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# Effects of dietary additives on growth performance, immunity and mucosal barrier defences of salmonids

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**UNIVERSITY OF  
PLYMOUTH**

**Effects of dietary additives on growth performance, immunity and mucosal barrier  
defences of salmonids**

By

**Nicola Pontefract**

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

**DOCTOR OF PHILOSOPHY**

School of Biological and Marine Sciences

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## **Dedication**

I dedicate this thesis to me.

To remind me that everything worth having is worth the fight.

## 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 any other establishment.

This study was financed with the aid of a studentship and in collaboration with Lallemand Animal Nutrition.

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- Marine Environmental Research Laboratory of the University of Stirling, located in Machrihanish, Scotland
- Marine Harvest, based in Loch Ailhort on the Ardnish peninsular, Scotland.
- INRA Research Centre (Institut National de la Recherche Agronomique), based in Toulouse, France.

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## Abstract

Effects of dietary additives on growth performance, immunity and mucosal barrier defences of salmonids

*Nicola Pontefract*

The mucosal surfaces of fish are considered highly active immunological sites that work to maintain homeostasis and play a key role in the defence against pathogens (Yu *et al.*, 2020). While disease represents a major constraint to the aquaculture industry, the potential influence of dietary additives upon the mucosal barriers and defences of fish is the subject of increasing amounts of research.

RT-q-PCR gene expression analysis and histological examinations of experiment one jointly indicate the potential of a dietary prebiotic mixture, a parabiotic and their synbiotic to positively influence the mucosal barrier defences of the Gill-(GIALT), Skin-(SALT) and Gut-associated lymphoid tissues (GALT) of rainbow trout. Significant increases in goblet cell area fraction (CAF) were observed in the SALT, in addition to significantly elevated expressions of tight junction-, mucosal- and immunity-associated genes, within the GIALT, SALT and GALT.

Similar results were observed for Atlantic salmon fed prebiotic- and probiotic-supplemented diets in experiment two: differences in goblet cell abundance and CAF were observed between treatments, while RT-q-PCR analysis revealed transcriptional regulation of mucosal- and immune-related genes. Additionally, following pathogen challenge, significantly fewer sea-lice (*Lepeophtheirus salmonis*) were observed on fish fed the additive supplemented diets.

Investigations of experiment three were conducted on Atlantic salmon housed in open sea pens, reflective of commercial aquaculture practices. Significant reductions in sea-lice prevalence and changes to epidermal mucous secretions were observed between treatments. Furthermore, Microfluidic q-PCR analysis allowed for the evaluation of 59 gene targets within the GIALT, SALT, and GALT at two different time-points, providing a holistic view of the expression alterations occurring in response to the prebiotic and probiotic additives investigated, and the latent copepod infection.

Through the implementation of *in vivo* feeding trials, results of the experiments conducted during this research collectively support the proposed interconnectivity of the MALT tissues in both rainbow trout and Atlantic salmon, and serve to highlight the potential of dietary additives to enhance the mucosal barrier defences and reduce sea-lice prevalence.

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## List of Abbreviations

AB-vG	Alcian Blue - van Gieson
AFOS	Animal feed optimisation software
APCs	Antigen-presenting cells
BT	Blank titre
BW	Body weight
Ct	Threshold cycle
CW	Crucible weight
EU	European Union
FCR	Feed conversion ratio
FL	Fork length
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GIALT	Gill-associated lymphoid tissue
HKWCPs	Heat-killed whole-cell products
IFC	Integrated fluidic circuit
FCR	Feed conversion ratio
FL	Fork length
FOS	Fructooligosaccharides
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgM	Immunoglobulin M
IgT	Immunoglobulin T
IgZ	Immunoglobulin Z
ILT	Inter-brachial lymphoid tissue
IMS	Industrial methylated spirit
JAM	Junction adhesion molecules
K-F	K-Factor
LAB	Lactic acid bacteria
LP	Lamina propria
LPS	Lipopolysaccharide
LW	Lipid weight
MALT	Mucosal-associated lymphoid tissues
MAMPs	Microbial-associated molecular patterns
MF-q-PCR	Microfluidic q-PCR
MOS	Mannanligosaccharides
MR	Mannose receptor
NALT	Nasopharynx-associated lymphoid tissue
NGS	Next generation sequencing
OTUs	Operational taxonomic units
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PGs	Prostaglandins

PRRs	Pattern-recognition receptors
ROS	Reactive oxygen species
SALT	Skin-associated lymphoid tissue
SBM	Soybean meal
SCFAs	Short-chain fatty acids
SGFM	Secreted gel forming mucin
SGR	Specific growth rate
SOD	Superoxide dismutase
SPC	Soy protein concentrate
SR	Sample residue
SR	Survival rate
ST	Sample titre
SW	Sample weight
TB	Tindalised bacteria
Th	T helper
TIL	Trypsin inhibitor-like
TJ	Tight junction
TLR	Toll-like receptor
UN	United Nations
VWD	von Willebrand-D
WHO	World Health Organisation
WTSS	Whole transcriptome shotgun sequencing

## List of Genes

<b>Abbreviation</b>	<b>Gene Name</b>
Arg2	Arginase 2
Cal	Calreticulin
Cal-like	Calreticulin-like
Casp3b	Caspase 3b
CATA	Catalase
Cath	Cathelicidin
CD4+	Cluster of Differentiation 4+
CD8 $\alpha$	Cluster of Differentiation 8 alpha
Cldn 10e	Claudin 10e
Cldn 12	Claudin 12
Cldn 15	Claudin 15
Cldn 25b	Claudin 25b
Cldn 27a	Claudin 27a
Cldn 28a	Claudin 28a
Cldn 28b	Claudin 28b
Cldn 30	Claudin 30
Cldn 6	Claudin 6
Cldn 7	Claudin 7
COX2	Cyclooxygenase-2
Ef1 $\alpha$	Elongation factor alpha
FoxP3	Fork-head box P3
GATA3	GATA Binding Protein 3
GOS2	G0/G1 Switch Regulatory Protein 2
GSH-Px	Glutathione peroxidase
HSP70	Heat shock protein 70
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17 $\alpha$	Interleukin 17 alpha
IL-1R1	Interleukin 1 receptor-1
IL-1 $\beta$	Interleukin-1 beta
IL-4/13 $\alpha$	Interleukin 4/13alpha
I-Muc	Intestinal mucin
INF- $\gamma$	Interferon gamma
iNOS	Inducible Nitric oxide synthase
IRAK-4	Interleukin-1 Receptor-Associated Kinase 4
MHC Class I	Major Histocompatibility Complex I
MHC Class II	Major Histocompatibility Complex II
MMP13	Matrix metalloproteinase 13
MMP9	Matrix metalloproteinase 9
Muc 2-like	Mucin 2-like
Muc 5ac-like	Mucin 5ac-like

Muc 5b-like	Mucin 5b-like
Muc13	Mucin 13
Muc18	Mucin 18
Muc19	Mucin 19
Mx-1	Myxoma Resistance Protein 1
MyD88	Myeloid Differentiation Primary Response 88
Occ #1	Occludin #1
Occ #2	Occludin #2
PCNA	Proliferating Cell Nuclear Antigen
pIgR	Polymeric Immunoglobulin Receptor
Prxs/ NKEF	Peroxiredoxins/Natural Killer Enhancement Factor
SOD1	Superoxide dismutase
Stat6	Signal transducer and activator of transcription 6
Tbet	T-box transcription factor
TGFβ	Transforming growth factor beta
TLR3	Toll-like receptor 3
TLR8b2	Toll-like receptor 8b2
TNFα	Tumour necrosis factor alpha
Tollip1	Toll interacting protein 1
Tollip2	Toll interacting protein 2
TRF	Transferrin
β-actin	Beta actin

## Conferences attended and work presented

AQUA 2018: World Aquaculture Society; August 2018, Montpellier, France

Oral presentations:

1. Development of a high throughput real-time qPCR assay technique for the targeted gene expression analysis of Atlantic salmon (*Salmo salar*) mucosal tissue responses.
2. Effect of a single-strain yeast fraction product on Atlantic salmon skin mucosal barrier and susceptibility to the sea lice *Lepeophtheirus salmonis*.

## Publications

E, Leclercq., **N, Pontefract.**, M, Rawling., V, Valdenegro., E, Aasum., L, Vera., H, Migaud., M, Castex., D, Merrifield (2020). Dietary supplementation with a specific mannan-rich yeast parietal fraction enhances the gut and skin mucosal barriers of Atlantic salmon (*Salmo salar*) and reduces its susceptibility to sea lice (*Lepeophtheirus salmonis*). *Aquaculture* 529. DOI: <https://doi.org/10.1016/j.aquaculture.2020.735701>

## General Introduction

### 1.1 Aquaculture; bridging the gap

According to the United Nations (UN), the current global human population of 7.6 billion is expected to reach 8.6 billion in 2030, escalating to 9.8 billion in 2050 (UN, 2017). If these predictions are realised, the inevitable increases in demand for high-quality sources of protein will also escalate dramatically. Fish currently provide an important source of animal protein and nutrients to a large proportion of the world's population. In 2015, fish accounted for approximately 17% of all animal protein consumed by the global population, providing 3.2 billion people with almost 20% of their average per capita intake of animal protein (FAO, 2018). However, the poor management of wild capture fisheries over the last 40-50 years has resulted in relatively static landings since the late 1980s. Global capture fisheries production in 2016 was reportedly 90.9 million tonnes, having peaked at approximately 94 million tonnes in the late 1990s (FAO, 2002 and 2018).

Over recent decades, the aquaculture industry has expanded across the globe to become a successful producer of a wide variety of marine and freshwater species. In fact, aquaculture production now exceeds that of wild capture fisheries and is becoming an increasingly important source of protein for human consumption, "bridging the gap" between the low supply from capture fisheries and high demand from the ever-increasing human population (FAO, 2018). Global aquaculture production in 2016 included 80.0 million tonnes of food fish, 30.1 million tonnes of aquatic plants, and 37,900 tonnes of non-food products (FAO, 2018). However, in order for the aquaculture industry to produce the required amount of protein for the predicted human population in 2030, it is vital that the industry continues to grow,

expand and refine its production processes to become more economically viable and sustainable on both a socioeconomic and environmental level (FAO, 2014).

Globally, Atlantic salmon (*Salmo salar* L.) is one of the most intensively farmed and highly valued fish, with the majority of the world's production originating from Norway, Chile, the United Kingdom, and Canada (Pettersen et al., 2015). Most recent estimates published by the Food and Agriculture Organisation (FAO) suggest that these countries collectively produced approximately 2.38 million metric tonnes of Atlantic salmon in 2017 (FAO-FIGIS, 2020). Native to the cold-water tributaries of the Pacific Ocean in Asia and North America, rainbow trout (*Oncorhynchus mykiss*) have been introduced to and cultured on all continents, with the exception of Antarctica (Khodadadi et al., 2019). Due to its rapid and high growth potential, ease of captive breeding and tolerance to a wide range of environmental and production conditions, rainbow trout has become one of the most commercially important species within the aquaculture industry today (Singh et al., 2016).

### 1.2 The immune system of teleosts

Teleost fish exhibit a well-developed immune system which is particularly strong with respect to barrier and mucosal defences (Foey and Picchiatti, 2014). In order to provide protection against pathogenic insults, vertebrates have evolved immune responses which are classically divided into two components: the innate immune response and the adaptive immune response (Castro and Tafalla, 2015). The innate immune system of teleosts is efficient, constituting the first line of defence against infection and including not only physical barriers but also humoral and cellular responses (Castro and Tafalla, 2015). The adaptive immune response is characterised by a specific antigen recognition system that drives a secondary pathogen-specific immune response: compared to mammalian counterparts, this reaction of

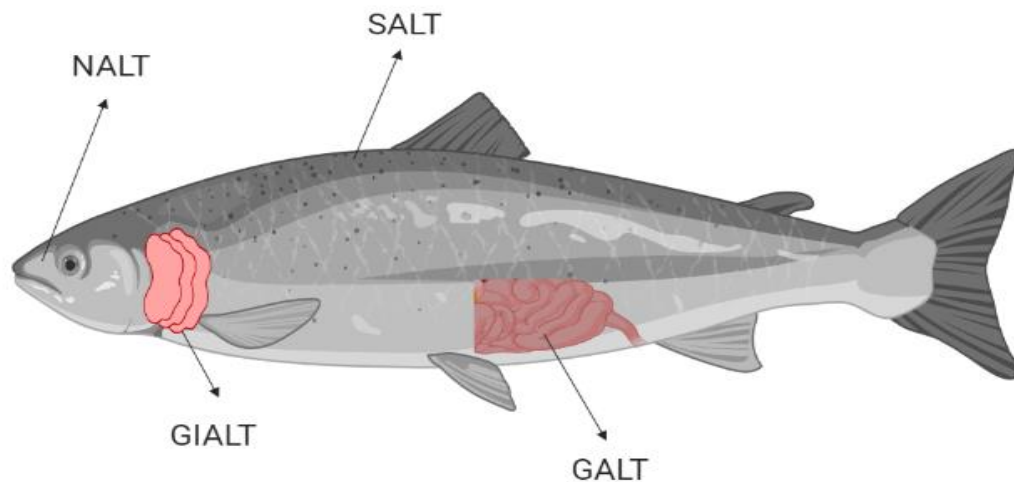
the adaptive immune system to antigenic stimuli in fish occurs at a much slower rate, in part due to lower environmental temperatures (Foey and Picchietti, 2014; Castro and Tafalla, 2015). Research over the last decade has led to a far deeper understanding of the immune system of teleosts, highlighting its intricacy, and drawing attention to the many cellular components and associated immune mechanisms. Recently, the interrelationship between the innate and adaptive components of the immune response have become evident and are currently recognized as being far more complicated than initially assumed: many of the cells and molecules traditionally assigned to either the innate or adaptive classifications in fact perform specific roles within both systems (Martin & Król, 2017).

In higher vertebrates, the immune system consists of primary (lymphocyte generating) and secondary (immune response-generating) lymphoid organs. Lacking bone marrow and lymph nodes, the organization of immune organs in fish differs slightly; the primary lymphoid organs are the thymus and the head kidney, while the secondary lymphoid organs are the spleen, the kidney, and the mucosal-associated lymphoid tissues (MALT) present in peripheral immune tissues (Castro and Tafalla, 2015).

### 1.3 The Mucosal-Associated Lymphoid Tissue (MALT)

Vertebrate mucosal surfaces contain associated lymphoid tissues known as MALT (Salinas, 2015). Mainly based on morphological distinctions, the MALT of teleosts is further subdivided into four distinct associations, as shown in Figure 1.1: the gut-associated lymphoid tissue (GALT) with the lamina propria and intraepithelial compartments (Salinas *et al.*, 2011), the skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT), and the recently discovered nasopharynx-associated lymphoid tissue (NALT) (Salinas, 2015).





**Figure 1.1.** Schematic representation of the four main mucosa-associated lymphoid tissues (MALT) described to date in teleosts and their anatomical locations. GALT: gut-associated lymphoid tissue; SALT: skin-associated lymphoid tissue; GIALT: gill-associated lymphoid tissue; NALT: nasopharynx-associated lymphoid tissue.

As a consequence of their inherently antigen-rich habitat, fish are continually challenged by a bombardment of both commensal and pathogenic microbes and environmental stressors which are able to circulate throughout the body, reaching every epithelial barrier surface (Salinas *et al.*, 2011; Salinas, 2015). In response, teleosts have developed highly effective mucosal surfaces as their strategy to protect themselves against such antagonisms (Salinas *et al.*, 2011). In addition to being physical barriers, these mucosal surfaces are also considered highly active immunological sites, composed of both innate and adaptive immune cells that work in concert to maintain homeostasis at the mucosa (Salinas, 2015); they therefore play a key role in defence mechanisms against pathogens (Lazado and Caipang, 2014). At the forefront of these barrier defences is the epidermal mucous layer, beneath which lie the epithelial cells which function as both a physical barrier and an immune instructor, capable of maintaining either immune tolerance in homeostatic conditions or immune activation in

response to pathogen invasion (Foey and Picchiatti, 2014). If these initial barrier defences are compromised however, the pathogen faces a barrage of underlying defences, including an abundance of cellular and soluble components, ranging from cells involved in phagocytosis, complement production and expression of inflammatory cytokines (Foey and Picchiatti, 2014).

Although their physiological roles vary depending upon their location, all of these mucosal tissues share structural similarities (see Table 1.1), notably the presence of an organized epithelial surface with supporting stromal tissues or lamina propria, a vascular supply network, musculature, and resident immune cells (Peterson, 2015).

**Table 1.1** Summary table of the main characteristics of teleost MALT. ILT = inter-brachial lymphoid tissue; ? = unknown, not studied. Adopted from Salinas (2015).

Characteristic	GALT	SALT	GIALT	NALT
Anatomical localization	Intestine	Skin	Gills	Olfactory organ
Organisation	Diffuse only	Diffuse	Diffuse with one organised tissue in salmon (ILT)	Diffuse
Presence of goblet cells	Yes	Yes	Yes	Yes
Approx. IgT/IgM B cell ratio	1:1	1:1	?	1:1
Total % of B cells	4-5%	4-5%	?	35-40%
Expression of pIgR	Yes	Yes	?	Yes
Presence of bacterial microbiota	Yes	Yes	Yes	Yes
Microbiota coated by secretory immunoglobulins	Yes	Yes	?	Yes

### 1.3.1 *The Gut-Associated Lymphoid Tissue (GALT)*

The gut-associated lymphoid tissue is strikingly diverse across vertebrate groups (Salinas *et al.*, 2011). Fish lack an organised GALT and the majority of the essential components of the mammalian mucosal immune system, such as Peyer's patches and mesenteric lymph nodes (Torrecillas, 2011; Rombout *et al.*, 2014; Castro & Tafalla, 2015). Instead, teleosts possess a rather more diffusely distributed GALT; lining the epithelium or distributed within the lamina propria (LP) are a variety of immune cells including, but not limited to lymphocytes, antigen-presenting cells (APCs), plasma cells, and macrophages (Foey and Picchiatti, 2014; Lazado and Caipang, 2014). Their diffuse organisation throughout the intestinal tract is suggestive of both innate and adaptive system readiness to respond to pathogens that infiltrate the mucosal barrier defences (Foey and Picchiatti, 2014).

### 1.3.2 *The Skin-Associated Lymphoid Tissue (SALT)*

The skin-associated lymphoid tissue is the largest mucosal tissue with respect to surface area and is continuous with the linings of all body openings and also enveloping the fins (Peterson, 2015). While the structure and function of fish skin reflects the adaptation of the organism to the physical, chemical and biological properties of its aquatic environment, the skin is also considered to have the most interspecies diversity of the four mucosal tissues (Peterson, 2015). Teleost diversity, with regard to structural organisation and dynamic function, is mirrored in a variety of skin morphologies (in terms of the presence or absence of scales), scale type, cell composition (including those of specialised cells), mucous characteristics and the molecules present in the mucosal secretions (Salinas *et al.*, 2011; Foey and Picchiatti, 2014). One example of this diversity was demonstrated in the work of Fast *et al.*, (2002), where the skin of three salmonid species was demonstrated to be biochemically and

histologically different: the outer epidermis of Atlantic salmon is significantly thinner, with fewer layers of epithelial cells, and fewer mucous cells than rainbow trout and Coho salmon (*Oncorhynchus kisutch*). Yet despite these differences, the general organization and microanatomic structures of the SALT are conserved along with the cell types (Peterson, 2015).

Being unique and histologically very different to that of mammals, the outer most layer of teleosts epidermis is a stratified but non-keratinized epithelium of variable thickness, that has retained the capacity to divide and secretes mucous involved in immune functions and protection (Salinas, Zhang and Sunyer, 2011; Ángeles-Esteban, 2012). As a metabolically active tissue, the SALT of fish and its components serve important roles not just in protection but also for communication, respiration, ion regulation, sensory perception, locomotion, excretion and thermal regulation, made possible by its complex structure and cell composition (Ostrand, 2000; Ángeles-Esteban, 2012; Lazado and Caipang, 2014). In addition to secretory cells, leukocytes such as granulocytes, macrophages and lymphocytes have all been observed in the skin of different teleost species; however, very little information is available on the function of each cell type during an immune response (Salinas *et al.*, 2011).

The importance of the skin as an immune organ was clearly demonstrated when transcript analysis of common carp (*Cyprinus carpio*) skin revealed 82 orthologues of genes of immune relevance previously described in other organisms, 61 of which had never before been described in carp (Gonzalez *et al.*, 2007). Similarly, micro-array analysis of Atlantic salmon skin following infection with *Lepeophtheirus salmonis* exhibited alterations in the expression of genes relating to immune response, oxidative stress, protein folding and cytoskeletal/structural proteins (Skugor *et al.*, 2008).

### 1.3.3 The Gill-Associated Lymphoid Tissue (GIALT)

Comprising the main respiratory surfaces of the fish, teleost gills are large mucosal surfaces and considered an important immune organ capable of mounting robust immune responses (Foey & Picchietti, 2014). The gills interact closely with the external environment. Forming a semi-permeable barrier between the organism and the external milieu, only two or three cell layers separate the blood and external water (Koppang *et al.*, 2015). Combined with the large surface area, this short distance not only promotes highly efficient gas exchange but also allows the gills to be major sites of osmoregulation, nitrogenous waste excretion, pH regulation and hormone production (Evans *et al.*, 2009). Conversely, and to the potential detriment of the fish, this feature also facilitates the leakage of ions and water, the uptake of foreign substances, and possible invasion by infectious agents (Koppang *et al.*, 2015). For instance, one of the portals of entry of two common fish pathogens *Vibrio anguillarum* and *Aeromonas salmonicida* is through the gills (Lazado and Caipang, 2014). In order to offset any invasion or attack by foreign pathogens the GIALT has developed into an immunologically active tissue consisting of potent immune factors and immunoreactive cells including antibody secreting cells, neutrophils, eosinophilic granulocytes, macrophages, small and large lymphocytes, and anti-microbial peptides (Salinas *et al.*, 2011; Lazado & Caipang, 2014).

### 1.4 The Epidermal Mucosal Layer

The external constituent of the teleost MALT is a mucous layer that forms a thin protective layer of adherent mucous over the living epithelial cells (Ángeles-Esteban and Cerezuela, 2015). In the SALT and GIALT and GALT, this mucosal layer separates the organisms internal milieu from the external aquatic environment, whilst simultaneously acting as a semipermeable, physical, chemical, and biological barrier (Ángeles-Esteban, 2012). At

mucosal sites, mucous secretion is one of the most important innate defence mechanisms and the first line of defence against pathogens (Lowrey, 2011).

Mucous is composed of around 95% water and 5% glycoprotein and produced by certain specialised secretory cells, mainly goblet cells and club cells, but also sacciform cells present within the epidermis (Ángeles-Esteban and Cerezuela, 2015; Salinas, 2015). Additionally, while the mucous composition is very complex and varies among fish species, endogenous factors such as sex and developmental stage, and exogenous factors such as stress and pathogenic infections, influence the mucous secreting cells and the composition of the mucous they produce (Ángeles-Esteban, 2012).

Capable of entrapping and immobilizing particles, virus or bacteria before they can reach the epithelial surfaces, the mucous layer is not static but continuously secreted and replaced to prevent the stable colonisation of potentially infectious microorganisms and invasion by parasites (Cone, 2009; Ángeles-Esteban, 2012; Ángeles-Esteban & Cerezuela, 2015). Mucous composition, structure, and thickness of the mucosal layer can vary depending on the mucosal territory and the physiological, immunological, or environmental conditions (Castro & Tafalla, 2015). Although the complete range of immune factors present in the mucous is poorly understood, a wide variety of biologically active substances and defensive molecules of both the innate and adaptive immune system function within the mucous (Ángeles-Esteban 2012; Ángeles-Esteban and Cerezuela, 2015). For example, lectins, lysozyme, mucins, transferrin, proteolytic enzymes, C-reactive protein, antimicrobial peptides, and secretory immunoglobulins, are present among others (Lowrey, 2011; Koshio, 2016). The antimicrobial properties of epidermal mucous against infectious pathogens has been demonstrated in a variety of different species (Ellis, 2001; Nagashima *et al.*, 2003; Chinchar *et al.*, 2004;

Kuppulakshmi *et al.*, 2008; Wei *et al.*, 2010; Su, 2011), while its active immunological role against parasitic infection has also been documented (Sigh *et al.*, 2004; Gonzalez *et al.*, 2007a, 2007b).

### 1.4.1 Mucins

The most predominant molecules present within the mucous layer are mucins (Gomez *et al.*, 2013). Mucins belong to a heterogeneous family of high molecular weight filamentous glycoproteins composed of a long peptide chain with a large number of tandem repeats that form the so-called mucin domain (Perez-Sanchez *et al.*, 2013; Salinas and Parra, 2015). These domains are particularly rich in threonine, serine and proline residues, whose hydroxyl groups are in *O*-glycosidic linkage with oligosaccharides and can differ significantly in length and sequence from one mucin to another: some are small, containing a few hundred amino acid residues, whereas others contain several thousand residues and are among the largest known proteins (Perez-vilar and Hill, 1999). Glycosylation of the mucin's carbohydrate chains (which can account for up to 90% of its weight) is necessary for the role of mucous as a mechanical barrier as well as for its interactions with the environment (Perez-vilar and Hill, 1999). Thus, changes in glycosylation levels of mucins due to pathogenic infection, for example, modulate bacterial attachment to the mucous layer (Salinas & Parra, 2015). The difference in the extent of glycosylation between mucins of different MALT tissues has been reported in Atlantic salmon, where skin mucins are less glycosylated and carry a lower diversity of structures than intestinal mucins: the skin glycan repertoire is dominated by linear structures whereas intestinal mucin *O*-glycans are predominately branched (Jin *et al.*, 2015). Within the scientific literature, it is known that pathogens may bind to specific carbohydrate structures present on both glycolipids and mucins: the balance between adherences to these different but

structurally related molecules is likely important to the outcome of the disease (Padra *et al.*, 2019).

It has been previously postulated that the ability of mucins to trap pathogens on human gastric mucins will likely benefit the host by keeping the pathogens closer to the lumen centre, away from the epithelial surface, thereby minimising host-pathogen interactions (Lindén *et al.*, 2008; 2010). While this concept potentially applies to fish gastrointestinal mucins also, for tissues such as the SALT and GIALT that are inherently exposed to the fluid velocity of the surrounding water, it has been hypothesised that a weaker mucin-pathogen interaction would be of greater benefit, allowing pathogens to be removed by the water flow and thus minimising pathogen attachment (Lindén *et al.*, 2009; Padra *et al.*, 2014). Once contact between the mucin and pathogen has been established, high mucin affinity for the pathogen may aid in the continued entrapment of the pathogen within the mucous, preventing infection through immunological and mechanical defences (Padra *et al.*, 2019). For example, the mammalian pathogen *Helicobacter pylori* has been observed to downregulate mucin-binding adhesins when cultured in the presence of mucins with high binding affinity but not when cultured in the presence of non-binding mucins (Skoog *et al.*, 2012). This may be an avoidance mechanism utilised by the pathogen in order to prevent its removal from the niche. Scientific understanding of complex mucous-pathogen interactions remains in its infancy but should, nevertheless be kept in mind when trialling different “mucous enhancing” feeds and treatments.

Based on their structural and functional features, mucins are grouped into two structurally distinct families (Hicks *et al.*, 2000). Secreted Gel Forming Mucins (SGFMs) are characterized by the presence of several domain structures, namely the von Willebrand D (VWD), cysteine



rich (C8) and trypsin inhibitor like (TIL) cysteine rich domains which, via oligomerization of the mucin proteins through disulphide bond formation, provide the gel-forming properties of mucous (Malachowicz *et al.*, 2017). On the other hand, the membrane-bound cell-surface mucins contain long segments of highly repetitive sequences rich in proline, threonine and serine residues, known as the PTS-domain: the length and amino acid sequence of this domain varies between mucins and provides rigidity and solubility to the protein and may act as ligands for microbial adhesions (Salinas and Parra, 2015; Sveen *et al.*, 2017). Several other non-mucin proteins also contain the VWD domain and the domain structure VWD-C8-TIL, confounding the verification of newly identified gene codes as a mucin (Sveen *et al.*, 2017). These two main classes of mucins therefore have both unique and shared structural features, that influence co-ordinated cell proliferation, differentiation, and apoptosis among other cell responses (Perez-Sanchez *et al.*, 2013).

It stands to reason that several mucins are likely awaiting discovery yet have so far eluded detection due to their large size and repetitive nature, as reported by previous authors (Perez-Sanchez *et al.*, 2013). To date, more than 20 mucin genes have been described in higher vertebrates (Marcos-López *et al.*, 2018). Based on biochemical characterisation, mucin genes are currently assigned to the mucin family and are named “MUC-number” for humans or “Muc-number” for other species (van der Marel *et al.*, 2012). In humans, our knowledge of mucins and their patterns of distribution in tissues and organs is such that they are now employed as prognostic and diagnostic markers in malignant diseases involving epithelial cells (Perez-Sanchez *et al.*, 2013), however knowledge of mucin functionality in lower vertebrates such as fish is still in its infancy.

#### 1.4.1.1 Teleost mucins

Despite publications concerning mucous-producing cells and the protective role of the mucous layer in fish, little knowledge exists about mucin-encoding genes in fish, which have only just begun to be described in several species within the last 10 years (Sveen *et al.*, 2017). Fish mucin gene expression studies therefore remain scarce, yet immuno-cytochemical, cytochemical and biochemical techniques have been applied to determine the effect of environmental pollutants and pathogens on mucins and mucin-producing cells (Perez-Sanchez *et al.*, 2013).

Van der Marel and colleagues (2012) were among the first to describe mucin genes in fish. Owing to the large size of the tandem repeats, attempts to fully sequence a number of mucin genes in common carp were unsuccessful during their investigation. However, two partial mucin-like sequences were discovered and proved to have high homology with the mammalian and avian gel forming mucins: Muc2 and Muc5B. Muc2 was highly expressed in both the first and second intestinal segments and marginally in the gills. Muc5B expression however, was not observed in the intestine but was highly abundant in the gills and even more so in the skin (van der Marel *et al.*, 2012). Additionally, the dietary effect of  $\beta$ -glucan administration upon the expression of the mucin genes was also assessed in the mucosal tissues: a significant 2.7-fold increase in the abundance of Muc5B was detected in the skin, yet a decrease in Muc2 and Muc5B expression was observed in the gills following  $\beta$ -glucan feeding.

A similar study by Pérez-Sánchez *et al.* (2013) was concerned with the identification and tissue specific expression pattern of mucins in the SALT, GIALT, and GALT of gilthead sea bream (*Sparus aurata*) and successfully identified six sequences. Three were membrane bound

mucins: intestinal mucin (I-Muc), mucin 13 (Muc13), and mucin 18 (Muc18); and a further three were SGFMs: mucin 2 (Muc2), mucin 2-like (Muc-2-like), and mucin 19 (Muc19) (Perez-Sanchez *et al.*, 2013). A very different pattern was found for each of the membrane-bound mucins in terms of their tissue-specific gene expression (as described in Table 1.2). Although consistently detected in all the sampled organs, Muc18 was found to be most abundant in the gills and even more so in the skin. The authors propose that the deduced amino acid sequence of Muc18 indicates that it is a member of the immunoglobulin superfamily, showing the greatest sequence similarity to a group of neutral cell adhesion molecules expressed during organogenesis (Perez-Sanchez *et al.*, 2013). This is in agreement with the speculation that human-MUC18 may be developmentally regulated and a mediator of intracellular adhesion: this characteristic would be of particular relevance in fish skin and gills directly exposed to turbulence of the surrounding water (Johnson *et al.*, 1996). The membrane-bound mucin gene candidate, the so-called I-Muc, was again expressed in all the studied organs with the exception of the anterior and middle intestine, yet it was mostly expressed in the posterior intestine. In contrast to this, Muc13, was not only found to be present along the entirety of the intestinal tract but was also the most abundant mucin, exhibiting an antero-posterior increasing trend, similar to the increasing expression pattern from the small intestine to the rectum in humans (Perez-Sanchez *et al.*, 2013). Overall, Muc19 was by far the highest expressed mucin, predominantly present in the oesophagus and only scarcely in the stomach. In humans, MUC-19 is one of the major components of salivary glands, yet since fish lack these structures, the authors suggest that Muc19 could be homologous to those of saliva in higher terrestrial animals and contribute to food digestion (Perez-Sanchez *et al.*, 2013).

**Table 1.2.** Summary table listing known mucins in different species of fish, and in which tissues they are found to be present and most abundant. AI = anterior intestine, MI = middle intestine, PI = posterior intestine. Compiled from van der Marel *et al.*, 2012; Perez-Sanchez *et al.*, 2013; and Sveen *et al.*, 2017.

Species	Mucin	Tissues present in	Most abundant tissue
<b>Sea bream</b>	Muc18	Skin, gills, oesophagus, stomach, intestine	Skin
	I-Muc	Skin, gills, oesophagus, stomach, PI	PI
	Muc13	Entire intestine	Entire intestine
	Muc19	Oesophagus and stomach	Oesophagus
	Muc2	Stomach and entire intestine	AI
	Muc2-like	Stomach and entire intestine	AI
<b>Common carp</b>	Muc2	Gill, AI and MI	AI and MI
	Muc5B	Gill and skin	Skin and gills
<b>Atlantic salmon</b>	Muc2	Anterior, middle, and posterior intestine	Posterior intestine
	Muc5B	Skin, tongue, gills and operculum	Skin
	Muc5ac	Tongue, skin, operculum and gills	Gills

Concerning the remaining SGFMs, Perez-Sanchez and colleagues (2013) observed that, together with Muc13, Muc2 and Muc2-like were predominant mucins in the whole of the intestinal tract, the profiles of which were both affected, and significantly so, in the posterior intestine following parasitic infection. In humans and mice, Muc2 is the predominant mucin produced by intestinal goblet cells and also has a function as a tumour suppressor (Velcich *et al.*, 2002; Yonezawa *et al.*, 2011). Furthermore, patients with ulcerative colitis exhibited decreased Muc2 expression, supporting the data of other studies and the proposition that Muc2 is essential for the protection of intestinal epithelium against commensal bacteria and potential pathogens (Sheng *et al.*, 2012; Pérez-Sánchez *et al.*, 2013). Moreover, Adamek *et*

*al.*, (2013) were the first to demonstrate the down-regulation of Muc5B expression in the skin of common carp infected with the viral pathogen herpesvirus-3.

Based on their homology with other species, many predicted Atlantic salmon mucin sequences can be found in the National Centre for Biotechnology Information (NCBI) databases (Marcos-López *et al.*, 2018). Only recently has a genome-wide analysis of Atlantic salmon mucin genes been conducted. The results of the study were in agreement with those of previously performed studies in different species; two mucin genes annotated as Muc2 were predominantly described in the intestine, while two genes annotated as belonging to the Muc5 family were mainly transcribed in the skin (Muc5ac) and gills (Muc5B) of Atlantic salmon (Sveen *et al.*, 2017). Additionally, the transcriptional regulation of mucins during stress conditions was assessed: handling-stress induced mucin transcription in the gill, whereas long-term intensive rearing conditions interrupted by additional confinement led to increased transcription of mucin genes in the skin (Sveen *et al.*, 2017). Taken together, the results of these studies would suggest at the comparative functionality of mucins within different species, the important role mucins play in maintaining the homeostasis and integrity of the mucosal layer, and further highlight the potential influence of pathogens, stress and nutrition upon their expression.

Historically, mucins have not been identified in proteomic studies concerned with the epidermal skin mucous of fish because, before gel or even gel-free analysis can commence, sample preparation necessitates centrifugation and/or filtration which removes mucins due to their high molecular weight and complex structure (Brinchmann, 2016). Transcriptomics is a powerful tool for the discovery of new genes related to mucosal immunity and holds the potential to reveal important aspects of mucosal functions. However, as with the other

techniques, it remains challenged, primarily depending upon the availability of well-annotated genomes which remain uncommon in non-model and non-commercial fish species (Reverter *et al.*, 2018), and by limitations imposed by the size, highly repetitive nature and sequence similarity of mucin genes (Pérez-Sánchez *et al.*, 2013). Nonetheless, several studies have reported interesting results concerning mucin gene expression in several species of fish.

#### 1.4.2 Immunoglobulins

At present, immunoglobulins are generally accepted as the primary humoral component of the adaptive immune system, playing a pivotal role in the maintenance of mucosal homeostasis (Ángeles-Esteban & Cerezuela, 2015). Mainly produced by plasma blasts and plasma cells, the function of immunoglobulins is to very specifically target and neutralise any foreign antigens present within the organism: after binding to a pathogen or cognate, antigen-specific immunoglobulins mediate a number of different actions, including complement activation, phagocytosis by opsonisation, neutralisation, antibody dependant cellular cytotoxicity, and agglutination (Mashoof and Criscitiello, 2016). To date, three major immunoglobulin isotypes have been reported in teleost fish: IgM was the first to be discovered, followed by IgD (Wilson *et al.*, 1997) and finally IgT/IgZ in 2005 (Danilova *et al.* 2005; Hansen *et al.*, 2005).

IgM is the most common immunoglobulin in the plasma of teleosts and is the principal immunoglobulin involved in systemic immune responses (Ángeles-Esteban, 2012). In contrast to early studies in which no apparent differences between the different isotypes of immunoglobulins present within skin mucous and serum were reported, new investigations have revealed that while IgM is usually found in high concentrations in fish serum, it is only present at very low levels in the skin mucous of several fish species (Zhao *et al.*, 2008; Ángeles-

Esteban & Cerezuela, 2015). It is also reported that IgM antibodies possess a limited antigen spectrum in fish, while their presence depends upon their spatial distribution: immunoglobulin levels in channel catfish (*Ictalurus punctatus*) were found to be highest on lateral skin, lower between pectoral and anal fins, and lowest on the tail and ventral skin (Salinas *et al.*, 2011). It should be mentioned however, that it is challenging to accurately evaluate the concentration of IgM in fish mucous as it varies between different individuals and is highly regulated by certain factors. For example, IgM levels increase with increasing water temperature, yet purified IgM from serum was rapidly digested in gut mucous at 4°C (Ángeles-Esteban, 2012). It has also been reported to vary in response to water quality and seasons of the year, as well as stress stimulation, or immunisation (Salinas *et al.*, 2011). Typically, the amounts of detected IgM are extremely small compared to the detected amounts of IgA in mammalian secretions (Ángeles-Esteban, 2012). The critical role IgM plays in the defence against surface infection was demonstrated by a study in channel catfish skin, in which the number of IgM-secreting cells increased 20-fold following immunisation with the protozoan parasite *Ichthyophthirius multifiliis* (Zhao *et al.*, 2008).

While IgD has been successfully described in a variety of teleosts, its involvement in the mucosal response has not yet been clarified and remains only partially understood, despite the knowledge that it can indeed be secreted (Rombout *et al.*, 2014). Total IgD levels range from 2 to 80µg/mL in the plasma of rainbow trout but may be very low or even below detection levels in mucosal secretions (Salinas, 2015b). In Atlantic salmon overall expression of IgM and IgT are, respectively, up to 200 and 20 times more abundant than IgD, while the abundance in the gills and other organs was measured to be <10% than that of the kidney and spleen (Tadiso *et al.*, 2011a). Future functional experiments will be critical in ascertaining the functional role of IgD in the mucosal adaptive immune response of fish.

IgT shares many functional similarities with mammalian IgA (Rombout *et al.*, 2014) and has been described in a number of different teleost species, including salmonids (Danilova *et al.*, 2005; Hansen *et al.*, 2005). In a study on rainbow trout, Zhang and colleagues (2010), observed that while the concentration of IgM in serum was much higher than that in gut mucous, the concentration of IgT in gut mucous was double to that of serum demonstrating the important role of IgT in gut mucosal immunity (Zhang *et al.*, 2010; Gomez *et al.*, 2013). Additionally, it has been revealed that IgT plays a prevailing role in skin mucosal immunity, being the most abundant isotype in the skin mucosa of rainbow trout (Xu *et al.*, 2013), while it is also abundantly secreted in gill mucous (Tadiso *et al.*, 2011a). Acting as a mucosal antibody, IgT is now generally accredited as being the only teleost immunoglobulin isotype with a specialized mucosal function (Koppang *et al.*, 2015; Parra *et al.*, 2016).

### *1.4.3 The polymeric immunoglobulin receptor*

The polymeric immunoglobulin receptor (pIgR) is a vital component of the mucosal immune system in teleost fish, playing an essential role in mucosal immunity and in the transport of immunoglobulin molecules (Foey and Picchiatti, 2014; Rombout, Yang and Kiron, 2014). The primary function of the pIgR is to mediate the trans-epithelial transport of IgM and IgT across mucosal barriers in order to protect the organism from environmental pathogens (Gomez, Sunyer and Salinas, 2013; Yu *et al.*, 2018a). The expression of pIgR has been reported in the four mucosal tissues of teleosts (Salinas, 2015b). In the gut for example, the pIgR receptor is expressed by mucosal epithelial cells, allowing the binding of mucosal immunoglobulins onto the basolateral surface, transcytosis through the epithelial cell and secretion into the luminal compartment of the gut: in doing so the extracellular domain of the receptor is cleaved off



with the antibody and serves as a proteolytic cleavage protection secretory component (Foej and Picchiatti, 2014).

#### 1.4.4 Lysozyme

It is well known that the mucous secretions of fish contain a wide variety of innate immune molecules (Gomez *et al.*, 2013). Lysozyme is one such molecule: an anti-microbial protein associated with innate immune defences and ubiquitous in its distribution among all vertebrates (Marsh and Rice, 2010). As a mucolytic enzyme, lysozyme degrades the peptidoglycan hetero-polymers found within the cell walls of bacterial pathogens by catalysing the hydrolysis of the  $\beta$ -1,4 glycosidic linkages between *N*-acetyl-muramic acid and *N*-acetyl-D-glucosamine (Castro and Tafalla, 2015; Carbone and Faggio, 2016). Mainly produced by monocyte-macrophages and neutrophils, lysozyme is consequently abundant in lymphoid tissues, mucous and the serum of fish (Castro and Tafalla, 2015).

Lysozyme level or activity is considered an important index of innate immunity of fish (Saurabh and Sahoo, 2008). In addition to the antibacterial function against Gram-positive bacteria, lysozyme promotes phagocytosis by directly activating leucocytes and macrophages or indirectly via an opsonic effect (Saurabh and Sahoo, 2008), and may additionally affect Gram-negative bacteria when acting in association with complement and other enzymes that expose the inner peptidoglycan layer (Saurabh and Sahoo, 2008; Marsh and Rice, 2010). Lysozyme activity is influenced by a range of physiological and environmental factors, including stress and infection; season and sexual maturity; environmental conditions such as water temperature, salinity and pH; and nutrition (Saurabh and Sahoo 2008). The potential effect of nutrition upon lysozyme activity is of particular interest to this thesis and will be described and discussed in following sections.

### 1.5 The mucosal microbiota

In addition to the unique array of innate and adaptive immune cells and molecules that work in concert to protect the host against pathogens, the mucosal surfaces are also colonised by complex and diverse microbial populations (Gomez *et al.*, 2013; Lazado & Caipang, 2014). These microbial communities are present at sites in which surfaces and cavities are open to the environment, such as the skin, gill and intestine, and are collectively termed as the “microbiota”, alongside the more historically used term the “microflora” (Romero *et al.*, 2014).

