spp., *Weissella cibaria* and *Pediococcus* spp., with the genera of warm water species (tilapia, zebrafish, cobia for example) thought to also comprise of *Leuconostoc* species and *Cetobacterium* species (Merrifield *et al*. 2014; Li *et al*. 2015; Xia *et al*. 2020). Among these genera in fish species, *Weissella* is a Gram-positive genus comprising of non-motile, usually non-pathogenic species that are found within the GIT of fish, and is part of the *Latobacillaceae* family (Welch *et al*. 2017). For the relative abundance reads assigned to *Weissella* within this chapter, the relative abundance of this genus was

significantly greater in the 4g kg^{-1} diet fed fish than in the 6g kg^{-1} group, however, this difference is not significant from the control fed fish. Xia *et al*. (2020) demonstrated that juvenile Nile tilapia fed probiotics containing either *B. subtilis* or *B. cereus* or a mix of both after 6 weeks had significant improvements in their microbiomes compared to control fed fish, with the composition of *Weissella* spp. increased in *B. subtilis* fed fish. Previous research has demonstrated the potential for *W. cibaria* and *W. confusa* as probiotics when isolated from human faeces (Lee *et al*. 2012), and so the results of the present chapter suggest that the addition of 4g kg^{-1} B-GOS® may help to modify the intestinal microbiota in tilapia by enhancing its diversity (Egerton *et al*. 2018). Given that this genus has been found as part of the core microbiome in Nile tilapia, it is worth exploring how this taxonomic group may further benefit other fish species by its increased abundance upon addition of feed additives. Further research should focus on how the novel B-GOS® prebiotic tested in the current chapter at 4g kg^{-1} inclusion level may further benefit economically valuable fish species such as tilapia by the upregulation of potentially new probiotics.

For relative abundance of reads assigned as *Enterococcus* during this study, the abundance of fish fed the 4g kg^{-1} diet was greater than fish fed the 6g kg^{-1} diet, however, this difference is not significant from the control fed fish. This taxonomic group comprises of a large genus of Gram-positive LAB, which have been previously highlighted as good candidates for probiotics in aquaculture settings, as discussed in Chapter 3, section 3.4.8. Previous research has shown evidence of enterococci providing benefits to the host should their populations become upregulated by the presence of a pro/prebiotic (Peggs 2015; Cavalcante *et al*. 2020). These benefits may be presented as competition for adhesion sites within the GIT (Cavalcante *et al*. 2020), such that

potentially harmful opportunistic pathogens from the genera *Aeromonas*, *Pseudomonas*, *Flavobacterium* and *Clostridium* may exhibit reduced relative abundance (Ramos *et al*. 2013; Peggs 2015). *Enterococcus* spp. are not usually pathogenic within the GIT, and species that have been utilised as probiotics such as *E. facealis* and *E. faecium* comprise of a normal part of the microbiome in humans (Gilmore *et al*. 2002). There are numerous studies of *Enterococcus* spp., such as *E. faecalis*, improving growth performance, immunity and resistance to pathogen challenges (Avella *et al*. 2011; Sorroza *et al*. 2012; Peggs 2015). Liu *et al*. (2021) observed that tilapia (*O. mossambicus*) fed probiotics containing strains of *B. subtilus*, *E. faecalis*, or both, demonstrating significant improvements to survivability and immune response when fed these treatments over 42 days, and after exposure to *S. agalactiae* challenge. Within the present chapter, the significantly elevated *Enterococcus* levels in tilapia fed 4g kg^{-1} B-GOS® could be considered potentially beneficial given their positive attributes demonstrated in other feed additive studies (Ramos *et al*. 2013; Peggs 2015; Cavalcante et al. 2020). This genus and other LAB abundances may provide greater immunity in fish fed the novel prebiotic B-GOS®, and further research should focus on understanding how the prebiotic tested in the present study may further improve the performance of tilapia or other economically important species once challenged.

As the significant changes to the microbiome abundance of *Bacillus*, *Weissella* and *Enterococcus* were still relative; caution is advised when comparing abundances. In some genera, the 16S rRNA copy numbers differ between bacterial species, and so the bacterial sequence reads may be unreliable in samples sequenced in this manner (Fogel *et al*. 1999; Peggs 2015). Therefore, future work should focus on combining more recent developments in 16S rRNA sequencing with absolute methods of identification to

ensure that the microbiome is fully explored in fish fed this novel dietary GOS prebiotic tested in this study.

5.5 Conclusions

The results reported in the present study provides evidence that a novel GOS does not meaningfully alter the health and growth of Nile tilapia relative to fish fed an already highly nutritious and optimally formulated diet. There were marginal improvements at a localised level of the histomorphology of GIT mucosal folds, modulation of the GIT microbiota and immune and stress-related gene expression exhibited in tilapia fed a basal diet supplemented with 4g kg^{-1} B-GOS®, which is in agreement with the results presented in Chapters 3 and 4.