The microbial communities present at the SALT, GIALT and GALT confer many physiological, metabolic and immunological benefits upon the host (Gomez *et al.*, 2013, Merrifield and Rodiles, 2015). These commensal microbiota aid in the facilitation and mediation of host defences and barrier functions by competing with pathogens for nutrients and binding sites on epithelial surfaces, modulating epithelial cell growth and apoptosis turnover, increasing epithelial barrier strength by modulating tight junction proteins, advancing epithelial cell mucous production, initiating the production of defensin and other anti-microbial peptides from epithelial cells, producing various compounds that may antagonise pathogens, and via the modulation of the underlying innate and adaptive immune components of the epithelial layer (Foey and Picchietti 2014; Romero *et al.*, 2014; Merrifield and Rodiles, 2015).

The microbiota can be subcategorized into two major groups: allochthonous microbiota are considered to be transient and non-adherent to the host tissues, whereas the autochthonous microbiota are potentially resident and intimately associated with the host tissues (Romero *et al.*, 2014; Caipang and Lazado, 2015). While the close contact of the mucosal surfaces to the immediate environment plays an important role in shaping the overall microbiological

make-up of the mucosal surfaces, it should be mentioned that the microbiota also contains opportunistic pathogens that may be considered a liability if the opportunity arises for them to overpopulate and out-compete the beneficial commensal population, eventually disrupting the homeostasis of the microbiota (Lazado and Caipang, 2014). For example, the administration of antibiotics to Olive flounder (*Paralicthys olivaceus*) had a large impact on the gut microbial community, resulting in severe proliferation of opportunistic pathogens, including *Vibrio scophthalmi*, *Photobacterium damsela* and *Streptococcus parauberis* within the intestinal microbiota (Kim *et al.*, 2019).

The composition and structure of taxa within the microbiota can in turn influence its effectiveness at conferring benefits upon the host (Schmidt *et al.*, 2015). For example, Rawls and colleagues (2006) demonstrated that gnotobiotic animals inoculated with microbiomes from zebrafish (*Danio rerio*) and mice (*Mus spp.*) exhibited reduced immune function compared to animals inoculated from conspecifics. Antibiotic disruptions of microbiota communities can also reduce host disease resistance (Ng *et al.*, 2013; Theriot *et al.*, 2014), while nutritional uptake and survival ability can be limited in the absence of specific symbionts (Franzenburg *et al.*, 2013). Collectively these findings highlight the significance of the microbiota to host physiology and the importance of community composition to the function of the microbiota.

The skin of fish harbours a complex and diverse microbiota that closely interacts with the microbial communities of the surrounding water (Boutin *et al.*, 2014). To date, research on the microbiota associated with the skin of fish has experienced challenges when attempting to identify the genera present and in determining their abundance. When sampling, avoiding contamination of the epidermal mucous is the first challenge, while standardisation of the

precise sampling site on the skin also poses complications when attempting to compare research investigations: given the observations in mammals, it is not unreasonable to suggest that variations in the microbial communities may be present on different regions of the body surface (Merrifield and Rodiles, 2015). It has also been proposed that culture-based surveys that are commonly used to investigate microbial community composition, vastly underestimate diversity as <10% of bacteria can be isolated and cultured under laboratory conditions (Larsen *et al.*, 2013). Nevertheless, bacterial populations on the skin of Atlantic salmon and rainbow trout are reportedly in the range of  $10^2$  to  $10^4$  CFU  $\text{cm}^{-2}$ , composed of a wide range of microorganisms belonging to a range of phyla, including *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* (Merrifield and Rodiles, 2015). Although the majority of information available on the skin microbiota of fish pertains to the bacterial components, fungi and yeast have been observed within the skin microbiota of certain species also (Merrifield and Rodiles, 2015).

At present, the commensal communities associated with fish gills remain poorly understood in terms of microbial diversity, the factors that structure the diversity, and the extent to which this diversity is specific to the gill or individual, and are far less studied than those of the gut and skin (Reverter *et al.*, 2017; Pratte *et al.*, 2018). Notwithstanding, high populations of a variety of bacterial genera, in the range of  $10^6$  CFU/g of tissue, have been reported in the gills of both marine and freshwater species: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* in addition to certain fungi (Merrifield and Rodiles, 2015). A number of environmental and dietary factors, such as nutrition, stress, seasonality and surrounding environmental parameters have been reported to affect the composition and abundance of the skin and gill microbiotas (as described in Table 1.3) (Legrand *et al.*, 2018; Tapia-Paniagua *et al.*, 2018).

In comparison to the skin and gills, the microbiota of the gastro-intestinal tract is the subject of a far greater number of investigations, yet still remains only partly described and understood in terms of its interaction and influence upon the host. The microbiota of the gut is typically several orders of magnitude greater than the microbial communities of the skin and gills (Rodiles *et al.*, 2018). In terms of abundance, while yeasts, protozoa, archaea and viruses are reportedly present in the gastro-intestinal tract of fish, bacteria are typically the dominant microbes present, comprising 100s-1000s of Operational Taxonomic Units (OTUs) (Merrifield and Rodiles, 2015). The microbiota actively contributes to digestive function and process: a wide range of microbes are capable of producing extracellular digestive enzymes that the host is often unable to produce itself (or only able to produce in low concentrations), such as cellulose, chitinase and phytase (Ray *et al.*, 2012). The production of vitamins, such as vitamin B12 (cobalamin), and short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate have also been described in the intestine, reportedly produced by the microbiota (Hoseinifar *et al.*, 2019). The SCFAs contribute considerably to the host energy requirements, supplying energy either directly to the enterocytes or to other organs via the vascular system, while the resulting decrease in pH may increase the solubility of minerals and make the intestinal tract an unfavourable environment for opportunistic pathogens (Merrifield and Rodiles, 2015).

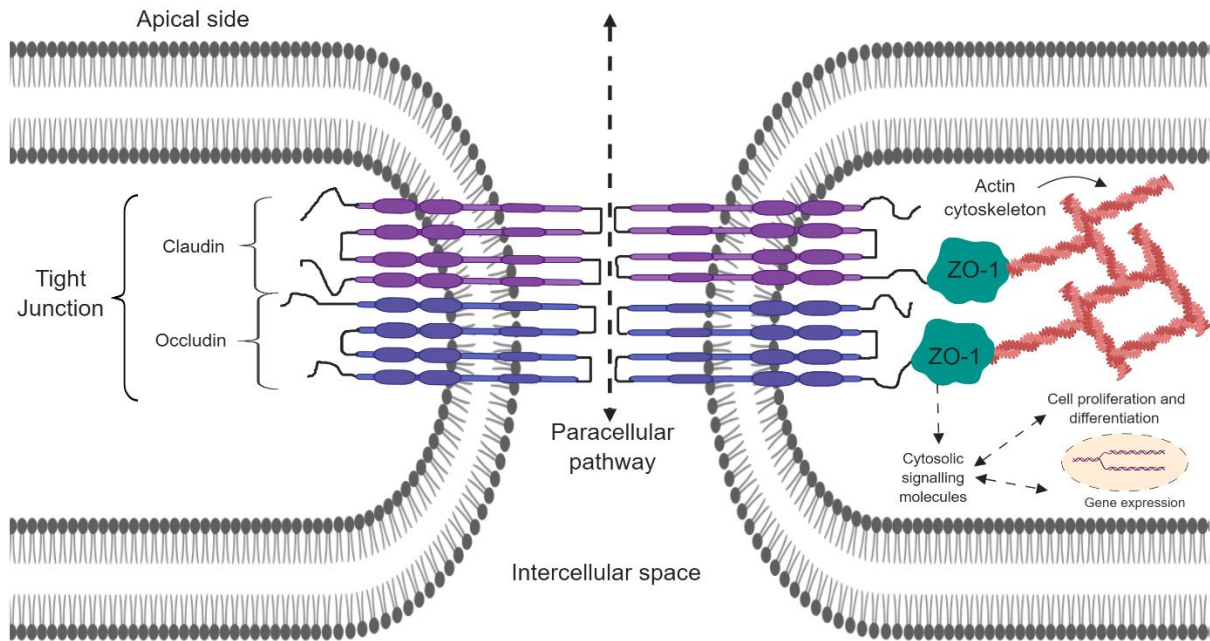
**Table 1.3.** Factors influencing the microbiota of fish, modified from (Merrifield and Rodiles 2015, Legrand *et al.*, 2018, Tapia-Paniagua *et al.*, 2018). GIT= gastro-intestinal tract.

Factor	Example	Details	Microbiota affected
<b>Environmental</b>	Seasonality	-	GIT, gills
	Temperature	-	GIT
	Water salinity	-	GIT
	Water quality	-	Eggs, skin, gills, GIT
	Toxicants	Metal nanoparticles, heavy metals	GIT
	Wild vs. farmed fish	-	GIT
<b>Dietary</b>	Form	Pellet vs. natural diet	GIT, skin
	Trophic level	-	GIT
	Food deprivation	Fed vs. unfed fish	GIT, skin
	Dietary lipid	Lipid level, lipid sources	GIT
	Protein sources	Plant-derived proteins, yeast protein concentrates, insect meals	GIT
	Carbohydrates	Plant-derived carbohydrates, chitin	GIT
	Feed additives	Probiotics	GIT
	-	Prebiotics	GIT
	-	Antibiotics/ disinfectants	Eggs, GIT, skin
	-	Phytobiotics	GIT
-	Immunostimulants	GIT	
<b>Other</b>	Genetics	Different fish families within a species, transgenic individuals	GIT, gills, skin
	Hierarchy/ stress	Dominant vs. subordinate individuals	GIT
	Stocking density	-	GIT

As a result of the increased research efforts over the last decade and the utilisation of culture-independent analysis techniques, it is becoming increasingly apparent that the microbiota of the mucosal-associated tissues are intimately involved in several aspects of homeostasis maintenance, diseases prevention, mucosal barrier integrity, nutrition, and host development at the larval stages (Rodiles *et al.*, 2018). As already confirmed in the GALT, the co-existence of these commensals in a dynamic equilibrium is maintained through continued and active signalling, a mechanism thought to be operational at the SALT and GIALT also (Lazado & Caipang, 2014). However, despite the importance of these microorganisms in development, homeostasis and protection of the fish, particularly in the gut, the commensal microbiota cannot be considered as part of the MALT in its strictest anatomical sense. Instead, the distinction of being an “extra organ” of the host is generally accepted (Lazado & Caipang, 2014; Caipang & Lazado, 2015).

### 1.6 Tight junctions

Beneath the mucosal layer, the epithelial layer serves as a physical barrier to the external environment. A fundamental function of the epithelia is to separate different compartments within the organism and to regulate the exchange of substances across membranes (González-Mariscal *et al.*, 2003). A complex known as the tight junction (TJ) constitutes the barrier to the passage of ions and molecules through the paracellular pathway of a membrane (i.e. TJ “gate” function), and to the movement of proteins and lipids between the apical and basolateral domains (i.e. TJ “fence” functions) (Gonzalez-Mariscal *et al.*, 2003; Kolosov *et al.*, 2013). As such, they are important determinants of ion selectivity and general permeability of the epithelia (Gauberg *et al.*, 2017).



**Figure 1.2** Generalised model of the tight junction (TJ) complex between epithelial cells. Tetraspan transmembrane TJ proteins such as occludin (shown in blue) and claudins (shown in purple) directly regulate the permaselectivity characteristics of the TJ by bridging the intracellular space (via their extracellular domains) to form the TJ barrier. Zonula Occludins (ZO)-1 (shown in green) provides structural support to TJs by linking the transmembrane TJ proteins to the actin cytoskeleton. ZO-1 also participates in intracellular signalling pathways related to gene expression, cell proliferation and differentiation by interacting with cytosolic signalling molecules. Adapted from Chasiotis *et al.* (2012).

Over the last 30 years, the identification of TJ-associated proteins has been a key area of cell biology. The TJ complex is now known to be composed of multiple transmembrane proteins including occludin, junction adhesion molecules (JAM), and claudins (Engelund *et al.*, 2012). Cytosolic proteins, such as Zonula-occludins, provide structural support to the complex by linking the transmembrane proteins to the actin cytoskeleton of the epithelial cell (as depicted in Figure 1.2) (Chasiotis *et al.*, 2012). Within the last 20 years, an unprecedented expansion of information has highlighted the functional diversity of TJs: once considered to



be a mere a paracellular barrier, TJs are now recognised as complex structures involved in signalling cascades, cellular differentiation, proliferation, migration, and gene expression (González-Mariscal *et al.* 2003, Cummins 2012).

### 1.6.1 Occludin

Occludin was the first transmembrane TJ protein to be identified and isolated from vertebrate epithelia and currently represents the most comprehensively characterised TJ protein in teleost fish (Chasiotis *et al.*, 2012). Being a tetraspan protein, occludin localises exclusively to TJ fibrils at sites of cell-cell contact and is found in a wide variety of epithelial and endothelial tissues (González-Mariscal *et al.*, 2003). In mammalian models, several lines of evidence suggest a role for occludin in the formation and enhancement of the TJ barrier via its extracellular domains, which “occlude” the intracellular space and therefore restrict the paracellular movement of solutes (Gonzalez-Mariscal *et al.*, 2003; Cummins, 2012; Chasiotis *et al.*, 2012).

While expression profiles in fish have demonstrated widespread occludin mRNA distribution among the mucosal tissues, gill tissues have been shown to exhibit the highest levels of occludin transcript abundance, where it likely contributes to a “tightening” of the gill epithelium (Chasiotis *et al.*, 2012). Mechanistic studies utilizing *in vitro* gill models have confirmed the barrier-forming role of occludins in teleost gill, a finding in accord with the functional characterisation of mammalian occludin (reviewed by Cummins, 2012). Alterations in gill occludin transcript have been reported in fish following food deprivation (Chasiotis & Kelly, 2008), upon exposure to lower pH (Kumai *et al.*, 2011), and when circulating levels of cortisol are chronically elevated (Chasiotis & Kelly, 2012a), suggesting that gill occludin may be sensitive to a number of environmental and/ or systemic variables (Chasiotis *et al.*, 2012).

### 1.6.2 *Zonula occludens protein*

Zonula occludens-1 (ZO-1) is frequently described as a TJ “scaffolding” or “adaptor” belonging to the family of proteins that contain different types of protein/ protein-interaction domains such as three PDZ and an SH3 domain (Balda & Matter, 2009). Found on the inner cytosolic surface of TJs with several binding domains which target other TJ proteins (e.g. occludins and claudins), cytosolic signalling molecules (e.g. transcription factors) and cytoskeletal actin, it is widely accepted that ZO-1 plays a dual role: (a) to provide indispensable structural support to TJs by linking occludin and claudins to the actin cytoskeleton and; (b) to transduce signals relating to gene expression, cell proliferation and differentiation via intracellular signalling pathways (Bauer *et al.*, 2010; Chasiotis *et al.*, 2012). While our knowledge about the functional role of ZO-1 at TJs remains incomplete, *in vitro* studies using primary cultured gill models have linked alterations in ZO-1 mRNA abundance with reductions in paracellular permeability, while it is also equally accepted that ZO-1 supports the structure and organisation of TJs in all teleost epithelia (Walker *et al.*, 2007; Chasiotis *et al.*, 2012).

### 1.6.3 *Claudins*

A major breakthrough in the understanding of TJ function was the discovery of claudin proteins: they are by far the most diverse group of the TJ proteins and make up a far greater part of the TJ complex architecture than the other associated proteins (Engelund *et al.*, 2012; Kolosov *et al.*, 2013). Claudins are the major structural and functional components of TJs that largely determine TJ permeability by bridging the intracellular space between epithelial cells (Chasiotis *et al.*, 2012; Perez-Sanchez *et al.*, 2015). From research conducted to date, it is clear that members of the claudin family play a crucial role in the regulation of physiological functions within the epithelial and endothelial tissues (Kolosov *et al.*, 2013).

The permeability and selectivity of TJs towards charged and uncharged molecules is complex, involving a number of TJ strands, and influenced by the differential expression of the TJ proteins (Sundh and Sundell, 2015). In mammals, two main groups of claudins have been identified, based on their barrier-forming capabilities: 'barrier-building' or 'tightening' claudins (claudin- 1, -3, -4, -5, -6, -8, -9, -11, -14, and -19) hinder paracellular solute movement, while 'pore-forming' or 'leaky' claudins (claudin- 2, -10, -15, and 17) augment the paracellular permeation of charged solutes, particularly small ions (Chasiotis *et al.*, 2012; Sundh & Sundell, 2015). These contrasting properties together with: (1) the number of members within the claudin superfamily; (2) the numerous possible combinations of claudin associations (e.g. homo- and heterotropic) within and between TJ fibrils; and, (3) the diversity of claudin permaselectivity properties, have resulted in the general understanding that claudins bestow the wide-ranging and distinct paracellular permeability properties, exhibited within and between different types of vertebrate epithelia (Chasiotis *et al.*, 2012).

Since the first claudin protein was reported in a teleost fish some 20 years ago, over 60 claudins have been described in 16 different teleost species (Gauberg *et al.*, 2016). The most comprehensive account of claudins in fishes to date is provided by Loh and colleagues (2004) who describe the characteristics and tissue specific distribution patterns of ~56 claudin genes in the Japanese pufferfish (*Fugu rubripes*). Although ~38% of the claudin genes are yet to be described in mammals and may in fact be specific to the teleost lineage, the further characterisation and distribution of a number of claudins have been reported in several alternative fish models (Loh *et al.*, 2004; Chasiotis *et al.*, 2012; Kolosov *et al.*, 2013), including 26 claudin isoforms in Atlantic salmon (Engelund *et al.* 2012).

The results of recent studies collectively suggest that the physiology of fish gill TJs is not straightforward and a small number of reports further suggest that the molecular architecture and physiological properties of TJs in the skin of adult teleosts may also be more complex and perhaps more environmentally responsive than might have been previously expected (Bui and Kelly, 2014). The gene expression patterns of teleost claudin TJ proteins have been reported to vary between different tissues, a pattern which is also observed for claudin TJ proteins investigated in all vertebrates studied to date (Kolosov *et al.*, 2013). It is also important to note here that claudin abundance is also reported to vary spatially within tissues (Caipang *et al.*, 2011; Chasiotis *et al.*, 2012). Variations between species have also been documented, confounding insight into the potential function of certain claudin genes. For example, claudin 27a was found to be largely unchanged in the gills of Atlantic salmon acclimated from fresh water to sea-water (Tipsmark *et al.*, 2008) yet significantly increased in the gills of the Fugu (*Tetraodon nigroviridis*) undergoing the same environmental acclimation (Bagherie-Lachidan *et al.*, 2009). Taken together and in view of the considerable size of the claudin superfamily, it is understandably rather challenging to pinpoint the exact function of a specific claudin in a specific tissue of a specific species in the absence of more data and functional investigations.

### 1.6.3.1 Claudins of the gills

The number of claudins reported in the gills of teleosts is approximately 44, yet some species express claudins in their gills that others do not. For example, claudin-29a is found in the gill tissue of zebrafish and goldfish (*Carassius auratus*), but not in Japanese pufferfish or fugu (Kolosov *et al.*, 2013). It is worth noting again here that the epithelia of the skin and gill are constantly in direct contact with the external environment and play critical roles in the maintenance of mucosal homeostasis. Both the skin and gills possess a large complement of

claudins, with recent evidence suggesting that many are highly sensitive to environmental change (Kolosov *et al.*, 2013). The claudin expression profile of a freshwater fish may therefore differ greatly to that of a marine fish.

The majority of studies conducted to date on gill claudins have focussed upon the role these proteins potentially play in the “tight” freshwater vs. “leaky” salt-water fish gill paradigm, which is associated with basic strategies of teleost fish osmoregulation (Kolosov *et al.*, 2013). Tipsmark *et al* (2008) evaluated the expression patterns of five gill-claudin isoforms during acclimation of freshwater salmon to seawater and during the smoltification process. Acclimation to saltwater reduced the expression of claudins 27a and 30, but had no overall affect upon claudin 28a or 28b, suggesting this reduction in expression may change the permeability conditions in favour of the ion secretory mode of the saltwater gill (Tipsmark *et al.*, 2008). Conversely, saltwater induced a four-fold increase in expression of claudin 10e leading the authors to propose claudin 10e as an important component of cation selective channels in the gill (Tipsmark *et al.*, 2008).

While functional insight is lacking for many claudins of the gill, and indeed those of the skin and intestine also, a number of transcriptional studies have reported on the sensitivity of gill claudins to alterations in environmental acidity, as being responsive to endocrine factors, and casually linked to measured changes in gill epithelium paracellular permeability (Kumai *et al.*, 2011; Chasiotis & Kelly, 2011; Sandbichler *et al.*, 2011).

### 1.6.3.2 Claudins of the skin

Relatively little attention has been paid to the roles of claudins found in teleost skin, and very little is known about how they are distributed within the tissue (Kolosov *et al.*, 2013). Nevertheless, investigations have revealed the presence of a considerable number of genes encoding tight-junction proteins in the skin of several species of adult fish, some of which significantly alter in abundance upon exposure to varying ionic environments. This would infer a more dynamic role for this tissue in the osmoregulatory physiology of adult teleost fish than simplistic views of simple barrier function would imply (Bui and Kelly, 2014).

The differential expression of numerous genes have been documented along the dorso-ventral axis of fish (Caipang *et al.*, 2011), of which claudins appear to be no exception (Gauberg *et al.*, 2017). Considering the morphological and functional differences that are acknowledged to occur along the dorso-ventral axis of fish skin, it follows that spatial differences in the abundance and/ or presence of claudin genes should vary also. Regional comparisons indicated that 12 of the 20 mRNA encoding claudin sequences investigated by Gauberg and colleagues (2017) exhibited differences in abundance along the dorso-ventral axis of rainbow trout skin. This will have implications upon the chosen sampling site of tissue samples in studies attempting to investigate claudins of the skin via gene expression analysis and upon the determination of claudin function and influence by external factors.

Furthermore, observations in pufferfish (*Tetraodon*) skin have strengthened the theory that claudins may be involved in the regulation of epidermal permeability following changes in environmental conditions. The demonstration of co-localisation between claudin 6 and Na<sup>+</sup>-K<sup>+</sup>-ATPase-immunoreactive cells in the skin of green spotted pufferfish (*Dichotomyctere nigroviridis*) was found to be sensitive to changes in environmental salt concentration, further

underlining the importance of the skin and its associated claudins in osmoregulatory control mechanisms (Bui and Kelly, 2014).

Interestingly, Adamek and colleagues (2013) further demonstrated the reduced mRNA expression of genes encoding several important components of the mucosal barrier, in particular Muc5B, beta defensin 1 and 2, and the tight junction proteins claudin 23 and 30 in the skin of common carp infected with herpesvirus-3. This has important implications on the ability of the infected host fish to defend itself against a pathogen as changes in the expression of proteins from the paracellular junctional complex can lead to an opening of the skin mucosa for pathogens, toxins or ions and, affecting the homeostasis of the fish (Adamek *et al.*, 2013). The downregulation of the Muc5B also has important inferences, as a reduction in expression would suggest a reduction in abundance and therefore presence within the mucous, of which mucins play an important role as one of the first lines of humoral innate defence.

### 1.6.3.3 Claudins of the intestine

At least 30 members of the claudin family are reportedly present in the gastrointestinal (GI) tracts of teleost fish, yet only a handful have been examined, with even fewer functional studies completed (Perez-Sanchez *et al.*, 2015). While we understand that the abundance of certain claudins can vary spatially along the GI tract, we know very little about the role these proteins play in the GI tract physiology (Kolosov *et al.*, 2013).

In the intestine of Atlantic salmon, claudin 15 and 25b have been observed as the most prominent tight junction proteins in terms of abundance, however the functions of these proteins throughout different animal groups remain unclear as they vary depending on the system studied (Tipsmark *et al.*, 2010). In humans, claudin 15 is considered to be pore-

forming, yet in Atlantic salmon, claudin 15 mRNA abundance was found to increase in the intestine in response to seawater acclimation (Kolosov *et al.*, 2013; Perez-Sanchez *et al.*, 2015). Claudin 25b exhibits a progressive increase in mRNA abundance along the GI tract of Atlantic salmon also, and due to its sequence similarity with the barrier-forming mammalian Claudin 4, is suggested to be involved in “tightening” the intestinal epithelium (Tipsmark *et al.*, 2010).

Very few investigations have addressed the effects of pathogens on intestinal TJ integrity. Morphological response changes have been reported in fishes including Atlantic salmon and rainbow trout after pathogen exposure (Del-Pozo *et al.*, 2010; Ringo *et al.*, 2004; Ringø *et al.*, 2007). Previously, the effect of pathogen presence upon claudin transcript abundance in the intestine of common carp was investigated: following cyprinid herpesvirus 3 infection, mRNA encoding for claudins 2, 3c, 11, and 23 was significantly elevated in the intestine of common carp, in conjunction with an upregulation of mRNA encoding for genes involved in the inflammatory response (Syakuri *et al.*, 2013). In light of this, it was proposed that alterations in claudin abundance may contribute to mechanisms that compensate for a possible disruption of proteins by nitric oxide produced during the host immune response to virus-induced tissue damage (Kolosov *et al.*, 2013). Future studies concerned with the effect of pathogen presence upon the mucosal barriers and associated tight junction proteins of the epithelia are of vital importance to aid our understanding of the mechanisms at work and the potential impact they have on immunological defences.



## 1.7 Dietary additives

Functional feeds contain both digestible and non-digestible components, and include probiotic and prebiotic additives, nucleotides, vitamins, immunostimulants and algal/plant extracts (Micallef *et al.*, 2017). The FAO and WHO jointly define probiotics as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Morelli and Capurso, 2012). This is the most widely accepted definition of a probiotic across scientific disciplines. More recently, Merrifield *et al.* (2010) proposed a more inclusive and broader definition for a probiotic intended for use in aquaculture: “any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or fish consumer, which is achieved, in part at least, by improving the microbial balance of the fish”. In this context the direct benefits to the host are regarded as, among others, immune-stimulation, improved disease resistance, reduced stress response, and improved morphology of the gastro-intestinal tract and benefits to the farmer and consumer as improved fish appetite, growth performance, feed utilization, improvement to the flesh and carcass quality, and reduced malformations (Merrifield *et al.*, 2010). On the other hand, prebiotics were first described by Gibson and Roberfroid, in (1995) as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improving host health”. This definition remained unchanged for 15 years until the 6<sup>th</sup> Meeting of the International Scientific Association of Probiotic and Prebiotics defined “dietary prebiotics” as “a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health” (Davani-Davari *et al.*, 2019).

Today, the success of the aquaculture industry depends largely upon minimising the impact of diseases in farmed fish. As an alternative to the traditional disease-control treatments such as chemotherapeutants, antibiotics, and vaccines, the use of natural immunomodulants, probiotics, and prebiotics to prevent or control pathogenic organisms constitute one of the most promising techniques of controlling diseases in aquaculture (Cerezuela *et al.*, 2013). These additives not only minimise the risk associated with the overuse of chemical treatments, but their potential to increase resistance to infectious diseases by enhancing non-specific defence mechanisms have also been demonstrated in numerous studies in recent years (Dawood *et al.*, 2018). The additional potential these additives hold to influence growth rates, improve the balance of the intestinal microflora and even digestion and absorption of nutrients in the gut of a variety of fish species has also been documented within the scientific literature (Dawood *et al.*, 2018).

The concept of maintaining fish health through the best possible nutrition is well accepted in modern aquaculture and as a result, most additives are usually included as dietary additives (Cerezuela *et al.*, 2013; Caipang & Lazado, 2015). A large number of substances are under evaluation to assess their suitability and applicability as either an immunomodulant, probiotic or prebiotic. Although research in this area has been ongoing for some years now, we have only recently begun to appreciate the importance of the mucosal tissues in fish and their prominent role as the first line of defence. As a result, few studies have investigated the effect dietary supplements and additives have upon the mucosal tissues, the interactions that take place there, and whether or not they hold potential as manipulators of these sites.

### 1.7.1 Probiotics

Following the research of Tissier (1900) on the importance of bifidobacteria in the gut of infants, Elie Metchnikoff, the Russian Nobel laureate, introduced the concept of probiotics in the early 1900s, postulating a key hypothesis that lactic acid bacteria (LAB) were capable of promoting longevity through their offered health benefits (Caipang & Lazado, 2015). Probiotics are now widely used to promote human well-being and alleviate health issues, yet it is only within the last 15 years that their relevance in animal husbandry, including fish, has generated substantial research applications (Syngai *et al.*, 2016). Following the 2006 EU ban on antimicrobial growth promoters to reduce the use of antibiotics in agri-business productions, new opportunities for the development of probiotic products have arisen (Lauzon *et al.*, 2014). Since then, the administration of probiotics has appeared as a very promising biological control for aquaculture. In order to compete with endogenous microbes in an effort to establish populations within the mucosa, probiotics employ a variety of mechanisms; these include the production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, inhibition of virulence gene expression or the disruption of quorum sensing (Rodiles *et al.*, 2018). Further positive effects of probiotic-host interactions include modulation of both the local and systemic immune response, in addition to enhanced survival, growth, and development (Lauzon *et al.*, 2014). Numerous investigations have evaluated the feasibility of supplementing aquaculture diets with a range of potentially probiotic bacterial species, including *Aeromonas* spp., *Bacillus* spp., *Pseudomonas* spp., *Vibrio* spp., *Saccharomyces* spp., and LAB (Lauzon *et al.*, 2014).

### 1.7.1.1 Lactic Acid Bacteria

LAB are classified within the phylum Firmicutes, class Bacilli, and order Lactobacillales (Ringø *et al.*, 2018). As Gram-positive, often non-motile and non-sporing bacteria that are tolerant to bile and pancreatic enzymes, LAB mainly produce lactic acid as a major product of their fermentative metabolism, in addition to other growth-inhibition substances such as bacteriocins, hydrogen peroxide, and diacyls (Stanier, Doudoroff and Adelberg, 1975; Ringø *et al.*, 2018). LAB fall among the most commonly studied probiotics with the most commonly studied and utilised LAB probionts for applications in fish belong to the *Carnobacterium*, *Enterococcus*/*Streptococcus*, *Pediococcus*, *Lactobacillus*, *Lactococcus* and *Leuconostoc* genera (Merrifield and Carnevali, 2014; Merrifield *et al.*, 2014). Within the last 20 years, an impressive amount of knowledge has been published on LAB in finfish intestine, their potential as probiotics and their effect upon the immune system as reviewed by Merrifield *et al.* (2014), Hoseinifar *et al.* (2016) and Ringø *et al.* (2018). Table 1.4 summarises recent investigations conducted to evaluate the probiotic properties of different genus and species of LAB.

**Table 1.4** An overview of various LAB investigated as a probiotic for aquaculture applications in various aquatic species. Modified from Ringø *et al.*, (2018).

Probiotic	Dose and administration duration	Fish species	Parameters examined	Reference
<i>Lb. plantarum</i>	2 x 10 <sup>7</sup> CFU g <sup>-1</sup> for 72 days	Rainbow trout	Growth performance and immune parameters	(Soltani <i>et al.</i> , 2019)
	10 x 10 <sup>9</sup> CFU/kg for 90 days	European seabass ( <i>Dicentrarchus labrax</i> )	Growth performance and serum biochemical parameters	(Piccolo <i>et al.</i> , 2015)
	10 <sup>8</sup> CFU g <sup>-1</sup> for 60 days	Rainbow trout	Serum biochemical and immune responses	(Kane <i>et al.</i> , 2016)
	1.2 x 10 <sup>6</sup> , 0.9 x 10 <sup>6</sup> and 0.56 x 10 <sup>6</sup> CFU/g for 80 days	Common carp	Growth performance, immune parameters and disease resistance	(Soltani <i>et al.</i> , 2017)
	1.81 x 10 <sup>7</sup> CFU g <sup>-1</sup> for 58 days	Nile tilapia ( <i>Oreochromis niloticus</i> )	Growth performance, haemato-immunological parameters and gut microbiota	(Yamashita <i>et al.</i> , 2017)
	10 <sup>8</sup> CFU g <sup>-1</sup> for 28 days	Nile tilapia	Intestinal microbiota, growth performance and resistance against Cd exposure Growth performance and resistance against waterborne aluminium exposure	(Zhai <i>et al.</i> , 2017) (Yu <i>et al.</i> , 2017)
<i>Lb. plantarum</i> + <i>B. Subtilis</i> + <i>P. aeruginosa</i>	0.5 x 10 <sup>8</sup> CFU g <sup>-1</sup> for 60 days	Rohu ( <i>Labeo rohita</i> )	Immune parameters, antioxidant defences and disease resistance	(Giri <i>et al.</i> , 2014)

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<i>Lb. plantarum</i> + <i>L. lactis</i>	log <sub>10</sub> 7.0 CFU/g for 30 days	Olive flounder	Immune parameters and disease resistance	(Beck <i>et al.</i> , 2015)
<i>Lb. plantarum</i> + LMWSA	10 <sup>8</sup> CFU g <sup>-1</sup> for 60 days	Nile tilapia	Growth performance, immune parameters and disease resistance	(Van Doan <i>et al.</i> , 2016a)
<i>Lb. plantarum</i> + Jerusalem artichoke	10 <sup>8</sup> CFU g <sup>-1</sup> for 12 weeks	Pangasius catfish ( <i>Pangasius bocourti</i> )	Growth performance, immune parameters and disease resistance	(Van Doan <i>et al.</i> , 2016b)
<i>Lb. plantarum</i> + Eryngii mushroom ( <i>Pleurotus eryngii</i> )	10 <sup>8</sup> CFU g <sup>-1</sup> for 90 days	Pangasius catfish	Growth performance, immune parameters and disease resistance	(Van Doan <i>et al.</i> , 2017)
<i>Lb. acidophilus</i>	1.5 x 10 <sup>8</sup> , 3 x 10 <sup>8</sup> and 6 x 10 <sup>8</sup> CFUg <sup>-1</sup> for 70 days	Black swordtail ( <i>Xiphophorus helleri</i> )	Growth performance, mucosal immunity and intestinal microbiota	(Hoseinifar, <i>et al.</i> , 2015)
	1.5 x 10 <sup>8</sup> , 3 x 10 <sup>8</sup> and 6 x 10 <sup>8</sup> CFUg <sup>-1</sup> for 56 days	Prussian carp ( <i>Carassius auratus gibelio</i> )	Skin mucous protein profile and immune parameters, appetite and immune related gene expression	(Hosseini <i>et al.</i> , 2016)
	10 <sup>6</sup> CFU g <sup>-1</sup> for 15 days	Nile tilapia	Immune related gene expression and disease resistance	(Villamil <i>et al.</i> , 2014)
<i>Lb. acidophilus</i> + <i>B. cereus</i> + <i>Clostridium butyricum</i>	1.0 x 10 <sup>9</sup> CFU g <sup>-1</sup> for 60 days	Hybrid grouper ( <i>Epinephelus lanceolatus</i> × <i>Epinephelus fuscoguttatus</i> )	Growth performance, digestive and antioxidant enzyme activities	(He <i>et al.</i> , 2017)
<i>Lb. casei</i>	5 x 10 <sup>6</sup> , 5 x 10 <sup>7</sup> and 5 x 10 <sup>8</sup> CFU g <sup>-1</sup> for 60 days	Shirbot ( <i>Barbus gryprus</i> )	Growth performance and digestive enzyme activity	(Mohammadian <i>et al.</i> , 2017)
	1.0 x 10 <sup>8</sup> cells/g for 28 days	Zebrafish	Reproductive performance and related gene expression	(Qin <i>et al.</i> , 2014)

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<i>Lb. casei</i> + apple cider vinegar	10 <sup>8</sup> CFU g <sup>-1</sup> for 56 days	Common carp	Serum and mucous immune parameters, immune and antioxidant defence related gene expression	(Safari <i>et al.</i> , 2017)
<i>Lb. paracasei</i>	10 <sup>6</sup> CFU g <sup>-1</sup> for 66 days	Rainbow trout	Growth performance and intestinal microbiota	(Lopez <i>et al.</i> , 2015)
<i>Lb. delbrueckii</i>	1 x 10 <sup>5</sup> , 1 x 10 <sup>6</sup> , 1 x 10 <sup>7</sup> and 1 x 10 <sup>8</sup> CFU g <sup>-1</sup>	Common carp	Intestinal immune parameters immune related gene expression, antioxidant defence and disease resistance	(Zhang <i>et al.</i> , 2017)
<i>Lb. delbrueckii</i> spp. <i>bulgaricus</i>	5 x 10 <sup>7</sup> CFU g <sup>-1</sup> for 60 days	Shirbot	Immune parameters and disease resistance	(Mohammadian <i>et al.</i> , 2016)
<i>Lb. rhamnosus</i>	10 <sup>3</sup> , 10 <sup>5</sup> and 10 <sup>6</sup> CFU/g for 63 days	European eel ( <i>Anguilla anguilla</i> )	Sperm quality and quantity, expression of genes related to spermatogenesis	(Vílchez <i>et al.</i> , 2015)
	1 x 10 <sup>2</sup> , 1 x 10 <sup>4</sup> and 1 x 10 <sup>6</sup> cells g <sup>-1</sup> for 56 days	Red sea bream ( <i>Pagrus major</i> )	Plasma and mucous parameters	(Dawood <i>et al.</i> , 2017)
	10 <sup>7</sup> and 10 <sup>8</sup> CFU g <sup>-1</sup> for 56 days	Rainbow trout	Intestinal microbiota and histology, biochemical parameters, and antioxidant defence	(Popovic <i>et al.</i> , 2017)
<i>Lb. rhamnosus</i> + <i>Lb. lactis</i>	10 <sup>6</sup> x cell/g for 56 days	Red sea bream	Immune parameters and antioxidant defence	(Dawood <i>et al.</i> , 2016)
<i>P. acidilactici</i>	10 <sup>6</sup> CFU/g for 10 days	Zebrafish	Expression of genes related to male and sperm quality	(Valcarce <i>et al.</i> , 2015)
	1g kg <sup>-1</sup> for 56 days	Green terror ( <i>Aequidens rivulatus</i> )	Innate immune parameters and resistance to hypoxia stress	(Neissi <i>et al.</i> , 2013)
<i>P. acidilactici</i> + galactooligosaccharide (GOS)	7.57 log CFU g <sup>-1</sup> for 56 days	Rainbow trout	Growth performance, immune parameters and disease resistance	(Hoseinifar <i>et al.</i> , 2015a; 2015b; 2017)

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<i>P. acidilactici</i> + GOS	7.57 log CFU g <sup>-1</sup> for 56 days	Common carp	Immune parameters and related gene expression	(Modanloo <i>et al.</i> , 2017)
<i>P. pentosaceus</i>	6 x 10 <sup>10</sup> , 1,6 x 10 <sup>11</sup> , 1.6 x 10 <sup>12</sup> and 3.2 x 10 <sup>12</sup> cells g <sup>-1</sup> for 56 days	Red sea bream	Skin mucous and serum immune parameters, resistance to low-salinity stress	(Dawood <i>et al.</i> , 2016a)
	2 x 10 <sup>7</sup> , 2 x 10 <sup>8</sup> and 2 x 10 <sup>9</sup> CFU g <sup>-1</sup> for 56 days	Siberian sturgeon ( <i>Acipenser baerii</i> )	Intestinal and body composition	(Moslehi <i>et al.</i> , 2016)
	10 <sup>9</sup> CFU g <sup>-1</sup> for 21 days	Orange-spotted grouper ( <i>Epinephelus coioides</i> )	Growth performance, immune related gene expression and disease resistance	(Huang <i>et al.</i> , 2014)
<i>W. cibaria</i>	1.18 x 10 <sup>7</sup> CFU g <sup>-1</sup> for 45 days	Brazilian native surubins	Growth performance, haemato-immunological parameters and intestinal morphology	(Jesus <i>et al.</i> , 2017)
<i>Lc. Mesenteroides</i> + <i>E. faecalis</i> + <i>Lb. fermentum</i>	10 <sup>5</sup> , 10 <sup>7</sup> and 10 <sup>9</sup> CFU g <sup>-1</sup> for 56 days	Javanese carp ( <i>Puntius gonionotus</i> )	Growth performance, intestinal microbiota and body composition	(Allameh <i>et al.</i> , 2017)
<i>L. lactis</i> WFLU12	10 <sup>9</sup> CFU g <sup>-1</sup> for 56 days	Olive flounder	Growth performance, immune parameters and disease resistance	(Nguyen <i>et al.</i> , 2017)
<i>E. faecium</i>	10 <sup>7</sup> CFU/g for 35 days	Javanese carp	Digestive enzymes activity, intestinal short chain fatty acids, and disease resistance	(Allameh, 2015)
<i>E. gallinarum</i> L-1	10 <sup>6</sup> , 10 <sup>7</sup> and 10 <sup>8</sup> CFU mL <sup>-1</sup> for 28 days	Sea bream, European sea bass, meagre ( <i>Argyrosomus regius</i> ) and red porgy ( <i>Pagrus pagrus</i> )	Immune parameters and peroxidase content	(Román <i>et al.</i> , 2015)
<i>E. casseliflavus</i>	10 <sup>7</sup> , 10 <sup>8</sup> , and 10 <sup>9</sup> CFU g <sup>-1</sup> for 56 days	Rainbow trout	Intestinal microbiota, humoral immune parameters and disease resistance	(Safari <i>et al.</i> , 2017)



### 1.7.1.2 Effect of probiotics on the MALT of teleosts

The interactions that occur between the host and probiotic organisms at the mucosal barriers are complex and only partly described in fish, yet are known to share certain molecules and immune processes with mammals, where the depth of knowledge of this topic has been elucidated to a far greater extent (Rodiles *et al.*, 2018).

Vertebrate hosts are able to recognise pathogen-associated molecular patterns (PAMPs) and microbial-associated molecular patterns (MAMPs) present on the outer surface of foreign microbial cells via the expression of pattern-recognition receptors (PRRs), of which the toll-like receptor (TLR) and nucleotide-binding oligomerization domain-like receptor (NOD) families are the best characterised in fish (Covello *et al.*, 2012; Akhter *et al.*, 2015). Binding of the PAMPs/MAMPs to host PRRs triggers a series of intracellular signalling cascades and molecular pathways, including those of the adaptor molecule MyD88 and the transcription factor NF- $\kappa$ B, leading to the subsequent production of cytokines involved in the inflammatory responses, such as interleukin (IL)-1 $\beta$ , TNF- $\alpha$ , IL-10 and IL-8 (Rodiles *et al.*, 2018). A number of studies have reported the transcriptional modulation of immune-related genes within the GALT of several species of fish following probiotic administration, including elevated expression of IL-1 $\beta$  (Standen *et al.*, 2016), TNF- $\alpha$  (Liu *et al.*, 2013), and IL-8 (Pérez-Sánchez *et al.*, 2011). These observed changes in mRNA expression suggest that probiotics may be assisting the molecular immune repertoire of the intestinal mucosa in preparation for a more effective and anticipated immune response (Caipang & Lazado, 2015).

In contrast, the lower intestinal transcript levels of IL-1 $\beta$ , IL-10, COX-2 and transforming growth factor (TGF)- $\beta$  was reported in the intestinal tissue of European seabass fed a diet supplemented with *Lactobacillus delbrueckii* (Picchiatti *et al.*, 2009). Confounding results

presented by different investigations may be attributable to any number of isolated or interconnected factors such as fish species and age; dosage or inclusion level of the dietary additive; length of supplementation; culture conditions; basal diet formulation and production technique; and the type or species of additive used (Perez-Sanchez *et al.*, 2015; Torrecillas *et al.*, 2015).

Beyond the localised intestinal responses, a number of studies have reported increased systemic or peripheral immune responses. For example, respiratory burst and phagocytic activity have both been demonstrated to increase in response to the administration of probiotics in fish (Rodiles *et al.*, 2018). Phagocytes such as neutrophils and macrophages play an important role in antibacterial defence, killing and ingesting foreign bacteria via the production of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide and hydroxyl radicals of the respiratory burst (Akhter *et al.*, 2015). Dietary additions of a range of probiotics have been reported to increase the phagocytic activity of head kidney leucocytes in several species of fish, including Nile tilapia (Van Doan *et al.*, 2017), olive flounder (Beck *et al.*, 2015) and rainbow trout (Rodriguez-Estrada *et al.*, 2013), while the dietary administration of *Bacillus subtilis* E20 to orange-spotted grouper exhibited significantly increased respiratory burst activity compared to the control treatment (Liu *et al.*, 2012).

Lysozyme is one of the most important bactericidal enzymes of intrinsic immunity that has also exhibited significantly increased serum activity in a variety of species including rainbow trout following 30 days of feeding with *Lactobacillus rhamnosus* (Panigrahi *et al.*, 2004), olive flounder fed diets supplemented with *L. lactis* spp. *lactis* I2 for 5 weeks (Heo *et al.*, 2013), Huanghe common carp (*Cyprinus carpio* Huanghe var.) fed *Lb. Delbrueckii*-supplemented diets for 8 weeks (Zhang *et al.*, 2017) and rainbow trout administered a range of LAB within

the diet for 2 weeks (Balcazar *et al.*, 2007). Dietary additions of *Pediococcus acidilactici* for a 56-day period resulted in significantly increase complement (C3) activity in green terror: the complement cascade is the main component of the innate humoral immune response, which plays an important role in alerting the host immune system to the presence of potential pathogens and their clearance (Neissi *et al.*, 2013).

The influence of probiotic microorganisms on the ultrastructure of the GALT has also been investigated. The uniformity, density and/or length of the microvilli that constitute the apical brush border of the intestine have been reported in several species, including rainbow trout (Merrifield *et al.*, 2010), tilapia (Standen *et al.*, 2015), and zebrafish (Falcinelli *et al.*, 2016). Bactocell® was the first commercially available probiotic mixture to be qualified for use in aquaculture within the EU (Caipang & Lazado, 2015). Several studies have reported on the ability of Bactocell® to influence the cellular components of the GALT. In both tilapia and Atlantic salmon, the number of intestinal intraepithelial leukocytes increased significantly following Bactocell® feeding, while substantial increases in goblet cell abundance, and morphological structural changes to the gut in terms of villi height and fold length were also observed (Merrifield *et al.*, 2010; Abid *et al.*, 2013; Standen *et al.*, 2013).

Few studies have explored the influence of probiotics on the SALT of fish, especially with regard to describing the morphological alterations or transcriptional alterations that occur following probiotic feeding (Caipang & Lazado, 2015). However, Lazado & Caipang (2014) were able to show that probiotics are capable of modulating the transcription of immune-related genes and protecting the epidermal skin cells during pathogenic infection *in vitro* in Atlantic cod (*Gadus morhua*). To date, there have been numerous demonstrations of probiotics protecting the surfaces of fish against a range of bacterial pathogens, including

*Aeromonas bestiarum* and *I. multifiliis* (Pieters *et al.*, 2008), *Edwardsiella tarda* (Das *et al.*, 2013) *A. salmonicida* (Rodriguez-Estrada *et al.*, 2013), *A. hydrophila* (Mohammadian *et al.*, 2016), *Streptococcus iniae* (Beck *et al.*, 2015) and *V. anguillarum* (Lee *et al.*, 2017).

Concerning the GIALT, it is widely hypothesised that probiotics may act through either direct or indirect mechanisms when administered as dietary additives. As with aqueous applied probiotics, if dietary probiotics become diffused and suspended within the rearing water, it is not inconceivable that the gills may be exposed to the probiotic in this manner as they are in constant contact with the surrounding environment and could eventually provoke an immune response. However, an indirect mechanism is also plausible. When probiotics are ingested through the diet and illicit immune responses from the GALT, the immune-related signals from this response could then be transmitted to the GIALT via the blood, prompting an immune response in the gills also (Caipang & Lazado, 2015). To date, few studies have been conducted into the effects of probiotic dietary supplementation on the GIALT, but it is undoubtedly an area of research worthy of further attention.

Despite the wealth of research publications demonstrating the benefits of probiotic applications to fin fish in aquaculture, Rodiles and colleagues point out that there are “an equal, or greater number of studies” that did not observe any effect, either positive or negative, from probiotic administration, and further highlight the difficulties of obtaining reproducible outcomes from investigations (Rodiles *et al.*, 2018).

### 1.7.2 Parabiotics

Despite a plethora of scientific evidence advocating the beneficial effects of administering certain probiotic microbes within the diets of aquatic animals, concerns have been raised pertaining to the practical applicability and functionality of releasing live micro-organisms into

the natural environment during commercial aquaculture practices (Piqué *et al.*, 2019). Some important concerns include the viability of probiotic species in product/feed, their different colonization patterns and persistence in the gut, and the potential for virulent gene acquisition from pathogenic bacteria via horizontal gene transfer (Choudhury and Kamilya, 2019). The majority of scientific reports define probiotics according to the definition proposed by the FAO and the WHO in 2002, stating that a probiotic is “a live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Lauzon *et al.*, 2014). This definition specifies that a probiotic must be “live”, a stipulation supported by an extensive number of studies that suggest probiotic microorganisms must be viable in order to confer health benefits (Taverniti and Guglielmetti, 2011). However, recent research indicating that non-viable microbes can be equally beneficial to the host in a manner similar to their viable counterparts, have prompted the inception of “Parabiotics”. Taverniti and Guglielmetti (2011) introduced the concept of parabiotics, defining them as “non-viable microbial cells (intact or broken) or crude cell extracts (i.e. with complex chemical composition), which, when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal consumer”. “Postbiotics” are very similar to parabiotics and were proposed at a similar time, yet their definition is broader: “non-viable bacterial products or metabolic by-products from probiotic microorganisms that have biological activity in the host” (Patel and Denning, 2013).

Parabiotics are obtained via the inactivation of viable probiotic microorganisms through various methods such as, ionizing radiation, inactivation by ultraviolet rays, heat-inactivation, high pressure techniques and sonication (de Almada *et al.*, 2016). Each method has a different mode of action for inactivation, however it is important that the parabiotic retain the beneficial attributes of the probiotic organism following inactivation.

Among the numerous inactivation techniques, heat-inactivation is the most commonly used method for parabiotic preparation destined for aquaculture practices (Choudhury and Kamilya, 2019). Involving the application of heat for an extended period of time, the elevated temperatures to which the microorganisms are subjected can greatly affect different components of the cell structure of which disruption of membrane integrity, loss of nutrients and ions, ribosome aggregation, DNA filament rupture, inactivation of essential enzymes and coagulation of proteins are of particular relevance (Gould, 1989). The effectiveness of heat inactivation may be influenced by many factors including the type of microorganisms, whether it is in a vegetative or spore form, its stage of growth, the growth medium and its pH, and the mode of heating (Choudhury and Kamilya, 2019).

As inactivated microorganisms, the extent to which parabiotics are able to interact with the natural environment and wild organisms is greatly reduced and aquatic animal diets supplemented with parabiotics have, so far, exhibited several positive results regarding growth performance, immunity and disease resistance of several species, as described in Table 1.5. It can therefore be reasonably hypothesized that inactivated bacteria may constitute an effective alternative to live bacterial supplementation in aquatic animal diets. Although the potential application of para- and pro-biotics in higher vertebrate models has been relatively well studied and reviewed (de Almada *et al.*, 2016), the concept of their applicability within the aquaculture sector is only beginning to gain momentum; further research and more rigorous investigations are warranted.

### *1.7.2.1 Effect of parabiotics on the MALT of teleosts*

Early studies in numerous aquatic species have demonstrated that the oral administration of heat-inactivated bacteria may confer advantages upon the host (Díaz-Rosales *et al.*, 2006).

**Table 1.5** Biological modifications in response to heat-inactivated parabiotics administered to various aquatic species. Adapted from Choudhury and Kamilya (2019).

Test organism	Fish species	Mode of assay	Principle observations	Reference
<i>Lactococcus lactis</i> spp. <i>lactis</i>	Turbot ( <i>Scophthalmus maximus</i> )	<i>In vivo</i> and <i>in vitro</i>	<ul style="list-style-type: none"> <li>↑ <i>In vitro</i> and <i>in vivo</i> macrophage chemiluminescent response</li> <li>↑ <i>In vitro</i> and <i>in vivo</i> nitric oxide production</li> <li>↑ <i>In vitro</i> intestinal colonisation</li> <li>↓ <i>In vivo</i> macrophage phagocytosis</li> <li>↓ <i>In vivo</i> lysozyme activity</li> <li>↓ <i>In vivo</i> bactericidal activity</li> </ul>	(Villamil <i>et al.</i> , 2002)
<i>Lactobacillus rhamnosus</i> JCM 1136	Rainbow trout	<i>In vivo</i>	<ul style="list-style-type: none"> <li>↑ Phagocytic activity of head kidney leucocytes</li> <li>↑ Complement activity</li> <li>↑ Plasma immunoglobulin activity</li> <li>↑ Blood triglycerides</li> <li>↑ Alkaline phosphatase activity</li> <li>↑ Plasma protein compared</li> <li>↑ Expression of immune genes (TGF-β, TNF-α, INF-γ, and Ig genes)</li> </ul>	(Panigrahi <i>et al.</i> , 2005 Panigrahi <i>et al.</i> , 2010) (Panigrahi, Viswanath and Satoh., 2011)
<i>L. delbrueckii</i> ssp. <i>Lactis</i> and <i>Bacillus subtilis</i>	Gilthead sea bream	<i>In vitro</i> and <i>in vivo</i>	<ul style="list-style-type: none"> <li>↑ Leucocyte peroxidase content</li> <li>↑ Respiratory burst activity</li> <li>↑ Head kindey leucocyte phagocytosis</li> <li>↑ Cytotoxicity</li> <li>↑ Natural complement</li> <li>↑ Serum peroxidase</li> </ul>	(Salinas <i>et al.</i> , 2006; 2008b)

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			<ul style="list-style-type: none"> <li>↑ Total serum IgM</li> <li>↑ Number of gut IgM+ cells</li> <li>↑ Acidophilic granulocytes in the gut</li> </ul>	
<i>Clostridium butyrium</i> CB2	Chinese drum ( <i>Miichthys miiuy</i> )	<i>In vivo</i>	<ul style="list-style-type: none"> <li>↑ Phagocytic activity of head kidney macrophages</li> <li>↑ Lysozyme activity of serum and intestinal mucosa</li> <li>↑ Ig level</li> <li>↑ Survival of <i>V. anguillarum</i> or <i>A. hydrophila</i> challenged fish</li> </ul>	(Pan <i>et al.</i> , 2008)
<i>Pseudomonas</i> spp. (GP21) and <i>Psychrobacter</i> spp. (GP12)	Atlantic cod ( <i>Gadus morhua</i> )	<i>In vitro</i>	<ul style="list-style-type: none"> <li>↑ Transcription level of bacterial defense genes</li> <li>Diverse expression (with time and strains) of cell-mediated immunity genes</li> <li>↑ Pro-inflammatory cytokines expression: IL-1 and IL-8 when fed with killed GP21</li> <li>↔ Antioxidant defense genes: catalase and glutathione peroxidase</li> </ul>	(Lazado <i>et al.</i> , 2010)
<i>Psychrobacter</i> spp. (strain GP11), <i>Schwanella</i> spp. (GS11), <i>Photobacterium</i> spp. (GP31) and <i>Vibrio</i> spp. (GV11)	Atlantic cod	<i>In vitro</i>	<ul style="list-style-type: none"> <li>↑ Bactericidal/permeability increasing protein/lipopolysaccharide-binding protein</li> <li>g-type lysozyme expression is strain dependant</li> <li>↑ Expression of IL-1<math>\beta</math> but not IL-10</li> </ul>	(Caipang, Brinchmann and Kiron., 2010)
<i>L. paracasei</i> spp. <i>paracasei</i> (strain 06TCa22) and <i>L. plantarum</i> (strain 06cc2)	Japanese puffer	<i>In vitro</i> and <i>in vivo</i>	<ul style="list-style-type: none"> <li>↑ Expression of pro-inflammatory cytokines (IL-1<math>\beta</math>, IL6, IL17A/F3, TNF<math>\alpha</math> and TNF-N)</li> <li>↑ Expression of cell-mediated immune regulators IL12-p35, IL12-p40, and IL-18</li> <li>↑ Expression of antiviral cytokine (I-IFN-I and IFN-g)</li> </ul>	(Biswas <i>et al.</i> , 2013a; 2013b)



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			<p>↑ Expression of other regulatory cytokines (IL-2, IL-7, IL-15, IL21, IL-10 and TGF-b1)</p> <p>↑ Superoxide anion production, phagocytic activity and pathogen resistance (<i>in vivo</i>)</p>	
<i>Psychrobacter</i> spp. SE6	Orange-spotted grouper	<i>In vitro</i>	<p>↑ Expression of TLR2, but not MyD88 and cytokines (IL-1β, IL-8 and TGF-β)</p> <p>↑ Expression of antibacterial epinecidin-1 and IgM</p>	(Sun <i>et al.</i> , 2014)
<i>L. plantarum</i>	Red seabream	<i>In vivo</i>	<p>↑ Feed intake</p> <p>↑ Feed efficiency ratio</p> <p>↑ Protein retention</p> <p>↑ Apparent digestibility coefficient</p> <p>↑ Growth performance</p> <p>↑ Total plasma and serum protein, glucose</p> <p>↑ Serum bactericidal activity</p> <p>↑ Serum alternative complement pathway</p> <p>↑ Serum lysozyme activity</p> <p>↑ Mucous secretion</p> <p>↑ Tolerance against low salinity stress</p>	(Dawood <i>et al.</i> , 2015a; 2015b)
<i>L. plantarum</i>	Giant river prawn ( <i>Macrobrachium rosenberii</i> )	<i>In vitro</i>	<p>↑ Weight gain</p> <p>↑ Specific Growth Ratio</p> <p>↔ Feed conversion efficiency, FCR, PER</p> <p>↑ Total haemocyte count</p> <p>↑ Respiratory burst activity</p>	(Dash <i>et al.</i> , 2015)
<i>B. pumilus</i> SES	Orange-spotted grouper	<i>In vivo</i>	<p>↑ Final weight, weigh gain, specific growth ratio, feed conversion ratio</p> <p>↑ Phagocytic activity of serum leucocytes</p> <p>↑ Serum complement C3</p> <p>↑ IgM levels</p>	(Yan <i>et al.</i> , 2016)

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			↑ SOD ↑ Expression of TLR2, IL-8 and IL-1β	
<i>Pseudomonas aeruginosa</i> VSG2	Rohu	<i>In vitro</i>	↑ Expression of IL-1β, TNF-α, COX-2, NF-kB, IFN-a, IFN-γ, IL12P35, IL-12p40, IL-10 ↑ Phagocytic activity of head kidney macrophages ↑ Superoxide anion production ↑ Lysozyme activity	(Giri <i>et al.</i> , 2016)
<i>B. subtilis</i> , <i>L. lactis</i> and <i>S. cerevisiae</i>	Rohu	<i>In vivo</i>	↔ Growth, Protein efficiency ratio, nutrient retention, digestibility and FCR ↔ Gut colonization	(Mohapatra <i>et al.</i> , 2012)

Growth performance has been demonstrably improved in several species, including rainbow trout fed heat-inactivated *Gordonia bronchialis* (Shabanzadeh *et al.*, 2016), Amberjack fed heat-killed *L. plantarum* (Dawood *et al.*, 2015a), and red seabream fed heat-inactivated *Lactobacillus plantarum* (Dawood *et al.*, 2015b) and *Pediococcus pentosaceus* (Dawood *et al.*, 2016).

Enhanced disease resistance has been documented in several species challenged with various pathogens, including Japanese pufferfish fed *L. paracasei* spp. *paracasei* (strain 06TCa22) and subsequently challenged with *Vibrio harveyi* (Biswas *et al.*, 2013b), and Rainbow trout fed inactivated *E. faecalis* and thereafter challenged with *A. salmonicida* (Rodriguez-Estrada *et al.*, 2013).

Numerous studies have also reported positive stimulation of the innate immune system, including increase phagocytic, complement and plasma immunoglobulin activity, in addition to increased expression of several immune-related genes such as TGF- $\beta$ , TNF- $\alpha$ , IFN- $\gamma$  and immunoglobulin genes in rainbow trout (Panigrahi *et al.*, 2005; Panigrahi, Viswanath and Satoh, 2011).

More recently in orange-spotted grouper, Yan *et al* (2016) reported that phagocytic activity, serum complement C3 and IgM levels as well as SOD activity elevated significantly in fish fed heat-inactivated *B. pumilus* SE5 for 60 days, compared to fish fed the control regime. The same study also reported significant upregulation of the pro-inflammatory cytokines IL-1 $\beta$  and IL-8, and TLR2 in the head kidney of fish fed heat-inactivated bacteria, leading the authors to propose heat-inactivated *B. pumilus* as an effective stimulant of immune responses in grouper (Yan *et al.*, 2016). A notable *in vitro* study in which the head-kidney macrophages of Rohu were incubated with heat-killed whole-cell products (HKWCPs) of the probiotic

*Pseudomonas aeruginosa* VSG2 strain, investigated the response of various cytokine genes via quantitative real-time PCR. Among pro-inflammatory cytokines, significantly higher expression of IL-1 $\beta$  and TNF- $\alpha$  was observed, in addition to COX-2 and NF- $\kappa$ B, the anti-viral cytokines IFN- $\alpha$  and IFN- $\gamma$ , the cell-mediated immune factor genes IL-12p35 and IL-12p40, and finally the anti-inflammatory cytokine IL-10 (Giri *et al.*, 2016). Thus, the authors draw attention to the *in vitro* stimulatory capability of HKWCPs of the probiotic *P. aeruginosa* VSG2 and advocate its applicability as a vaccine adjuvant in aquaculture (Giri *et al.*, 2016).

### 1.7.3 Prebiotics

Gibson & Roberfroid (1995) first introduced the prebiotic concept: a non-digestible food ingredient that selectively stimulates the growth and/or metabolism of the beneficial bacteria community in the gut, thus improving host wellbeing and health (Merrifield *et al.*, 2010; Caipang & Lazado, 2015). Unlike probiotics, prebiotics are not “micro-organisms”, although some may be micro-organism derived, and therefore have little-to-no influence upon the environment, meaning particular precautions and authorization for their use as feed additives are not required (Caipang & Lazado, 2015).

$\beta$ -glucans (specifically  $\beta$ -1,3/1,6-glucans) are polysaccharide chains of mono-carbohydrates bound by glycosidic bonds with a wide range of molecular structural diversity, while mannan-oligosaccharides (MOS) are glucomannoprotein complexes (Song *et al.*, 2014). Both are prebiotic compounds derived from the outer cell wall of the yeast *Saccharomyces cerevisiae* and fall among the best-studied and most-applied prebiotics used in aquaculture (Petit and Wiegertjes, 2016; Jami *et al.*, 2019). Due to their chemical structure and the subsequent inability of the host to digest them, these “functional saccharides” remain intact throughout the upper part of the digestive tract of fish until their arrival in the distal intestine (Carbone

and Faggio, 2016). At this point, commensal bacteria are able to utilise the prebiotics as sources of energy, metabolising them to produce a variety of short-chain fatty acids: the consequent reduction in pH improves digestibility, nutrient and mineral absorption and eventually the growth of the fish (Lauzon *et al.*, 2014; Najdegerami, Tokmachi and Bakhshi, 2017).

The application of prebiotics to human subjects and terrestrial livestock have been studied extensively (Gioia and Biavati 2017, Markowiak and Ślizewska 2017). While information concerning the pertinence and suitability of their inclusion into the diets of aquatic animals is rapidly accumulating, the first reported investigative study into the potential applicability of prebiotics in fish is widely accredited as being that of Hanley *et al* (1995). However, results from investigations often report contradictory results. These disparities may arise from any number of isolated or interconnected factors and may be explained, in part, due to the structural differences of the prebiotic used, the supplemented dose or inclusion level of the product, the formulation and production technique of the basal diet, the length of administration, or the age, species and culture conditions of the fish (Torrecillas *et al.*, 2014; Perez-Sanchez *et al.*, 2015).

#### *1.7.3.1 Effect of prebiotics on the MALT of teleosts*

To date, dietary supplementations of MOS and  $\beta$ -glucans have been documented as having numerous beneficial influences upon fish hosts. Several studies have reported the increased growth rate of several species of fish fed MOS and  $\beta$ -glucan-supplemented diets, including Nile tilapia (Pilarski *et al.*, 2017), rainbow trout (Ji *et al.*, 2017), Koi carp (*Cyprinus carpio koi*) (Lin *et al.*, 2011) and pompano fish (*Trachinotus ovatus Linnaeus*) (Do Huu *et al.*, 2016). The mechanism by which prebiotics promote the growth of aquatic animals is not clear; however,

there are two separate hypotheses concerning their function in aquaculture. The first proposes that the production of glucose enzymes by the fish would serve to break-down the prebiotic, generating energy which can subsequently be used for protein synthesis, thus promoting growth (López *et al.*, 2003). The second argues that the enhanced growth of fish fed  $\beta$ -glucan supplemented diets is a result of improved intestinal immune response and therefore increased disease resistance to pathogens, that would otherwise result in a decreased weight and possible disease, and therefore promoting the growth of the fish (Dalmo and Bøggwald, 2008). However, in other experiments, no significant growth increases have been observed following dietary prebiotic inclusions (Bagni *et al.*, 2005; Whittington *et al.*, 2005; Welker *et al.*, 2007).

Enrichment of intestinal morphology is one of the significant influences of dietary prebiotics on the GALT in a variety of species. Increased microvilli density and height, increased fold length, and increases in the frequency of mucous-secreting cells have been reported in a number of species following dietary supplementation with prebiotics, including Atlantic salmon (Dimitroglou *et al.*, 2011), juvenile hybrid striped bass (*Morone chrysops X Morone saxatilis*) (Anguiano *et al.*, 2017), rainbow trout (Khodadadi *et al.*, 2019), Nile tilapia (Selim and Reda, 2015), and channel catfish (Zhu, 2012). Regulation of the fish's gastrointestinal microflora has also been observed as a result of prebiotic administration (Dimitroglou *et al.*, 2009; Torrecillas *et al.*, 2012), in addition to improved nutrient digestion and absorption (Burr *et al.* 2008, Gültepe *et al.*, 2011) and increased activity of digestive enzymes (Anguiano *et al.*, 2013). Yet, perhaps one of the most favourable and beneficial advantages of prebiotic inclusion into aquatic animal feeds is their ability to modulate the immune status of the host, via both direct and indirect methods (Caipang and Lazado, 2015).

It has been well established that the production of useful by-products following the fermentation of prebiotics stimulates both the commensal and symbiotic bacteria, leading to their proliferation within the microflora of the GI tract (Song *et al.*, 2014, Dawood *et al.*, 2018). This indirectly enhances pathogen resistance by promoting the competitive exclusion of pathogens (Lauzon *et al.*, 2014). Similarly MOS, in particular, is capable of reducing the frequency of adhesion and the subsequent invasion of pathogenic organisms by competing for the same glycol-conjugates found on the surface of intestinal epithelial cells (Akhter *et al.*, 2015). As a result of the mannose sugar within its structure, MOS acts as a high affinity ligand, offering a competitive binding site for bacteria and therefore serving as a blocking agent to intestinal cells by interfering with the colonization of pathogens (Caipang and Lazado, 2015).

Certain prebiotics are also capable of acting in a more direct immunomodulatory capacity within the host. Glucans are able to bind to a c-type lectin receptor expressed on the surface of host macrophages, neutrophils and natural killer cells: dectin-1 (Song *et al.*, 2014, Wang *et al.*, 2017). Once bound, the  $\beta$ -glucan/dectin-1 complex activates macrophages in combination with TLR2 and its signalling pathway, causing a pro-inflammatory response, beginning with the expression of TNF $\alpha$  (Song *et al.*, 2014). MOSs are also capable of triggering potent innate immune responses from the host fish. Macrophages and endothelial cell subsets contain an endocytic mannose receptor (MR) which is capable of recognising both microbial glycans and self-glycoproteins; in the presence of MOS, intracellular signalling cascades may be induced in dendritic cells by mannose-containing ligands through the MR, leading to the stimulated production of pro-inflammatory cytokines (Caipang and Lazado, 2015).

Often referred to as immunosaccharides or immunostimulants, MOS and  $\beta$ -glucans have been documented as having a measurable impact upon several innate immune parameters in

numerous species, including: alternative complement activity; lysozyme activity; natural haemagglutination activity; respiratory burst; superoxide dismutase activity; and phagocytic activity (Covello *et al.*, 2012). The role and potential influence of  $\beta$ -glucans and MOS on immune-related gene and protein expression in numerous fish species has been presented by many authors (for review see Wang *et al.*, 2017). As a result, prebiotics are now considered to be a valid and alternative method for the prevention and control of various diseases in aquaculture (Carbone and Faggio, 2016).

A notable study by Cerezuela *et al.* (2013a) is one of only a few studies discussing the negative impacts of prebiotics/synbiotic feeding on the intestinal morphology of fish. Different signs of oedema and inflammation were observed in gilthead sea bream fed with an inulin-supplemented diet, in addition to a reduction in the number of goblet cells. A similar but earlier study by the same research group also observed that an increase in immune parameters following dietary administration of the prebiotic did not correlate with disease resistance (Cerezuela *et al.*, 2012).

To date, there are only a handful of studies in fish documenting the effects of prebiotic supplementation on the SALT. A study by Rodrigues-Estrada and colleagues (2008) administered 0.4% MOS-product in the diets of rainbow trout and found a remarkable increase in the skin mucous weight after 12 weeks of feeding. In European sea bass however, these results were not reproduced even though the same dosage of the same prebiotic was administered (Torrecillas *et al.*, 2011). Taken together, contradictory results such as these indicate that prebiotic property should not be extrapolated and assumed between species and highlights the importance of primarily demonstrating actual effect, before claiming its potential use without reference from another fish host (Caipang & Lazado, 2015).



To the author's knowledge, there have been no investigations completed to date concerning the effect of dietary prebiotics upon the GIALT. The possibility that dietary supplemented prebiotics come into contact with the gills is almost nil, yet as with probiotic applications, there remains the possibility that the GIALT may be affected by prebiotic feeding through an indirect mechanism of immunostimulation (Caipang & Lazado, 2015). Further research is warranted.

#### *1.7.4 Synbiotics*

Synbiotics are nutritional supplements that combine a mixture of probiotics and prebiotics in a form of synergism that work together and beneficially affect the host by improving the survival and implantation of live microbial dietary additives in the GI tract (Cerezuela et al., 2011; Ringø & Song, 2016). While synbiotics have become rather broadly used in both human and veterinary medicine, research into the potential use of synbiotics for aquaculture species was non-existent prior to 2009 and remains scarce today (Ringo and Song, 2016). Following the first published study on synbiotics in finfish by Rodriguez *et al.*, (2009), several studies have emerged focusing on a wide range of parameters, from growth performance, feed utilization, and body composition, to immunological responses, disease resistance, and modulation of the GI microbiota (Cerezuela *et al.*, 2011; De *et al.*, 2014; Ringø *et al.*, 2014). A notable study by Abid *et al* (2013) fed a mixture of short-chain fructooligosaccharides (FOS) and *P. acidilactici* to Atlantic salmon, and reported encouraging results in fish exposed to the synbiotic compared to the control fish: microbial community diversity and richness were significantly higher, mucosal fold (villi) and infiltration of epithelial leucocytes were significantly greater, and the expression of pro-inflammatory cytokines IL-1 $\beta$ , IL-8 and TNF $\alpha$  were significantly upregulated by synbiotic administration. This observed increased in the

expression of pro-inflammatory cytokines indicates that the innate immune system of the fish is activated, contributing to the postulation that synbiotics offer an alternative strategy with innumerable advantages that may overcome the limitations and side effects of antibiotics and other drugs in aquaculture (Cerezuela *et al.*, 2011; RingØ & Song, 2016).

### 1.8 Conclusions and future work

The historic global mismanagement of wild fisheries has led to significantly declined fish stocks (FAO, 2002 and 2018). Coupled with the ever-increasing worldwide human population (UN, 2017), the growth and expansion of the aquaculture industry has been necessary in order to meet the growing global demand for seafood. However, the industry must strive to become more economically viable and environmentally sustainable as finite dietary ingredients and disease represent the two biggest constraints to the industry's continued expansion (FAO, 2014).

Conventional methods of disease prevention and treatment, such as antibiotics and vaccines, are no longer acceptable due to the serious implications they have on environmental and human health. Since the ban preventing the extensive use of antibiotics within the EU in 2003, the search for alternative strategies for disease control has grown into a large and fast-paced area of research (RingØ and Song, 2016). One of the most promising alternatives is the use of dietary additives, such as probiotics, prebiotics and synbiotics: the potential benefits of these products have been documented across a range of aquacultured species, and in some regions, the use of these products is becoming commonplace (Lauzon *et al.*, 2014; Hoseinifar *et al.*, 2019). However, this remains a relatively new field of research and limited knowledge is available concerning the precise mechanisms governing the interactions between feed additives and host health.

In recent years, there has been increasing literature advocating the importance of the mucosal-associated lymphoid tissues in maintaining host health and as a first line of defence against disease and pathogenic insults (Foey and Picchiatti 2014, Salinas 2015). A growing body of research is accumulating concerning the potential influence of feed additives upon the gut-associated lymphoid tissue and their associated microbiota. However, very little attention has been paid to the other associated-lymphoid tissues, such as those found at the skin and gills, despite the important implications of their close proximity to the external environment and as portals of entry for several pathogens. Knowledge concerning the extent of inter-connectivity between the mucosal-associated lymphoid tissues is limited at best, while our understanding concerning the potential of feed additives to modulate the immune system and influence the mucosal barrier defences at these sites remains scarce. Research in this area is vital to determine the true potential of incorporating feed additives into the diets of aquacultured species and will be instrumental to combating the disease within the industry.

### 1.9 Thesis aims and objectives

The aim of the research presented here is to assess the effects of various dietary additives on the health of salmonids through the implementation of a series of in vivo feeding trials.

**Objective one.** Determine whether the investigated feed additives significantly affect the growth of fish over the course of a feeding trial, in comparison to fish fed basal control diets.

**Objective two.** Determine whether the feed additives affect the mucosal barrier defences of the mucosal-associated lymphoid tissues, either directly by influencing epidermal mucous quantity and composition, or indirectly by influencing the morphology and production of mucous-producing cells.

**Objective three.** Determine whether feed additives effect the innate immune responses of the fish at the mucosal-associated lymphoid tissues by modulating the expression of a range of target genes of interest.

**Objective four.** Determine whether the dietary additives effect the susceptibility of fish to sea lice (*Lepeophtheirus* and *Caligus spp.*) under controlled pathogen challenge conditions and *in situ* sea cage aquaculture.

## **General Materials and methodologies**

### 2.1 Licences and consumables

All experimental analyses were carried out with the following protocols unless otherwise stated. Chemicals and reagents were sourced from Fisher Scientific, BioRad, Sigma Aldrich, MP Biomedicals, Qiagen or Life Technologies, unless otherwise indicated. All experiments were conducted under Institution Home Office licences as stated for each experimental trial.

### 2.2 Experimental trials

Three experimental trials were conducted on salmonids at three different institutions, the specifics of which are described in the following experimental chapters. The first trial was conducted on rainbow trout at the Aquaculture and Fish Nutrition Research Aquarium at the University of Plymouth. The second trial was performed on Atlantic salmon at the Marine Environmental Research Laboratory of the University of Stirling, located in Machrihanish, Scotland. The third and final trial was conducted on Atlantic salmon at the sea-cage facilities of Marine Harvest, based in Loch Ailhort on the Ardnish peninsular, Scotland.

### 2.3 Samples and fish dissections

For all trials, fish were euthanised by an overdose (200mg/L water for 5 minutes) of MS222 (Pharmaq, UK) followed by destruction of the brain. It should be noted that for practical and logistical reasons, and due to the different objectives of each experimental trial, not all samples were collected from the same fish, while the types of samples taken and the sampling time points differed between the experiments. Where required, samples were collected as follows.

- **Morphometric measurements:** The body weight (BW) and fork length (FL) of each individual fish was recorded (following sea-lice assessment if necessary).
- **Skin mucous collection:** Skin mucous was collected from the left flank, preserved from any unnecessary handling disturbance and following the removal of sea-lice where necessary. A spatula was wiped from the edge of the operculum to the anal pore. The accumulated mucous at the tail-end of the fish was transferred into a 1mL pre-weighed syringe, quantified ( $\pm 10\text{mg}$ ), and snap frozen at  $-80^{\circ}\text{C}$  until further analysis.
- **Gene Expression:** ca. 200mg of gill tissue from the 2nd gill arch was collected for gene expression, in addition to a standard  $1\text{cm}^2$  area of skin from the right flank, and a 5mm transversal segment of the posterior intestine. All tissues were immediately stored in RNAlater for 24 hours at  $4^{\circ}\text{C}$  and thereafter stored at  $-80^{\circ}\text{C}$  until further analysis.
- **Histological Examination:** the entire 3rd gill arch was excised, along with approximately  $1\text{cm}^2$  sample of skin from the right flank, dorsolaterally adjacent to the dorsal fin, and a 5mm segment of the posterior intestine, and stored in 10% Natural Buffered Formalin for 1 week before being subsequently transferred to 70% ethanol for storage until processing.

#### 2.4 Epidermal mucous analysis

Epidermal mucous secretions were collected from fish as described above in section 2.2 and immediately quantified.

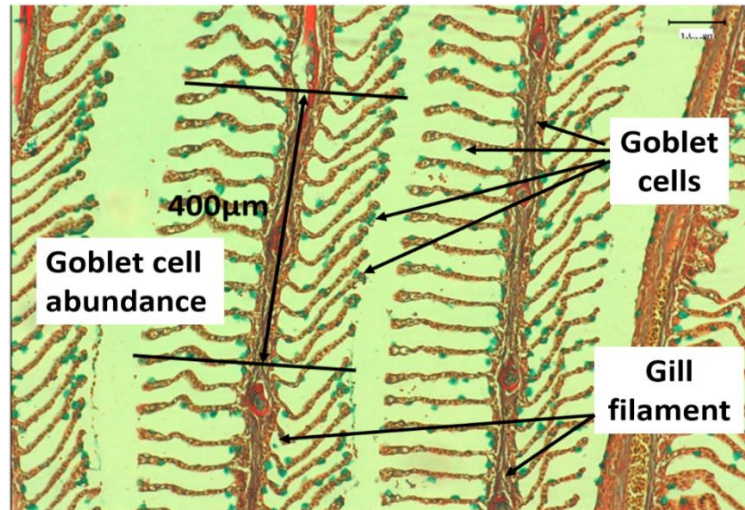
#### 2.5 Histological examinations

Following storage in 70% ethanol, samples were first dehydrated in a graded ethanol series using a Leica TP 1020 tissue processor, and then embedded in paraffin wax according to

standard histological techniques (Suvarna *et al.*, 2019). Ultrathin sections of 4µm thickness were cut on a Leica RM2235 microtome, dried in an incubator overnight and thereafter stained manually with Alcian Blue – van Gieson (AB-vG) to ensure visible contrast between mucin cells and the surrounding tissue. Briefly, slides were “taken to water” via sequential submersion in Histolene, 100% Industrial Methylated Spirit (IMS) and distilled water, and then left in 0.5% Celestine Blue for 5 minutes. Following rinsing in tap water, the slides were placed in Harris’s Haematoxylin for approximately 10 minutes and then rinsed in tap water for a further 5 minutes. After leaving to “blue” in Lithium Carbonate for 5 minutes, the slides were again washed in tap water and then submerged in Alcian blue for 10 minutes. Following a final rinse in tap water, the slides were placed in Curtis van Gieson for 3 minutes before dehydrating rapidly in 100% IMS. Slides were immediately mounted with cover slips using DPX and left to dry. Micrographs of stained samples were captured on a Leica DMD 108 digital microscope and the images analysed using Image J 1.47v or Fiji v5.2 (National Institutes of Health, Bethesda, Maryland, USA) software.

### *2.5.1 Gill measurements*

Images were captured at x40 magnification. Goblet cell abundance was measured across 400µm within the middle range of six single lamellae of 2 fish as demonstrated in Plate 2.1.



**Plate 2.1** Example image to illustrate method of measuring goblet cell abundance in the gill of Atlantic salmon gill tissue. AB/vG stained. Scale bar represents 100µm.

### 2.5.2 Intestinal measurements

For intestinal samples, images were captured at x40 magnification and used to measure lamina propria width, mucosal fold length, goblet cell abundance, and goblet cell area fraction. Mucosal fold lengths were determined from four complete mucosal folds per fish. From the same folds, lamina propria widths were averaged from three points per fold (bottom, middle and top of the fold), while goblet cell abundance was measured across a distance of 200µm from the apex of five mucosal folds per fish as demonstrated in Plate 2.2a. To calculate the goblet cell area fraction, scripts were written for use in the Fiji software programme to determine the total area covered by intestinal tissue within each of 5 images per fish as shown in Plate 2.2b and the total area covered by goblet cells, as shown in Plate 2.2c. The goblet cell area fraction was calculated as follows:

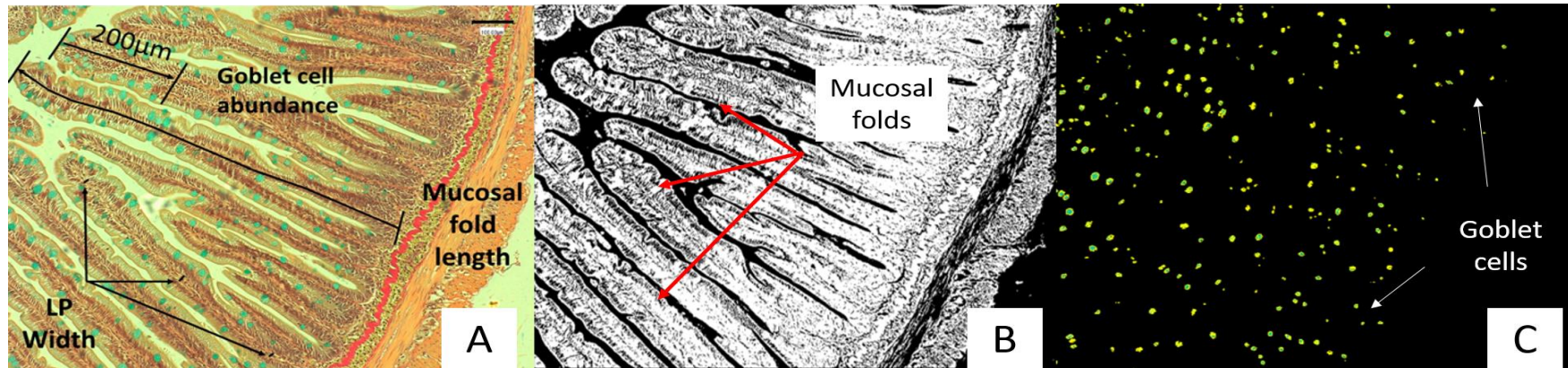
$$\text{Goblet cell area fraction (\%)} = (\text{area of goblet cells} \div \text{area of intestinal tissue}) \times 100$$



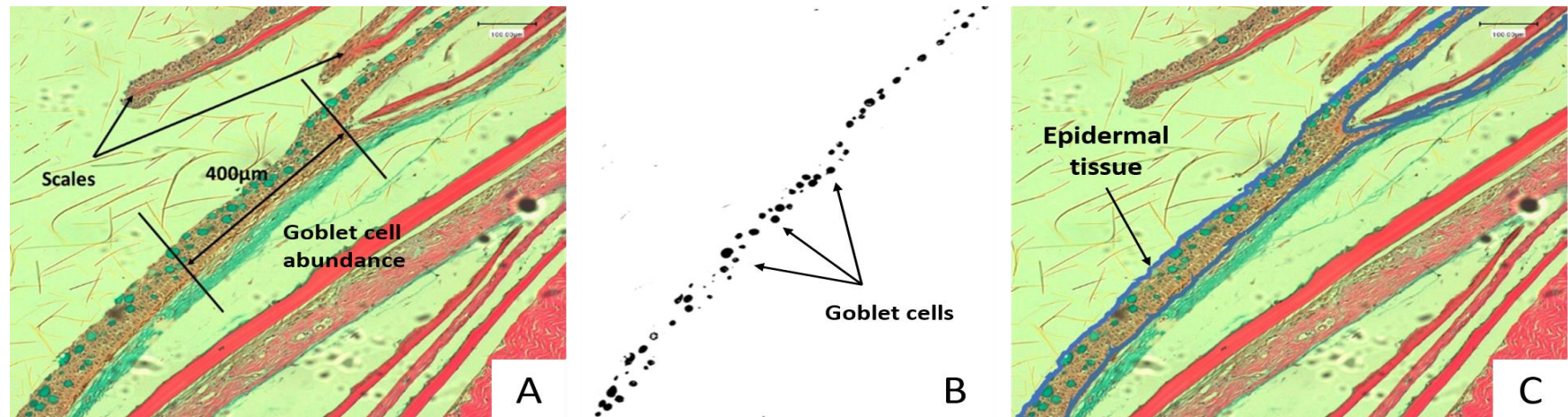
### *2.5.3 Skin measurements*

For the skin samples, goblet cell abundance was measured across 400µm from the edge of 5 scale pockets for each of two fish as demonstrated in Plate 2.3a. The goblet cell area fraction was calculated using three complete images per fish: a Fiji script was written to determine the total area covered by goblet cells by manipulating the contrast, threshold and bit-usage of the image, as shown in Plate 2.3b. The area of the epidermis was then measured using the freehand-draw tool as shown in Plate 2.3c. The goblet cell area fraction (CAF) was calculated as follows:

$$\text{Goblet cell area fraction (\%)} = (\text{total area of goblet cells} \div \text{epidermal tissue area}) \times 100$$



**Plate 2.2.** Atlantic salmon distal intestine. A) Example image to illustrate methodology of different anatomical measurements: Lamina propria (LP) width, mucosal fold length, and goblet cell abundance. B) Example image to illustrate contrast manipulation of image to determine total tissue area. C) Example image to show image manipulation to determine goblet cell area. AB/vG stained. Scale bar represents 100 μm.



**Plate 2.3** Atlantic salmon skin. A) Illustrating the method used to measure goblet cell abundance. B) Illustrating image transformation in order to determine total goblet cell area. C) Showing measurable epidermal area outlined in purple. Tissue is AB/vG stained. Scale bars represents 100 μm.