Administration of 4g kg^{-1} B-GOS® resulted in increased lamina propria within the mucosal folds of the intestine, modulation of pro-inflammatory and stress responses via improved expression in key immune-related cytokine genes *IL-10*, *TNFα*, *HSP70* and *PCNA*, in addition to a higher abundance of *Bacillus*, *Enterococcus* and *Weisella* in the GIT microbiota. These data suggest that this inclusion rate of 4g kg^{-1} prebiotic may modulate the microbiota of the intestine at a localised level and help to improve immune readiness within Nile tilapia fed this novel GOS.

However, as not all parameters were significantly improved compared to the control fed fish at the whole organism level, further experiments must be conducted to ascertain if the consistent rearing conditions and optimally formulated diet were factors that affected the prebiotic efficacy. Recent reports in the literature demonstrate that the health benefits to immunity and growth from prebiotics administered in tilapia aquaculture rely on a number of factors (Mugwanya *et al*. 2021). Prebiotic effectiveness

appears to be dose-dependent and is heavily impacted by the age and species of fish, the system used for rearing, and if there is the addition of a/biotic stressors in the form of challenge trials (Mugwanya *et al*. 2021). As the fish tested in this study were at a crucial point in their development, it is vital to determine how this additive may improve the performance of Nile tilapia and other commercially valuable warm-water species at a high risk and vulnerable age.

The research conducted in Chapters 3 and 4 has illustrated that there is the potential for greater utilisation of B-GOS® within the important salmonid species, as marginal improvements in health and performance were evident. Similar results are concluded in this chapter, with the inclusion rate of 4g kg^{-1} determined to be the most suitable concentration of this prebiotic, and as a candidate for further testing in conjunction with challenge trials. Abiotic and biotic challenges introduced to the rearing environment of juvenile tilapia may provide additional insight as to how changes in water quality, overcrowding or seasonal shifts in temperature may induce pathogenic infections and stress within fish, thus mimicking the challenges faced in an aquaculture facility. Such challenges may allow for greater utilisation of the prebiotic in the mucosal tissues of Nile tilapia.

General Discussion

The aquaculture industry has the potential to enable sustainable food production and provide the world with a reliable source of protein, fats and omega 3's to complement what may be for many a solely vegetable based diet (Ibrahem *et al*. 2010; FAO 2020; Koehn *et al*. 2022). Historically, global mismanagement of wild capture fisheries has led to declined fish stocks, and so the aquaculture industry must expand to meet demand for seafood and work towards becoming more environmentally and economically sustainable (FAO 2018; Pontefract 2021). Disease still remains one of the most critical challenges to overcome within the aquaculture industry (Mzula *et al*. 2021), and research has focused on refining methods of achieving enough food to meet demand, whilst also providing effective means of preventing pathogenic infection in cultured species. Feed additives have demonstrated significant improvements to teleost fish health and growth within the literature, and can be an alternative to the overuse of antibiotic growth promoters (Dawood *et al*. 2018). There is a diverse range of feed additives tested for commercial use in teleost aquaculture, such as exogenous enzymes or immune stimulants; these can improve digestibility, growth performance and important immune response functions found within important mucosal organs such as the intestine or skin (Encarnação 2016).

The teleost immune system is comprised of innate and adaptive sub-systems that function to respond to infection and provide long-lasting immunity to disease (Smith *et al*. 2019). Whilst a great deal of research has concentrated on how feed additives affect immune responses within key organs, such as the mucosal-associated tissues like the skin and GIT (Gatesoupe 1999; Gómez & Balcázar 2007; Merrifield *et al.* 2010a,b,c;

Dimitroglou *et al*. 2009, 2011a,b; Merrifield & Ringø 2014; Merrifield & Rodiles 2015; Hoseinifar *et al.* 2016a; FAO 2020; Vargas-Albores *et al*. 2021), there is still much to be gained within this field of enquiry into the effects of prebiotics within commercially valuable fish species.