## 2.6 Gene expression

### 2.6.1 RNA extraction and cDNA synthesis

Total RNA was extracted from samples using TRI reagent (Ambion, Life technologies, UK) following the manufacturer's instructions, with some modifications. Briefly, 50-100mg of skin, gill or intestine samples were removed from the RNAlater solution and pressed between sterile tissues to remove excess solution. The samples were transferred into Lysing Matrix D tubes containing 1mL TRI reagent and homogenised for 40 seconds using the FastPrep-24™ 5G instrument (MP Biomedicals). Following the addition of 200µl of chloroform, samples were centrifuged at 12,000 x g for 15 min at 4°C. The upper aqueous phase was transferred into a tube containing 500 µl of isopropanol. Mixtures were vortexed and centrifuged at 14,000 x g for 15 min at 4°C. Supernatants were discarded and the precipitated RNA pellets were washed using 1ml of 70% ethanol. Total RNA was dissolved in diethylpyrocarbonate (DEPC) water and stored at -80°C. Any contaminating genomic DNA was removed during Part I of the QuantiTect Reverse Transcription Kit (Qiagen) in which 2µl of gDNA Wipe-out Buffer was added to template RNA and incubated for 2 minutes at 42°C. The concentration and quality of RNA in each sample were then determined by measuring 260/280 nm and 260/230 absorbance ratios (NanoDrop Technologies, Wilmington, USA). The integrity of RNA was also confirmed by running samples on a 1% Agarose gel. Using Part II of QuantiTect Reverse Transcription Kit (Qiagen), a 20µl reaction volume containing of 1µg of total RNA was prepared as described in the kit protocol and used for cDNA synthesis. The reaction was run at 42 °C for 15 minutes and inactivated at 95 °C for 3 minutes.

### 2.6.2 Quantitative Real-time PCR

PCR reactions were performed with the SYBR green method using a Quantstudio 12k Flex Real-time PCR thermal cycler (Applied Biosystems). Duplicate PCR reactions were carried out for each sample analysed. Each PCR reaction was set on a 384-well plate by mixing 2 $\mu$ L of diluted (1/10) cDNA with 5.5 $\mu$ L 2 $\times$  concentrated iQ SYBR Green Supermix (Bio-Rad), containing SYBR Green as a fluorescent intercalating agent, 0.3 $\mu$ M forward primer, and 0.3 $\mu$ M reverse primer. The thermal profile for all reactions was 10 min at 95 °C and then 40 cycles of 15 seconds at 95 °C, 60 seconds at 60 °C with fluorescence monitoring occurring at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases one single peak. No amplification product was observed in negative controls and no primer–dimer formations were observed in the control templates. The threshold cycle (Ct), defined as the point at which the fluorescence rises appreciably above the background fluorescence, was determined manually for each run.

PCR efficiencies for each set of primers were determined using serial dilutions of pooled cDNA and resulting plots of Ct versus the logarithmic cDNA input, using the equation  $E = 10(-1/\text{slope})$  (Rasmussen, 2001). For information relating to the housekeeping genes, genes of interest and methods for analyses refer to individual chapters.

**The effect of prebiotic, parabiotic and synbiotic dietary supplementation upon the growth, mucosal health and barrier defences of juvenile rainbow trout (*Oncorhynchus mykiss*)**



### 3.1 Introduction

With the continuing growth and expansion of the aquaculture industry, increasing attention must be paid to fish welfare (Oliva-Teles, 2012). Influencing health, growth, stress response and disease resistance, the overall welfare status of fish in aquaculture ultimately affects the sustainability of the industry (FAO, 2019). Under intensive culture conditions, fish are subjected to increased stress owing to a variety of environmental conditions and operational events, including pH, salinity and temperature fluctuations, high stocking densities, grading, net changing, and handling during vaccination events (Oliva-Teles, 2012). As the principle corticosteroid of teleost fish, stressful events induce the secretion of cortisol, which has been documented to effect the immune system (Engelsma *et al.*, 2003; Castillo *et al.*, 2009), energy metabolism (Bernier and Peter, 2001; Aluru and Vijayan, 2009), haematological parameters (Barton, 2002), and the proliferation, differentiation and tight junctions of gill epithelial cells (Gauberg *et al.*, 2017). As such, plasma cortisol levels are used as a marker of acute stress in fish (Krasnov *et al.*, 2012). The resulting immunosuppression, reduced feed intake, and reduced growth rate of the fish in response to increased cortisol levels, contribute to the increased susceptibility of the fish to pathogens and disease (Anderson, 1996).

Much of the aquaculture industry is increasingly threatened by emerging infectious diseases which have caused considerable problems and costs, and represent one of the main constraints to the industry's continued expansion (Pettersen *et al.*, 2015). While significant progress has been made in terms of identification, diagnostics, treatments, and zone management of disease in certain sectors (e.g. the European Atlantic salmon (*Salmo salar*) industry), recalcitrant issues, (such as those associated with sea lice infestations), have the potential to remain significant barriers to expansion (Groner *et al.*, 2016). Infectious diseases

caused by viral, bacterial and eukaryote pathogens continue to impose major yield-limiting effects on production: currently, industry-wide losses to aquatic animal diseases exceed US\$6 billion per annum (Stentiford *et al.*, 2017). This is reflective of the wider aquaculture sector, where infectious diseases are also causing devastating economic and social impacts, with total losses exceeding 40% of global capacity (Stentiford *et al.*, 2017).

In recent years, the detrimental and potentially dangerous effects of the overuse of traditional disease-control treatments, such as chemotherapeutic agents and vaccines, in both agriculture and aquaculture, has become widely apparent (Cerezuela *et al.*, 2013). In response, a major research effort was launched in an attempt to not only further our understanding of fish immunity, including the different immune pathways fish possess and the interactions between pathogen and fish host, but also to identify alternative methods of controlling diseases in aquaculture. Functional feeds represent one such alternative that have proven successful in a number of situations, as evidenced by their extensive use in the industry today (Dawood, Koshio and Esteban, 2018; Dawood *et al.*, 2019; Hoseinifar *et al.*, 2019). Scientific evidence gathered over the last 60 years has served to highlight the crucial role nutrition plays in maintaining the health status of fish and as a result, the concept of maintaining fish health through the best possible nutrition is well accepted in modern aquaculture (Kiron, 2012).

Functional feeds contain both digestible and non-digestible components, and include probiotic and prebiotic additives, nucleotides, vitamins, immunostimulants and algal/plant extracts (Micallef *et al.*, 2017). Both probiotics and prebiotics have demonstrated beneficial effects not only upon the gut microbiota, immunity, and disease resistance, but also upon growth performance, feed utilisation, and water quality in a range of different species

(Dawood *et al.*, 2018 and 2019; Jami *et al.*, 2019; Rawling *et al.*, 2019). A growing body of research is now focussing on the potential of pro- and pre-biotics to act as immunomodulants in the diets of a number of different teleost species.

While the majority of prior studies have investigated the dietary effect of a single prebiotic, the combined use of multiple yeast saccharides in fish diets has received increased attention in recent years. This combinational approach is theorised to exert additional beneficial effects on the health and growth performance of fish farmed under intensive culture conditions; several commercial preparations have since been formulated for aquaculture use (Selim and Reda 2015; Najdegerami *et al.*, 2017). One such commercial product is B-Wyse, a non-living formulation of yeast cell wall fractions, mannanoligosaccharides (MOS) and yeast metabolites, derived from *Saccharomyces cerevisiae*. This cocktail takes advantage of the different modes of action of both  $\beta$ -glucans and MOS working simultaneously within the host, potentially resulting in what is termed a “synergistic effect”: increased efficacy of both components working in concert to benefit the host. Within the literature, several investigations have documented the effects of commercial MOS +  $\beta$ -glucan products on a wide variety of aquatic species. For example, experimenting on the sea cucumber (*Apostichopus japonicus*) Gu *et al.*, (2011) reported a synergistic effect between dietary  $\beta$ -glucan and MOS as evidenced by prolonged elevations of certain immune indices, relative to individual  $\beta$ -glucan or MOS supplementation. Zhu *et al.*, (2012) reported that the dietary supplementation of yeast polysaccharides (primarily  $\beta$ -glucan and MOS) had significant effects upon the histomorphology of channel catfish, increasing the number of goblet cells and the height of the mucosal folds within the intestine. In Nile Tilapia, Selim and Reda (2015) demonstrated the potential of a  $\beta$ -glucan and MOS mixture to improve the crude protein and fat content of fish when supplemented into the diet at an inclusion level of 0.3%.



The concept of “parabiotics” is relatively new. Coined within the last decade, the term refers to inactivated or non-viable probiotic microorganisms that hold the potential to be equally beneficial to the host in a manner similar to their viable counterparts (Choudhury and Kamilya, 2019). “Tindalised bacteria” (TB) is the name given to an experimental parabiotic compound produced by the European Animal Nutrition company Lallemand® which is currently under rigorous investigation for its applicability and suitability for commercial aquaculture-related practices. It is a cocktail of heat-killed and therefore non-viable bacteria and associated cell components, and will be investigated for its suitability as a dietary supplement in this investigation, alongside the commercial prebiotic B-Wyse.

The importance of the mucosal-associated lymphoid tissues present in the gut, gills and skin of fish has become well established in recent years (Peterson, 2015). Due to their direct contact with the external environment, these MALTs represent one of the primary barriers to pathogens, hindering access to the internal milieu of the fish (Lazado and Caipang 2014). The majority of research into the effect of orally administered prebiotics and probiotics in salmonids has focused almost exclusively on the physiological and immunological reactions that occur in the intestine and in concert with the GALT and its concomitant microbiome (Ringø *et al.*, 2014; Davani-Davari *et al.*, 2019). The potential interconnectivity of the gut-, skin- and gill-associated lymphoid tissues is now receiving increased attention, particularly with regard to how feed additives may exert a beneficial influence upon them to improve the mucosal barrier defences.

A notable study, demonstrated the effect of  $\beta$ -glucan oral administration upon the expression of mucin and  $\beta$ -defensin genes in common carp (van der Marel *et al.*, 2012). Following a 14-day trial, Muc5B was significantly up regulated in the skin, while  $\beta$ -defensin genes were

significantly up regulated in the gills and skin. The regulation of these genes following glucan-feeding is suggestive of an interconnection between the mucosal tissues and further serves to highlight the potential of feed additives to improve fish skin health in particular (van der Marel *et al.*, 2012). More recently, Micallef *et al.*, (2017) reported on the ability of a yeast cell wall extract to influence changes in the proteomic profile of Atlantic salmon skin mucous. The authors nominated a calreticulin-like protein as a possible biomarker for future research into yeast cell wall derived feed additives due to consistent increases in its abundance at both the protein and transcript level (Micallef *et al.*, 2017).

Rainbow trout is considered one of the most commercially important species within the aquaculture industry today (Singh *et al.*, 2016). According to recent FAO estimates, 810,000 metric tonnes of rainbow trout are produced globally by the aquaculture industry each year, worth an estimated £3.6 billion (FAO-FIGIS, 2020). It is therefore unsurprising that rainbow trout are among the most well documented fish species in respect to dietary additive applications (Ringø, *et al.*, 2014; Khodadadi *et al.*, 2019).

The first aim of the research within this chapter was to assess the effect of a commercially available MOS +  $\beta$ -glucan product (B-Wyse) and a paraprobiotic product (TB), upon the growth indices of juvenile rainbow trout; the products were added, both individually and in combination, to a basal diet consisting of a relatively high combined soybean meal (SBM) and soy protein concentrate (SPC) content (~50%). The second aim was to determine the effect of these products upon the mucosal responses of the fish, in terms of not only the quantity and quality of epithelial mucous but also via histological appraisal of the lesser-studied mucosal tissues, the GALT and SALT. The third aim was to investigate the expression of a selection of 10 mucous-associated and immunology-related genes in the MALT tissues to further inform

existing knowledge and understanding relating to the mechanisms underpinning alterations at the mucosal tissues in response to dietary additives.

### 3.2 Materials and methodologies

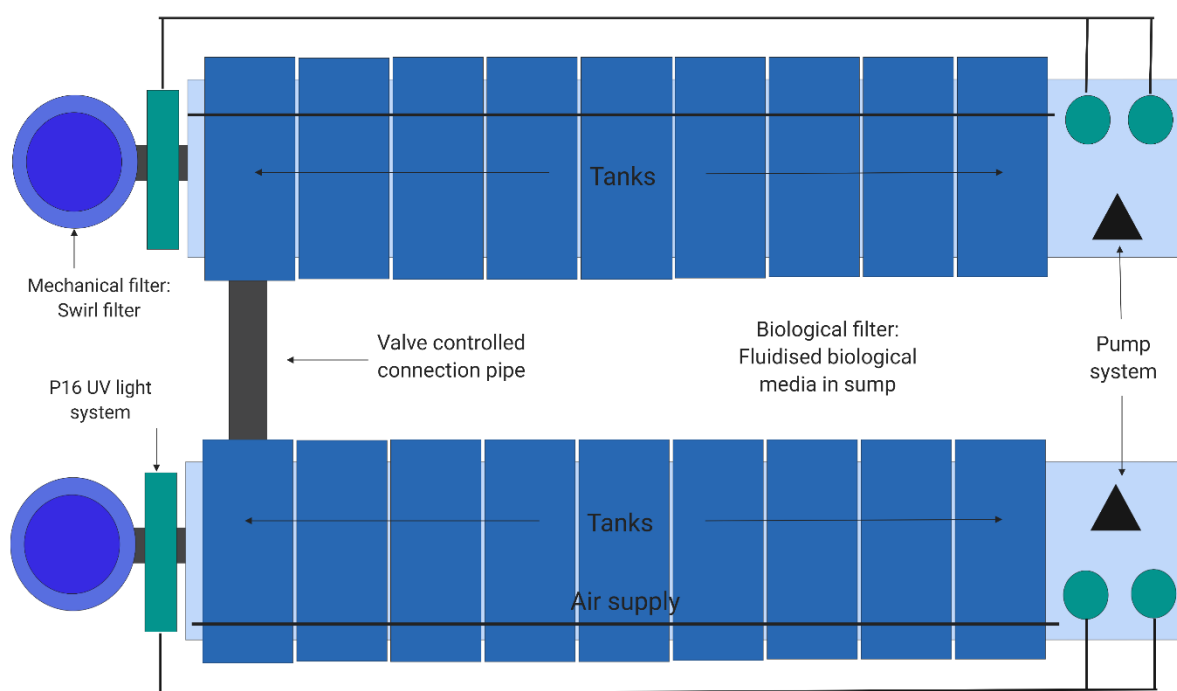
All experimental work involving fish was conducted under the UK Home Office institute licence of the University of Plymouth and in accordance with the UK Animals Scientific Procedures Act of 1986 and the University of Plymouth Animal Welfare and Ethical Review Committee.

#### *3.2.1 Experimental design*

The PhD candidate was solely responsible for the set-up and the day-to-day running of the trial, conducted at the Aquaculture and Fish Nutrition Research Aquarium at the University of Plymouth. A closed recirculation system, comprising 18 x 135L rectangular fiberglass tanks, each provided with recirculated fresh water at a rate of 900L per hour was employed (see Figure 3.1). Mechanical filtration was provided via a large swirl filter incorporating Japanese nylon filter media. Biological filtration was provided via fluidised biological media (Kaldnes K1) in the sump, with vigorous aeration ensuring adequate turbulence. Sterilisation treatment was provided by a P16 commercial UV light system. A 12-hour light/dark photoperiod was maintained throughout via an LED AquaRay control system, incorporating sunrise and sunset simulations.

The pH, dissolved O<sub>2</sub> and temperature were monitored daily using a portable reader (Hach HQ40D Multi meter, HACH, Dusseldorf, Germany). The water temperature was maintained at 15 ± 0.5 °C. The system was buffered with sodium bicarbonate (NaHCO<sub>3</sub>) as necessary to maintain ~pH 7. Dissolved O<sub>2</sub> levels were maintained >80% saturation with additional aeration

provided by an air stone supplied via a side channel air blower. Ammonium (Hach Lange LCK 304), nitrite (Hach Lange LCK 341) and nitrate (Hach Lange LCK 340) were monitored on a weekly basis using a Hach Lange DR 2800, with acceptable levels considered to be  $< 1.0 \text{ mg L}^{-1}$ ,  $< 0.1 \text{ mg L}^{-1}$  and  $< 30 \text{ mg L}^{-1}$  for ammonia, nitrite and nitrate, respectively. Water exchanges were incorporated as required to control nitrate levels.



**Figure 3.1** Schematic depiction of the system facilities of the Aquaculture and Fish Nutrition Research Aquarium at the University of Plymouth in which the trial was conducted.

### 3.2.2 Experimental fish and feeding rates

Rainbow trout were acquired from Exmoor Fisheries, Somerset, UK. A routine quarantine period of 10 days was observed in which twice-daily administrations (30ml) of an anti-parasite and anti-fungal treatment (F-M-G Mixture, NT Labs, Kent, UK) were added to the surface of the water. Following a further 3-week on-growing period during which the fish were fed a standard commercial diet suitable for their size, the fish were graded and separated into 12

tanks at a stocking density of 35 fish per tank. A preliminary grading was carried out approximately one week prior to final grading where batch tank weights were graded to within 2% of each other. At the start of the trial, each of the four dietary treatments were randomly assigned to tanks in triplicate and fish were fed at a rate of 1.1-2.2% of total tank biomass spread across three to five meals per day. The feeding rate was adjusted after weighing (as tank biomass) every two weeks. Feed was reduced to half ration the day before weighing.

### *3.2.3 Sampling schedule and specifics*

Sampling occurred after five weeks (35 days) and eight weeks (56 days) of feeding the experimental diets. Two (at 5 weeks) or four (at 8 weeks) fish per tank were randomly netted and humanely euthanised according to ethical practices. Mucous collection and individual morphometric measurements (body weight and fork length) occurred immediately, followed by skin, gill and distal intestine sample collection for histological examination and gene expression, as described in chapter 2, section 2.2.

### *3.2.4 Diet preparation*

Four iso-nitrogenous and iso-lipidic diets were formulated (as described in Table 3.1) to meet the known nutritional requirements of rainbow trout using Animal Feed Optimisation Software (AFOS) and the “linear least cost” scenario.

**Table 3.1** Ingredient inclusion levels (%) of the 4 experimental diets formulated for rainbow trout in addition to their analysed composition (no significant differences, n=3).

Ingredient (% inclusion)	Experimental diet			
	Control	B-Wyse	Tindalised Bacteria	B-Wyse + Tindalised Bacteria
Soy protein concentrate <sup>a</sup>	37.36	37.36	37.36	37.36
Fishmeal <sup>a</sup>	12.50	12.50	12.50	12.50
Soybean meal <sup>a</sup>	12.00	12.00	12.00	12.00
Vegetable oil <sup>a</sup>	9.31	9.31	9.31	9.31
Fish oil <sup>a</sup>	9.00	9.00	9.00	9.00
Cornstarch <sup>a</sup>	8.34	8.34	8.34	8.34
Wheat gluten <sup>a</sup>	8.20	8.20	8.20	8.20
Vitamin and mineral premix <sup>b</sup>	2.00	2.00	2.00	2.00
Lysine HCL <sup>c</sup>	0.70	0.70	0.70	0.70
CMC binder <sup>c</sup>	0.50	0.50	0.50	0.50
DL methionine <sup>c</sup>	0.38	0.38	0.38	0.38
B-Wyse	-	0.15	-	0.15
Tindalised Bacteria	-	-	0.03	0.03
<b><i>Analysed composition (% of feed)</i></b>				
Crude protein	46.88 ± 0.91	46.90 ± 0.25	45.51 ± 0.86	45.53 ± 0.28
Crude lipid	21.11 ± 0.15	ND	20.18 ± 0.90	20.67 ± 0.18
Ash	8.00 ± 0.14	7.45 ± 0.07	6.22 ± 0.51	6.69 ± 0.10
Moisture	3.90 ± 1.26	ND	4.60 ± 1.84	3.24 ± 0.23

Control: No inclusion of additives; B-Wyse: 1.5g/kg<sup>-1</sup> inclusion; Tindalised bacteria: 0.3g/kg<sup>-1</sup> inclusion; B-Wyse + TB: 1.5g/kg<sup>-1</sup> and 0.3g/kg<sup>-1</sup> respective inclusion.

<sup>a</sup> Skretting, Invergordon, Easter Ross, UK

<sup>b</sup> PNP Fish 2%, Premier Nutrition, UK

<sup>c</sup> Sigma-Aldrich, Merck, Darmstadt, Germany

ND= not determined due to technical issues with machines and unusable remaining samples

For the experimental diets, the additive was first mixed in to the designated amount of cornstarch, which acted as a lightweight carrier. Dry ingredients were then added one at a time to a Hobart food mixer (Hobart Food Equipment, Sydney, Australia, Model number: HL 1400-10STDA) and mixed thoroughly for approximately 2 hours to ensure homogeneity of the additive and all other ingredients throughout. Measures of oil and warm water were added

gradually to the mixer prior to cold press extrusion (PTM P6 extruder, Plymouth, UK) to produce 2mm pellets and subsequently dried to ca. 5% moisture in an air convection oven at 45°C for 48 hours. Once dry, pellets were broken up to the appropriate size and subsequently stored in airtight containers at 4°C.

#### *3.2.4.1 Chemical proximate analysis*

Experimental diets were subjected to analysis for the determination of moisture, lipid, protein, and ash content following standard AOAC (2016) protocols, as described below.

#### *3.2.4.2 Moisture*

Approximately 4.0g of sample was weighed into a metal crucible and air dried in a fan assisted oven at 105°C until a constant weight was achieved. The percentage moisture was then calculated as:

$$\text{Moisture content} = ((\text{WW}-\text{DW})/\text{WW}) * 100,$$

where WW is the wet weight (g) and DW is the dry weight (g).

#### *3.2.4.3 Lipid*

Lipid content was determined via the rapid Soxhlet extraction method. Approximately 2.5g of sample was weighed into a cellulose thimble, lightly plugged with cotton wool and inserted into glass beakers containing bumping granules. Before being placed into the Soxhlet unit (Gerhardt Laboratory Instruments, Bonn, Germany), 140 ml of petroleum ether was added to each beaker. The samples were then heated to 150°C for 30 minutes and rinsed for 45 minutes. Upon completion, the solvent was left to evaporate overnight in a fume hood, after which the extracted lipid was weighed.

The lipid content was determined as:

$$\text{Lipid content} = (\text{LW} / \text{SW}) \times 100,$$

where LW is the lipid weight determined from the weight increase of the beaker, and SW is the initial sample weight (g).

#### 3.2.4.4 Crude protein

The Kjeldahl method was used to determine crude protein levels by establishing the total nitrogen content of samples. This amount was then multiplied by a factor of 6.25 to calculate content on the assumption that animal proteins contain 1% nitrogen (AOAC, 2016). Approximately 150mg of sample was added to a kjedahl digestion tube in addition to a catalyst tablet (3g K<sub>2</sub>SO<sub>4</sub>, 105 mg CuSO<sub>4</sub>.5H<sub>2</sub>O and 105mg TiO<sub>2</sub>; BDH Ltd UK) and 10ml of concentrated (98%) H<sub>2</sub>SO<sub>4</sub> (Sp.Gr. 1.84, BDH Ltd UK). To correct for the efficiency of nitrogen extraction two samples of acetanilide (nitrogen content 10.36%) were used. Casein was used to validate the nitrogen content. The digestion was performed using a Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) at 105°C for 15 minutes, increasing to 225°C for 60 minutes and 380°C for 45 minutes. Following digestion, the samples were distilled using a Vapodest-40 automatic distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany). Crude protein content was then determined as:

$$\text{Crude protein content} = (((\text{ST}-\text{BT}) \times 0.2 \times 1.4007 \times 6.25)/\text{SW}) \times 100,$$

where ST is sample titre (ml), BT is blank titre (ml), SW is sample weight (mg), 0.2 is the acid molarity and 1.4007 is the molecular weight of nitrogen.



#### 3.2.4.5 Ash

Ash content (total mineral or inorganic content) was determined by adding approximately 500mg of sample to a pre-weighed porcelain crucible and incinerated in a muffle furnace (Carbolite, Sheffield, UK) at 550°C for 12 hours. The ash content was then determined as:

$$\text{Ash content} = ((\text{SR} - \text{CW}) / \text{SW}) * 100,$$

where SR is the sample residue weight (g), CW is the crucible weight (g) and SW is the original sample weight (g).

#### 3.2.5 Fish growth and condition

Calculations to determine feed efficiency, growth performance and fish condition were performed as follows:

- Fulton's condition factor (K-factor) =  $(100 * \text{weight (g)}) / (\text{total fish length (cm)})^3$
- Feed Conversion Ratio (FCR) =  $\text{feed intake (g)} / \text{weight gain (g)}$
- Specific Growth Rate (SGR) =  $((\text{Ln}W2 - \text{Ln}W1) / T) \times 100$ : where  $\text{Ln}W1$  and  $\text{Ln}W2$  are the initial and final natural logarithmic weights, respectively, and  $T$  is the number of days in the feeding period.
- Survival Rate (SR) (%) =  $(\text{final number of fish} / \text{initial number of fish}) \times 100$ .

#### 3.2.6 Epidermal mucous analysis

Mucous quantity was determined from individual fish, as described in chapter 2, section 2.2. Protein concentration and lysozyme activity were determined from duplicate measurements of each fish sampled per tank resulting in n=6 per treatment at 5 weeks and n=12 at 8 weeks, as described below.

### 3.2.6.1 Lysozyme activity

Lysozyme activity of the epidermal mucous was determined using a turbidimetric assay based upon the lysing activity of *Micrococcus Lysodeikticus* according to Ellis, (1990). Briefly, solutions of  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  were mixed with distilled water to produce a 0.04 molar solution with a pH of 6.2, to which *M. lysodeikticus* was added at  $0.2\text{mg ml}^{-1}$ . On a flat-bottom well plate  $190\mu\text{m}$  of solution and  $10\mu\text{l}$  of sample were combined and the absorbance measured at 540nm on a Spectrophotometer (SpectraMax190, Molecular Devices) after 1 minute and 15 minutes. Samples were run in duplicate per fish. The lysozyme activity was then calculated as follows:

$$\text{Lysozyme activity (U/ml)} = ((\text{Abs 1} - \text{Abs 15}) * 900) * 60,$$

where Abs 1 is the absorbance after 1 minute and Abs 15 is the absorbance after 15 minutes.

### 3.2.6.2 Protein concentration

Epidermal mucous protein concentration was determined using a Protein Assay kit (Pierce™ BCA, ThermoFisher Scientific) in accordance with the manufacturer's recommendations, after Rawling *et al.*, (2019). According to the manufacturer's protocol, the sensitivity range of this assay is  $20\mu\text{g/ml}$  -  $2000\mu\text{g/ml}$ . Briefly, the first step is a biuret reaction, in which copper is chelated with protein in an alkaline environment, followed by a colour-development second reaction which is influenced by the presence of specific amino acid residues found in protein.  $200\mu\text{L}$  of working solution is added to  $25\mu\text{l}$  of sample, in duplicate, and incubated for 30 minutes at  $37^\circ\text{C}$ . After leaving to cool to room temperature, the absorbance is then measured at 562nm on a Spectrophotometer (SpectraMax190, Molecular Devices). After calculating the averages from duplicate samples per fish, the protein concentration of the sample was then

determined from the standard curve produced from the Blank standard replicates provided, calculated as follows:

$$\text{Protein concentration (mg/ml)} = ((\text{Absorbance at 562nm} - 0.0903) / 0.0013) / 1000$$

### 3.2.7 Histological analyses

Light microscopy analysis of skin and gill tissue were carried out on 2 fish per tank sampled at 5 weeks (n=6 per treatment) and 4 fish per tank sampled at 8 weeks (n=12 per treatment) as described in chapter 2, section 2.5. For goblet cell abundance, 6 measurements were taken per fish, while 3 measurements per fish were taken to calculate the CAF in the skin.

### 3.2.8 Gene expression

#### 3.2.8.1 RNA extraction, cDNA synthesis and real time qPCR

RNA extraction, cDNA synthesis and real time qPCR (RT-q-PCR) was carried out as described in chapter 2, sections 2.6.1 and 2.6.2 on 2 fish per tank (n=6 per treatment). All individual samples were checked for quality control purposes at multiple stages and were removed from analysis if considered above or below acceptable limits.

#### 3.2.8.2 Reference genes, genes of interest and analyses

$\beta$ -actin and Elf1 $\alpha$  were used as reference genes for each sample in order to standardise the results by eliminating variation in mRNA and cDNA quantity and quality (Bustin *et al.*, 2009). The stability and suitability of  $\beta$ -actin and Elf1 $\alpha$  as reference genes were confirmed by generating an expression stability measure “M” for each reference gene using the calculations outlined in Vandesompele *et al.*, (2002). No amplification product was observed in negative controls and no primer–dimer formations were observed in the control templates.

Modification of gene expression was represented with respect to the control group being sampled at the same time as the treatment group. A further 10 genes of interest were selected for each of the sample tissues, including pro-inflammatory, immune-regulatory and Th2-associated cytokines; tight-junction-associated claudins; and genes associated with mucosal composition. Primers designed by the PhD candidate are presented in Table 3.2.

### *3.2.9 Statistical analyses*

Statistical analyses were performed using R Studio version 1.2.5042 (R Studio PBC, Boston USA). All statistical analysis for RT-qPCR data were carried out using the permutation tests in R following Röhmel (1996). All other statistical differences (growth performance, mucous quantity and quality, and light microscopy data) were assessed by one-way ANOVA tests in R following Shapiro-Wilk test for normality, and with Tukey high significant difference (HSD) post-hoc tests where differences occurred. Where data could not be transformed into normality, permutation tests were conducted.

The level of significance was accepted at  $p < 0.05$ . All gene expression data is presented as mean  $\pm$  standard error (SE), while all other data is presented as mean  $\pm$  standard deviation (SD).

**Table 3.2** Primer pair sequences, Accession number, annealing temperature (°C), amplicon size (bp), and primer efficiency (E-value) for genes used for real-time PCR. Dashes represent primers not analysed in designated tissue.

Gene name	Accession number	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Annealing Temp (°C)	Amplicon size	E-value		
						Gill	Skin	Gut
$\beta$ -actin	AF254414	AGCCCTCCTTCCTCGGTATG	GGATGTCCACGTCACACTTCAT	60	81	2.01	2.01	1.93
EIF 1 $\alpha$	NM_001124339	TGCGGAGGCATTGACAAGAG	TCCAGCACCCAGGCATACTT	60	92	1.93	1.97	1.93
IL-1 $\beta$	NM_001124347.2	GGACATGCAGCAGGACTACA	GCTGGATGGTGAAGGTGGTA	60	83	2.05	2.10	1.93
TGF $\beta$	X99303.1	CCCACTGGCTACTTTGCTAAC	TGCTTATACAGAGCCAGTACCT	60	95	2.10	2.10	2.05
IL-4/13a	FN820500.1	GCGTTTGGTGAGGAGGGAAA	CCTGGTCTTGGCTCTTCACA	60	84	1.97	2.01	1.93
GATA 3	NM_001195792.1	ACCTCGGCCACTCGTACAT	GGTTGCCCTGTGAGTCGATA	60	87	2.05	2.10	1.86
Calreticulin	AY372389	GAACCCGGACCAGACATACG	ATGGTCTTTGCGGGCAGAAT	60	96	1.97	2.01	1.93
Muc17-like	XM_021610915	CCACTAAGACCTGGACTGACA	CGCTCCAATCCAGACCCTTA	60	108	2.01	1.97	2.05
Claudin 10d	KP233888	GGTGTCCGACTGCAAAGAGT	AGCGAAGAACCCAAGGATGA	60	103	1.97	2.01	-
Claudin 12	BK007967	CCCTGCTGATGTTTATGTGGTA	TGGGTGGAGAGGGAGTTAGG	60	94	1.93	2.10	-
Claudin 10e	KP233889	ATTGTGAGGGGCCTGCTGAT	AGGTGCACTCCATCCCAATG	60	82	2.01	2.10	-
Claudin 28b	NM_001195160	GGGTCGTCTTCCTCATTGCT	TGTAGAAGTCTCGGATGATGGT	60	81	1.93	-	-
Claudin 30	NM_001246273	CAACTGCGTGGAGGATGAGA	ACAGGATGCCAGCGATGATG	60	80	-	2.01	-
IL- 17a	NM_001124619	ACATTCACACACGCTCTCTGT	CTCCACATGGTCTGAGGAA	60	80	-	-	2.10
Tollip1	NM_001124420.1	GACGACAAGGAGGGCATGAT	GGACAACGGGTGGAGACATA	60	81	-	-	2.01
IRAK4	FN598575.1	CCGAGGTA CTCTCAGCAACAT	CTCCACGGTGCAGTTAGAT	60	112	-	-	2.10
pIgR	FJ940682	GCATGGTGAGTGGAGAGGAA	ACACCAACGCTTCTCATGCT	60	86	-	-	1.93

## 3.3. Results

## 3.3.1 Fish growth and condition

Fish readily accepted all dietary regimes and appeared healthy throughout the trial, with survival rate above 98% across all treatments. Growth parameters were not statistically different between fish fed the different dietary treatments. Results are presented in Table 3.3.

**Table 3.3** Growth indices of rainbow trout over the duration of the trial. Data presented as mean  $\pm$  SD; no significant differences  $p < 0.05$ ;  $n = 3$ .

Time-point	Parameter	Control	B-Wyse	Tindalised Bacteria	B-Wyse + TB
Initial	Body-weight (g)	36.82 $\pm$ 1.28	36.53 $\pm$ 0.20	36.72 $\pm$ 0.86	36.57 $\pm$ 0.40
	Biomass (kg)	1.29 $\pm$ 0.04	1.28 $\pm$ 0.01	1.29 $\pm$ 0.03	1.28 $\pm$ 0.01
5 Weeks	Body-weight (g)	64.05 $\pm$ 0.98	62.63 $\pm$ 4.16	66.27 $\pm$ 2.37	62.42 $\pm$ 4.70
	Fork length (cm)	18.03 $\pm$ 1.27	18.12 $\pm$ 1.02	17.87 $\pm$ 1.29	17.85 $\pm$ 0.95
	K-factor (au)	1.46 $\pm$ 0.13	1.46 $\pm$ 0.09	1.44 $\pm$ 0.17	1.36 $\pm$ 0.09
8 weeks	Body-weight (g)	94.49 $\pm$ 1.31	91.81 $\pm$ 8.72	96.80 $\pm$ 1.68	92.09 $\pm$ 5.55
	Fork length (cm)	19.93 $\pm$ 1.14	19.53 $\pm$ 0.76	20.12 $\pm$ 1.10	19.90 $\pm$ 1.44
	K-factor (au)	1.48 $\pm$ 0.13	1.47 $\pm$ 0.12	1.47 $\pm$ 0.06	1.44 $\pm$ 0.11
	Biomass (kg)	2.80 $\pm$ 0.08	2.72 $\pm$ 0.26	2.90 $\pm$ 0.05	2.76 $\pm$ 0.17
Overall performance	Total feed intake (kg)	4.91	4.81	5.05	4.85
	SGR (%/day)	1.70 $\pm$ 0.05	1.60 $\pm$ 0.18	1.71 $\pm$ 0.03	1.60 $\pm$ 0.09
	FCR (au)	0.85 $\pm$ 0.03	0.91 $\pm$ 0.04	0.87 $\pm$ 0.01	0.91 $\pm$ 0.04
	Survival Rate (%)	98.9	98.9	100	100

### 3.3.2 Epidermal mucous analysis

No significant differences were observed between fish fed the different experimental diets in relation to epidermal mucous quantity, lysozyme activity or protein content at either time point compared to the control. Results are presented in Table 3.4.

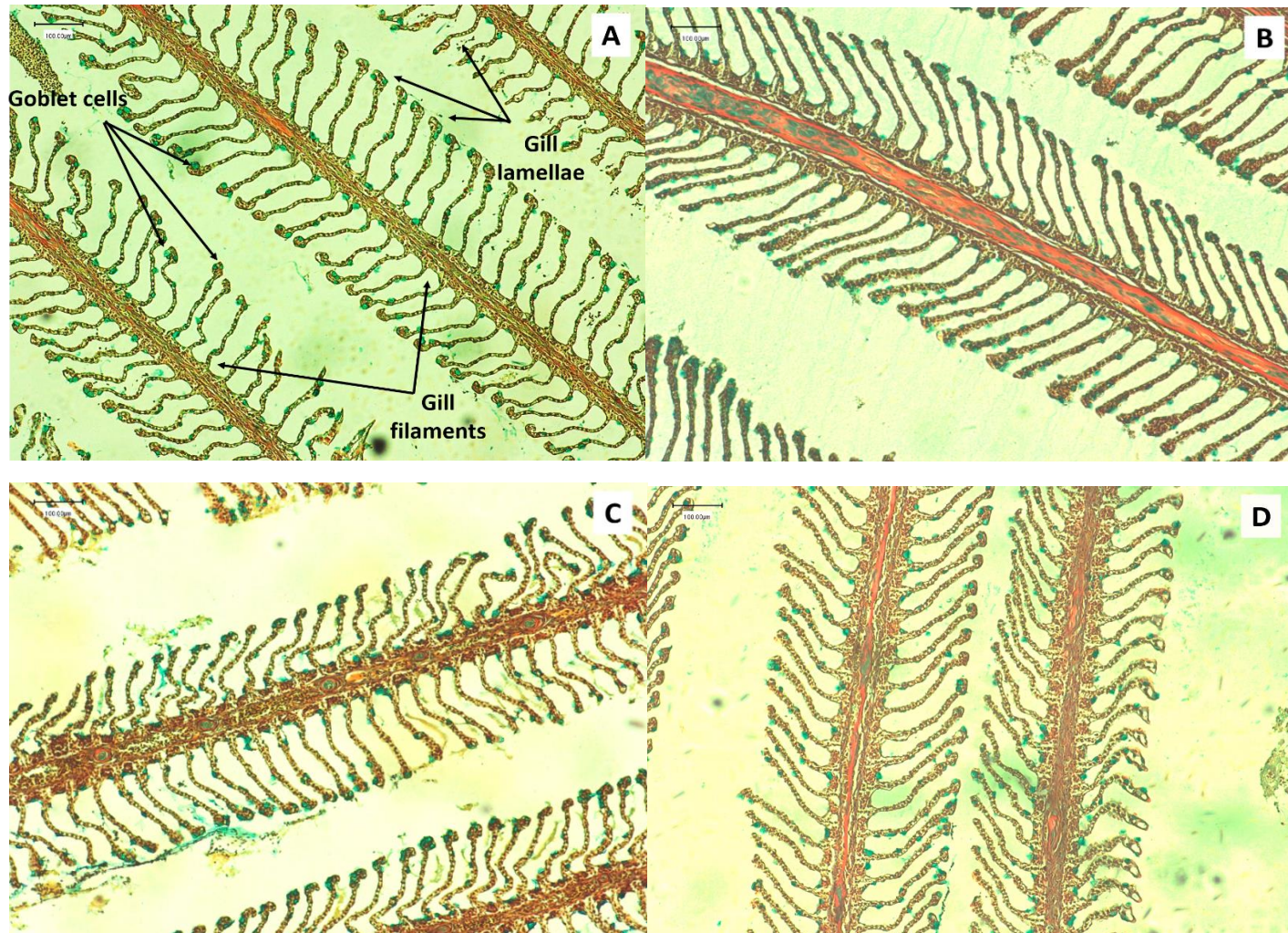
**Table 3.4** Skin mucous measurements for the different dietary regimes. Data presented as mean  $\pm$  SD; no significant differences,  $p < 0.05$ ;  $n = 3$ .

Mucous parameter	Sampling point	Treatment			
		Control	B-Wyse	TB	BWyse + TB
Quantity (mg/cm)	5 weeks	0.82 $\pm$ 0.44	0.95 $\pm$ 0.36	0.95 $\pm$ 0.41	0.90 $\pm$ 0.20
	8 weeks	1.01 $\pm$ 0.18	0.85 $\pm$ 0.40	1.11 $\pm$ 0.23	0.86 $\pm$ 0.40
Lysozyme activity (U/ml)	5 weeks	384.37 $\pm$ 53.56	459.29 $\pm$ 63.79	563.60 $\pm$ 41.95	356.63 $\pm$ 21.20
	8 weeks	131.33 $\pm$ 72.60	137.59 $\pm$ 47.26	137.61 $\pm$ 47.26	139.21 $\pm$ 56.34
Protein content (mg/mL)	5 weeks	1.21 $\pm$ 0.18	1.93 $\pm$ 0.15	1.19 $\pm$ 0.07	1.28 $\pm$ 0.19
	8 weeks	2.45 $\pm$ 0.56	2.69 $\pm$ 0.20	2.48 $\pm$ 0.39	2.73 $\pm$ 0.27

### 3.3.3 Histological analysis

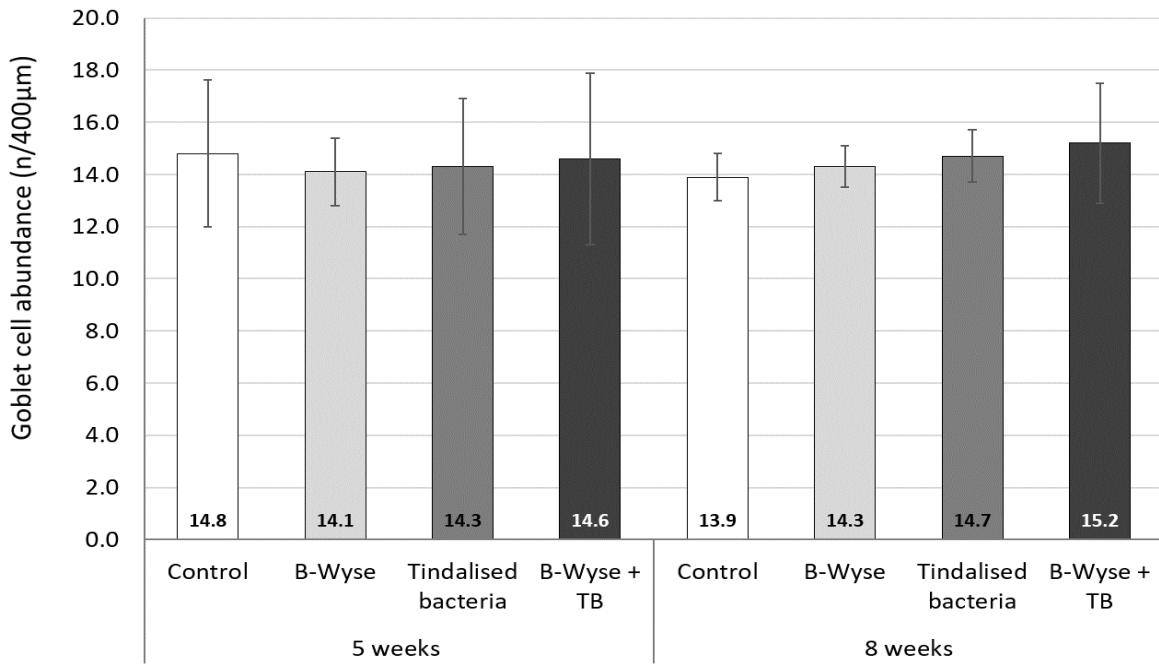
#### 3.3.3.1 Gill

The abundance of goblet cells present in the GIALT did not significantly differ between fish fed the different dietary regimes at either of the sampled time points, 5 or 8 weeks. Representative micrographs used for light histological analysis of the gill are presented in Plate 3.1 and results are presented in Figure 3.2.