The body of research presented within this thesis comprises the results of *in vivo* feeding trials assessing the efficacy of a novel galactooligosaccharide B-GOS® to promote fish health and robustness within three commercially important fish species; its aim is to contribute to the industry's growing knowledge of prebiotic effects, with a focus on potential wider prebiotic use in aquaculture. The first experimental trial (Chapter 3) investigated how B-GOS® addition may affect the immune responses and growth in rainbow trout fed this prebiotic over 8 weeks. The second trial (Chapter 4) focused on how Atlantic salmon, a closely related species of the salmonids and an incredibly important commercial species, would perform under the same dietary conditions and trial length. The third trial (Chapter 5) investigated how a commercially important warm-water species, Nile tilapia, would be affected by B-GOS® inclusion, and was conducted over 29 days using the mid-range of prebiotic concentrations investigated from the first two experimental chapters. Measurements covered overall growth performance, immunological, histological and haematological appraisal, gene expression of a range of immune-related genes, and intestinal microbiota analysis, and results from each Chapter are summarised in Table 6.1. This work therefore provides

valuable insight into the response of teleost immunity to dietary B-GOS® supplementation.

Table 6.1 Table summarising the results from Chapter 3 (rainbow trout), Chapter 4 (Atlantic salmon) and Chapter 5 (Nile tilapia). A green upwards arrow represents a significant increase in metric between the diet regimes; a red downwards arrow represents a significant decrease in metric between the diet regimes; and a black line represents no significant differences detected between any treatment groups. N/A = not applicable for this species; C = Control diet; numbers in grams (e.g. 4g) within the table represent the amount of g kg^{-1} B-GOS® for dietary regimes.

Parameter Meausred	Rainbow Trout	Atlantic Salmon	Nile Tilapia
Growth Performance			
Initial Weight	—	—	—
Final Weight	—	—	—
FCR	—	—	—
SGR	—	—	—
PWG	—	—	—
K-Factor	—	—	—
Survival	N/A	N/A	—
Haematology			
Hb	—	—	N/A
Hct	—	N/A	N/A
RBC	—	—	N/A
WBC	—	—	N/A
MCV	—	N/A	N/A
MCH	—	—	N/A
MCHC	—	N/A	N/A
Lymphocytes	—	—	N/A
Basophilic Granulocytes	—	—	N/A
Neutrophilic Granulocytes	—	—	N/A
Immunology			
Mucus Lysozyme	—	N/A	N/A
Serum Lysozyme	—	2g ↑ C	N/A

Mucus Protein	—	N/A	N/A
Serum Protein	—	2g, 8g ↓ C	N/A
Normalised Mucus Lysozyme	—	N/A	N/A
Normalised Serum Lysozyme	—	2g ↑ C	N/A
Histology			
Muscularis Thickness	—	—	—
Mucosal Fold Length	—	—	—
Lamina Propria Width	—	—	4g ↑ C
Intestinal Goblet Cell Count	—	—	—
Skin Goblet Cell Count	—	—	N/A
Body Composition			
Moisture	—	—	4g ↓ C
Ash	—	—	—
Protein	—	—	—
Lipid	—	—	—
Gene Expression			
IL-1β	4g ↑ C. 10g ↑ C.	2g ↑ 10g. 8g ↑ C, 10g.	—
TNFα	2g ↓ C, 4g, 8g, 10g. 4g ↑ C, 6g, 10g. 6g ↓ C, 8g, 10g. 8g ↑ C, 10g.	2g, 4g ↑ all diets.	4g ↑ 6g.
IL-10	2g ↓ C, 4g, 6g. 4g ↑ C, 6g, 8g.	2g ↑ all diets except C. 4g ↓ 2g. 6g ↓ all diets. 8g, 10g ↓ C, 2g, 4g.	4g ↑ C, 6g. 6g ↑ C.
TGF-β	2g ↓ C, 4g, 8g. 4g ↑ all diets.	4g ↑ 2g, 6g, 10g. 6g ↓ C, 4g, 8g. 10g ↓ C, 4g.	—
Calreticulin	4g ↓ all diets.	2g ↑ C, 6g. 4g ↑ C, 6g. 6g ↓ 2g, 4g, 8g. 8g ↑ C, 6g.	N/A
CASP3	N/A	N/A	—

MYD88	N/A	N/A	—
PCNA	N/A	N/A	4g ↓ C.
HSP70	N/A	N/A	6g ↑ 4g, C.
Microbiome Analysis			
OTUs	—	—	—
Good's Coverage	—	—	—
Chao1	—	—	—
Shannon	—	—	—
Relative Abundance	*Macrococcus*: 4g ↑ all diets except C. *Aerococcus*: 4g ↑ C; 6g ↑ C, 4g, 10g.	*Rummeliibacillus* 8g ↑ 2g, 4g. *Aerococcus* 6g ↑ all diets except 8g. *Lactobacillus* 4g ↑ 2g.	*Bacillus*, *Enterococcus*, *Weissella* 4g ↑ 6g.

Observations of growth performance in rainbow trout from Chapter 3 revealed no significant improvement in individual final weight, FCR, SGR or PWG between fish fed the control or experimental diets. All growth performance metrics indicated that there was good growth in trout fed all dietary regimes. Previous research has demonstrated that prebiotics supplemented to teleosts may improve growth and overall performance (Zhou *et al*. 2010; Talpur *et al*. 2014; Hoseinifar *et al*. 2017a), however, there is also evidence from previous studies of no overall improvement relative to control groups (Grisdale-Hellend *et al*. 2008; Burr *et al*. 2010; Yousefi *et al*. 2018). The results from Chapters 4 and 5 were also in accordance with the growth performance seen in rainbow trout, as Atlantic salmon and Nile tilapia fed the experimental and control diets all exhibited a doubling of biomass, yet no significant improvements in the same growth performance metrics between dietary regimes. All three fish trials presented K-factors

of 1.2 – 1.8, suggesting that there was adequate nutritional availability within the diets, and the optimal rearing conditions utilising recirculating aquaculture systems (RAS) afforded excellent growth. Previous research has demonstrated that RAS offer more control over the feed input, thus improving and in some cases lowering the FCR, and may also reduce the occurrence of disease when compared with open-net pen systems (Philis *et al*. 2019; Bergman *et al*. 2020). The results from this thesis suggest that there was little scope for the prebiotic to further improve growth performance metrics in trout, salmon and tilapia at the whole organism level under the conditions tested during this thesis.

Haematological assessment of haemoglobin and PCV, RBC, WBC, MCV, MCH and MCHC in rainbow trout and Atlantic salmon supplemented with B-GOS® revealed no significant differences between experimental and control fish, suggesting that there was negligible modulation in the immune response after 8 weeks of feeding. These trends were similar in the metrics measuring differential leucocyte counts, and the haematological data reported for each species were within the acceptable ranges, demonstrating that the fish were healthy during the length of each trial. An increase in differential cell types, such as lymphocytes and RBCs, may indicate a cellular response to stress and the need for higher oxygen demand, potentially indicating the need for pathogenic defence (Braun *et al*. 2010; Liu *et al*. 2017b), or priming the immune response to a state of readiness to better response to future threats (Talpur *et al*. 2014). The results from both the trout and salmon trials demonstrate that fish fed the dietary regimes were unlikely to be presenting an immune response through elevation of differential cell types in response to the addition of the prebiotic, and so it is unlikely

that B-GOS® can modulate haematological parameters in salmonids at the concentrations tested here.

Variations across species were however seen in the results for serum and mucus lysozyme activity and protein concentration. These were not significantly affected by the addition of B-GOS® at any inclusion level in rainbow trout; however, the Atlantic salmon fed 2g kg^{-1} B-GOS® presented significantly increased serum lysozyme activity and decreased protein concentration than control fed fish. Lysozyme activity is incredibly important in the innate immune system response, and when elevated, produces antibacterial effects and triggers anti-inflammatory responses to destroy non-self cellular material (Kiron 2012; Akhter *et al*. 2015; Hoseinifar *et al*. 2015). Previous research has demonstrated that prebiotics may increase serum lysozyme activity in fish, such as Caspian fry (Soleimani *et al*. 2012), Caspian salmon (Aftabgard *et al*. 2019) and goldfish (Miandare *et al*. 2016), which can heighten the immune response in preparation for potential pathogenic insult. The increased lysozyme activity demonstrated in salmon fed 2g kg^{-1} B-GOS® suggest that this inclusion rate may have modulated this aspect of the immune response, whereas this prebiotic was not able to induce positive responses within the blood serum of rainbow trout.

The serum protein concentration was lowest in fish fed this same concentration of B-GOS®, which may suggest a downregulation in protein production within the serum for salmon fed this inclusion level. These findings are contrary to previous literature, some of which has demonstrated that prebiotics can elevate serum protein content in snakehead fingerlings and Nile tilapia challenged by *A. hydrophila* (Talpur *et al*. 2014; Cavalcante *et al*. 2020, respectively). A range of immunoglobulins, cytokines,

transferrin proteins and complement proteins are found in the serum and skin mucus of teleosts (Karimi *et al*. 2020), however, the present study was not able to elucidate the exact nature of the proteins measured within trout and salmon. To understand how serum protein content may decrease with increased serum lysozyme activity, further work must first be conducted into the specific proteins that are present by the use of proteomic assays, in addition to more traditional approaches using spectrophotometric measurements of *M. lysodeikticus* lysing. There was no scope for blood collection from the juvenile Nile tilapia studied in Chapter 5 of this thesis, as the fish were too small to extract enough blood for valid downstream processes. Future work should also consider testing older life stages of Nile tilapia when investigating B-GOS® and the effect on key organs and tissues, so that the results produced can be compared with the salmonids investigated in Chapters 3 and 4.