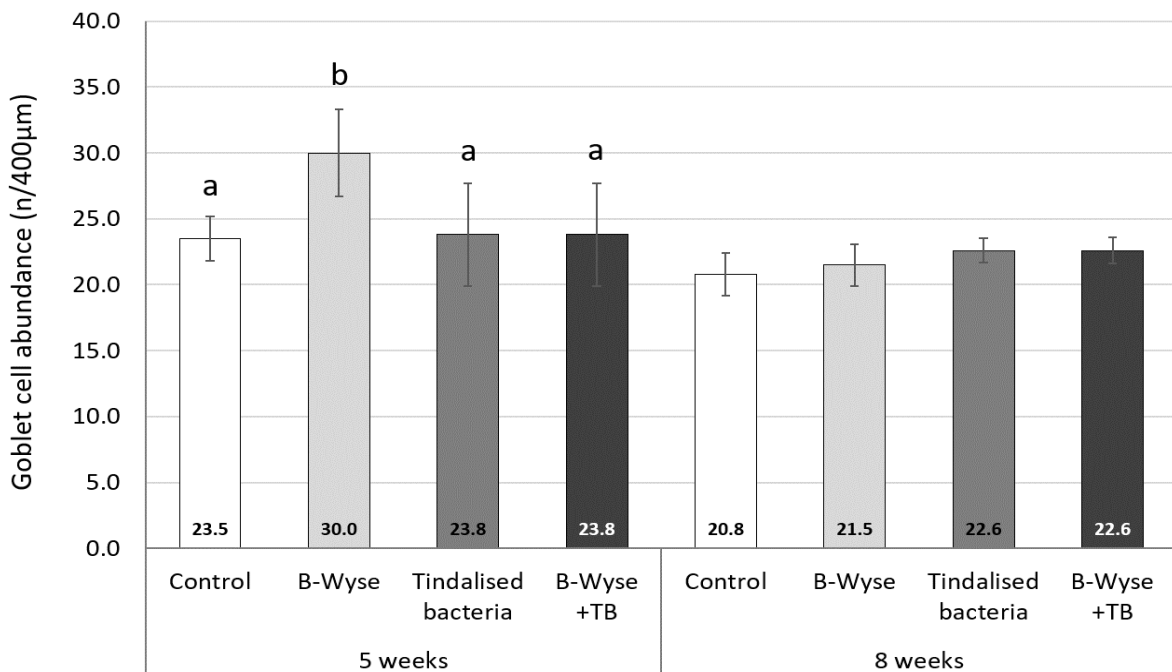


**Plate 3.1** Light micrographs of the GIALT of fish fed A) the control, B) B-Wyse-, C) TB-, and D) B-Wyse + TB-supplemented diets for 8 weeks. No significant differences in goblet cell abundance were observed, where  $p < 0.05$  Scale bar represents 100 $\mu$ m.





**Figure 3.2.** Goblet cell abundance in the GIALT of fish fed the four experimental diets. No significant differences, where  $p < 0.05$ . Data presented as mean  $\pm$  SD;  $n=6$  at 5 weeks;  $n=12$  at 8 weeks.



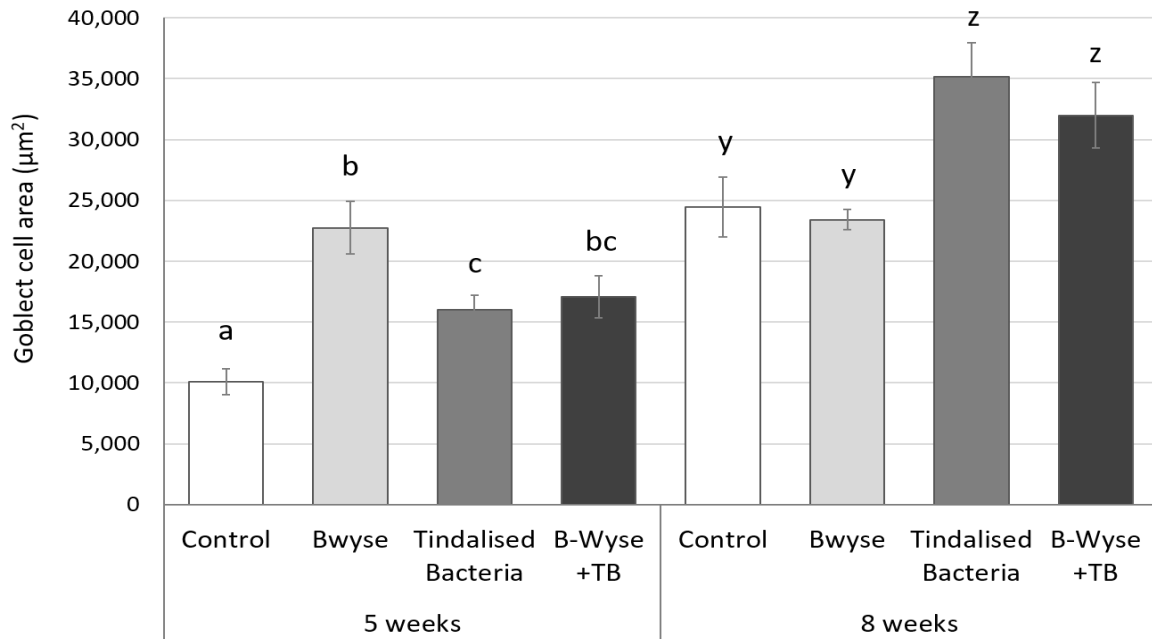
**Figure 3.3** Goblet cell abundance in the SALT of fish fed the four experimental diets. Results displayed as mean  $\pm$  SD;  $n=6$  after 5 weeks;  $n=12$  after 8 weeks. Bars with different letters within the same time-point denote significant differences between treatments ( $p < 0.05$ ).

### 3.3.3.2 Skin

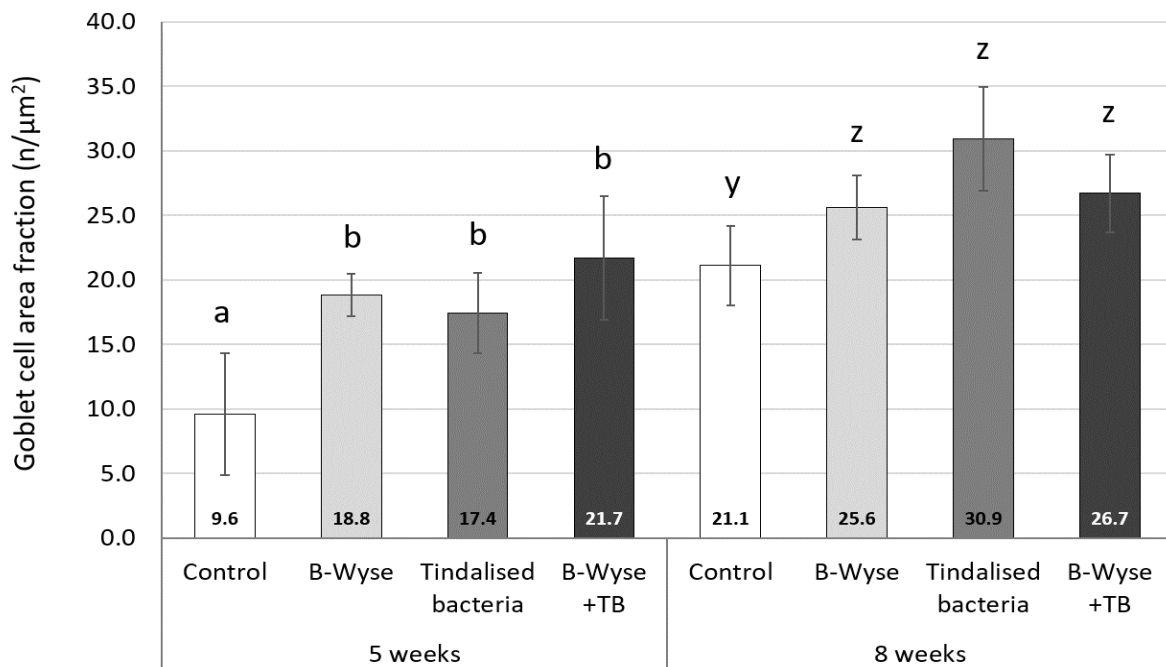
The abundance of goblet cells ( $n/400\mu\text{m}$ ) in the skin of fish fed the B-Wyse treatment ( $30.0 \pm 3.28$ ) were significantly higher after 5 weeks compared to fish fed the control ( $23.48 \pm 1.67$ ,  $p= 0.032$ ), TB ( $23.83 \pm 3.94$ ,  $p= 0.023$ ), and B-Wyse + TB ( $23.83 \pm 3.94$ ,  $p= 0.002$ ) treatments. However, no differences in abundance were detected between treatments after 8 weeks, as presented in Figure 3.3.

The goblet cell area coverage ( $\mu\text{m}^2$ ) was significantly increased in fish fed the B-Wyse ( $22,744 \pm 9,208$ ,  $p<0.001$ ), TB ( $15,989 \pm 5,241$ ,  $p= 0.001$ ), and B-Wyse + TB ( $17,072 \pm 7,433$ ,  $p= 0.002$ ) treatments compared to fish fed the control treatment ( $10,094 \pm 4,626$ ) after 5 weeks. While the TB ( $35,188 \pm 11,631$ ,  $p= 0.006$ ) and the B-Wyse + TB treatments ( $31,988 \pm 11,347$ ,  $p=0.044$ ) continued to exhibit significantly increased goblet cell area coverage after eight weeks compared to the control ( $24,4546 \pm 10,339$ ), no significant difference was observed between fish fed the B-Wyse ( $23,416 \pm 3,515$ ,  $p= 0.692$ ) and control treatments, as presented in Figure 3.4.

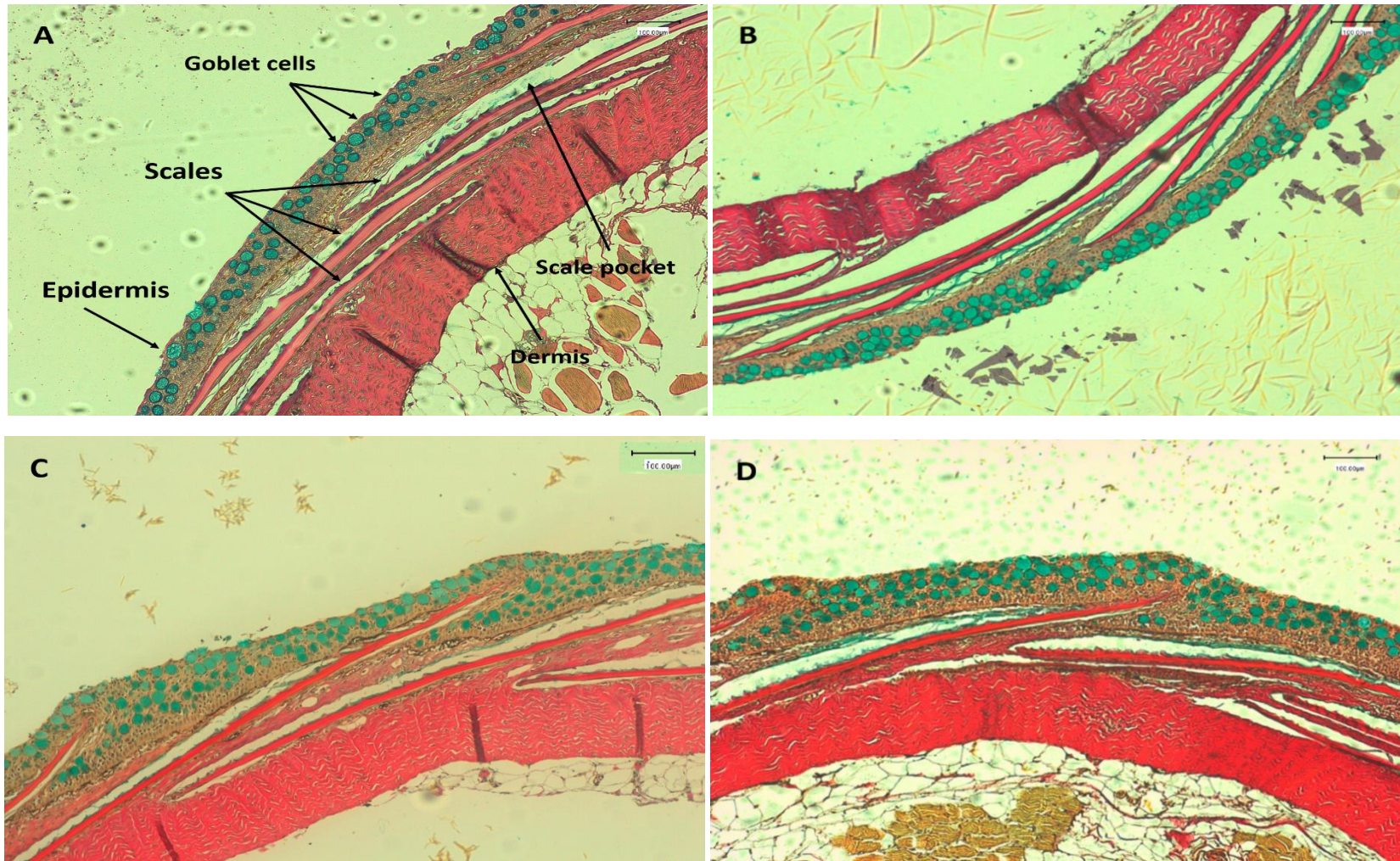
The goblet cell area fraction ( $n/\mu\text{m}^2$ ) (CAF) was observed to be significantly greater in the skin of fish fed the B-Wyse ( $18.82 \pm 3.59$ ,  $p= 0.002$ ), TB ( $17.44 \pm 2.82$ ,  $p= 0.002$ ) and the B-Wyse + TB ( $21.54 \pm 4.66$ ,  $p= 0.002$ ) treatments compared to the control ( $9.60 \pm 2.68$ ) treatment after 5 weeks. Similarly after 8 weeks, the goblet cell area fraction was significantly larger in fish fed the B-Wyse ( $25.64 \pm 2.5$ ,  $p= 0.030$ ), TB ( $30.89 \pm 3.95$ ,  $p= 0.002$ ) and the B-Wyse + TB ( $26.74 \pm 2.99$ ,  $p= 0.006$ ) treatments compared to the control ( $21.14 \pm 3.10$ ) treatment, as presented in Figure 3.5. Representative micrographs used for histological analysis of the skin are presented in Plate 3.2.



**Figure 3.4** Goblet cell area coverage in the SALT of fish fed the four different experimental diets. Results displayed as mean  $\pm$  SD; n=6 after 5 weeks; n=12 after 8 weeks. Different letters within the same time-point denote significant differences between treatments ( $p < 0.05$ ).



**Figure 3.5** Goblet cell area fraction in the SALT of fish fed the four experimental diets. Data presented as mean  $\pm$  SD; n=6 after 5 weeks; n=12 after 8 weeks. Different letters within the same time-point denote significant differences between treatments ( $p < 0.05$ ).



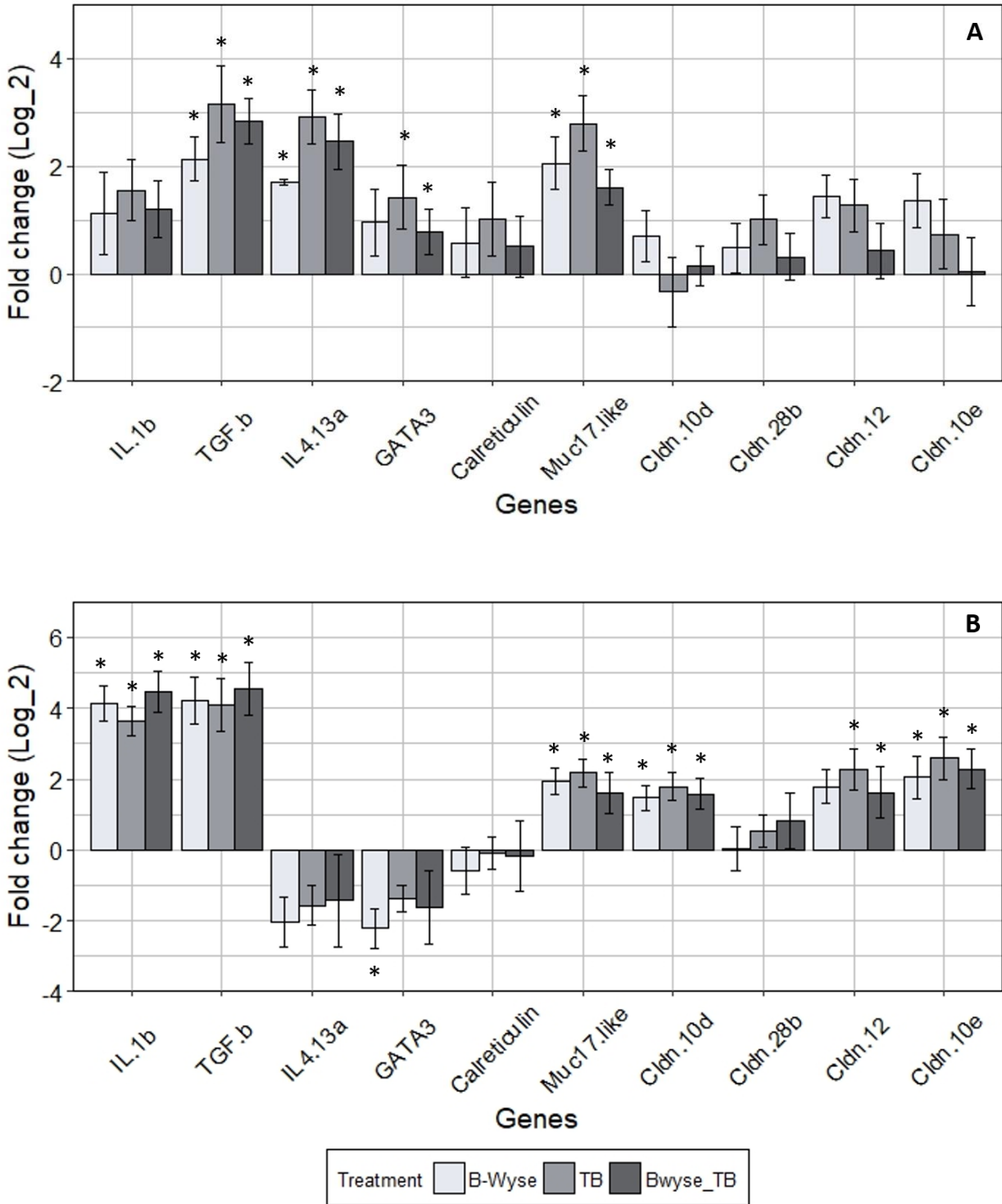
**Plate 3.2** Light micrographs of the SALT of fish fed A) the control, B) B-Wyse-, C) TB-, and D) B-Wyse + TB-supplemented diets for 8 weeks. Measurements were taken to determine goblet cell abundance, area coverage and area fraction. Scale bar represents 100µm.

### 3.3.4 Real time qPCR gene expression

#### 3.3.4.1 Gill

Fold changes relative to the control of the 10 selected genes in gill tissue after 5 and 8 weeks are presented in Figures 3.6 A) and B) respectively. After 5 weeks, fish fed the B-Wyse ( $p = 0.029$ ), TB ( $p = 0.027$ ) and the B-Wyse + TB ( $p = 0.030$ ) treatments exhibited significant increases in the expression of TGF- $\beta$  compared to the control-fed fish. Additionally, significant elevations in the expression of IL4/13a were observed in fish fed the B-Wyse ( $p = 0.029$ ), TB ( $p = 0.029$ ) and the B-Wyse + TB ( $p = 0.029$ ) treatments and in the expression of GATA3 for fish fed the TB ( $p = 0.057$ ) and the B-Wyse + TB ( $p = 0.029$ ) treatments, compared to the control-fed fish. Expression levels for the Muc17-like gene were also significantly elevated in the gill tissue of fish fed B-Wyse ( $p = 0.029$ ), TB ( $p = 0.029$ ) and the B-Wyse + TB ( $p = 0.029$ ) treatments compared to the control, however, no significant increases in the expression of the claudin genes were observed in fish fed any of the experimental treatments.

After 8 weeks of feeding, TGF- $\beta$  expression levels remained significantly elevated in the gills of fish fed the B-Wyse ( $p = 0.004$ ), TB ( $p = 0.004$ ) and the B-Wyse + TB ( $p = 0.002$ ) treatments compared to the control. In addition, significantly elevated expression of IL-1 $\beta$  was also exhibited by fish fed the B-Wyse ( $p = 0.002$ ), TB ( $p = 0.002$ ) and the B-Wyse + TB treatments ( $p = 0.002$ ), in comparison to the control-fed fish. The expression of Muc17-like continued to be significantly upregulated in the B-Wyse ( $p = 0.002$ ), TB ( $p = 0.006$ ) and the B-Wyse + TB treatments ( $p = 0.011$ ) compared to the control treatment after eight weeks. Additionally, the expression of Claudin 10d was significantly upregulated in fish fed the B-Wyse ( $p = 0.004$ ), TB ( $p = 0.006$ ), and B-Wyse + TB ( $p = 0.004$ ) treatments. Fish fed the B-Wyse ( $p = 0.013$ ), TB ( $p = 0.006$ ), and B-Wyse + TB treatments ( $p = 0.006$ ) also exhibited significant upregulation of



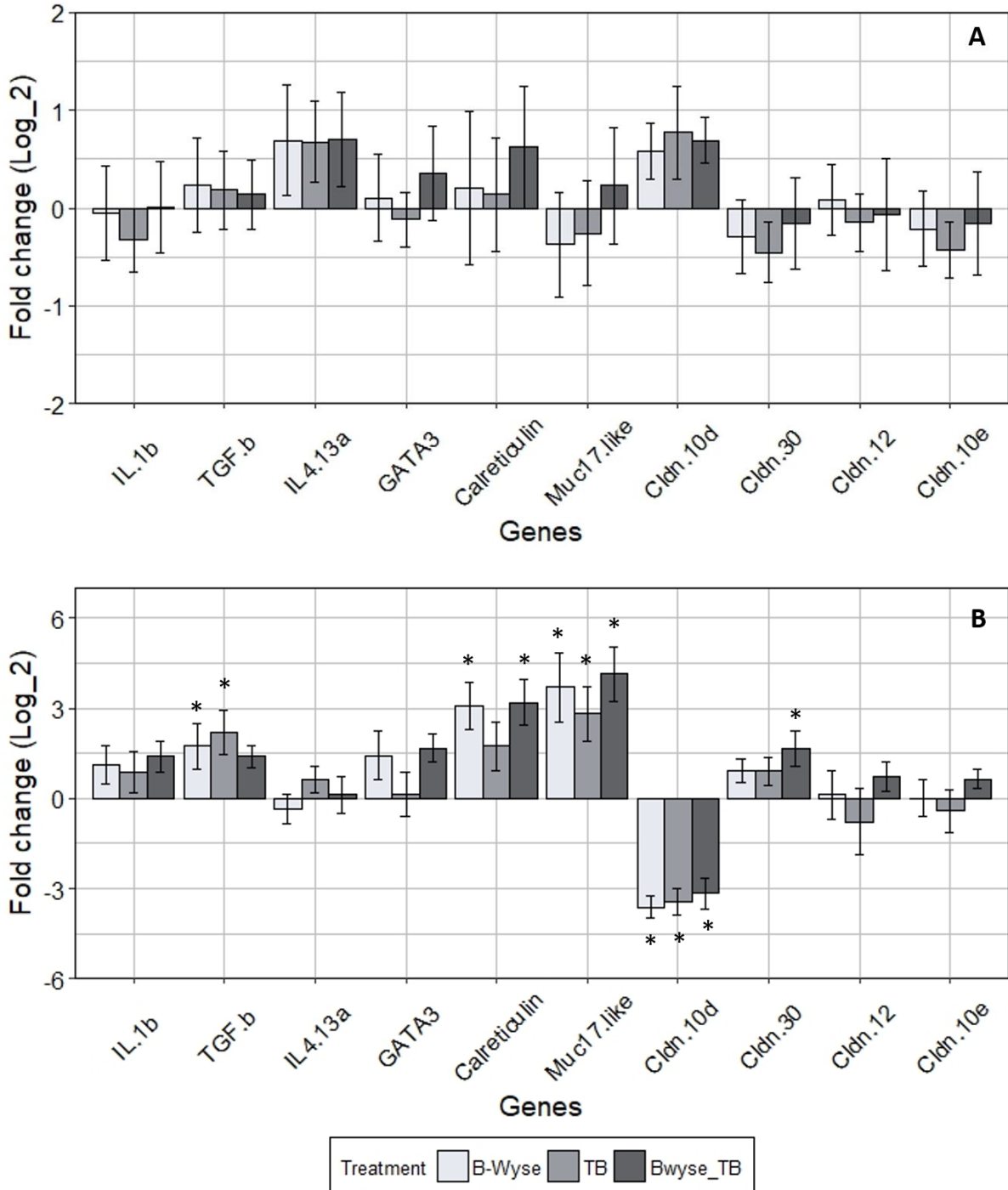
**Figure 3.6** Gene expression data (fold change ( $\log_2$ )) of 10 genes of interest in the GIALT of fish fed three different experimental dietary regimes after A) five weeks ( $n=4$ ) and, B) 8 weeks ( $n=6$ ). Data presented as mean  $\pm$  SEM. Asterix denote a significant difference between the experimental and control dietary regimes ( $p<0.05$ ).

Claudin 10e also, in comparison to the control-fed fish. The expression of Claudin 12 was also observed to be significantly increased in the TB ( $p = 0.008$ ) and B-Wyse ( $p = 0.035$ ) fed fish, compared to the control-fed fish.

#### 3.3.4.2 Skin

Fold changes relative to the control of the 10 selected genes in skin tissue after five and eight weeks are presented in figures 3.7 A) and B). After 5 weeks of feeding the experimental diets, very few significant differences in expression level were observed between fish fed any of the three experimental treatments and the control.

After 8 weeks however, fish fed the B-Wyse ( $p = 0.015$ ) and B-Wyse + TB ( $p = 0.006$ ) treatments were observed to have significantly increased expression of calreticulin compared to fish fed the control treatment. Significantly increased expression of Muc17-like was observed in the skin of fish fed the B-Wyse ( $p = 0.004$ ), TB ( $p = 0.028$ ), and B-Wyse + TB ( $p = 0.004$ ) treatments compared to the control, while the B-Wyse + TB ( $p = 0.022$ ) treatment was also found to have significantly increased expression of Claudin 30 compared to the control. Interestingly however, fish fed the B-Wyse ( $p = 0.002$ ), TB ( $p = 0.002$ ), and B-Wyse + TB ( $p = 0.006$ ) treatments demonstrated a significant down-regulation in the expression of Claudin 10d compared to the control fish. Additionally, fish fed both of the B-Wyse ( $p = 0.032$ ) and TB ( $p = 0.006$ ) treatments showed significant up-regulation of TGF- $\beta$  compared to the control-fed fish.



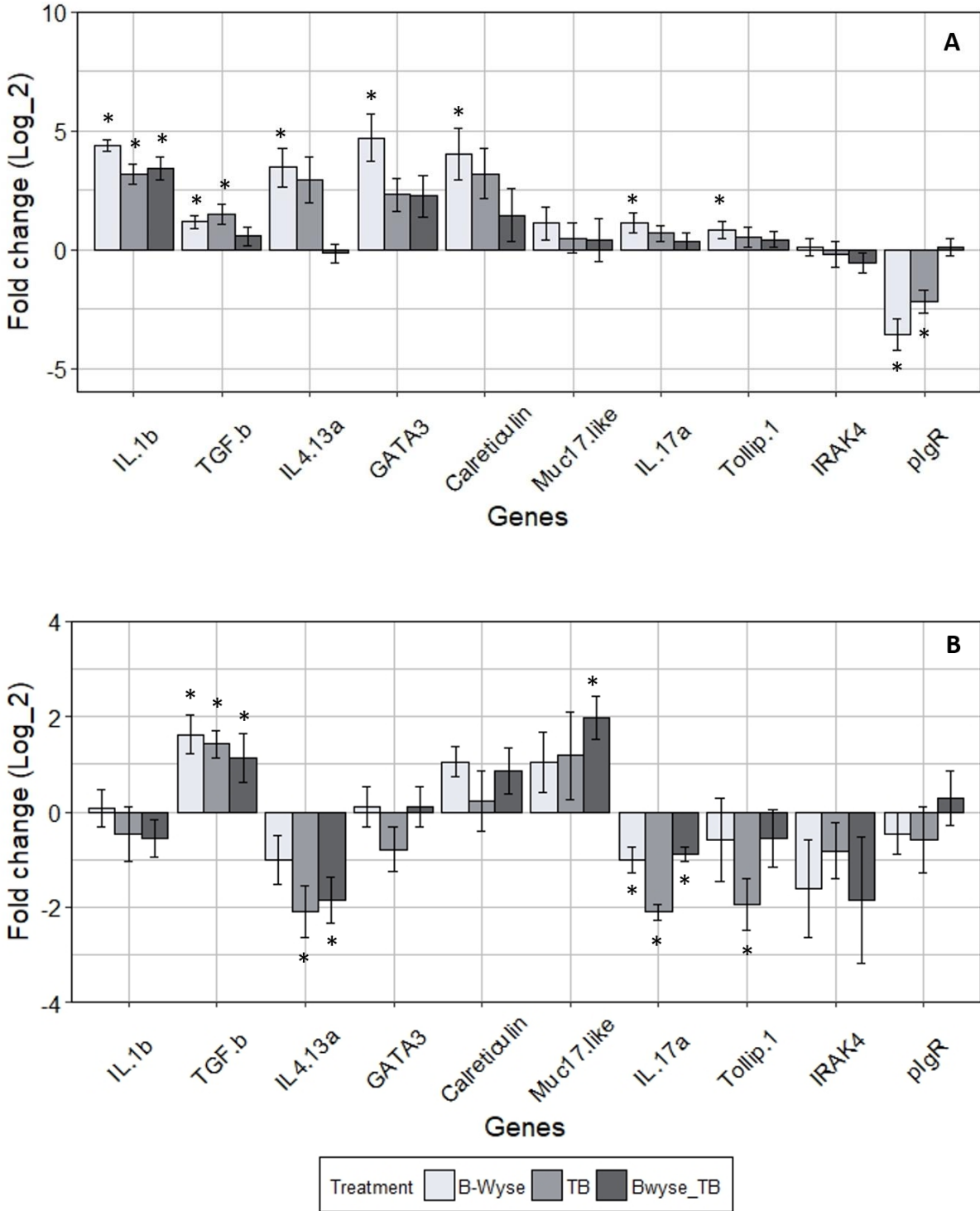
**Figure 3.7** Gene expression data (fold change (log<sub>2</sub>)) of 10 genes of interest in the SALT of fish fed three different experimental dietary regimes after A) five weeks and, B) 8 weeks. Data presented as mean  $\pm$  SEM; n=6. Asterisk denote a significant difference between the experimental and control dietary regimes ( $p < 0.05$ ).



### 3.3.4.3 Intestine

The relative fold changes compared to the control of the 10 selected genes in intestinal tissue after 5 and 8 weeks are presented in Figures 3.8 A) and B) respectively. In the distal intestine, fish fed the diets supplemented with B-Wyse ( $p = 0.002$ ), TB ( $p = 0.006$ ), and B-Wyse + TB ( $p = 0.013$ ) treatments exhibited significant upregulation in the expression of the pro-inflammatory cytokine IL-1 $\beta$ , and of TGF- $\beta$  for the B-Wyse ( $p = 0.032$ ) and TB ( $p = 0.006$ ) treatments after 5 weeks, compared to the control-fed fish. After 8 weeks, significant upregulation in the expression of TGF- $\beta$  was observed in fish fed all three of the experimental diets (B-Wyse  $p = 0.004$ ; TB  $p = 0.022$ ; B-Wyse + TB  $p = 0.039$ ), compared to the control-fed fish. Fish fed the B-Wyse treatment exhibited significantly increased expression of IL4/13a ( $p = 0.013$ ), GATA3 ( $p = 0.004$ ), and IL-17a ( $p = 0.041$ ) compared to control-fed fish after 5 weeks of feeding, while no significant differences in the expression of these genes were observed for fish fed the TB or B-Wyse + TB diets. After eight weeks, the TB ( $p = 0.002$ ) and B-Wyse + TB ( $p = 0.006$ ) treatments exhibited significantly reduced expression of IL4/13a compared to the control fish, while all experimental treatments were observed to have significantly reduced expression of IL-17a (B-Wyse  $p = 0.006$ ; TB  $p = 0.002$ ; B-Wyse + TB  $p = 0.002$ ), and the TB treatment alone showed significant downregulation in the expression of the Tollip1 gene ( $p = 0.015$ ), in comparison to the control-fed fish.

Concerning the mucosal-associated genes, after 5 weeks, fish fed the B-Wyse treatment demonstrated significantly increased mRNA expression of calreticulin ( $p = 0.017$ ) compared to the control, while both the B-Wyse ( $p = 0.004$ ) and TB ( $p = 0.019$ ) treatments exhibited significant downregulation in the expression of the plgR gene. After 8 weeks, only the B-Wyse + TB treatment exhibited significant increases in the expression of Muc17-like ( $p = 0.039$ ).



**Figure 3.8** Gene expression data (fold change (log<sub>2</sub>)) of 10 genes of interest in the GALT of fish fed three different experimental dietary regimes after A) five weeks and B) 8 weeks. Data presented as mean ± SEM; n=6. Asterix denote a significant difference between the experimental and the control dietary regimes ( $p < 0.05$ ).

### 3.4 Discussion

For several decades, the administration of various additives within the diets of numerous aquacultured species have been documented to influence a number of wide-ranging beneficial effects (as described in chapter 1; Ringo and Song, 2016; Hoseinifar *et al.*, 2019). Among those currently under investigation, there exists a wealth of published investigations documenting the health promoting properties of prebiotics and probiotics, in addition to their stimulatory ability to promote changes within the host that are deemed beneficial (Merrifield *et al.*, 2010; Ringø *et al.*, 2014; Ringø *et al.*, 2018). The present study investigated the individual and combined effects of two commercially available dietary additives upon i) growth performance indices of the fish, ii) epidermal mucous quality and quantity, iii) the structure and integrity of the gill and skin mucosal-associated lymphoid tissues and iv) the expression of 10 immune-related and mucous-associated genes within the skin-, gill- and gut-associated lymphoid tissues.

#### *3.4.1 Growth indices of rainbow trout*

Growth, nutrient utilization and survival rates of fish are the basic criteria that determine the production, productivity and profitability of fish culture operations. Over the 8-week duration of the current trial, the overall performance of the fish was excellent: mean body weight increased 2.5-fold, mortality was consistently low with a survival rate above 98% across all treatments, in addition to exceptional FCR values within the range of 0.85-0.91. No significant differences were observed in growth performance indices between fish fed the experimental dietary regimes and the control treatment. Under the excellent rearing conditions of the current study and the administration of high quality diets that induce good animal

performance, there appeared to be little scope for the experimental dietary additives investigated here to induce any positive benefits on the growth metrics.

These results differ from the findings of other studies. In a recent study of similar length on Caspian trout (*Salmo trutta caspius*), fish fed a diet supplemented with a  $\beta$ -glucan + MOS mixture (3g and 4g inclusion of  $\beta$ -glucan and MOS respectively, per kg of basal diet) demonstrated significantly elevated final weight, weight gain and significantly reduced FCR compared to fish fed the basal diet (Jami *et al.*, 2019). Similarly, a study assessing the effect of the commercial prebiotic product “mainly composed of  $\beta$ -glucan and MOS derived from the yeast cell wall of *S. cerevisiae*” on rainbow trout, reported significant increases in final weight and weight gain, and significantly reduced FCR values in fish fed the prebiotic-supplemented dietary regime, compared to those fed the basal dietary regime (Khodadadi *et al.*, 2018). However, the FCR values for the control-fed fish in both of the mentioned studies were reported to be  $1.65 \pm 0.33$  for the Caspian trout and  $1.39 \pm 0.06$  for the rainbow trout study. These poor FCR values would suggest that the basal control diets were not of optimal nutrient quality, resulting in low FCR values for the control-fed fish but also allowing for the improvement of nutrient utilisation, growth and weight gain in fish fed diets containing the experimental additives. Caspian trout fed the  $\beta$ -glucan + MOS supplemented diet exhibited a significant improvement in FCR value of  $1.07 \pm 0.15$  (Jami *et al.*, 2019), while the FCR of the prebiotic-fed rainbow trout significantly improved to  $1.04 \pm 0.06$  (Khodadadi *et al.*, 2018). Drawing comparisons between the results of different investigations is challenging as a large number of environmental, biological and nutritional factors and variables may differ between studies, all of which may have an impact upon the observed results. Providing as much detail as possible in research publications allows for the prospective reproducibility of results and a means of reference for determining potential reasons for differing results.

While information concerning the effect of dietary supplementation of heat-inactivated bacteria on the growth of aquacultured species is rare, a handful of studies have been performed. However, the results are predictably contentious, with some studies reporting similar (Dawood *et al.*, 2015b; Wang *et al.*, 2017) and others dissimilar (Dawood *et al.*, 2015c; 2016; Yan *et al.*, 2016) findings to those of the present study. Wang *et al.* (2018) fed juvenile orange-spotted grouper either a basal diet or one that included  $1.0 \times 10^8$  CFU  $g^{-1}$  of live *B. clausii* DE5 or one that included  $1.0 \times 10^8$  CFU  $g^{-1}$  of heat-inactivated *B. clausii* DE5 for 60 days. Similar to the findings of the present study, the final weight, weight gain and SGR of the fish remained unaffected by both viable and heat-inactivated bacterial inclusion, compared to the control. As growth performance is a factor of cellular and tissue growth, and is therefore highly influenced by the quality and quantity of the dietary protein fed to the fish, the authors proposed that the relatively high protein content (506g/kg) of the experimental diets may have masked the potential growth promoting effects of the bacteria, regardless of viability (Wang *et al.*, 2018). The same limiting mechanisms could be a potential reason for the lack of differences in growth performance observed in the present study, as the protein content of the dietary regimes was optimal at approximately 460 g/kg. Further, the dietary formulation was overall reflective of a high quality commercial diet, theoretically further limiting the scope for benefits.

In a similar study of the same length in which the application of a different *Bacillus* species was assessed in orange-spotted grouper, Yan *et al.* (2016) supplemented a basal diet with  $1.0 \times 10^8$  CFU  $g^{-1}$  of either live *Bacillus pumilus* SE5 or one that included  $1.0 \times 10^8$  CFU  $g^{-1}$  of heat-inactivated *B. pumilus* SE5. Perhaps surprisingly, fish fed the parabiotic demonstrated significantly improved weight gain, final weight and SGR compared to those fed the control and viable probiotic diet (Yan *et al.*, 2016). Although the precise mechanisms remain unclear,

the authors have previously reported on the ability of heat-inactivated *B. pumilus* SE5 to influence the intestinal microbiota and the mucosal immunity of orange-spotted grouper (Yang *et al.*, 2014). This maintenance of intestinal homeostasis is considered a benefit, aiding digestion and metabolism within the intestine and may therefore explain, at least in part, the enhanced growth performance. Furthermore, a pioneer study of note, reported that the probiotic *L. rhamnosus* decreased feed intake in zebrafish larvae in addition to the simultaneous increase in the expression of genes responsible for reducing feed intake (leptin and mc4r), and decreased expression of cb1 and npy genes, which are responsible for enhancing appetite stimulus (Falcinelli *et al.*, 2016), suggesting towards the potential of the probiotic to modulate the expression of genes involved in appetite control in zebrafish larvae (Wang *et al.*, 2018). Additionally, Hosseini *et al.* (2016) investigated the effect of *L. acidophilus* as a probiotic in gold fish and reported the downregulated expression of genes relating to appetite (Ghrelin) in fish fed the probiotic-supplemented diet, compared to fish fed the control regime. The specific mechanisms by which suppression of appetite correlates with improved growth performance and digestive function remains unclear, yet it remains possible that this effect may not be specific to the *Lactobacillus* genus (Rodiles *et al.*, 2018). Further investigations into the potential of bacterial probiotics to modulate the expression of genes involved in appetite control and metabolism are warranted.

#### 3.4.2 Epidermal mucous measurements

Acting as the first line of defence, fish possess unique physical barriers that prevent the body from being directly exposed to pollutants, pathogens and stressors present within the surrounding environmental water. At the mucosal sites, mucous secretion is one of the most important innate defence mechanisms (Lowrey, 2014). Previous studies have demonstrated

the capacity of LAB (Salinas *et al.*, 2008) and MOS (Torrecillas *et al.*, 2011) to modify epidermal mucous production in fish. More recently, Rodriguez-Estrada *et al.* (2013) reported that mucous production was significantly increased in rainbow trout fed diets containing heat-inactivated *Enterococcus faecalis*, MOS, and a combination of the two. Similarly, mucous production was increased in red sea bream (*Pagrus major*) fed a diet supplemented with heat-killed *L. plantarum* +  $\beta$ -glucan (Dawood *et al.*, 2015c) and a heat-inactivated *Pediococcus pentosaceus* preparation strain D3286 (Dawood *et al.*, 2016). In the present study and in contrast to the studies mentioned, while numerical increases in the amount of secreted mucous were observed for fish fed all three supplemented diets, these differences were not significantly greater than the amount produced by the control-fed fish. At present, there remains a lack of definitive explanation as to how dietary additives and supplements work to effect epidermal mucous secretion in fish. The composition of mucous is very complex and varies among fish species, while endogenous factors (such as sex and developmental stage), and exogenous factors (such as stress and infections), have demonstrable influence (Dash *et al.*, 2018). These factors may cause significant changes to the composition, viscoelasticity, rheology and functionality of the mucous and to modifications of the mucosa, including the number, size and distribution of skin mucous cells (Dash *et al.*, 201; Fæste *et al.*, 2019). Similarly, the method of sample collection may also affect characteristics of the skin mucous. In a study in which epidermal mucous samples were collected via three different methods (absorption to medical wipes, wiping from head to tail with a medical wipe, and scraping from head to tail with the back of a blunt scalpel blade), the consistency, volume and overall protein content of the samples were found to differ between collection method in addition to the proteomic profile of individual proteins (Fæste *et al.* 2019). In light of these results Fæste *et al.* (2019) therefore proposed employing the absorption technique for investigations where

it is important that the mucous be devoid of proteins from the underlying epithelium, and promote the wiping method when protein yield is of importance or when the proteome of the outer epithelium is of specific interest.

#### *3.4.3 Histological appraisal of the GIALT and SALT*

The quantitative assessment of epidermal mucous remains technically challenging (Jensen *et al.*, 2015). The use of histological techniques is becoming a more commonly employed method of appraisal, as the number of mucous producing cells has been suggested to reflect the health status of the mucosal tissues and as a possible stress index for fish (Sveen *et al.*, 2017). Several adverse biotic and abiotic environmental factors such as reduced pH (Zuchelkowski *et al.*, 1981), high nitrate and low O<sub>2</sub> (Vatsos *et al.*, 2010a), aluminium exposure (Ledy *et al.*, 2003), and the presence of pathogens (van der Marel *et al.*, 2010) can reportedly affect the morphology and structure of fish skin, with particular effect on the mucous cells. In the gills, the presence of eco-toxins (Bols *et al.*, 2001) and changes in water quality (Hoole *et al.*, 2001) have also been reported to cause an increase in mucous cell numbers.

However, from just a handful of studies, it has become apparent that the method of morphological appraisal is in need of standardisation in order to allow comparisons between investigations and their results. Firstly is the matter of target site for sample collection. Different investigations have varied greatly in their chosen sample area including between the eyes (Vatsos *et al.*, 2010a), behind the head above the operculum (Jensen *et al.*, 2015), surrounding the caudal peduncle (Hallberg, 2018), and in the case of the present study and others, dorsolaterally adjacent to the dorsal fin. Moreover, the contrasting mucous cell densities at four different body sites of Atlantic salmon have been described: significantly denser and larger mucous cells are present in the dorso lateral skin, whereas the lowest mean



mucous cell density and smallest area are found on the head (Pittman *et al.*, 2013). These results have since been supported by results of other trials that caution comparisons between different sample sites on the fish (Jensen *et al.*, 2015; Hallberg, 2018). Earlier work by Fast *et al.* (2002) demonstrated the significantly thicker epidermis and significantly elevated mucous cell number of rainbow trout skin compared to Coho (*Oncorhynchus kisutch*) and Atlantic salmon skin; comparisons between species should therefore carry caution also.

Secondly are the processing methodologies. Thick scales can often impede histological sectioning and therefore necessitate decalcification of the tissue prior to processing. However, some studies claim this process may affect the absolute measure of mucous cell size, as may the embedding medium; skin samples that underwent Technovit embedding contained overall larger mucous cells compared to those embedded in paraffin wax (Pittman *et al.*, 2013).

The most common method of histological skin analysis involves transverse sections of the embedded sample to give a layered view of the skin and the cells within. Measurements of these cells is perhaps the most contentious factor effecting comparable and reliable results as the choice of where and what to measure are rather subjective. Often, measurements do not consider the epidermal areas folded under and around the scales where mucous cells have been shown to aggregate in species such as Japanese flounder (*Paralichthys olivaceus*) (Yamamoto *et al.*, 2011). Additionally, the cross-sectional area of the mucous cells can often be misleading as the profile of the cell may increase/decrease between successive tissue sections. The specialist technique of the investigator and their ability to section to a consistent level across samples are vital to provide confident results of a reliable nature. At present, technological advancements are under development with the aim of eliminating the

subjective nature of measurements as much as possible. The extent to how well this will be achieved is yet to be determined in the absence of a greater number of comparable studies concerned with the morphological responses of the mucosal tissues.

To date, only a handful of studies have investigated the effect of dietary additives upon micro-anatomical structure of the skin- and gill-associated lymphoid tissues; instead, there is far more interest into the effect of stressors and pathogens upon these tissues. However, considering the direct contact of the SALT and GIALT with the external environment and their subsequent role as a primary immunological barrier, there exists an urgency to generate knowledge that allows for the understanding of the influence of immune-supplemented diets in these tissues. Although there is a lack of direct or targeted contact between dietary additives and the SALT or GIALT, it has been proposed that an indirect mechanism of influence may be plausible (Caipang and Lazado, 2015), while recent research has suggested at the potential interconnectivity of the associated-lymphoid tissues and is a concept now receiving increased attention (van der Marel *et al.*, 2012).

In the present study, the abundance of goblet cells in the SALT and GIALT, in addition to the goblet cell area and CAF (in relation to epidermal thickness) of the SALT were measured. After 5 weeks, fish fed the prebiotic diet has significantly more goblet cells in their skin tissue than fish fed the other three dietary regimes, suggesting dietary stimulation as the cause of goblet cell proliferation within the tissue. Numerical density however is not a very informative measure because it does not consider the volumetric density of the cells. For example, five very small goblet cells could have a smaller combined area than one very large goblet cell. To this end, the combined total area of goblet cell coverage within the tissue was also calculated. Fish fed all three of the experimental diets exhibited significantly greater areas of goblet cell

coverage within the skin epidermis compared to fish fed the control regime after five weeks. This suggests that prebiotics, parabiotics and a combination of the two are capable of influencing the size of goblet cells within the epidermal tissue of rainbow trout at the dosages used here (B-Wyse = 1.5g/kg<sup>-1</sup> inclusion; TB = 0.3g/kg<sup>-1</sup> inclusion; B-Wyse + TB: 1.5g/kg<sup>-1</sup> and 0.3g/kg<sup>-1</sup> respective inclusion).

While this volumetric measure of goblet cells is far more informative than numerical density alone, it does not consider the tissue as a whole. As a uniquely complex and metabolically active tissue, the skin of fish and its components orchestrate several vital responses and processes, such as communication, sensory perception, respiration and ion regulation (Ángeles Esteban, 2012; Lazado and Caipang, 2014). It therefore follows logically that the epidermis as a whole should also be taken into consideration when investigating one of its most predominant constituents, the goblet cells. In order to place the total area of goblet cell coverage into the context of the tissue, the total epidermal area was also quantified and used to calculate the goblet cell area fraction within the skin. Fish fed all three of the supplemented dietary regimes exhibited significantly elevated goblet cell area fractions compared to those fed the un-supplemented control regime, after five weeks and eight weeks. These results further support the proposal that dietary inclusion of prebiotics, parabiotics and a combination of the two, act in a stimulatory capacity upon goblet cell morphology within the skin tissue of rainbow trout. However, these results stand in contrast to those of the investigation on European sea bass in which the ratio of epithelium to mucous cells in the skin was not affected by dietary inclusion of MOS or soybean oil as an alternative to fish oil (Torrecillas *et al.*, 2015). Additionally, it should be noted that both the number of goblet cells and the chemical composition of the mucous they produce varies greatly among fish genera

(Peterson, 2015); these confounding results may therefore be a function of species physiology rather than efficacy of the dietary supplement. A challenge study was performed recently to examine the effect of an immunostimulant and essential oil supplementation on sea lice prevalence; four experimental diets were fed to Atlantic salmon for 4 weeks prior to challenge with sea lice copepodids (Jensen *et al.*, 2015). Unfortunately, MALT tissue samples were not obtained prior to challenge and following 4 weeks of feeding the experimental diets in order to ascertain whether a dietary effect was acting upon the morphology of the mucous-associated lymphoid tissues; studies of this nature should be encouraged to help better understand the effect and influence of immune-supplemented diets in these important tissues.

Calculating the goblet cell area fraction allows for a much more representative assessment as a large number of small cells may give the same mucosal density as a few large cells. Taking the area of epidermis into consideration also, allows for the true representation of the goblet cell coverage within the tissue; comparisons may then be drawn between individual fish and body areas. A definitive consensus regarding the optimum size of mucous cells remains to be elucidated. It has been proposed by Pittman *et al.*, (2018) that the size of the mucous cell is an indication of how quickly it can fill with mucous and migrate to the outer epidermal surface. It follows logically that smaller cells would have the advantage of becoming full with mucous at a faster rate and begin to migrate more rapidly through the tissue to expel their contents as the ease and rapidity of migration through the tissue may be limited by cell size. Once a threat is detected, necessitating the need for more epidermal mucous, smaller cells may be more beneficial in this respect. However, in the absence of a pathogenic threat of environmental stressors, larger mucous cells may in fact be more beneficial as less mucous is unnecessarily produced at an energetic cost to the fish.

The chemical composition of the mucous also deserves consideration here. As the main constituent of mucous, mucins may be categorised as two different types: acidic and neutral. It has been postulated that acid mucous cells (cells producing mucous that is rich in acidic mucins) are associated with increased mucous viscosity (Vatsos *et al.*, 2010). This in turn would increase the protective role of the mucous, whilst simultaneously decreasing friction with the surrounding water, leading to reduced sloughing and reduced mucous turnover, leading to reduced demand for mucous and so less need for many or indeed bigger mucous cells. Therefore, more abundant or larger mucous cells should not necessarily be considered advantageous to the fish and future studies should work to determine the acidity/ chemical composition of the mucous. As already mentioned, the assessment of epidermal mucous remains technically challenging, yet a simple methodology exists to determine the acidity of the mucous cell (and by extension, the mucous they contain) upon histological examination with the use of Alcian Blue/ Periodic Acid Schiff stain. Staining acidic cells blue and neutral cells pink, the simultaneous collection of data concerned with the acidity of the mucous cells in addition to morphometric analysis of the cells and epidermal tissue would be greatly beneficial.

Drawing attention to the prebiotic treatment in this study, goblet cell abundance and total area coverage were found to be significantly elevated in fish fed the prebiotic regime after five weeks compared to the control regime, which would suggest the presence of a greater number of larger goblet cells. After eight weeks of feeding, these differences between the control and experimental treatment are no longer significantly different, yet in terms of goblet cell area fraction, the prebiotic treatment is significantly elevated above that of the control treatment at both five and eight weeks. Insignificant differences in abundance and area coverage, yet significant increases in area fraction are suggestive of a thinning of the

epidermal tissue, as a similar amount of goblet cells are present yet their density significantly increases. Whether or not this thinning is an additional effect of the dietary supplement or of the environment remains to be definitively elucidated. A recent study by Jensen *et al.*, (2015) proposed rearing temperature as an influencing factor on the epidermal morphology of Atlantic salmon: compared to fish reared at lower temperatures, fish reared at 16°C exhibited significantly elevated mucous cell density, and significantly reduced epidermal thickness. As no other variable differed between the treatments, these observed differences were attributed to the increased temperature (Jensen *et al.*, 2015). The inclusion of epidermal thickness measurements in response to dietary supplement administration in future studies may provide further clarity. A decrease in epidermal thickness would benefit the migration of mucous cells from the basal membrane, having a shorter distance to diffuse before reaching the outer epidermis to expel their contents. This would in turn benefit the fish in terms of response time to stressors or agitators.

The gills are an important immune-regulating organ, representing a considerable amount of the total surface area of the fish (Matey *et al.*, 2008). Numerous studies under laboratory conditions and in natural environments have demonstrated that the morphological evaluation of gill tissue may serve as an indicator for assessing the health status of the fish (Strzyżewska *et al.*, 2016). Changes in gill morphology occur much quicker than alterations in behaviour or exterior appearance (Yancheva *et al.*, 2015) and may cause further disturbances to basic physiological processes, such as osmoregulation or anti-oxidative defence mechanisms (Strzyżewska-Worotyńska *et al.*, 2017). Previously designated as “sentinel” organs, the gills of fish have been described as having two distinct populations of mucous cell: those located on the lamellar are typically small and of low density when the fish is healthy, while goblet cells located on the filament are larger, denser and reflect the systemic health of

the fish (Pittman *et al.*, 2018). Numerous studies have reported changes in the numbers of goblet cells present within the gill tissue of several species of fish in response to several environmental stressors, including acidic and aluminium infused rearing water (Jago and Haines, 1997), salinity (Roberts and Powell, 2003) and organophosphates (Moron *et al.*, 2018). Often and in addition to goblet cell changes, a variety of rapid cellular modifications also occur, including changes to the chemical composition of the mucous produced and stimulated mucous production (Ledy *et al.*, 2003; Moron *et al.*, 2018). Whether changes in goblet cell populations and in mucous quantity interfere with the protective actions of the gills against infectious organisms is still a matter of speculation (Bols *et al.*, 2001). Early research observed hyperplasia of gill epithelium in response to a deficit in pantothenic acid, while deficits in vitamin C were observed to caused gill deformations and functional impairment (Wilson *et al.*, 1983; Karges and Woodward, 1984). It therefore appears justifiable to infer that nutrient availability and feeding may affect gill physiology (Strzyzewska *et al.*, 2016), yet studies concerned with the effect of dietary additives on the goblet cells present within the GIALT are scarce. In the present study, no changes in the goblet cell abundance were observed in the gills of fish fed the three differing dietary regimes. Nevertheless, further studies should continue to explore the possible effect of dietary additives upon goblet cell populations and morphology within the GIALT.

#### *3.4.4 Expression of mucosal-associated genes*

The expression of certain genes relating to mucosal health and epithelial integrity can also be measured in order to assess the health status of the different MALTs. Mucins are well established as the main component of epidermal mucous, yet despite numerous publications concerned with the histological appraisal of mucous cells and the protective role of the

mucous layer, little knowledge exists about mucin-encoding genes in fish (Sveen *et al.*, 2017). The majority of current knowledge regarding mucins is based on mammalian studies. Several mucin isotgls have been predicted in some fish species, based on their homology to mammalian mucins, while only a handful of studies have been performed to date assessing mucin structure and tissue expression in carp (van der Marel *et al.*, 2012), gilthead sea bream (Pérez-Sánchez *et al.*, 2013), zebrafish (Jevtov *et al.*, 2015), Atlantic salmon (Sveen *et al.*, 2017), and sea trout (*Salmo trutta m. trutta*) (Malachowicz *et al.*, 2017).

One of the first studies to investigate the pattern of mucin distribution in fish tissues was that by Perez-Sanchez and colleagues (2013). Following the identification of six mucin gene sequences within the genome of gilthead sea bream, the levels of expression were analysed across seven tissues, including those of the GIALT, SALT and GALT. The authors further reported that the gene expression pattern of gut mucins is altered by dietary oils and additionally propose mucins as genetic markers for fish intestinal health (Perez-Sanchez *et al.*, 2013). Another study in in common carp, reported increased Muc5B expression in the skin of fish fed a  $\beta$ -glucan supplemented diet, alongside decreases in Muc2 and Muc5B expression in the gills (van der Marel *et al.*, 2012). It would therefore appear that dietary additives have the potential to regulate the expression of mucins not only in the intestine but also in the skin and gill, further underscoring the interconnection of mucosal tissues.

In the current study, the expression of Muc17-like, was assessed in the GALT, GIALT, and SALT in response to the three dietary additives. In the GIALT, after 5 and indeed 8 weeks, fish fed all three supplemented diets exhibited significantly elevated expression of Muc17-like compared to those fed the control regime. Significantly elevated expression of Muc17-like was observed in fish fed all three of the supplemented treatments in the SALT after 8 weeks,



whereas only the synbiotic diet exhibited significantly increased expression of Muc17-like in the GALT after 8 weeks. These results support those of the study in common carp (van der Marel *et al.*, 2012), the suggestion that dietary additives are able to influence mucin gene expression in the GALT and SALT, and that Muc17 may serve an important role within these tissues. The results of the GALT are suggestive of a beneficial synergistic effect of the synbiotic diet as a stimulant of Muc17-like expression in this tissue.

In humans, MUC17 is reportedly highly expressed in the small and large intestines and, as a transmembrane mucin, it is expected to have important functions in the control of the mucosal surface environment (Malmberg *et al.*, 2008). However, despite first being cloned more than 25 years ago, information pertaining to its precise function and scope of regulation remains largely unknown (Schneider *et al.*, 2019). That said, a recent study induced an inflammatory state in human Caco-2 cells via long-term stimulation with the pro-inflammatory cytokine TNF $\alpha$ , resulting in increased MUC17 expression (Schneider *et al.*, 2019). Furthermore, the authors proposed a new function of MUC17 in inflammation, where MUC17 acts as a second line of defence by preventing the attachment of bacteria to epithelial cells in the small intestine. Considering the gills and skin are important portals of pathogen entry, with a high blood flow and large surface area exposed to the external environment, if Muc17 performs a similar function in the gills and skin of fish as MUC17 in human intestine, the induced elevated expression of Muc17, as observed in the present study, would be extremely beneficial in maintaining the homeostasis of these important tissues.

Interestingly, murine Muc3 is now considered to be the orthologue of human MUC17 as they have high sequence similarity and are located close to the same gene (Malmberg *et al.*, 2008). They are so similar in fact that MUC17 is seemingly more similar to rat and mouse Muc3 than

to any other known human protein (Moniaux *et al.*, 2006). Previous investigations reported that the cysteine-rich domains of murine Muc3 play an active role in epithelial restitution (Ho *et al.* 2006), that is, the re-establishment of a superficial epithelium following damage: in the absence of mitosis, undamaged cells migrate across the wound, forming new tight junctions and repolarizing organelles (Lacy, 1988). Moreover, data from an alternative investigation indicates that cytokines and growth factors are capable of regulating Muc3 gene expression, suggesting that this protein may play an important role in intestinal mucosal defence (Shekels and Ho, 2003), while it has also been reported that a large number of inflammatory mediators can directly enhance mucin transcription and glycosylation pattern in mammals (Sveen *et al.*, 2019). Although Muc3 has not yet been identified within the teleost genome, considering the high degree of likeness previously observed between murine-Muc3 and human-MUC17, it is possible that human MUC17 and teleost Muc17 execute similar roles such as those described above. This would be most advantageous in teleosts especially during incidences of wound healing in tissues under constant exposure to the external environment and pathogenic insults such as the MALTs. The further identification and characterisation of teleost mucins is vital to broaden the knowledge of mucin function and distribution within the MALT tissues.

In accordance with a previous investigation involving the addition of a yeast cell wall extract to the diets of Atlantic salmon (Micallef *et al.*, 2017), the expression of calreticulin was significantly upregulated in the skin tissue of rainbow trout fed the prebiotic- and synbiotic-supplemented diets in the present study. Calreticulin is a multi-functional chaperone protein, which binds to glycoproteins in the endoplasmic reticulum and is involved in their folding and degradation: in the context of this study and that of Micallef *et al.* (2017), it is particularly interesting that calreticulin is directly involved in the synthesis of mucins. The results observed in this study support the proposition of calreticulin as a potential biomarker for

yeast-derived functional feeds as the prebiotic and synbiotic diets used in this study contain yeast cell wall extracts. However, the results of this study do not support the proposal of calreticulin as a skin-specific protein because a significant upregulation in expression of calreticulin was also observed in the intestine of fish fed the prebiotic treatment for five weeks. While, the study by Micallef and colleagues (2017) did not analyse the expression of calreticulin in the intestine, species differences and diet composition should also be taken into consideration when attempting to directly compare results of these different nutrition trials.

#### *3.4.5 Expression of TJ-associated genes*

Changes to mucin composition cause alterations to the structure of the mucous, which may in turn compromise the stability of the underlying epithelium (Parra *et al.*, 2015). The barrier function of the epithelium is maintained by the formation of complex protein-protein networks which mechanically interlink neighbouring epithelial cells via the formation of adhesive complexes: desmosomes, adherence-junctions and TJs are different types of cell-adjoining complexes and are considered vital to maintaining epithelial integrity and conformity (Nawaz *et al.*, 2018).

Since the first claudin protein was reported in a teleost fish some 20 years ago, we are now aware of a further 62 claudins in 16 different teleost species (Kolosov *et al.*, 2013a). The expression of claudin genes provides valuable information pertaining to the overall health of the epidermal tissue at the mucosal sites, whilst simultaneously providing further insight into the molecular architecture of these complexes. Yet, despite an increasing number of studies examining gill and skin epithelial TJ proteins in relation to changes in the external environment, such as environmental acidification (Kumai *et al.*, 2011) salinity and

corticosteroid induced stress (Tipsmark *et al.*, 2009), there exists a paucity of information regarding the ability of dietary ingredients and additives to regulate TJ-associated gene expression in the tissues of the MALT.

In the current study, claudins 10d and 10e exhibited significantly elevated transcription levels in the GALT of fish fed all three of the supplemented diets after eight weeks. However, claudin 10d was significantly down regulated in the skin of fish fed all three supplemented diets after eight weeks. In support of these results, previous research has documented the expression patterns of claudin isoforms to vary spatially within tissues, between different tissues of the same animal, and between different species, confounding insight into the potential function of certain claudin genes (Caipang *et al.*, 2011; Chasiotis *et al.*, 2012; Kolosov *et al.*, 2013b). For example, claudin 27a was found to be largely unchanged in the gills of Atlantic salmon acclimated from freshwater to seawater (Tipsmark *et al.*, 2008), yet significantly increased expression was observed in the gills of the green-spotted pufferfish (*Tetradon nigroviridis*) undergoing the same environmental acclimation (Bagherie-Lachidan *et al.*, 2009). Taken together and considering the considerable size of the claudin superfamily, it is understandably rather challenging to elucidate exact functions of a specific claudins in a specific tissue of a specific species in the absence of more data and investigations. Broadening the current knowledge of claudin function will allow for the further elucidation of the MALT tissues in response to dietary supplementations of functional feed additives and the immunopotentiating effects they may exert.

Earlier studies performed in Atlantic salmon demonstrated the salinity regulation of five gill specific claudins, including claudin 10e, leading the authors to propose remodelling of the gill epithelium in response to ambient salinity changes as a specific role for this claudin in the gill

(Tipsmark *et al.*, 2009). More recently, claudin 10d in addition to claudin 10e was documented as salinity responsive at a transcriptional level in the gill tissue of the green-spotted pufferfish (Bui and Kelly, 2014). Furthermore, the expression of claudins 10d and 10e have been observed as being at least one order of magnitude higher in the gill tissue of rainbow trout than any of the other nine tissues investigated (Kolosov *et al.*, 2014). Collectively, and in support of the result of this investigation, these findings infer the functional importance of these claudins within the gill tissue of rainbow trout. Although the definitive function of these claudins and the mechanisms by which the dietary additives induce their increased expression in gill tissue remains unknown, it appears reasonable to classify the up-regulation as beneficial.

The skin of adult fishes is classically regarded as a relatively static and passive barrier to diffusional loss in freshwater, as skin from the trunk of the fish is well documented as being devoid of any significant capacity for active ion movement (Gauberg *et al.*, 2016). Nevertheless, recent investigations have revealed the presence of a considerable number of genes encoding tight-junction proteins in the skin of several species of adult fish, some of which significantly alter in abundance upon exposure to varying ionic environments (Kolosov *et al.*, 2013). Although fish fed all three of the experimental diets in this study exhibited significant down-regulation of claudin 10d in the skin after 8 weeks of feeding, a differential expression along dorso-ventral axis of the fish has been documented in numerous genes (Caipang *et al.*, 2011) of which claudins appear to be no exception (Gauberg *et al.*, 2016). Skin samples in this study were collected from the dorsal side of the fish yet it is plausible that the upregulation of claudin 10d may be occurring in the ventral skin where osmotic control may indeed be occurring. Future studies should consider analysing multiple skin samples across the dorso-ventral axis to attain a better understanding of the transcriptional responsiveness

of claudin genes across the axis. Alternatively, the stimulated down-regulation of claudin 10d by the experimental additives may constitute an energy-saving mechanism, conserving energy to be used in the up-regulation of different claudins or other homeostatic/ metabolic processes that serve a greater importance in the skin than claudin 10d. Although the expression of claudin 10d and 10e has been reported in other species (Bui and Kelly, 2014), the lack of data and functional investigations concerning claudins in the skin of rainbow trout make definitive explanations difficult.

A significant upregulation in the expression of claudin 30 was observed in the skin of rainbow trout after 8 weeks of feeding the synbiotic diet. This demonstrably synergistic dietary effect significantly elevated expression levels beyond those observed for the control fed fish and indeed those fed diets containing the individual additives. However, numerous investigations have suggested that claudin 30 may serve a tissue-specific function in gill tissue (Chasiotis and Kelly, 2008; 2011; Tipsmark *et al.*, 2008). It has been previously suggested that claudin 30 enhances the barrier properties of the gill epithelium based on observational changes in mRNA or protein abundance in whole gill tissue following or during alterations in environmental conditions such as salinity (Tipsmark *et al.*, 2009) or in response to permeability altering endocrine factors (Kolosov *et al.*, 2013a). Utilising a flask-cultured gill epithelium model, Kolosov and colleagues further proposed that claudin 30 is important in establishing cell-to-cell contact, and further suggest it plays a significant role in the establishment of gill epithelial integrity (Kolosov *et al.*, 2014). The stimulated expression of claudin 30 in the skin of rainbow trout by the addition of a synbiotic, in the present study, would prove beneficial to the fish if it performs the same function as described in the gill, further serving to increase the epithelial integrity of the skin and the defences of the fish.

Additional investigations into claudin function, expression and regulatory ability in all three of the mucous-associated lymphoid tissues of fish are warranted to better our understanding.

#### *3.4.6 Expression of immune-related genes*

The role and potential influence of dietary additives including prebiotics, probiotics and their combination as synbiotics on immune-related gene expression in different fish species has been reported within the literature (for review refer to Martin and Król, 2017). It is a growing field of investigation and one that is beginning to provide much-needed answers to many functional and applicability-related questions regarding the use of dietary additives to improve the sustainability and productivity of the aquaculture industry. However, at present, our knowledge of the immune system of fish remains incomplete which is a limiting factor when attempting to link immune functions to nutrient and/or additive intake. In addition to the mucous- and TJ-associated genes discussed above, the responsive expression of certain immune-related genes to the dietary additives were also analysed in the present study. The results presented here are brief and serve to highlight the potential capacity of the experimental additives to influence immune-related gene expression not only at the GALT but also in the GIALT and SALT: a further, more in-depth analysis of a wider range of genes will be presented in the following chapters.

Several investigations have hypothesized that functional feed additives such as probiotics, prebiotics and their combination as synbiotics hold the potential to activate the innate immune system of aquatic animals in two ways: (1) by directly stimulating the innate immune system or (2) by enhancing the growth of commensal microbiota (Dawood *et al.*, 2018). Although our knowledge of the exact mechanisms by which this is achieved is in no way definitive in fish, remarkable progress has been achieved in characterizing piscine cytokine

genes in recent years, while it has been well established that the expression of these immune-related genes can be considered a useful tool for testing the immune response activities (Yan *et al.* 2016). Similarly, the mechanisms by which the administration of dietary additives effect gene expression profiles in tissues where direct contact to the compound does not occur remain to be definitively identified.

Fish fed all three of the experimental diets exhibited significant upregulation of IL-1 $\beta$  in comparison to the control fed fish in the intestine after five weeks of feeding. Following activation of the host pattern recognition receptors (PRRs) by the microbial associated molecular patterns (MAMPs), or pathogen associated molecular patterns (PAMPs), IL-1 $\beta$  may be produced by a wide range of cell types, serving diverse physiological functions (Zou and Secombes 2016). As a pro-inflammatory cytokine, IL-1 $\beta$  is an important effector cytokine of the inflammatory response and therefore one of the first cytokines to be released during inflammation (Engelsma *et al.*, 2002). Previously, the significant upregulation of IL-1 $\beta$  in response to dietary supplementation of a multi-strain yeast fraction has been reported in the intestine of European seabass (Rawling *et al.*, 2019), and in response to the dietary addition of the heat-inactivated probiotic (*B. clausii* DE5) in the intestine of orange-spotted grouper (Wang *et al.*, 2018). Interestingly, live dietary additions of the probiotic *B. pumilus* were not able to elicit a significant upregulation of IL-1 $\beta$  expression in the head-kidney of the orange-spotted grouper compared to control-fed fish after 60 days, whereas fish fed dietary additions of heat-inactivated *B. pumilus* demonstrated significantly increased expression of IL-1 $\beta$  compared to the control-fed fish (Yan *et al.* 2016). In contrast to the results of the present study however, the dietary addition of a synbiotic (MOS +  $\beta$ -glucan + live *L. plantarum*) did not elicit a significant upregulation of IL-1 $\beta$  expression in Caspian trout (Jami *et al.* 2019). In the absence of a greater number of *in vivo* trials, it remains unclear as to whether these



differing results are due to species differences, inclusion rate, the length of administration, or any number of differences in trial design. However, upregulations in the expression of IL-1 $\beta$  observed in the present study are indicative of dietary-induced activation of the inflammatory response within the fish.

While the role of transforming growth factor- $\beta$  (TGF- $\beta$ ) remains to be definitively determined in fish, within the context of the immune response, it is considered an important pleiotropic cytokine which may exert both pro-inflammatory and immune-suppressive effects, de-limiting the inflammatory response induced by IL-1 $\beta$  (Wang and Secombes, 2013; Zou and Secombes, 2016). In the present study, significantly increased expression of TGF- $\beta$  compared to the control fish was observed in the intestine of fish fed diets supplemented with the parabiotic and the prebiotic additives after five weeks. Similarly, Wang *et al.* (2018) observed a significant increase in the expression of TGF- $\beta$  in the head-kidney of fish fed with heat-inactivated *B. clausii*. An *in vitro* rainbow trout model cell line exposed to MOS in solution also exhibited significantly increased expression of TGF- $\beta$  (Wang *et al.*, 2019). Following 8 weeks of feeding in the current study, the expression of TGF- $\beta$  was significantly upregulated in the GALT of fish fed all three of the experimental diets. Moreover, the expression of IL-1 $\beta$  in the GALT of fish fed the three experimental treatments after eight weeks seemingly returned to levels of expression similar to those of the control fed fish, as no significant differences in expression could be detected. Considering the de-limiting ability of TGF- $\beta$ , the less pronounced expression of IL-1 $\beta$  may be a response to the significant up-regulation of TGF- $\beta$  and its anti-inflammatory actions, serving to protect the fish from potential immunopathology (Foey and Picchiatti, 2014). In order to confirm the extent of the inflammatory response suggested by these results, it would be prudent to analyse the

expression of other anti-inflammatory cytokines such as IL-10, pro-inflammatory COX-2, and interleukin receptors such as IL-1R1, in response to the dietary additives, in future studies.

Furthermore, several studies have focused on the ability of TGF- $\beta$  to drive T helper (Th) 17 differentiation in combination with other cytokines (Zou and Secombes, 2016). Recently identified as a distinct lineage from those of Th1 and Th2 (Yang *et al.*, 2008), Th17 cells are considered orchestrators of the mucosal defences, secreting proinflammatory cytokines IL-17 and IL-22: these interleukins stimulate epithelial tissues to secrete chemokines and an array of antimicrobial peptides which repel assault from a wide range of infectious agents (Zhang *et al.*, 2013). To date, the majority of studies focussing on Th17 in fish have been concerned with the reaction of this lineage to pathogen challenge (Ribeiro *et al.*, 2010) and vaccine development (Zhang *et al.*, 2013). Studies concerning the stimulation of Th17 in response to dietary additives remain scarce, yet the present study was able to demonstrate the significant upregulation of IL-17a in the intestine of fish fed the prebiotic-supplemented diet after five weeks of feeding, compared to the control-fed fish, from which a stimulation of the mucosal defences of the fish can be inferred. However, IL-17a expression was significantly down regulated in the intestine of fish fed all three of the experimental treatments after eight weeks. These somewhat confounding results would be better understood with additional data provided by the analysis of IL-22 expression in response to the dietary additives as IL-22 is known to be preferentially produced by Th17 cells and has been observed to exhibit high constitutive expression in mucosal tissues (Wang and Secombes, 2013). It would also be interesting to investigate whether the expression of IL-17a remained upregulated in response to the dietary additives and simultaneous pathogen challenge, as the down regulation of this gene after eight weeks may be due to the absence of an antagonist, rendering the expression of IL-17a unnecessary in times of maintained homeostasis.

IL-4/13 molecules have a relatedness to both IL-4 and IL-13, yet in teleosts they diverged into IL4/13 due to the duplication of the locus from the whole genome duplication event at the base of this fish lineage (Zou and Secombes, 2016). IL-4 and IL-13 have previously been shown to stimulate B-cell proliferation and induce epithelial cells to produce mucous in mammals (Martinez *et al.*, 2009). In fish, IL4/13a is strongly expressed by Th2 cells: while IL-4 promotes Th2 development in a positive feedback loop, it suppresses the development of Th1 and Th17 cells (Takizawa *et al.*, 2011). Th2 responses are especially important for parasite defence, the implications of which will be discussed in further detail in following chapters. A master regulator of the Th2 response is the transcription factor GATA binding protein 3 (GATA-3), which induces transcription of the genomically clustered Th2 cytokine genes (Ansel *et al.*, 2006). It therefore follows that significant upregulation of IL4/13a would be observed alongside the up-regulation of GATA-3. Rainbow trout administered with the prebiotic-supplemented diet in this study exhibited significant increases in the expression of IL4/13a and GATA-3 in the intestine after five weeks. These results are suggestive of a dietary-induced response to the prebiotic supplement and indicate the activation of Th2 immunological defences. An interesting addition for future studies would be to include histological examinations of the GALT to determine whether the mucous-producing cells were also influenced by the dietary additive supplementation in the GALT.

The prebiotic mixture of MOS and  $\beta$ -glucan (B-Wyse) would appear to have a potent ability to stimulate the expression of certain immune-relevant genes investigated here within the GALT of rainbow trout, beyond those elicited by the heat-inactivated probiotic and their combinational synbiotic. This may be due to certain architectures present on the cell wall of the multiple yeast components within the B-Wyse product, which activate a variety of PRRs, facilitating their interaction and influence upon host cells and the inflammatory gene

responses observed. The potential of the dietary additives to stimulate the innate immune system via the enhancement of the commensal microbiota was in no way measured in this study. As Rawling and colleagues point out, the possible contribution of the gut microbiota to the observed effects on the gene expression reported in this study should and cannot be excluded (Rawling *et al.*, 2019). Further studies would do well to include assessments on the impact the dietary additives have upon the commensal microbiota of the intestinal tract, alongside gene expression analysis.

In the gills, significant increases in the expression of IL-1 $\beta$  were observed in fish fed all three of the experimental treatments compared to those fed the control diet after eight weeks of feeding. TGF- $\beta$  was observed to be highly significantly upregulated in fish fed all three of the dietary regimes compared to the control at both five and eight weeks, while significant upregulation of IL4/13a and the transcription factor GATA3 after five weeks of exposure to all three dietary regimes compared to the control were also observed. Taken together, these results underline the potential of the dietary additives to influence the expression of certain genes within the GALT of the host fish. Given their relatedness, these results would further suggest an important role for Th2 responses in the gills: previous research proposed salmonid gills form “Th2/Treg-skewed” environments which serve as protection from parasites and from inflammatory Th1 and Th17 responses (Takizawa *et al.*, 2011).

Concerning the skin, the expression of the majority of genes analysed exhibited a wide level of variance within the treatment groups with very little differences in expression compared to the control treatment. This is perhaps indicative of a number of highly activated individuals within the treatments causing large variations. Future studies should consider sampling prior to the 5-week mark in order to determine whether a more immediate dietary response is

occurring in the skin, which becomes less pronounced with continued exposure. Significant up-regulation of TGF- $\beta$  was however observed in fish fed both the prebiotic and parabiotic treatments after eight weeks of feeding. Nevertheless, these results and those of the TJ-associated genes suggest that the dietary additives are able to influence the expression of certain genes within the skin tissue of rainbow trout. The further analysis of a wider variety of genes would be of benefit and provide a more holistic understanding of the alterations occurring in the mucosal tissues in response to the dietary additives.

### 3.5 Conclusions

Under the excellent rearing conditions of the present study and in addition to the administration of optimally designed and highly nutritious diets, little opportunity remained for the prebiotic, parabiotic or synbiotic additives to stimulate improvement of the growth indices of the rainbow trout beyond those observed for the control-fed fish. The overall performance of the fish was excellent: FCR values were exceptional falling within the range of 0.85-0.91, mean body weight increased 2.5-fold, and survival rate was above 98% across all treatments.

Although no significant dietary-induced differences were observed in the quantity and quality of the epidermal mucous, the combined results of the histological examinations and gene expression analysis indicate the potential of the dietary additives to influence the mucosal responses of the SALT, GIALT, and GALT of rainbow trout when administered at the inclusion levels of the current study. Significant increases in the CAF of goblet cells within the SALT were observed, in addition to significantly increased expression of TJ- and mucosal-associated genes within all three of the mucosal-associated tissues. Administration of the experimental diet supplemented with the prebiotic B-Wyse, demonstrated particular influence upon the

expression of Muc17-like within the GALT, SALT and GIALT, calreticulin in the SALT, and claudins 10d and 10e in the GIALT. Together with the significantly increased expression of several immune-related genes (IL-1 $\beta$ , TGF- $\beta$ , IL-17a and IL4/13a) within the GALT, SALT and GIALT, the results of the investigations of the present chapter further support the proposition of the interconnectivity of the MALT tissues, as demonstrated by the influence of the dietary additives upon tissues which do not come into direct contact with the product, such as the GIALT and SALT.

Within the scientific literature, it has been demonstrated that the beneficial influences of a dietary supplement within one species are not necessarily demonstrated within an alternative species of fish. Investigations to determine whether the positive stimulation of the mucosal barrier and immune defences of rainbow trout observed here can be stimulated in Atlantic salmon, and whether these benefits aid in the defences of the fish in response to pathogen challenge, will form the basis of the following chapter.

**The effects of prebiotic and synbiotic dietary inclusion upon the growth, mucosal health and barrier defences of Atlantic salmon (*Salmo salar*) following six weeks of feeding and in response to sea lice (*Lepeophtheirus salmonis*) challenge**



#### 4.1 Introduction

Investigations in chapter 3 revealed the potential of the tested dietary additives to positively influence the mucosal barrier defences of rainbow trout (*Oncorhynchus mykiss*), with particular reference to goblet cell area coverage and morphology in the skin, and the expression of TJ-associated and immune-related genes in the SALT, GIALT, and GALT. To date, very few studies have investigated the effect of dietary additives upon fish mucosal and barrier defences of the MALTs. Considering the important role these MALTs play in the defence against pathogenic organisms, there is a need to generate knowledge that allows for the understanding of the influence of immune-supplemented diets in these tissues. Previous investigations have reported differing responses to the same dietary additives administered to different fish species. For example, a study by Rodrigues-Estrada and colleagues (2008) supplemented 0.4% BioMOS® into the diets of rainbow trout and observed significant increases in the amount of epidermal skin mucous after 12 weeks of feeding. However in the European sea bass (*Dicentrarchus labrax*), these results were not reproduced even though the same dosage of the same product was administered (Torrecillas *et al.*, 2011). Taken together, contradictory results such as these indicate that prebiotic property should not be extrapolated and/or assumed between species and highlights the importance of primarily demonstrating actual effect, before claiming its potential use without reference from another fish host (Caipang & Lazado, 2015). To this end and in light of the beneficial responses to the dietary additives observed in freshwater rainbow trout in chapter three, this chapter will evaluate the ability of dietary additives to influence similar mucosal responses and barrier functions in Atlantic salmon (*Salmo salar*) post-smolts.



Belonging to the family Salmonidae, rainbow trout and Atlantic salmon are highly valuable fish species of particular importance to the aquaculture industry. Globally, Atlantic salmon is one of the most intensively farmed fish, with the majority of the world's production originating from Norway, Chile, the United Kingdom, and Canada (Pettersen *et al.*, 2015). Most recent estimates published by the FAO suggest that these countries collectively produced approximately 2.36 million tonnes of aquaculture-reared Atlantic salmon in 2017, worth an estimated \$16.7 billion USD (FAO-FIGIS, 2017).

In addition to carrying out a diverse array of critical physiological processes, including waste excretion, osmoregulation, and nutrient absorption, the MALTs are also highly active immunological sites, which constitute the first line of defence against pathogen invasion (Peatman and Beck, 2015). Atlantic salmon aquaculture is performed in open-sea cages, where disease represents a major constraint to production due to the uncontrollably ubiquitous nature of pathogens within environmental seawater (Torrissen *et al.*, 2013). Parasitic diseases attributable to obligate or opportunistic eukaryotic pathogens continue to have a major impact on global finfish and shellfish aquaculture (Shinn *et al.*, 2014).

The sea louse *Lepeophtheirus salmonis* is the most damaging parasite to the salmonid farming industry throughout Europe and North America, owing to its large scale host-parasite density dependence (Costello 2009; Kristoffersen *et al.*, 2018). Estimating the true impact of sea lice infestations is complicated as costs can be affected by a diverse assortment of environmental and management factors, ranging from direct losses in production, mortality due to secondary infections, reduced growth, and loss of carcass value, to the more indirect costs of longer-term control and management of infections, and the wider, downstream socioeconomic impact on livelihoods and satellite industries associated with the primary

producer (Johnson *et al.*, 2004; Shinn *et al.*, 2014). Sea lice related economic losses to worldwide salmonid aquaculture have been estimated at approximately \$430 million USD per annum (Robledo *et al.*, 2018) or circa 10% of the potential fish harvest size (Sitja-Bobadilla *et al.*, 2016).

The host response to salmon lice infestations include a combination of chronic stress, reduced swimming performance, impaired wound healing, and immunomodulation, the extent of which is largely dependent upon the number and developmental stage of the copepod (Skugor *et al.*, 2008; Torrissen *et al.*, 2013). Historical methods of control have largely involved the use of chemotherapeutant interventions and veterinary compounds, yet their repertoire has become limited of late due to the drift towards resistance of anti-parasitic compounds (Holm *et al.*, 2016). Emamectin benzoate (EMB) is a chemical compound belonging to the class of avermectins (Aaen *et al.*, 2015), capable of modulating specific glutamate- and gamma-aminobutyric acid-gated anion channels within the sea louse, leading to the disruption of nerve impulses, paralysis and death of lice at all developmental stages (Torrissen *et al.*, 2013). EMB proved to be highly efficacious, conferring protection on the host for up to 10 weeks, however increasing concerns about the development of pesticide resistance, the occurrence of treatment failures, and undesirable environmental impacts raise questions about the future of EMB as a control strategy (Tadiso *et al.*, 2011). More recently, the industry has undergone a dramatic shift away from antiparasitic drugs in favour of alternative non-medical interventions. Four species of wrasse (*Labridae* spp.) and one of lumpsucker (*Cyclopteridae*) have been widely employed as an alternative delousing method: the Norwegian salmon industry alone had more than 50 million cleaner fish in use in 2017 (Overton *et al.*, 2019). Despite representing a cost-effective alternative to the use of chemotherapeutants and reducing the degree of stress experienced by lice-infected fish, concerns over the

sustainability of cleaner fish use and their welfare have arisen (Powell *et al.*, 2017). Mechanical and thermal delousing systems are recently developed technologies also used as alternatives to chemotherapeutants. While these technologies have been used within the industry since 2015, there remains a scarcity of independently evaluated reports concerning the extent of their use and the broad scale effects of post-treatment outcomes for the salmon across the industry (Overton *et al.*, 2019). In a survey conducted in 2018 by the Norwegian Veterinary Institute, the number of thermal and mechanical treatments was the highest ever registered, yet salmon farmers reported increased levels of acute and delayed mortality following these delousing methods, compared to medicinal or freshwater treatments (Helgesen and Qviller, 2019).

Notwithstanding the recent advancements and success of these alternative methodologies, there remains a firm interest to improve the overall health status and resilience of the salmon stock to both infectious and non-infectious challenges, in an effort to reduce the frequency and biological impact of such delousing interventions. Several publications have reported the increased survival or disease resistance of several species of fish in response to dietary additive supplementation. For example, dietary MOS supplementation reportedly increased the survival of channel catfish challenged with *Flavobacterium columnare*: gene expression patterns in the gill indicated the recruitment of mannose-associated signalling pathways, inflammatory resolution and enhanced epithelial repair (Zhao *et al.*, 2015). Similarly, the upregulation of immune-related genes was observed in the intestine, gill and skin of greater amber jack (*Seriola dumerili*), while simultaneously reducing susceptibility to the “skin fluke” (*Neobenedenia girellae*), following a prebiotic addition to the diet (Fernández-Montero *et al.*, 2019). In rainbow trout, increased epidermal mucous secretions, circulating immunity and survival against *Aeromonas salmonicida* challenge was reported following MOS

administration (Rodriguez-Estrada *et al.*, 2013). Dietary supplementations with the probiotic *Pediococcus acidilactici*, the prebiotic galacto-oligosaccharide (GOS) and their combination as a synbiotic, significantly increased the resistance of rainbow trout against *Streptococcus iniae* infection (Hoseinifar *et al.*, 2017). Furthermore, fish fed diets supplemented with live *P. acidilactici* alone and in combination with the commercial prebiotic product exhibited reduced infection of *Vibrio anguillarum* compared to those fed the control diet, following 90 days of feeding (Torrecillas *et al.*, 2018).