In terms of intestinal health, previous research has demonstrated that the administration of prebiotics to teleosts can positively affect the histology of the GIT, for example by increasing mucosal fold height, the number of mucin-producing cells such as goblet cells, and improving uniformity of the microvilli (Yilmaz *et al*. 2007; Salze *et al*. 2008; Dimitroglou *et al*. 2010; Zhou *et al*. 2010; Anguiano *et al*. 2013; Ziółkowska *et al*. 2020; Pontefract 2021). From the results of this thesis, histological appraisal revealed that the muscularis width, lamina propria width, mucosal fold length, and goblet cell counts of sections of the skin and intestine in rainbow trout and Atlantic salmon were unchanged by the addition of B-GOS® compared to control diets. These results are supported by Guerreiro *et al*. (2016b) for gilthead sea bream fed scFOS, as well as by Dimitroglou *et al*. (2010) for the same species fed MOS. These data from this thesis indicate that B-GOS® tested at the inclusion rates studied did not appear to induce

elevations in the overall immune readiness within the mucosal skin surface and intestinal epithelium, and the likelihood of greater nutrient absorption from increased mucosal fold heights is lacking. Previous research by Dimitroglou *et al*. (2010) demonstrated that standard light microscopy did not reveal differences in histomorphology of gilthead seabream intestine when fed either control of MOS diets; however, the results of the electron microscopy conducted by the authors did reveal variations in the effects of the different diets on the ultrastructure of the intestine. The possibility of seeing a greater level of detail in the intestinal structures of the fish species tested in this thesis should be explored by combining light and electron microscopy to determine if B-GOS® would have any effects on the intestinal health of teleosts that may have been missed during these experiments.

Contrary to the results from the rainbow trout and Atlantic salmon B-GOS® feeding trials, the lamina propria of mucosal folds within the intestine of Nile tilapia fed 4g kg^{-1} prebiotic was wider than in the control fed fish. The lamina propria in teleosts, especially salmonids, has been studied in detail, providing evidence for potential IgM positive cell, T cell, antigen-presenting cell and mast cell recruitment (Bjørgen *et al*. 2020). The wider lamina propria within the intestinal mucosal folds of tilapia fed 4g kg^{-1} diet, as well as an overall increased final weight and longer mucosal fold heights compared with the other treatments groups (albeit not significant), suggest that this level of B-GOS® inclusion may increase nutrient absorption and may induce immune readiness within the intestine of tilapia fed B-GOS® at an early life stage.

There is evidence from previous studies that increased lamina propria width, coupled with leucocyte infiltration within this connective tissue, may be produced by

inflammation within the intestine of fish fed diets with high proportions of plant feedstuffs (Bjørgen *et al*. 2020). The diet formulations for each experiment in this thesis were designed to minimise the use of fishmeal and oil-based proteins and lipids, and so SBM was used as a supplement to increase the amino acid profile and protein content in each diet (Merrifield *et al*. 2011; Adeoye *et al.* 2016a,b). Previous research has demonstrated that high SBM can produce enteritis within the GIT of fish in as little as 15 days of feeding, and induce immune responses such as elevated goblet cell abundance, shortening of the microvilli and lymphoid cell infiltration of the lamina propria (van den Ingh *et al*. 1991; Merrifield *et al*. 2011; Guerreiro *et al*. 2015; see review Bjørgen *et al*. 2020). The results from each experiment of this thesis reinforce the conclusion that fish supplemented with B-GOS® in salmonids for 8 weeks and tilapia for 29 days did not suffer any enteric effects in the context of the parameters assessed from these tested diets, and therefore the SBM levels used in the trials for this thesis were appropriate. The fish were however unlikely to receive any extra benefit from the presence of the prebiotic, as the histomorphology of the intestine was likely to already be at a maximum for efficient nutrient absorption and pathogenic defence.

The results from this research have informed how B-GOS® influences some key immune-related gene expression within targeted organs of trout, salmon and tilapia. Gene expression modulation was varied across all species fed the prebiotic, and there were significant differences within expression of some target genes that did not follow any particular trend between dietary treatments, for example in *IL-10,* expression was upregulated in trout skin fed 4 and 10g kg^{-1} 2.5-fold, but downregulated in fish fed 2g kg^{-1} by the same level. Similar results were also seen in trout skin for *TNFα*, and *TGF-β* for salmon intestine, among other differences from each species. There appears to be a

great deal of sensitivity in regulation of the immune-related genes between dietary groups of fish from each species tested with B-GOS®, with incremental increases of 2g kg^{-1} prebiotic producing significantly different regulation within intestinal gene expression. These results further demonstrate that closely related fish species such as salmon and trout reared in a similar manner and supplemented with the same prebiotic will still express immune-related genes differently.

The results are difficult to interpret due to a lack of trend and low expression observed within the raw data that was then further analysed to generate gene expression as fold change relative to the control fed fish. However, there may be some potential gene expression modulation within fish fed the 4g kg^{-1} B-GOS® diet compared to other inclusion levels. Overall, this inclusion rate in trout, salmon and tilapia investigated in this thesis demonstrated the most potential for improved growth, histological modulation and intestinal microbiome diversity. It would be prudent to investigate how other immune and stress-related genes are affected by dietary inclusion of a prebiotic, as there is research to suggest that multiple genes act in a cascade in response to additive inclusion, as well as when challenged by parasitic invasion such as sea lice (Pontefract 2021).

There would be benefit in further research to explore how the trends in these gene expression levels are affected in fish that are supplemented with the 4g kg^{-1} B-GOS® inclusion level, but challenged by different rearing conditions to those within this thesis, for example by rearing these species *in situ* or by different dietary formulations or pathogens. To further determine how immune responses may be affected by the addition of dietary additives, it may be advantageous to conduct further research using

omics analysis, such as transcriptomics or proteomics, as these fields are rapidly expanding. These fields of research may also complement the results from the gene expression data examined in this thesis, and determine if there are any results which have been overlooked or not represented in the assays performed during this research.

Of the bacterial communities present within trout, salmon, and tilapia, a few key genera were significantly upregulated in B-GOS® fed fish in comparison to the control fed fish, which supported evidence from previous research conducted on each species demonstrating key genera of the core microbiome (Sullam *et al*. 2012; Wong *et al*. 2013; Standen *et al*. 2015; Lyons *et al*. 2016; Yukgehnaish *et al*. 2020). High-throughput 16S rRNA sequencing revealed that the salmonids were dominated by *Firmicutes*, *Proteobacteria*, *Actinobacteriota*, with the salmon displaying an increased abundance of *Firmicutes* that made up the majority of reads. These phyla have been previously sequenced in salmonids such as rainbow trout (Sullam *et al*. 2012; Wong *et al*. 2013; Lyons *et al*. 2016) and Nile tilapia (Standen *et al*. 2015), indicating that the presence of these taxa are part of a core microbiome similar across multiple teleost species. Within the tilapia fed control and B-GOS® diets, the relative abundance of reads assigned to *Proteobacteria*, *Fusobacteria* and *Firmicutes* and *Actinobacteriota* were present in smaller proportions, indicating a slightly different core microbiome in cichlids compared to salmonids, perhaps due to different life histories and geographical ranges. These taxa have been sequenced in species of tilapia, with some flexibility in the overall abundance of each taxa depending on age and location of each species reared (He *et al*. 2013; Baldo *et al*. 2015; Standen *et al*. 2015; Haygood & Jha 2018; Yukgehnaish *et al*. 2020). The results for this thesis indicate that the prebiotic diet formulations produced

for each trial within this thesis help to promote a diverse group of bacterial genera within the GIT microbiome of commercially important fish species.

The inclusion of B-GOS® also appeared to promote some potentially beneficial bacteria within the GIT of the salmonids and tilapia studied in this thesis. Within the intestine of rainbow trout, *Aerococcus* and *Macrococcus* presented significantly more assigned relative abundance reads in fish fed 6g kg^{-1} and 4g kg^{-1} fed fish, respectively, and these genera have been previously found as part of the core microbiome in rainbow trout (Michel *et al*. 2007), common carp (Zhang *et al*. 2017) and sea bream (Parlapani *et al*. 2015). Insufficient research has been conducted on these genera within the intestine of prebiotic fed teleosts, and the field would benefit from further work would benefit to determine if isolated species from these genera would provide beneficial effects to the microbiome and intestinal structures within rainbow trout.

The relative abundance of reads assigned to genera *Rummeliibacillus* and *Aerococcus* were more abundant in salmonids fed 6 – 8g kg^{-1} B-GOS®, and for *Lactobacillus* the highest relative abundance was recorded in fish fed 4g kg^{-1} prebiotic. Research on *Lactobacillus* spp. as probiotics has demonstrated how this genus can enhance mutualistic relationships between these species and the host; provide a source of nutrients by increased fermentation utilising prebiotics as a carbon source; and also provide a means of protection from pathogens for the host (Martín *et al*. 2013; Gajardo *et al*. 2016; Alonso *et al*. 2018). Previous studies have presented benefits from this genus to the health and immune function in the intestine of fish species popular in aquaculture (Nikoskelainen *et al*. 2001; Ley *et al*. 2008; Standen *et al*. 2015; Rodiles *et al*. 2018; Mohammadian *et al*. 2020), and so the results of Chapter 4 indicate that

Lactobacillus may be beneficial to salmon fed the 4g kg^{-1} inclusion rate of B-GOS®. Other results from Chapter 4 also support this, as the final weights, histological and immunological measurements for fish fed this inclusion rate had overall improved performance, despite a lack of significance.