Concerning sea lice, Refstie and colleagues (2010) reported a significant 28% reduction in the frequency of salmon infected with the sea lice *Caligus elongatus* following the addition of a  $\beta$ -glucan to an experimental fishmeal + sunflower meal + soybean meal (SBM) diet, reflective of commercial formulation. This sea lice preventing effect of the  $\beta$ -glucan is in accordance with previous work by (Ritchie 2000), who hypothesised that stimulation of the non-specific immune system of the fish as the reason for reduced parasitic infection, an effect that has since been demonstrated in other species, including Bluefin tuna (*Thunnus maccoyii*) (Kirchhoff *et al.*, 2011) and red nose snapper (*Lutjanus guttatus*) (Del Rio-Zaragoza *et al.*, 2011). Interestingly, no differences in the number of infected fish were observed following the addition of the  $\beta$ -glucan to a diet lacking sunflower meal, for which the authors suggest an influential interaction between the  $\beta$ -glucan and the antioxidant present within the sunflower meal or a reactionary difference to the SMB induced enteritis the fish were suffering (Refstie *et al.*, 2010; Jensen *et al.*, 2015). Additionally, this investigation failed to observe any differences in sea lice prevalence between fish fed the control diet and one supplemented with MOS but did have an observable effect on intestinal integrity and health beyond that of the  $\beta$ -glucan diet (Refstie *et al.*, 2010). It may therefore be of benefit to investigate the combined effect of the MOS and  $\beta$ -glucan additives in a single diet to assess

whether a synergistic effect may be observed, reducing sea lice burdens while simultaneously augmenting the intestinal health. An investigation conducted on Atlantic salmon smolts reared in open sea cages reported significantly increased microvilli density, microvilli length, and absorptive surface in response to 0.4% dietary MOS supplementation, yet demonstrated significantly reduced numbers of attached sea lice and reduced numbers of infected fish in response to MOS supplementation compared to the control (Dimitroglou *et al.*, 2011). The authors proposed that the different results presented here between the MOS treatment and control regime may be related to the quality and quantity of skin mucous production. Although the results of these two studies differ, it should be pointed out that while the study by Refstie and colleagues (2010) concerned only *Caligus* spp., infections of both *Caligus* and *L. salmonis* were reported in the investigation by Dimitroglou *et al* (2011). This may therefore be a function of species-specificity. In addition, both investigations were performed in open sea pens and therefore no influence over the intensity of lice infestation was afforded: it is possible that the infection rates differed between investigations and contributed to the differing results observed.

The expression of a macrophage mannose receptor-1 (MRC1) has been observed to be significantly up-regulated in skin tissue of Atlantic salmon at the point of sea lice attachment, in addition to the increased expression of mucins (Robledo *et al.*, 2018). Similarly, it has recently been confirmed that wound-healing in Atlantic salmon involves a strong mucous response in the form of mucous cell recruitment at the border of the healing wound, along with simultaneous secretion of an adherent mucous layer (Sveen *et al.*, 2019). These recent findings suggest an important role for the mucosal defences towards resistance of parasitic invasion.

The investigations of this chapter fall into two parts. The first is concerned with the effect of dietary additives upon the mucosal health of Atlantic salmon following a six-week feeding regime, while the second involves a subsequent controlled challenge with *L. salmonis*. The first aim of the research presented in this chapter was to determine whether any discernible differences in fish growth indices could be detected between four differing dietary regimes. Following encouraging results from chapter three concerning the increased mucosal and barrier defences in rainbow trout, the B-Wyse prebiotic product was additionally investigated in this chapter. As discussed above, the effect of a  $\beta$ -glucan and MOS combination upon the resistance of the fish to sea lice challenge would be of great interest and relevance to the industry, and would enable the further determination of any synergistic effect between the components to aid in sea lice resistance. The second dietary regime was supplemented with a synbiotic product containing a  $\beta$ -glucan + MOS + live *P. acidilactici* (as B-Wyse + the commercial probiotic Bactocell®). At present, Bactocell® (Lallemand SAS, France) is the only strain currently authorised for use as an aquaculture probiotic within the EU (Rodiles *et al.*, 2018). Considering the urgency under which the industry is attempting to implement alternative non-medicinal treatments for sea lice control, it follows logically to assess the influence of a product already approved for commercial use. As a commonly used product in commercial aqua feeds, and given that MOS is a component of both the prebiotic and synbiotic dietary additives tested here, it was deemed prudent to assess the effect of a singular MOS product administered within a diet. This serves to generate additional data regarding the beneficial influence MOS reportedly has on the MALT tissues and to further inform previous conflicting works on the increased resistance of Atlantic salmon to sea lice following administrations of dietary-MOS.

The second aim of the research was to determine to what extent the dietary additives influence the mucosal defences of the fish after six weeks, and post-challenge, involving the compositional assays of the secreted epidermal mucous and histological analysis of the goblet cells in the three MALT tissues. Thirdly, RT-q-PCR analysis was conducted on a selection of mucous-related and immunology-associated targets within the GALT, SALT and GIALT, in an attempt to further elucidate the changes taking place within the mucosal tissues in response to the dietary additives. Finally, data pertaining to the sea lice challenge was collected: settlement location and abundance were documented at both the chalimus and motile stages, in order to determine whether any observable differences in sea lice attachment were present between fish fed the different dietary regimes.

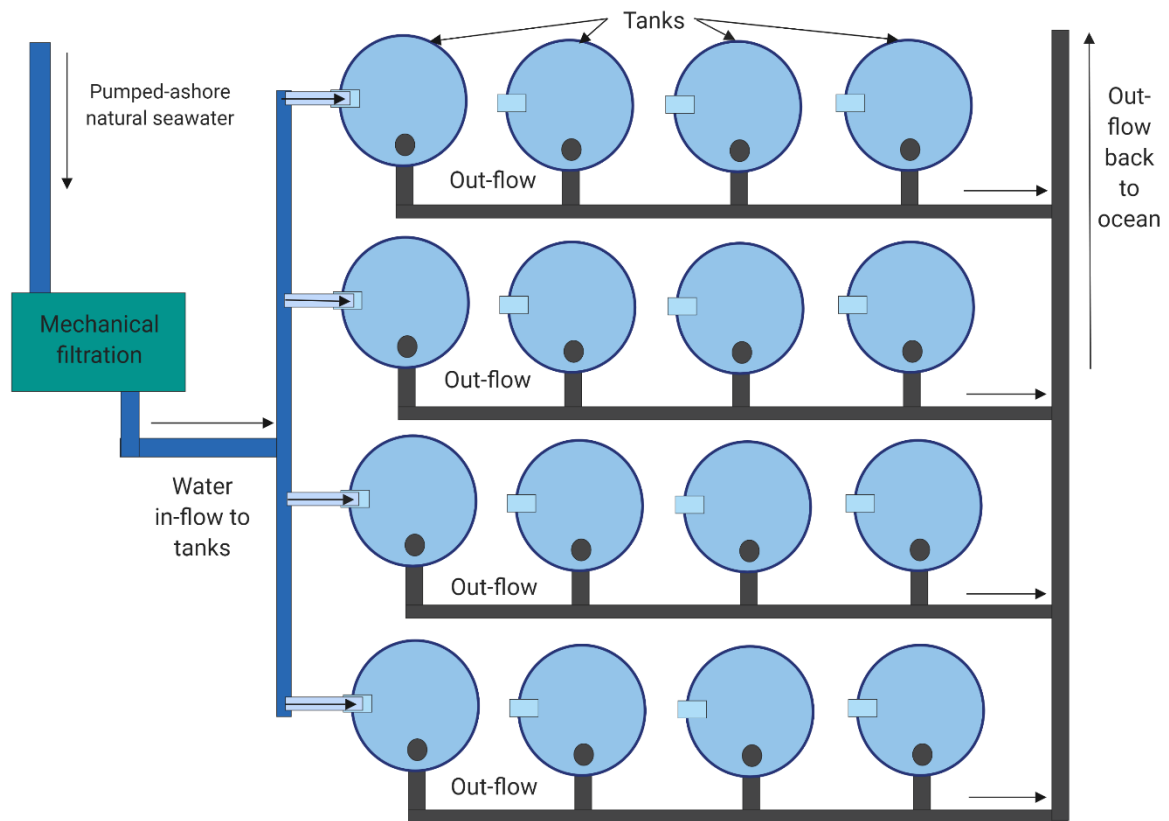
### 4.2 Materials and methodologies

All experimental work involving fish was conducted under the Personal Licence of David Bassett (60/4522), protocol number 7 [parasite efficacy studies] and conducted in accordance with the UK Animals Scientific Procedures Act 1986 and the Animal Welfare and Ethical Review Body of the University of Stirling.

#### *4.2.1 Experimental design*

The trial was conducted by animal technicians at the Marine Environmental Research Laboratory of the University of Stirling, located in Machrihanish, Scotland. A flow-through system was employed (see figure 4.1), comprising 16 x 400L circular fiberglass tanks, each provided with pumped-ashore natural seawater. Mechanical filtration (to 100µm) of incoming water was provided by a Hydrotech Drumfilter, type HDF 1203-IP. A 16-hour light: 8-hour dark photoperiod was maintained throughout the trial. The pH, dissolved O<sub>2</sub> and temperature were monitored daily. The system water temperature averaged 14 ± 0.5 °C.

Dissolved O<sub>2</sub> levels were maintained at above 80% saturation with additional aeration provided by an air stone supplied via a side channel air blower.



**Figure 4.1** Schematic depiction of the system facilities at the Marine and Environmental Research Laboratory, Machrihanish in which the trial was conducted.

#### 4.2.2 Experimental fish and feeding rates

Atlantic salmon were acquired from Howietoun Fishery, Stirling and were subjected to a 5-week acclimation period. Fresh water baths were administered on several occasions prior to the start of the trial to combat Amoebic Gill Disease. A preliminary grading was carried out by MERL animal technicians approximately one week prior to final grading where fish were separated into 16 tanks at a stocking density of 40 fish per tank and tank weights were within 3.0% of each other. At the start of the trial, each dietary treatment was randomly assigned to



the tanks in quadruplicate and fish were fed to visual satiation two or three times daily, avoiding waste. Feeding rate was adjusted weekly after weighing (as tank biomass) and was reduced to half ration the day before weighing.

#### *4.2.3 Pathogen challenge*

A sea-louse infection challenge (as previously described in Jensen *et al.*, 2015) was performed by expert Senior Animal Technician, Sally Boyd on day 46 of the trial using laboratory bred free-swimming *L. salmonis* copepodids as follows. Within each tank, fish were crowded to half the initial rearing volume (~200L) and approximately 3,000 copepodids were introduced to each tank with an expected minimum settlement of between 10 and 20 lice per fish. Low water volume and low water exchange for a 2-hour period following challenge favoured parasite settlement onto the hosts prior to returning the tanks to their original settings. Oxygen concentrations were monitored throughout the time of infection but no additional aeration was necessary. Successful copepodid settlement was confirmed the following day via the examination of two fish per tank under light anaesthesia: further challenges were unnecessary.

#### *4.2.4 Sampling schedule and specifics*

Preparation of the sampling protocol and sampling consumables was conducted by the PhD candidate. All reagents, labelled sampling tubes and necessary equipment needed to conduct sampling at all three time points was sent to the MERL site by courier approximately 1 week prior to each sampling date. At each sampling point, the sampling team comprised the PhD candidate, Dr Eric Leclercq from Lallemand, and either Dr Victoria Valdenegro from BioMar, Dr Luisa Vera of the University of Sterling, or Dr Mark Rawling from the University of

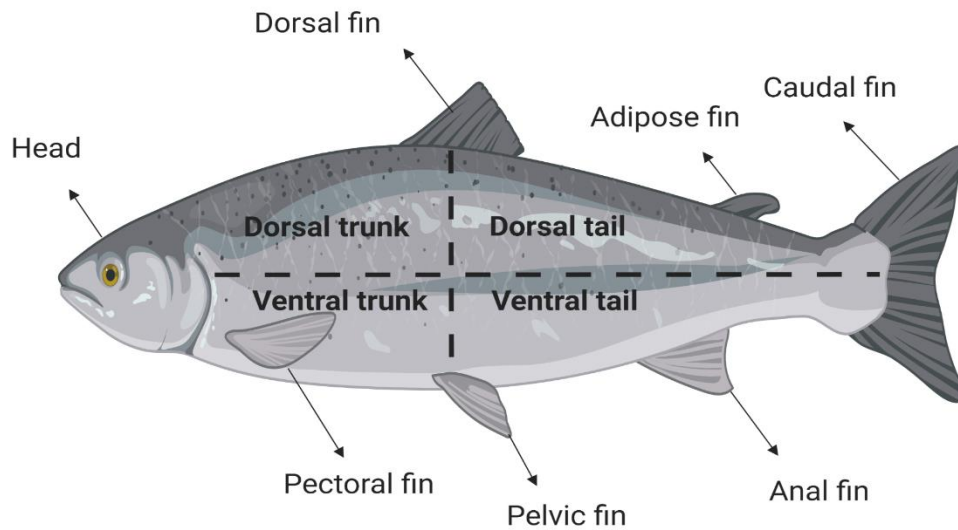
Plymouth, in addition to 2 MERL animal technicians responsible for the welfare of the fish over the course of the trial.

Sampling occurred after six weeks of feeding the experimental diets (T2): 10 fish per tank were randomly netted and sedated for body weight (BW) and fork length (FL) measurements. Four fish were then returned to their original tank following intermediary recovery holding, while the remaining 6 fish were sampled for epidermal skin mucous, in addition to skin, gill and intestinal tissues for histological and gene expression analysis. Further sampling occurred at one and three weeks post challenge with *L. salmonis* (T3 and T4, respectively): 15 fish per tank were randomly netted and sedated for BW, FL and sea lice assessment, of which 9 fish were returned to their tank and the remaining 6 were euthanised for skin mucous and tissue sampling. Samples for mucous, gene expression and histological analysis were collected from all euthanised fish at all time points, as described in chapter 2 section 2.3, while data pertaining to the prevalence and location of sea-lice attachment was also collected at sampling points that followed challenge.

Following each sampling event, all samples were shipped back to the University of Plymouth for analysis by the PhD candidate.

#### *4.2.5 Sea lice observations*

The assessment of sea lice infection was performed blindly by the same two trained scientists at all sampling points, with fish carefully examined under a bright-light microscope. For each fish examined, the number of sea lice per body-area was determined on a total of 22 designated body areas as described in Figure 4.2 below (Fins: pectoral, dorsal, adipose, caudal, anal, and pelvic; Body-proper: head, dorsal and ventral trunk, dorsal and ventral tail; recorded separately for the left and right flank).



**Figure 4.2** Schematic depiction of the different body regions identified to record settlement of *Lepeophtheirus salmonis* on Atlantic salmon, following challenge: a total of 22 body regions covering the entirety of the fish.

#### 4.2.6 Diet preparation

Meeting the known nutritional requirements of Atlantic salmon, four iso-nitrogenous and iso-lipidic dietary regimes were formulated, manufactured and supplied to the research facilities in Machrihanish by BioMar, following satisfactory quality-control checks. Dietary additives were added to the basal diet at the following inclusion levels: AgriMOS = 4kg/ton; B-Wyse = 1.5kg/ton; B-Wyse + Bactocell® = 1.5kg and 1kg/ton respectively.

#### 4.2.7 Fish growth and condition

Calculations to determine growth performance and fish condition were performed as follows:

- Fulton's condition factor (K-factor) =  $(100 * \text{weight (g)}) / (\text{total fish length (cm)})^3$
- Specific Growth Rate (SGR) =  $((\text{Ln}W2 - \text{Ln}W1) / T) \times 100$ : where  $\text{Ln}W1$  and  $\text{Ln}W2$  are the initial and final natural logarithmic weights, respectively, and  $T$  is the number of days in the feeding period.

#### 4.2.8 Epidermal mucous analysis

Mucous quantity was determined from 6 individual fish per tank (n=24 per treatment): refer to chapter 2, section 2.3 for methodology. Protein concentration and lysozyme activity were determined from duplicate measurements of each fish sampled (n=6 per tank / n=24 per treatment), as described below.

##### 4.2.8.1 Lysozyme activity

Lysozyme activity of the epidermal mucous was determined using a turbidimetric assay based upon the lysing activity of *Micrococcus lysodeikticus* according to Ellis (1990). Briefly, solutions of  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  were mixed with distilled water to produce a 0.04 molar solution with a pH of 6.2, to which *M. lysodeikticus* was added at  $0.2 \text{ mg ml}^{-1}$ . On a flat-bottom well plate,  $190 \mu\text{m}$  of solution and  $10 \mu\text{l}$  of sample were combined and the absorbance measured at 540nm on a Spectrophotometer (SpectraMax190, Molecular Devices) after 1 minute and 15 minutes. Samples were run in duplicate. The lysozyme activity was then calculated as follows:

$$\text{Lysozyme activity (U/ml)} = ((\text{Abs 1} - \text{Abs 15}) * 900) * 60,$$

where Abs 1 is the absorbance after 1 minute and Abs 15 is the absorbance after 15 minutes.

#### 4.2.8.2 Protein concentration

Epidermal mucous protein concentration was determined using a Protein Assay kit (Pierce™ BCA, ThermoFisher Scientific) in accordance with the manufacturer's recommendations, after Rawling *et al.* (2019). According to the manufacturer's protocol, the sensitivity range of this assay is 20µg/ml - 2000µg/ml. Briefly, the first step is a biuret reaction, in which copper is chelated with protein in an alkaline environment, followed by the colour development of the second reaction, which is influenced by the presence of specific amino acid residues found in protein. 200µL of working solution is added to 25µl of sample, in duplicate, and incubated for 30 minutes at 37°C. After leaving to cool to room temperature, the absorbance is then measured at 562nm on a Spectrophotometer (SpectraMax190, Molecular Devices). After calculating the averages from duplicate samples, the protein concentration of the sample was then determined from the standard curve produced from the Blank standard replicates provided, calculated as follows:

$$\text{Protein concentration (mg/ml)} = ((\text{Absorbance of sample at 562nm} - 0.0903) / 0.0013) / 1000)$$

#### 4.2.9 Histological examinations

Light microscopy analyses of skin, gill and intestinal tissue were carried out on two fish per tank (n=8 per treatment) across all time points as described in chapter 2, section 2.5. For

goblet cell abundance, six measurements were taken per fish, while three measurements per fish were taken to calculate the CAF in the skin and intestine. For fold length and lamina propria width, four measurements were taken per fish.

#### *4.2.10 Gene expression*

##### *4.2.10.1 RNA extraction, cDNA synthesis and real time qPCR*

RNA extraction, cDNA synthesis and real time qPCR (RT-q-PCR) was carried out as described in chapter 2, sections 2.6.1 and 2.6.2 on 2 fish per tank (n=8 per treatment). All individual samples were checked for quality control purposes at multiple stages and were removed from analysis if considered above or below acceptable limits.

##### *4.2.10.2 Reference genes, genes of interest and analyses*

$\beta$ -actin and Elf1 $\alpha$  were used as reference genes for each sample in order to standardise the results by eliminating variation in mRNA and cDNA quantity and quality (Bustin *et al.*, 2009). The stability and suitability of  $\beta$ -actin and Elf1 $\alpha$  as reference genes were confirmed by generating an expression stability measure “M” for each reference gene using the calculations outlined in Vandesompele *et al.*, (2002). No amplification product was observed in negative controls and no primer–dimer formations were observed in the control templates. Modification of gene expression was represented with respect to the control group being sampled at the same time as the treatment group. A further 10 genes of interest were selected for each of the sample tissues, including pro-inflammatory, immune-regulatory and Th2-associated cytokines; tight-junction-associated claudins; and genes associated with mucosal composition. Primers designed by the PhD candidate are presented in Table 4.1.

**Table 4.1** Atlantic salmon primer pair sequences, annealing temperature (Anneal temp in °C), amplicon size, and primer efficiency (E-value) for genes of interest used for RT-qPCR.

Gene name	Accession number	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Anneal Temp	Amplicon size	E-value		
						Gill	Skin	Gut
β-actin	NM_001123525.1	ACGGCATCGTCACCAACTG	CTCCTCTGGTGCCACTCTCA	60	83	2.05	1.97	2.10
Elf 1α	NM_001141909	GGCTGATTGTGCTGTGCTTAT	CACGAGTCTGCCCCGTTCTTT	60	80	2.05	2.15	2.05
IL-1β	AY617117	AGGAGGGAAGCAGGGTTCA	CATCAGGACCCAGCACTTGT	60	81	2.01	1.86	2.10
TGFβ	EU082211	AAGGACCTGGGCTGGAAATG	CCTGGGAGTACTTGTCTCTGT	60	106	2.05	2.05	2.10
IL-4/13a	AB574339	AGCATGGCGGAACAATACAC	GGTCCCAGTCAAATGTGTAACA	60	84	1.97	2.15	2.01
GATA3	NM_001171800	CAGCAACAGTCGCCAACAG	CGGCTGTGAGACTCCAGTTT	60	87	2.05	2.15	2.15
Calreticulin	BT058985	AGGCAAGAACCACCTCATCAA	GTGCCTGACTCCACCTTCTC	60	132	1.00	2.05	2.10
Muc5ac-like	XP_003206353.1	TCCGCAACTGGTCATGTTGTA	GCGGCTGGAGAGACACTATC	60	83	1.97	2.10	-
Muc2-like	XM_029770456.1	CRACTCAACGTGGATGTAGGA	GCGACCACTAGCCAGAAAGA	60	81	-	-	2.10
Occludin	XM_014143412	CCAGGGCATCTTCATCAACCA	GCAGGATGATCAGACCCACAA	60	110	1.90	2.01	-
Claudin 6	BK006386	TCATCGTCATCAGCATGGTACT	GGCTCAGGCAGTTGGTACAC	60	81	-	2.05	-
Claudin 7	BK006387	ACTATTGCCTGCTCGTGGTA	AGGCAATGAAGATGGCTGAAC	60	106	-	1.83	-
Claudin 12	BK006392	TGGCTACTCTGGCTGCTACT	GCAGAACTGGAGCAGTCTCA	60	82	-	1.97	-
Claudin 28a	BK006401	CTGGCTATCATCGGGTTCCT	ACGATGTTGGCTCCGATGAA	60	86	2.05	-	-
Claudin 28b	BK006402	GCCATCGCTAGTGGAGTCAT	AGAAGTCTCGGATGATGGTGT	60	91	1.86	-	-
Claudin 27a	BK006400	CCAGATGCAGTGTAAGGTCTAC	AGGAAGATGCCGACGATACC	60	111	2.01	-	-
IL-17a	KJ921972	CCACCAACAGCAGCCAGTA	CCTGGTGGCTGAAGTGTAGA	60	80	-	-	2.15
Tollip1	BT045489	TGAGAGAGGGCAGTGTGGTT	CAGGTTGATCATGCCCTCCTT	60	80	-	-	2.05
IRAK 4	NM_001141766	TCTTTCCACGAGCTGAAGAAGA	CAAAGCCTCCCTCACCAAGT	60	88	-	-	2.15
Arg 2	XM_014190234	TGTGGGCAGAGCTAACAACT	TCCTCAAGCATGATCGTAGTG	60	82	-	-	2.01

#### *4.2.11 Statistical analyses*

Statistical analysis were performed using R Studio version 1.2.5042 (RStudio PBC, Boston USA). All statistical analysis for RT-qPCR data were carried out using the permutation tests in R following Röhmel (1996). All other statistical differences (growth performance, mucous quantity and quality, and light microscopy data) were assessed by one-way ANOVA tests in R following Shapiro-Wilk test for normality, and with Tukey high significant difference (HSD) post-hoc tests where differences occurred. Where data could not be transformed into normality, permutation tests were conducted.

The level of significance was accepted at  $p < 0.05$ . All gene expression data is presented as mean  $\pm$  standard error (SE), while all other data is presented as mean  $\pm$  standard deviation (SD) unless otherwise stated

### 4.3 Results

#### *4.3.1 Fish growth indices*

All diets were accepted well throughout the trial. No statistically significant differences in growth or performance were observed between the different treatments before (T2) or after challenge (T3 and T4). Results are presented in Table 4.2.

#### *4.3.2 Epidermal mucous analysis*

Prior to pathogen challenge (T2), fish fed the AgriMOS, B-Wyse and B-Wyse + Bactocell® supplemented diets had significantly more epidermal mucous compared to fish fed the control diet (+46.2%, +49.9% and +70.4% respectively,  $p < 0.03$ ), however this trend did not continue after challenge (T3).



**Table 4.2** Growth and condition measurements of Atlantic salmon over the duration of the trial. Data presented as mean  $\pm$  SD; n=4. T1 = start of trial; T2 = 1<sup>st</sup> sampling at 6 weeks, prior to pathogen challenge; T3 = 1 week after challenge; T4 = 3 weeks after challenge.

Sampling point	Parameter	Unit	Control	AgriMOS	B-Wyse	B-Wyse + Bactocell®
T1	Body-weight	(g)	255.18 $\pm$ 3.3	248.94 $\pm$ 2.3	246.13 $\pm$ 6.9	255.64 $\pm$ 10.4
	Biomass	(kg)	40.83	39.83	39.38	40.90
T2	Body-weight	(g)	389.38 $\pm$ 41.0	383.18 $\pm$ 22.4	367.68 $\pm$ 17.1	411.18 $\pm$ 14.4
	Fork length	(cm)	30.70 $\pm$ 0.9	32.70 $\pm$ 0.4	32.10 $\pm$ 0.4	33.10 $\pm$ 0.4
	K-factor	(au)	1.10 $\pm$ 0.05	1.09 $\pm$ 0.02	1.10 $\pm$ 0.02	1.12 $\pm$ 0.03
T3	Body-weight	(g)	384.78 $\pm$ 10.8	375.75 $\pm$ 10.1	372.52 $\pm$ 20.8	376.80 $\pm$ 15.5
	Fork length	(cm)	33.30 $\pm$ 0.6	32.90 $\pm$ 0.3	33.00 $\pm$ 0.3	32.80 $\pm$ 0.3
	K-factor	(au)	1.04 $\pm$ 0.04	1.04 $\pm$ 0.02	1.03 $\pm$ 0.05	1.06 $\pm$ 0.02
T4	Body-weight	(g)	398.64 $\pm$ 38.4	408.00 $\pm$ 24.8	391.56 $\pm$ 17.6	420.83 $\pm$ 16.3
	Fork length	(cm)	33.70 $\pm$ 0.4	33.60 $\pm$ 0.6	33.10 $\pm$ 0.4	33.60 $\pm$ 0.5
	K-factor	(au)	1.04 $\pm$ 0.08	1.07 $\pm$ 0.01	1.08 $\pm$ 0.03	1.10 $\pm$ 0.01
	Biomass	(kg)	26.23	29.75	28.60	30.76
T1 to T2	SGR	(%/day)	0.95 $\pm$ 0.2	0.98 $\pm$ 0.1	0.91 $\pm$ 0.1	1.1 $\pm$ 0.1
T1 to T4	SGR	(%/day)	1.01 $\pm$ 0.2	1.12 $\pm$ 0.1	1.05 $\pm$ 0.1	1.13 $\pm$ 0.1

A significantly higher level of lysozyme activity was detected in the mucous of fish fed the AgriMOS (+5.3%), B-Wyse (+20.3%), and the B-Wyse + Bactocell®-supplemented (+21.3%) diets compared to those fed the control diet prior to challenge (T2) and one week after challenge (T3: AgriMOS = +181.4%; B-Wyse = +66.9%; and B-Wyse + Bactocell® = +40.7%). However, no significant differences in lysozyme activity levels were detected between any of the different treatments 3 weeks post challenge (T4).

No differences in protein content of the mucous were observed between any of the treatments either before or after challenge. Results are presented in Table 4.3

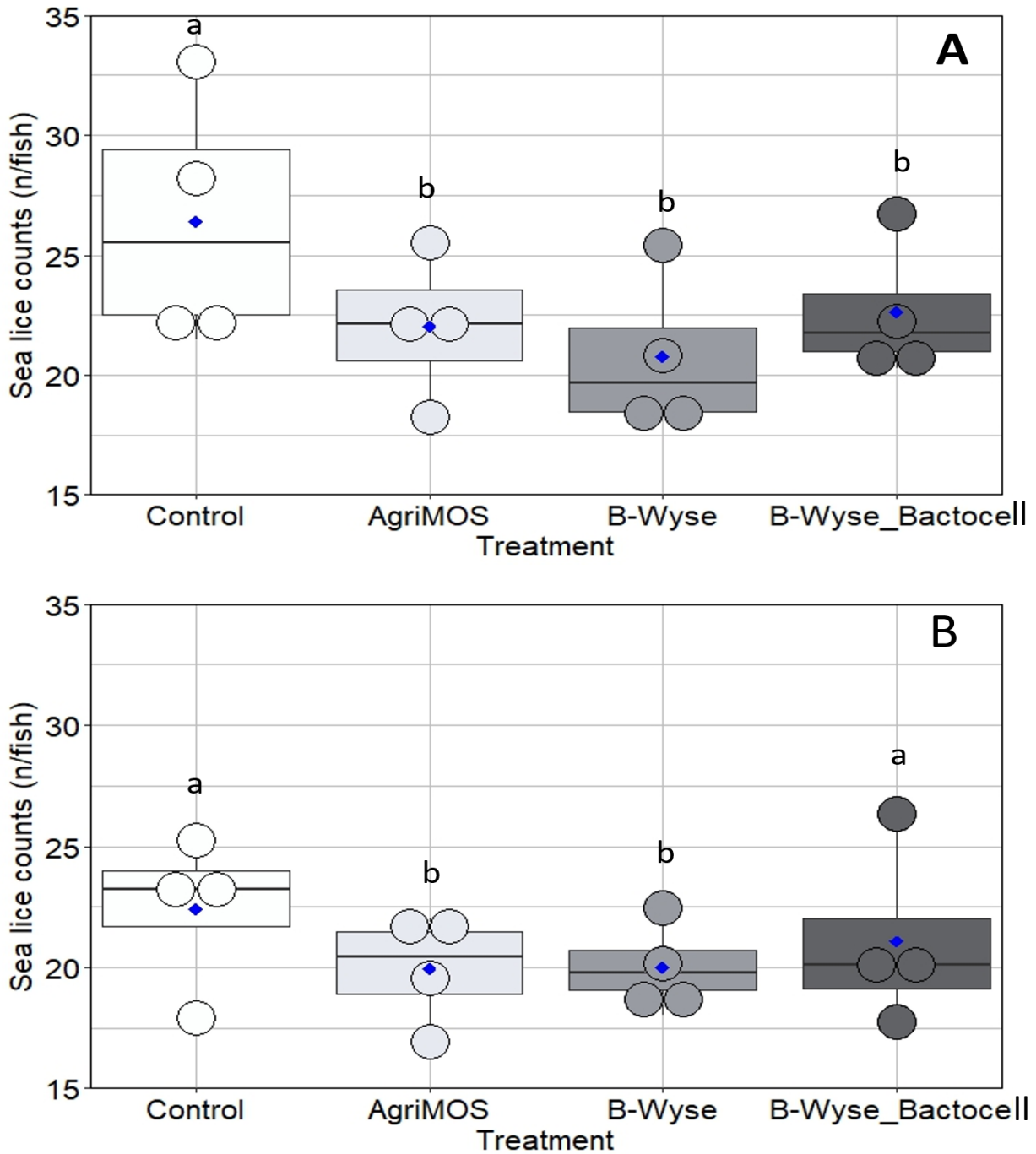
**Table 4.3** Epidermal mucous measurements of fish fed the different dietary regimes prior to challenge (T2), 1 week post challenge (T3) and three weeks post challenge (T4). Data presented as mean  $\pm$  SD; n=4; different letters between data on the same row denote significant difference between those of a different letter,  $p < 0.05$ .

Mucous parameter	Sampling point	Treatment			
		Control	AgriMOS	B-Wyse	B-Wyse & Bactocell®
Quantity (mg/cm)	T2	13.29 $\pm$ 1.26 <b>a</b>	19.42 $\pm$ 4.14 <b>b</b>	19.92 $\pm$ 1.88 <b>b</b>	22.64 $\pm$ 0.99 <b>b</b>
	T3	13.04 $\pm$ 0.17	13.48 $\pm$ 0.71	14.13 $\pm$ 1.54	12.73 $\pm$ 1.92
	T4	14.07 $\pm$ 1.32	16.61 $\pm$ 0.49	15.65 $\pm$ 0.49	17.26 $\pm$ 2.51
Lysozyme activity (U/ml)	T2	13.3 $\pm$ 5.8 <b>a</b>	14.0 $\pm$ 5.9 <b>b</b>	16.0 $\pm$ 8.2 <b>b</b>	21.3 $\pm$ 13.9 <b>b</b>
	T3	11.8 $\pm$ 7.1 <b>a</b>	33.2 $\pm$ 30.2 <b>b</b>	19.7 $\pm$ 15.6 <b>bc</b>	16.6 $\pm$ 7.6 <b>c</b>
	T4	27.9 $\pm$ 13.9	27.9 $\pm$ 17.6	31.5 $\pm$ 19.0	30.2 $\pm$ 22.2
Protein content (mg/ml)	T2	0.98 $\pm$ 0.11	1.06 $\pm$ 0.17	0.99 $\pm$ 0.12	1.05 $\pm$ 0.25
	T3	1.33 $\pm$ 0.18	1.23 $\pm$ 0.16	1.27 $\pm$ 0.17	1.34 $\pm$ 0.26
	T4	1.13 $\pm$ 0.10	1.08 $\pm$ 0.11	1.11 $\pm$ 0.11	1.11 $\pm$ 0.12

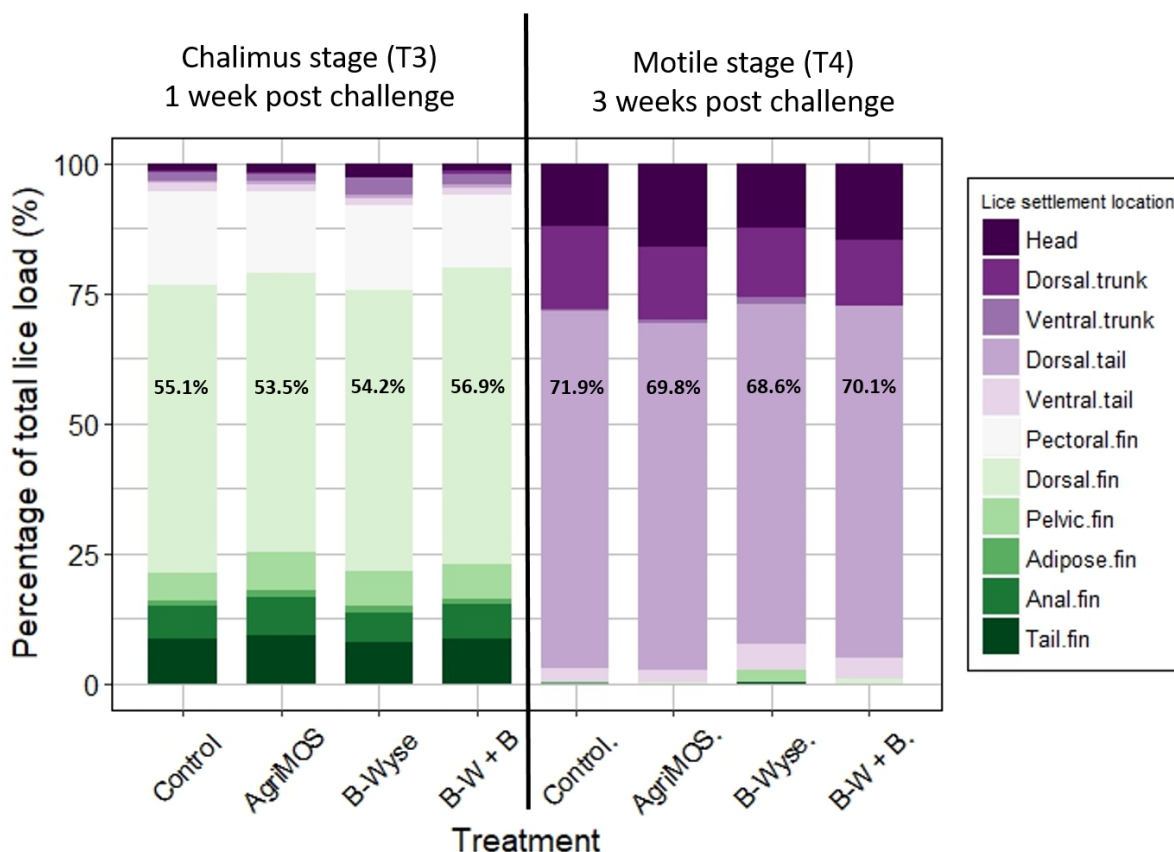
### 4.3.3 Sea lice analysis

Due to the nature and severity of the challenge, all fish presented with sea lice infection. Significantly fewer attached sea lice were observed on fish fed the AgriMOS (-16.6%;  $p = 0.010$ ), B-Wyse (-21.4%;  $p = 0.001$ ), and B-Wyse + Bactocell® (-14.3%;  $p = 0.024$ ) treatments, compared to those fed the control diet, 1 week post challenge (T3). After three weeks of exposure (T4), the B-Wyse (-9.6%,  $p = 0.016$ ) and AgriMOS (-9.8%,  $p = 0.022$ ) treatments continued to have significantly fewer attached sea lice compared to the control. The observed reduction in the B-Wyse + Bactocell® treatments after three weeks, while still present, was less profound and non-significant. Results are presented in Figure 4.3.

Additionally, the dietary treatments did not influence the settlement location of the sea lice on the fish: no significant differences were detected between the numbers of lice on the different body regions amongst the different treatments. As represented in Figure 4.4, at the chalimus stage (T3), only 5-10% of chalimus settled on the head and abdominal regions, while over 90% settled on fins with 50% settling on the dorsal fin alone. In contrast, when the lice reached the motile stage (T4), approximately 70% of lice were recorded on the dorsal tail section of the abdomen alone, mostly at the base of the dorsal and adipose fins.



**Figure 4.3** Dot plot presenting sea lice counts on fish fed the different experimental treatments at A) 1 week post infection (T3) and B) 3 weeks post infection (T4). Grey circles represent mean sea lice count of individual tanks (n=15); the blue dot represents treatment mean (n=4); the box plot represents median, upper and lower quartiles of the treatment. Different letters above different dot plots denote significant difference between those of a different letter,  $p < 0.05$ .



**Figure 4.4** Frequency of settled lice (presented as a percentage of total sea lice population) across the 11 different pre-designated body segments 1 week (T3) and 3 weeks (T4) after challenge,  $n=60$  per treatment. B-W + B = B-Wysey + Bactocell® treatment.

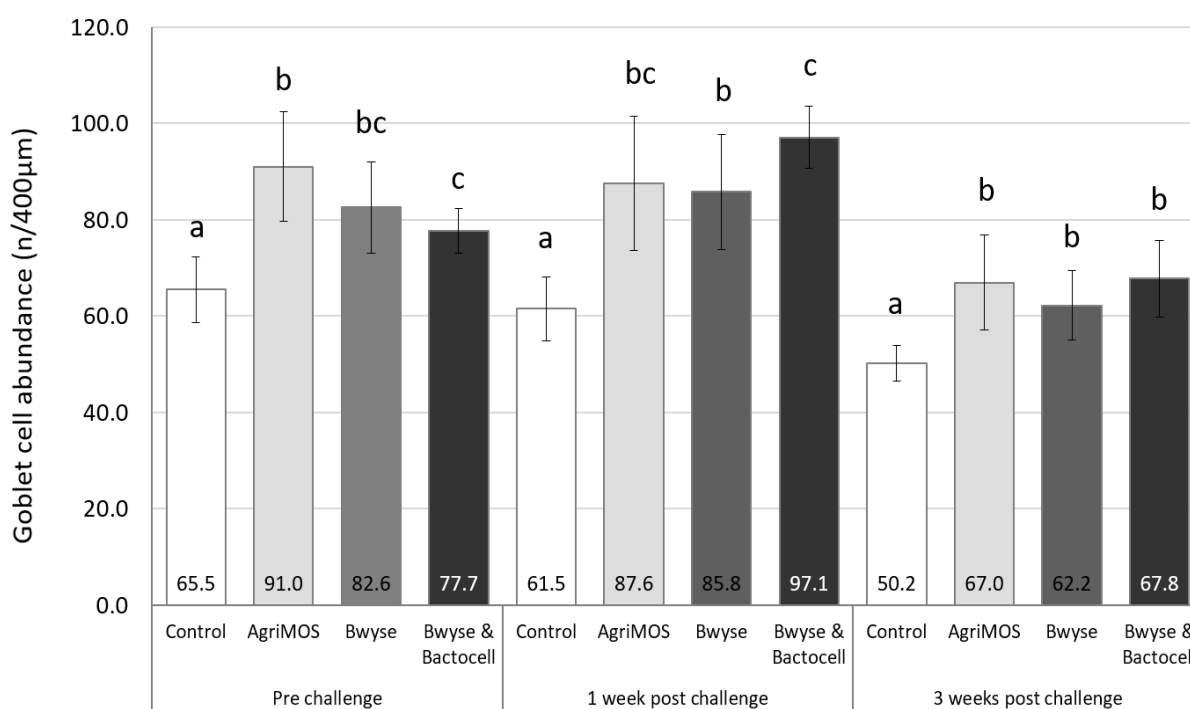
#### 4.3.4 Histological analysis

##### 4.3.4.1 Gill

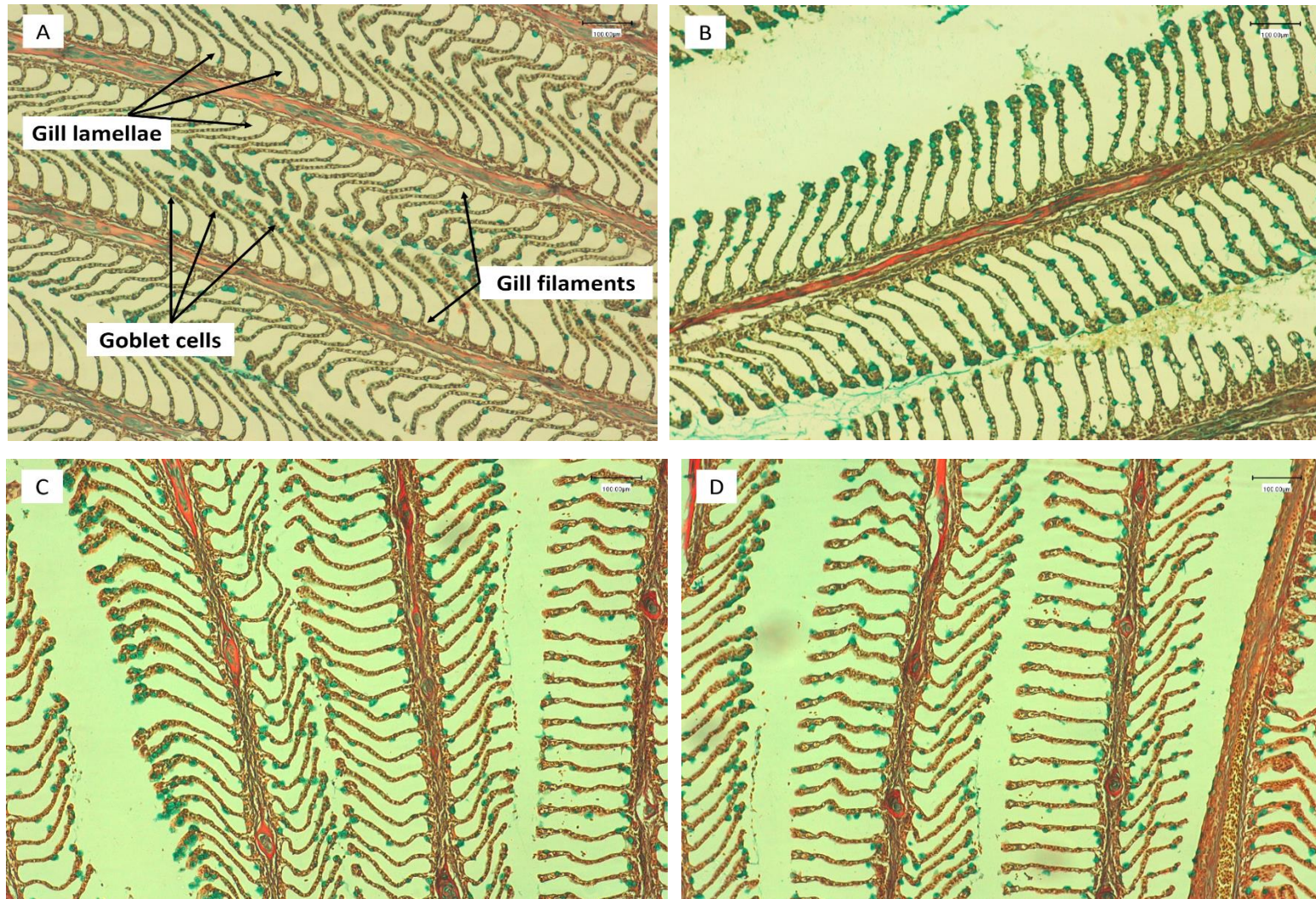
In the gill, the abundance of goblet cells ( $n/400\mu\text{m}$ ) were significantly elevated in fish fed the AgriMOS, B-Wysey, and B-Wysey + Bactocell® treatments compared to those fed the control diet both prior to infection (T2) ( $91.0 \pm 11.4$ ,  $p= 0.001$ ;  $82.6 \pm 9.4$ ,  $p= 0.003$ ;  $77.7 \pm 4.6$ ,  $p= 0.003$ , respectively) and post challenge at T3 ( $87.6 \pm 13.9$ ,  $p= 0.001$ ;  $85.8 \pm 12.0$ ,  $p= 0.001$ ;  $97.1 \pm 6.4$ ,  $p= 0.001$ , respectively) and T4 ( $67.0 \pm 9.9$ ,  $p= 0.002$ ;  $62.2 \pm 7.2$ ,  $p= 0.005$ ;  $67.8 \pm 8.0$ ,  $p= 0.001$ , respectively).