The relative abundances of genera *Bacillus*, *Weissella*, and *Entercoccus* were significantly elevated in tilapia fed 4g kg^{-1} B-GOS®, and previous research has provided evidence that these genera benefit tilapia and other fish species that host them within their GIT microbiota. *Bacillus* has been isolated and used as a probiotic supplement in Nile tilapia (Standen *et al*. 2015), rainbow trout (Merrifield *et al*. 2010a) and European seabass (Peggs 2015), with evidence from these trials suggesting that this genus can contribute toward greater nutritional utilisation within the GIT, and reduce the populations of prevalent pathogens. In addition, *Weissella* spp. have been used as potential probiotics to enhance the diversity of the microbiota (Ergerton *et al*. 2018), and *Enterococcus* spp. are members of the LAB which have been extensively tested in teleosts. The increased abundance of *Enterococcus* in the teleost intestine has demonstrably improved growth performance, immunity and resistance to pathogenic insult in previous research (Avella *et al*. 2011; Sorroza *et al*. 2012; Hoseinifar *et al*. 2013, 2016a, 2017b; Peggs 2015; Cavalcante *et al*. 2020). The prebiotic action in bacterial GIT communities is primarily fermentation by beneficial genera, such as *Lactobacillus*, which possess enzymes necessary to hydrolyse prebiotics that the host does not (Guerreiro *et al*. 2018a). The increase in LAB counts in fish fed prebiotics has been evidenced in several species (Guerreiro *et al*. 2018a,b). The results from Chapter 5 suggest that the inclusion rate of 4g kg^{-1} B-GOS® may modulate the diversity and relative abundance of microbes within the GIT by providing useful carbohydrates for

these beneficial bacteria to ferment and provide metabolites for host utilisation, whilst also potentially aiding the host's defence against pathogens.

The assignment of 16S rRNA sequencing reads to a defined taxonomy is a highly specialised method for characterising the microbiome of chosen teleosts, and while caution is advised when comparing genera, as some species have more copy numbers of the 16S rRNA gene than others, the results presented in this thesis provide evidence for a shared microbiota across each species in the form of *Firmicutes*, *Proteobacteria* and *Actinobacteriota*. While the specific genera differ in relative abundance, the significant increase in a few key immune and health-related taxa at a localised level in the GIT suggests that between trout, salmon and tilapia, the 4g kg^{-1} B-GOS® inclusion rate was the most favourable for these species. Further research should be warranted to determine how a pathogenic or environmental challenge may alter this diversity, and whether similar effects observed in this thesis would be elicited upon such challenges.

The results from the body of work in this thesis suggest that there was little scope for the additives to influence the health and growth indices of the fish species tested here, and so it would be energetically inefficient and demanding to induce an immune response where none is required (Pontefract 2021). This may however be in part related to the additive itself and the non-challenging conditions in which the fish were reared.

B-GOS® is a mixture of galactooligosaccharides produced from the activity of galactosyltransferases from *B. bifidum* NCIMB 41171 in the presence of lactose, and is typically not hydrolysed by mammalian digestive enzymes (Tzortzis *et al.*, 2005a, b; Depeint *et al.*, 2008; Gänzle 2011). This product has been tested within mammals, with attention to human health improvement within the GIT microbiota, and as this

prebiotic is derived from milk, it is entirely reasonable to assume that the gut microbiota within the fish species tested within this thesis are not capable of utilising B-GOS® as efficiently as other forms of oligosaccharide. Clinical evidence from testing of this prebiotic tested in humans and swine demonstrates improved digestive health and wellbeing, immune system modulation via an increase in bifidobacteria, antipathogenic activity and improved brain function (Tzortzis *et al.*, 2005a, b; Mao *et al*. 2015). This additive therefore may be a better dietary addition for mammals or humans based on some of the literature already presented. In mammals, prebiotics have been shown to interact with the gut autochthonous morphology and the gut microbiota, and the effects of prebiotic addition is often seen in the intestinal mucosa (Carbone & Faggio 2016). In teleosts, the intestinal structure and microbiome may not be able to efficiently utilise B-GOS®, or the bacterial species that ferment this additive in the GIT may not be able to compete with the microbiota already present within the fish, which will be based on an aquatic environment and uptake of bacterial species that are already established.

Further work should consider testing this GOS prebiotic against other well studied additives, such as MOS, β-glucans or FOS, and also compare how disease response is modulated, if at all, by these prebiotics. MOS is a popular additive in salmonid aquaculture, and has been shown to improve immune responses in teleosts when they are reared in normal or abnormal a/biotic conditions in a dose-dependent manner (Mugwanya *et al*. 2021). For β-glucans inclusion, there are similar results from multiple studies, whereby the stressor type paired with this type of prebiotic and dosage results in beneficial modulation of the immune response (Gewaily *et al*. 2021). With FOS, the size of fish and the duration of the trial with specific doses influences the growth performance metrics, gut morphology and immunity of teleosts in a significantly

positive way (Plongpunjong *et al*. 2011; Poolsawat *et al*. 2020; Mugwanya *et al*. 2021). Trials with these additives have demonstrated the capability to interact with the intestine of teleosts, and there would be value in researching how the different chemical structures within each oligosaccharide interact within the intestine of commercial fish species.

In terms of experimental conditions for this thesis, designs for all three trials were chosen to be suitable for growth, as each fish species was reared in RAS and water quality and temperatures were monitored closely, ensuring suitable environments for each species. This level of control over the rearing conditions, as well as the feed input, can reduce the FCR and the occurrence of pathogen invasion compared to *in situ* environments (Philis *et al*. 2019; Bergman *et al*. 2020). Fish subjected to challenges faced in open or semi-intensive systems, such as seasonal variation or reduced microorganism control, may therefore benefit significantly more from the addition of functional feeds to improve growth and overall performance when compared to fish that are reared in more controlled rearing environments such as RAS (Rud *et al*. 2017; Mugwanya *et al*. 2021).

Future work should be conducted to determine how *in situ* rearing conditions affect the growth of fish when tested with B-GOS® compared to RAS. The relatively reduced control in the presence of microorganisms within fish reared in open-net pens will produce drastically different microbiotas when compared to those for closed systems, as has been reported by Rud *et al*. (2017), wherein the authors reported through 16S rRNA deep MiSeq sequencing that the microbiotas in Atlantic salmon post-smolts were significantly different between RAS and semi-closed circulating systems.

In addition to environmental challenges, there are other experimental designs which will induce a challenge, such as formulating deliberately sub-optimal diets to induce a response, or introducing known fish pathogen strains to trialled fish. These designs are feasible within RAS under controlled conditions, and the results from previous studies suggest that feed additives used in conjunction with dietary or disease challenges may help increase performance and overall health in affected treated fish compared to controls (Salze *et al*. 2008; Hoseinifar *et al*. 2013; Hoseinifar *et al*. 2015; Khodadadi *et al*. 2018). By conducting further trials which deliberately provide challenge to induce a more pronounced immune response within fish fed dietary B-GOS® prebiotic supplementation, the responses of such trialled fish will complement the research conducted in this thesis, and allow for a comparison between the immune responses in rainbow trout, Atlantic salmon and Nile tilapia that have not been subjected to challenge, and those that have.

Dietary prebiotic addition has become a significant area of research that has ever-increasing coverage within the literature, and remains a key factor in improving the health and growth of fish and thus support the future expansion of the aquaculture industry, and increase its sustainability. However, there is still the need for more research within this field, with a particular focus on how novel GOS may influence the health and growth of commercially important species.

6.1 Conclusions

Without testing *in vivo*, it is not certain that the genera that make up the microbiota of a tested fish species will be abundant enough to be advantageous to the host. Therefore, it is paramount that prebiotic additives are reviewed in commercially valuable fish

species to confirm the relevance of these additives in today's ever-growing aquaculture industry. The present research therefore focused on the efficacy of B-GOS® to stimulate the health and growth of commercially important teleost species, and has provided valuable insight into specific responses related to the immune system in these fish, such as growth performance, histology, haematology, immunology, gene expression and intestinal microbiota analysis.

The results of the three experimental trials conducted on rainbow trout, Atlantic salmon and Nile tilapia juveniles suggest that there is little scope for improvement in the growth performance of these species supplemented with B-GOS® at the whole organism level, as body composition and growth metrics such as final weights, SGR and FCR, remained unaffected by treatment. However, despite a lack of significant differences between control fed fish and fish supplemented with B-GOS® of most health parameters, there is evidence of modulation of the intestinal barrier communities and intestinal gene expression of fish fed 4g kg^{-1} prebiotic inclusion. The results suggest that this inclusion level has the most potential to improve teleost health at a localised level, especially under *in situ* conditions.

Further research is recommended to support the ongoing development of the aquaculture industry, by determining how B-GOS® prebiotic at 4g kg^{-1} inclusion level would benefit fish with different life histories, and how this additive may enhance fish development in environments that mimic those challenges presented by the *in situ* rearing conditions typically found within global aquaculture facilities.

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