Additionally, the AgriMOS-fed fish ( $91.0 \pm 11.4$ ) had significantly more goblet cells ( $n/400\mu\text{m}$ ) than fish fed the B-Wyse + Bactocell treatment ( $77.7 \pm 4.6$ ,  $p= 0.013$ ) prior to challenge (T2), yet at 1 week post challenge (T3), no significant differences were detected between these two treatments. In contrast, a significant difference was observed between the B-Wyse ( $85.8 \pm 12.0$ ) and the B-Wyse + Bactocell® treatments ( $97.1 \pm 6.4$ ,  $p= 0.034$ ) 1 week post-infection (T3). No significant differences were observed between any of the experimental diets 3 week post-challenge (T4).

Results are presented Figure 4.5 and representative micrographs for histological analysis of the gill tissue are presented in Plate 4.1.



**Figure 4.5** Goblet cell abundance ( $n/400\mu\text{m}$ ) in the GIALT of fish fed the differing dietary treatments prior to challenge (T2), 1 week post challenge (T3) and 3 weeks post challenge (T4). Different letters denote significant differences between treatments with a different letter within the same time-point,  $p < 0.05$ ; data presented as mean  $\pm$  SD,  $n=8$ .



**Plate 4.1** Light micrographs of the gill of fish fed A) the control, B) AgriMOS-, C) B-Wyse-, and D) B-Wyse + TB-supplemented diets for 6 weeks (T2). Scale bar represents 100µm.

#### 4.3.4.2 Skin

After 6 weeks of feeding the experimental diets and prior to challenge (T2), the abundance of goblet cells ( $n/400\mu\text{m}$ ) within the SALT of fish fed the AgriMOS ( $15.68 \pm 2.0$ ,  $p= 0.022$ ), B-Wyze ( $15.2 \pm 1.1$ ,  $p= 0.001$ ), B-Wyze + Bactocell® ( $16.58 \pm 2.7$ ,  $p= 0.003$ ) were significantly increased compared to fish fed the control treatment ( $11.8 \pm 1.8$ ), as shown in Figure 4.6.

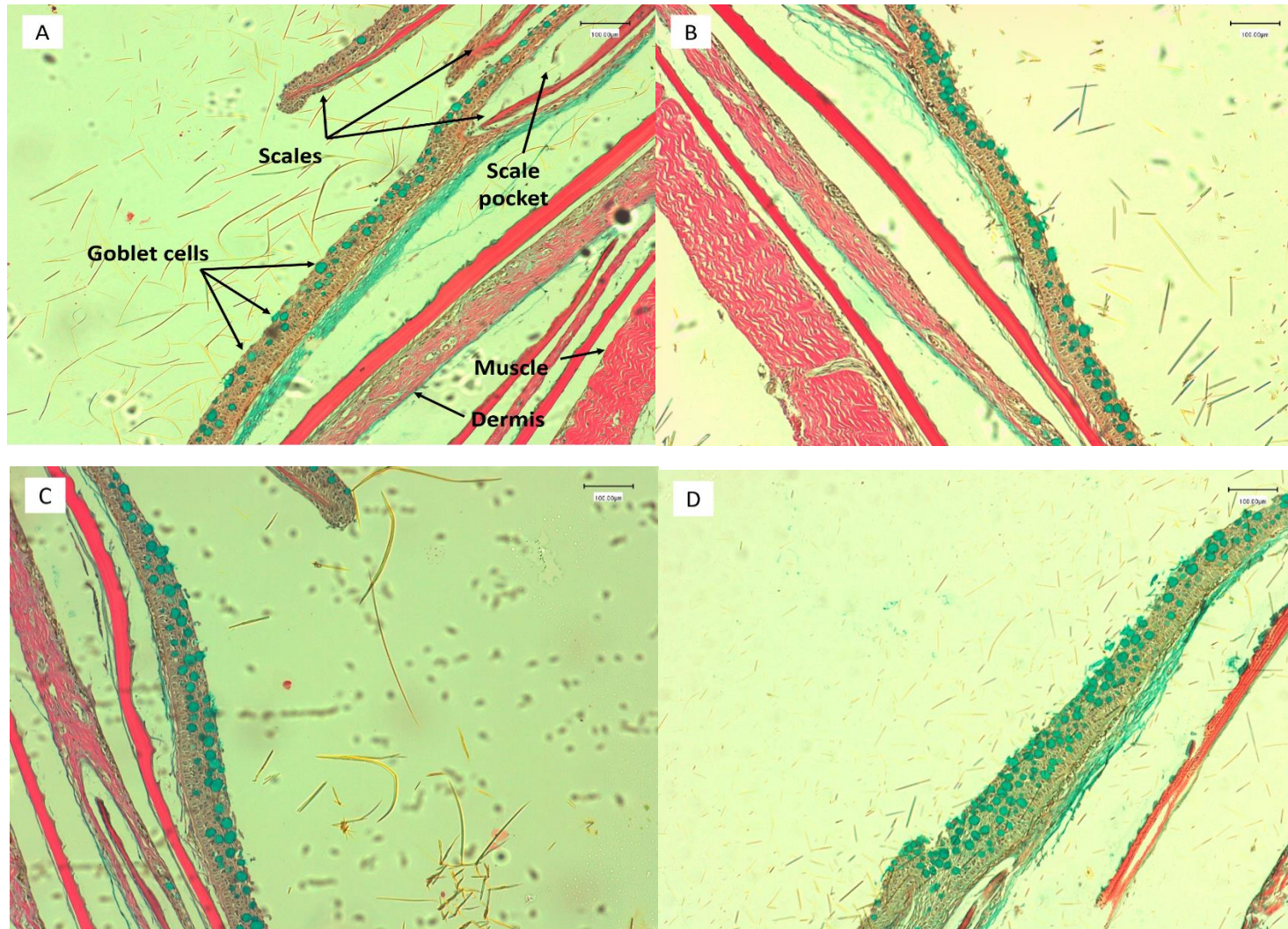
Also after 6 weeks, the area fraction of goblet cells ( $n/\mu\text{m}^2$ ) in the SALT of fish fed the AgriMOS treatment ( $16.3 \pm 4.4$ ,  $p= 0.001$ ), were significantly elevated compared to those fed the control diet ( $9.0 \pm 1.4$ ). Additionally, fish fed the B-Wyze ( $20.7 \pm 3.0$ ), and B-Wyze + Bactocell® ( $22.8 \pm 5.1$ ) treatments exhibited significantly elevated goblet cell area fractions ( $n/\mu\text{m}^2$ ) compared to those fed the control (B-Wyze  $p= 0.001$ , B-Wyze + Bactocell  $p= 0.001$ ) and AgriMOS (B-Wyze  $p= 0.041$ , B-Wyze + Bactocell  $p= 0.0017$ ) diets, as shown in Figure 4.7.

One week after challenge (T3), no differences in goblet cell abundance or area fraction were detected between any of the dietary treatments. At T4, three weeks post challenge, no differences were detected in goblet cell abundance, however the goblet cell area fraction ( $n/\mu\text{m}^2$ ) was observed to be significantly elevated in fish fed the AgriMOS ( $14.9 \pm 2.5$ ,  $p= 0.001$ ), B-Wyze ( $14.2 \pm 2.0$ ,  $p= 0.001$ ), and B-Wyze + Bactocell® ( $15.4 \pm 3.1$ ,  $p= 0.001$ ) treatments, compared to the control-fed fish ( $10.1 \pm 1.5$ ).

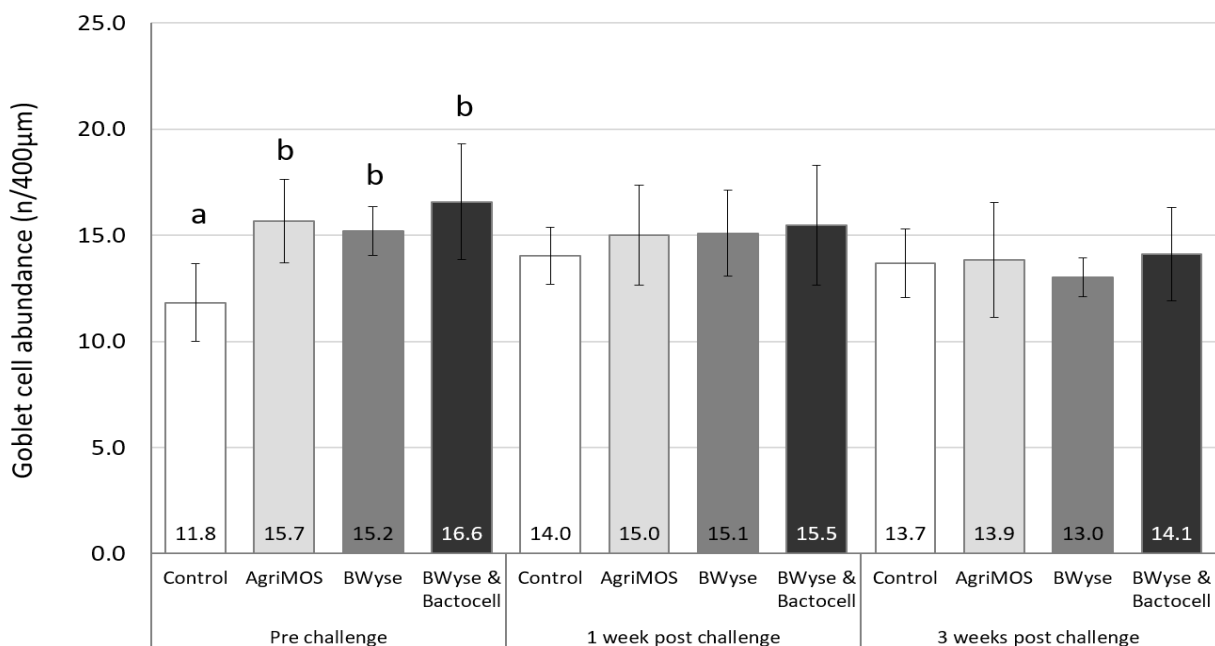
Additionally, a significant decrease was observed between the goblet cell area fraction of the control fed fish 1 week (T3) post challenge ( $14.4 \pm 2.2$ ) and 3 weeks (T4) post challenge ( $10.1 \pm 1.5$ ,  $p = 0.002$ ).

Representative micrographs used for histological analysis of the skin tissue are presented in Plate 4.2 and results are presented Figures 4.6 and 4.7.

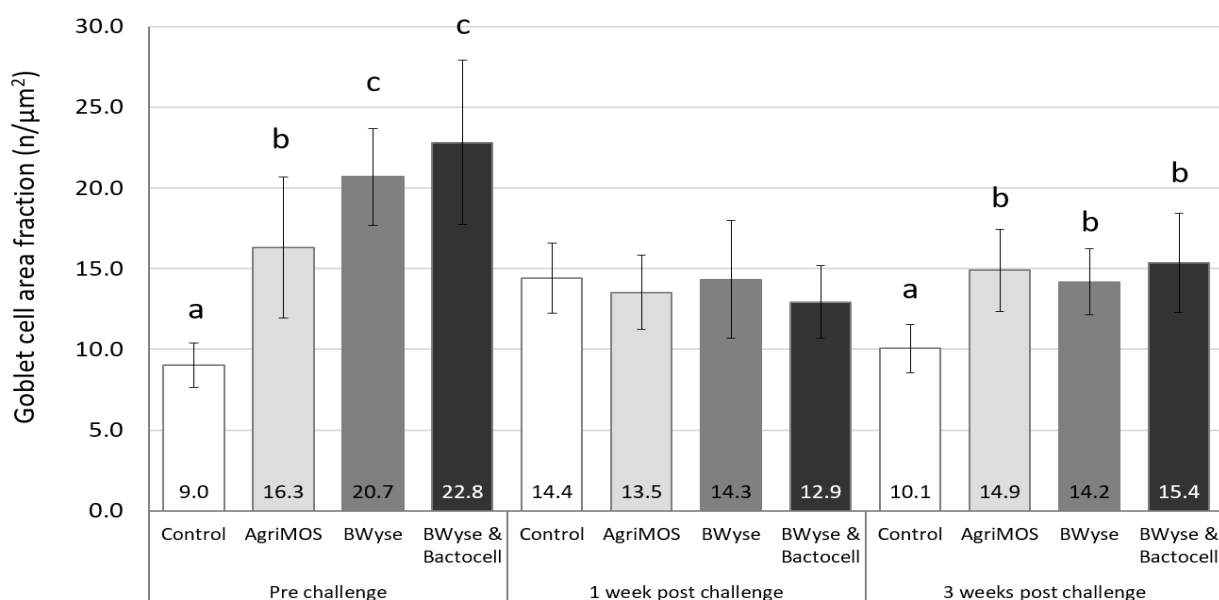




**Plate 4.2** Light micrographs of the SALT of fish fed A) the control, B) AgriMOS-, C) B-Wyse-, and D) B-Wyse + TB-supplemented diets for 6 weeks (T2). Scale bar represents 100µm.



**Figure 4.6** Goblet cell abundance (n/400µm) in the SALT of fish fed the differing dietary treatments before (T2) and after challenge (T3 and T4). Different letters denote significant differences between treatments with a different letter within the same time-point,  $p < 0.05$ ; data presented as mean  $\pm$  SD; n=8.



**Figure 4.7** Goblet cell area fraction (n/µm<sup>2</sup>) in the SALT of fish fed the differing dietary treatments before (T2) and after challenge (T3 and T4). Different letters denote significant differences between treatments with a different letter within the same time-point,  $p < 0.05$ ; data presented as mean  $\pm$  SD; n=8.

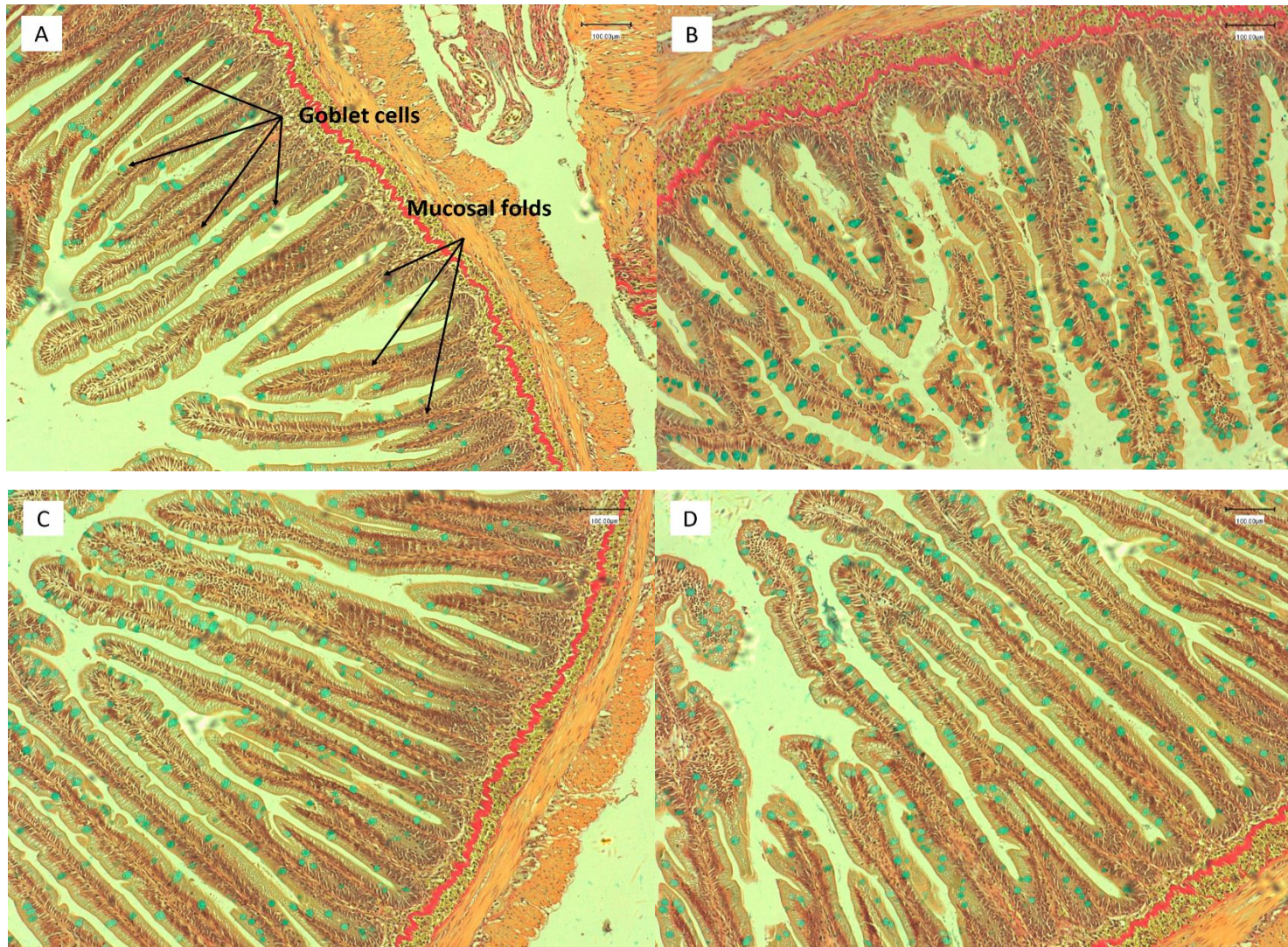
#### 4.3.4.3 Intestine

In the GALT, fish fed the AgriMOS, B-Wysey, and B-Wysey + Bactocell® treatments were observed as having significantly elevated goblet cell abundances (n/200µm) compared to those fed the control treatment prior to challenge (T2: 20.6 ± 3.9,  $p= 0.001$ ; 18.6 ± 1.4,  $p= 0.001$ ; and 18.3 ± 1.5,  $p= 0.001$ , respectively), 1 week following challenge (T3: 15.6 ± 1.8,  $p= 0.001$ ; 15.1 ± 1.9,  $p= 0.001$ ; and 16.0 ± 3.1,  $p= 0.001$ , respectively), and 3 weeks following challenge (T4: 15.2 ± 2.7,  $p= 0.001$ ; 15.0 ± 1.6,  $p= 0.001$ ; and 16.0 ± 1.8,  $p= 0.001$ , respectively).

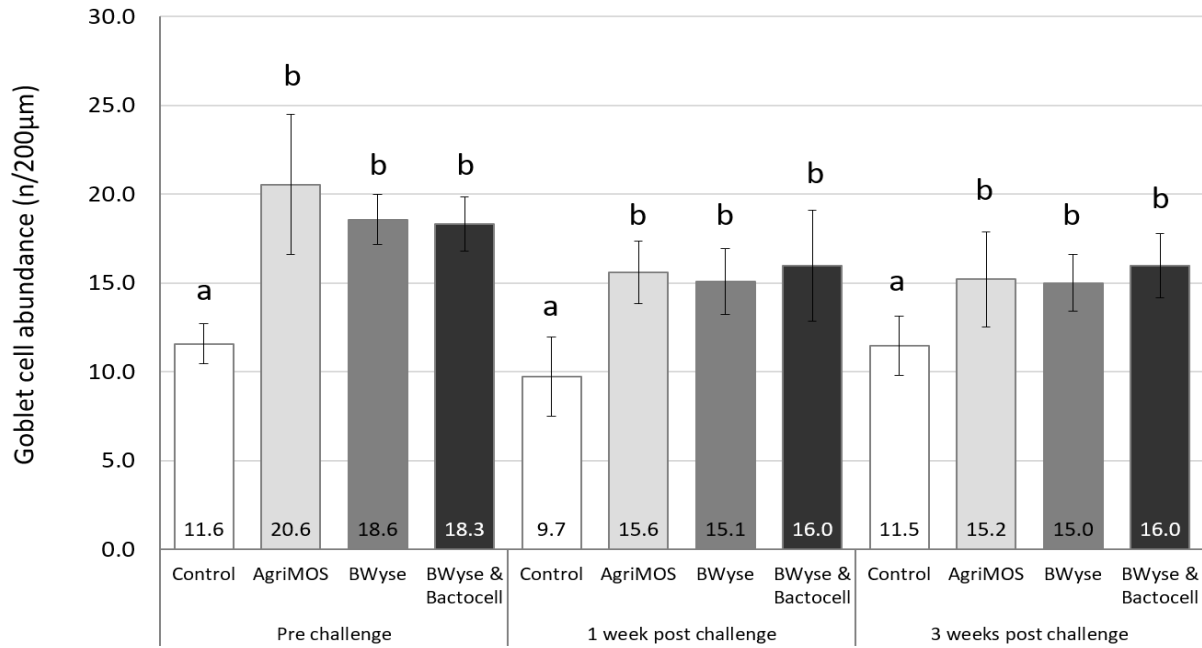
Prior to challenge (T2), fish fed the B-Wysey treatment (4.6 ± 0.5,  $p= 0.001$ ) had a significantly elevated goblet cell area fraction (n/µm<sup>2</sup>) in their intestine compared to those fed the control (3.5 ± 0.4), while the AgriMOS (5.7 ± 1.1) and B-Wysey + Bactocell® (5.7 ± 1.2) fed fish had significantly larger goblet cell area fractions compared to both the control (AgriMOS  $p= 0.001$ ; B-Wysey + Bactocell  $p= 0.001$ ) and the B-Wysey-supplemented (AgriMOS  $p= 0.009$ ; B-Wysey + Bactocell  $p= 0.030$ ) treatment.

At T3, 1 week following challenge, fish fed the AgriMOS (3.5 ± 0.4,  $p= 0.001$ ), B-Wysey (4.1 ± 0.5,  $p= 0.001$ ), and B-Wysey + Bactocell® (3.9 ± 0.3,  $p= 0.028$ ) treatments had significantly elevated goblet cell area fractions (n/µm<sup>2</sup>), compared to fish fed the control treatment (2.3 ± 0.7). No statistical variations were detected between the three additive supplemented diets.

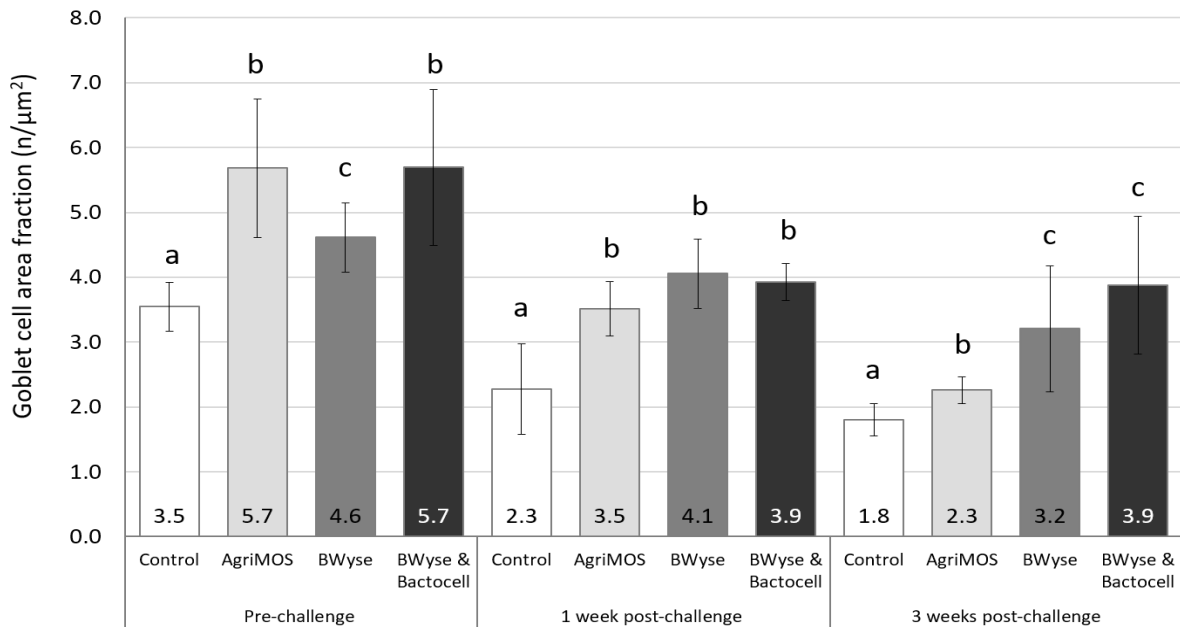
Three weeks following challenge (T4), the AgriMOS-fed fish (2.3 ± 0.2,  $p= 0.002$ ) exhibited significantly elevated goblet cell area fractions (n/µm<sup>2</sup>) compared to those fed the control regime (1.8 ± 0.2), while the B-Wysey (3.2 ± 1.0) and B-Wysey + Bactocell® (3.9 ± 1.1) treatments were observed as having goblet significantly elevated cell area fractions (n/µm<sup>2</sup>) compared to fish fed the AgriMOS (B-Wysey  $p= 0.022$ ; B-Wysey + Bactocell  $p= 0.001$ ) and control (B-Wysey  $p= 0.001$ ; B-Wysey + Bactocell  $p= 0.001$ ) regimes. Representative micrographs used for



**Plate 4.3** Light micrographs of the intestine of fish fed A) the control, B) AgriMOS-, C) B-Wyse-, and D) B-Wyse + TB-supplemented diets for 6 weeks (T2). Scale bar represents 100µm.



**Figure 4.8** Goblet cell abundance (n/200µm) in the GALT of fish fed the differing dietary treatments before (T2) and after challenge (T3 and T4). Different letters denote significant differences between treatments with a different letter within the same time-point,  $p < 0.05$ ; data presented as mean  $\pm$  SD; n=8.



**Figure 4.9.** Goblet cell area fraction (n/µm<sup>2</sup>) in the GALT of fish fed the differing dietary treatments before (T2) and after challenge (T3 and T4). Different letters denote significant differences between treatments with a different letter within the same time-point,  $p < 0.05$ ); data presented as mean  $\pm$  SD; n=8.

histological analysis of the skin tissue are presented in Plate 4.3 and results are presented in Figures 4.8 and 4.9.

Concerning the length of the mucosal folds, after six weeks of feeding and prior to pathogen challenge (T2), fish fed the AgriMOS ( $709.3 \pm 49.3$ ,  $p= 0.029$ ), B-Wyse ( $682.6 \pm 17.7$ ,  $p= 0.029$ ) and B-Wyse + Bactocell® ( $674.0 \pm 24.0$ ,  $p= 0.047$ ) regimes were observed as having significantly elevated fold lengths when compared to the control-fed fish ( $628.3 \pm 19.0$ ). However, no statistically significant differences were observed between treatments at either sampling point post challenge (T3 and T4). As presented in Table 4.4, no statistically significant differences were observed for lamina propria width between any of the sampled time-points for any of the experimental treatments.

**Table 4.4** Anatomical measurements of the intestine of fish fed the differing dietary treatments. Different letters in the same row denote significant differences between treatments with different letters ( $p<0.05$ ). Data presented as mean  $\pm$  SD;  $n=8$ .

Parameter	Sampling point	Treatment			
		Control	AgriMOS	BWyse	BWyse + Bactocell®
Fold length ( $\mu\text{m}$ )	T2	$628.32 \pm 19.0\text{a}$	$709.3 \pm 49.3\text{b}$	$682.6 \pm 17.7\text{b}$	$674.0 \pm 24.0\text{b}$
	T3	$659.8 \pm 54.2$	$692.9 \pm 70.2$	$760.3 \pm 53.4$	$762.8 \pm 27.8$
	T4	$634.6 \pm 19.9$	$724.7 \pm 59.8$	$689.0 \pm 17.8$	$756.5 \pm 69.7$
Lamina Propria Width ( $\mu\text{m}$ )	T2	$11.55 \pm 1.5$	$11.32 \pm 0.7$	$10.08 \pm 1.3$	$10.63 \pm 1.1$
	T3	$10.30 \pm 0.8$	$10.92 \pm 0.6$	$10.91 \pm 0.6$	$11.97 \pm 1.3$
	T4	$10.22 \pm 1.5$	$10.52 \pm 0.8$	$10.79 \pm 0.8$	$11.87 \pm 1.2$

### 4.3.5 Gene expression

#### 4.3.5.1 RT-q-PCR analysis of the GIALT

The relative fold change compared to the control of the 10 selected genes in GIALT prior to challenge (T2) and 3 weeks post challenge (T4) are presented in Figures 4.10 A) and B) respectively. Prior to challenge and following 6 weeks of feeding the experimental diets (T2), fish fed the AgriMOS ( $p = 0.002$ ) and B-Wyse ( $p = 0.002$ ) treatments exhibited significantly increased expression of calreticulin in comparison to fish fed the control treatment, while fish fed the diet supplemented with B-Wyse + Bactocell<sup>®</sup> demonstrated significantly increased expression of calreticulin in comparison to fish fed the control ( $p = 0.002$ ), AgriMOS ( $p = 0.015$ ) and B-Wyse-supplemented ( $p = 0.050$ ) diets.

Significantly increased expression of Muc5ac-like was also observed in fish fed the B-Wyse and B-Wyse + Bactocell<sup>®</sup>-supplemented diets, compared to fish fed the control ( $p = 0.011$  and  $p = 0.004$ , respectively) and AgriMOS-supplemented ( $p = 0.034$  and  $p = 0.037$ , respectively) diets. The expression of claudins 28b and 27a in the GIALT of fish fed the AgriMOS ( $p = 0.015$  and  $0.004$ , respectively) and B-Wyse + Bactocell<sup>®</sup>-supplemented ( $p = 0.019$  and  $p = 0.015$ , respectively) diets were significantly downregulated in comparison to the control-fed fish.

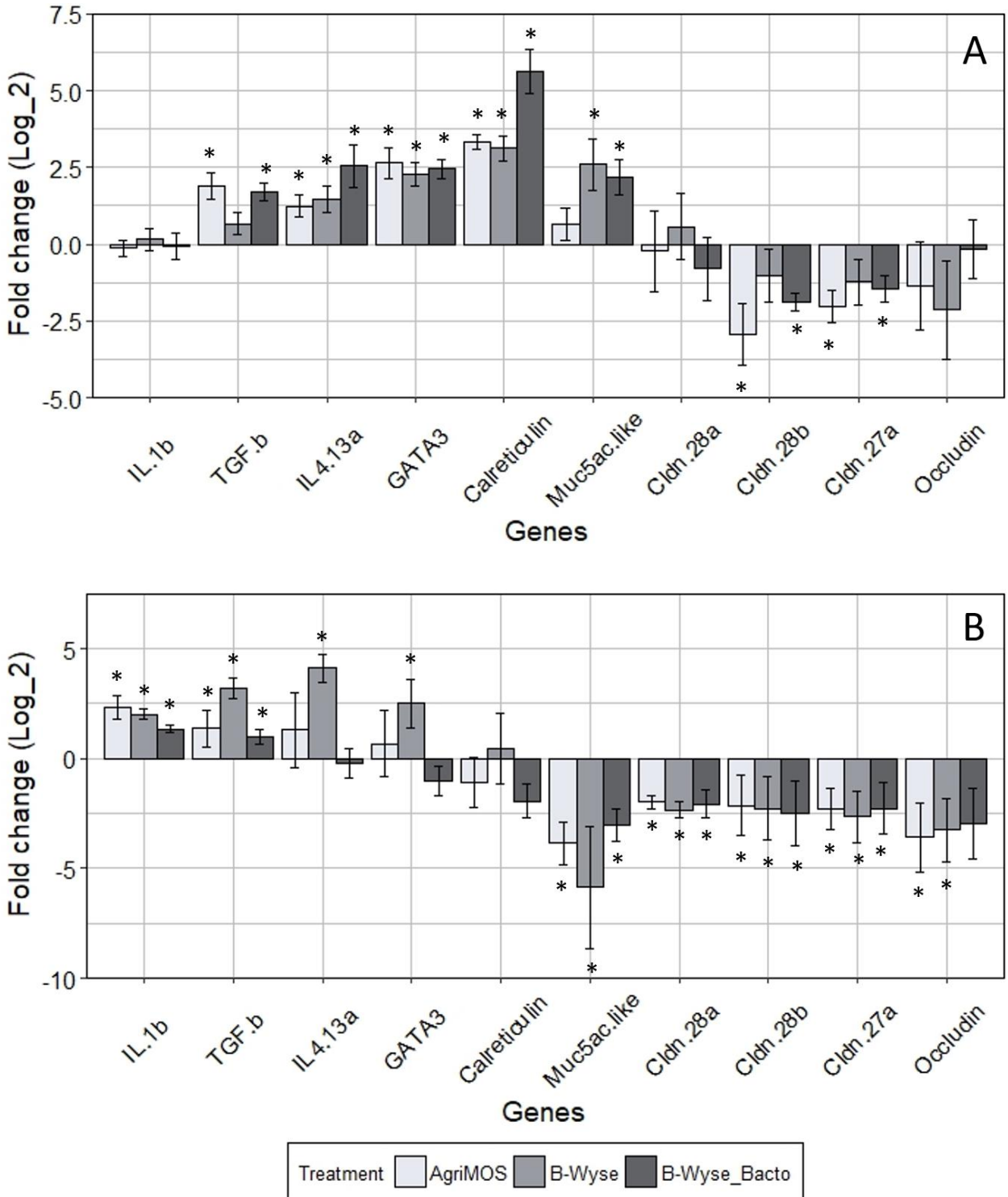
Expression levels for the IL4/13a gene were also significantly elevated in the gill tissue of fish fed the AgriMOS ( $p = 0.011$ ), B-Wyse ( $p = 0.032$ ), and B-Wyse + TB ( $p = 0.151$ ) treatments compared to the control, in addition to the significant upregulation in the expression of GATA3 in fish fed all three of the experimental treatments (AgriMOS  $p = 0.006$ ; B-Wyse  $p = 0.009$ ; B-Wyse + TB  $p = 0.002$ ), compared to the control-fed fish. Fish fed the AgriMOS-supplemented ( $p = 0.002$ ) diet demonstrated significantly elevated expression levels of TGF- $\beta$  in comparison to the control-fed fish, while fish fed the B-Wyse + Bactocell<sup>®</sup>-supplemented

diet exhibited significantly increased expression of TGF- $\beta$ , compared to fish fed the control ( $p = 0.006$ ) and B-Wyσε-supplemented ( $p = 0.045$ ) diets.

Three weeks post challenge (T4); fish fed all three of the experimental dietary treatments (AgriMOS  $p = 0.024$ ; B-Wyσε  $p = 0.016$ ; B-Wyσε + TB  $p = 0.016$ ) exhibited significantly reduced expression of Muc5ac-like in comparison to fish fed the control treatment. Additionally, fish fed all three of the experimental diets exhibited significantly reduced expression of claudin 28a (AgriMOS  $p = 0.024$ ; B-Wyσε  $p = 0.008$ ; B-Wyσε + TB  $p = 0.008$ ), 28b (AgriMOS  $p = 0.048$ ; B-Wyσε  $p = 0.048$ ; B-Wyσε + TB  $p = 0.048$ ), and 27a (AgriMOS  $p = 0.048$ ; B-Wyσε  $p = 0.048$ ; B-Wyσε + TB  $p = 0.048$ ), compared to fish fed the control treatment. Furthermore, significantly reduced expression of occludin was exhibited in fish fed the AgriMOS ( $p = 0.048$ ) and B-Wyσε-supplemented ( $p = 0.048$ ) diets, in comparison to those fed the control treatment.

The expression of IL-1 $\beta$  was also observed to be significantly elevated in the gills of fish fed the AgriMOS ( $p = 0.008$ ), B-Wyσε ( $p = 0.008$ ) and B-Wyσε + Bactocell<sup>®</sup> ( $p = 0.008$ ) treatments compared to fish fed the control treatment, in addition to the significantly increased expression of TGF- $\beta$  in fish fed all three of the experimental treatments (AgriMOS  $p = 0.008$ ; B-Wyσε  $p = 0.008$ ; B-Wyσε + TB  $p = 0.008$ ), compared to the control fed fish. Moreover, fish fed the B-Wyσε treatment exhibited significantly increased expression of IL4/13a ( $p = 0.008$ ) and GATA3 ( $p = 0.048$ ) compared to fish fed the control diet.





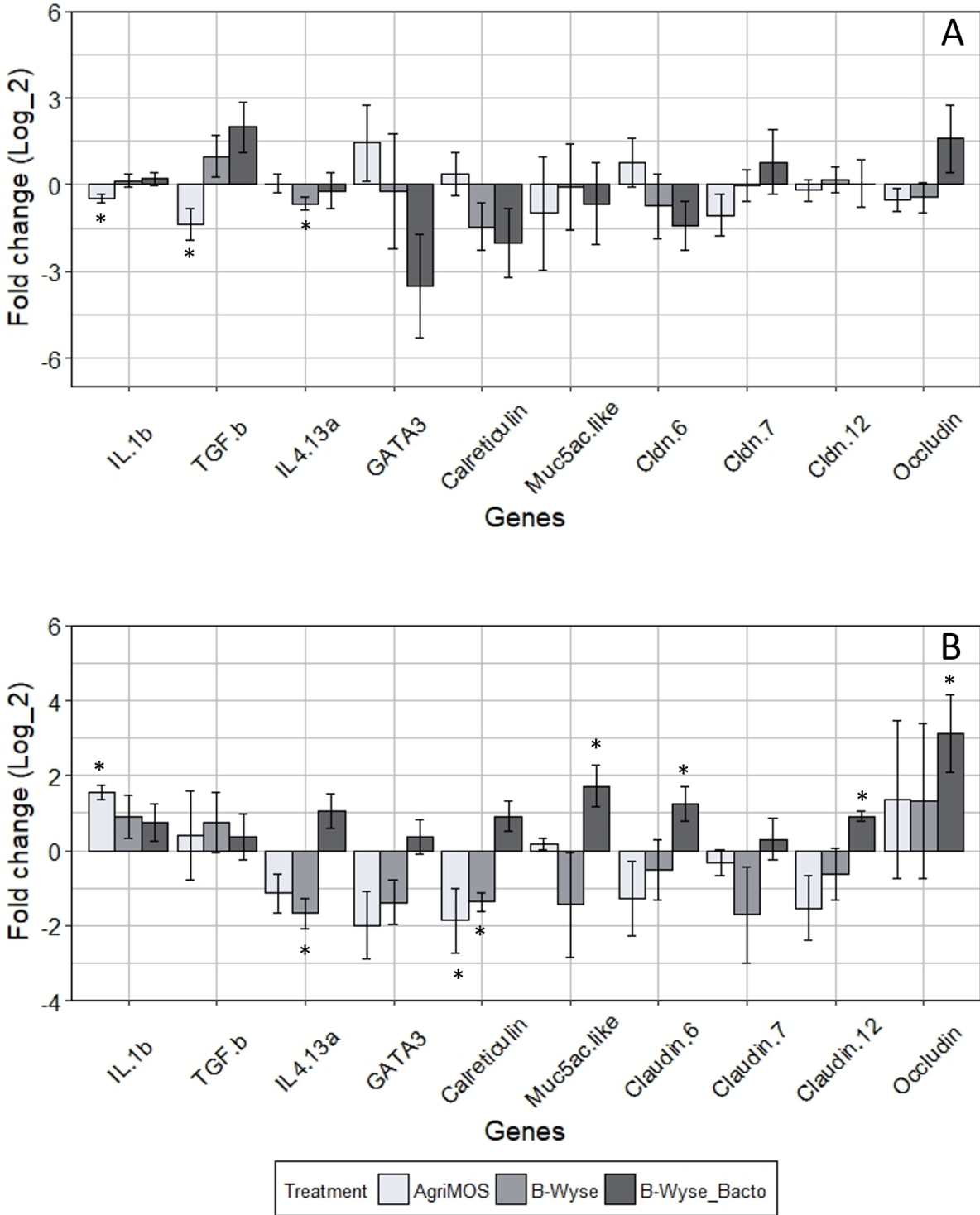
**Figure 4.10** Gene expression data (fold change ( $\log_2$ )) of 10 genes of interest in the GALT of fish fed the different dietary regimes A) prior to infection ( $n=6$ ) and B) 3 weeks post challenge ( $n=5$ ). Asterix denotes statistically significant difference to control,  $p<0.05$ ; data presented as mean  $\pm$  SE,

#### 4.3.5.2 RT-q-PCR analysis of the SALT

The fold change relative to the control of the 10 selected genes in SALT after 6 weeks of feeding and prior to challenge (T2) and 3 weeks post challenge (T4) are presented in Figures 4.11 A) and B), respectively. In the skin tissue after 6 weeks of feeding the experimental diets, very few significant differences in expression level were observed between fish fed any of the three experimental treatments and the control. However, in the SALT of fish fed the AgriMOS-supplemented diet, a significant downregulation in the expression of IL-1 $\beta$  and TGF- $\beta$  was observed in comparison to fish fed the control ( $p = 0.049$  and  $0.048$ , respectively), B-Wyse ( $p = 0.041$  and  $0.041$ , respectively) and B-Wyse + Bactocell<sup>®</sup> ( $p = 0.035$  and  $0.015$ , respectively) treatments.

Three weeks post challenge (T4), fish fed the AgriMOS-supplemented diet presented with significantly elevated levels of IL-1 $\beta$  ( $p = 0.006$ ) in the skin compared to fish fed the control treatment, while fish administered the B-Wyse + Bactocell<sup>®</sup> treatment exhibited significantly elevated expression of Muc5ac-like ( $p = 0.026$ ), claudin 6 ( $p = 0.024$ ), claudin 12 ( $p = 0.006$ ) and occludin ( $p = 0.015$ ), compared to fish fed the control treatment.

Furthermore, the significant downregulation in the expression of calreticulin was observed in the SALT tissue of fish fed AgriMOS ( $p = 0.001$ ) and B-Wyse-supplemented ( $p = 0.011$ ) diets, compared to those fed the control, in addition to the significant downregulation of IL4/13a in the SALT of fish administered the B-Wyse ( $p = 0.011$ ) diet, compared to the control-fed fish.

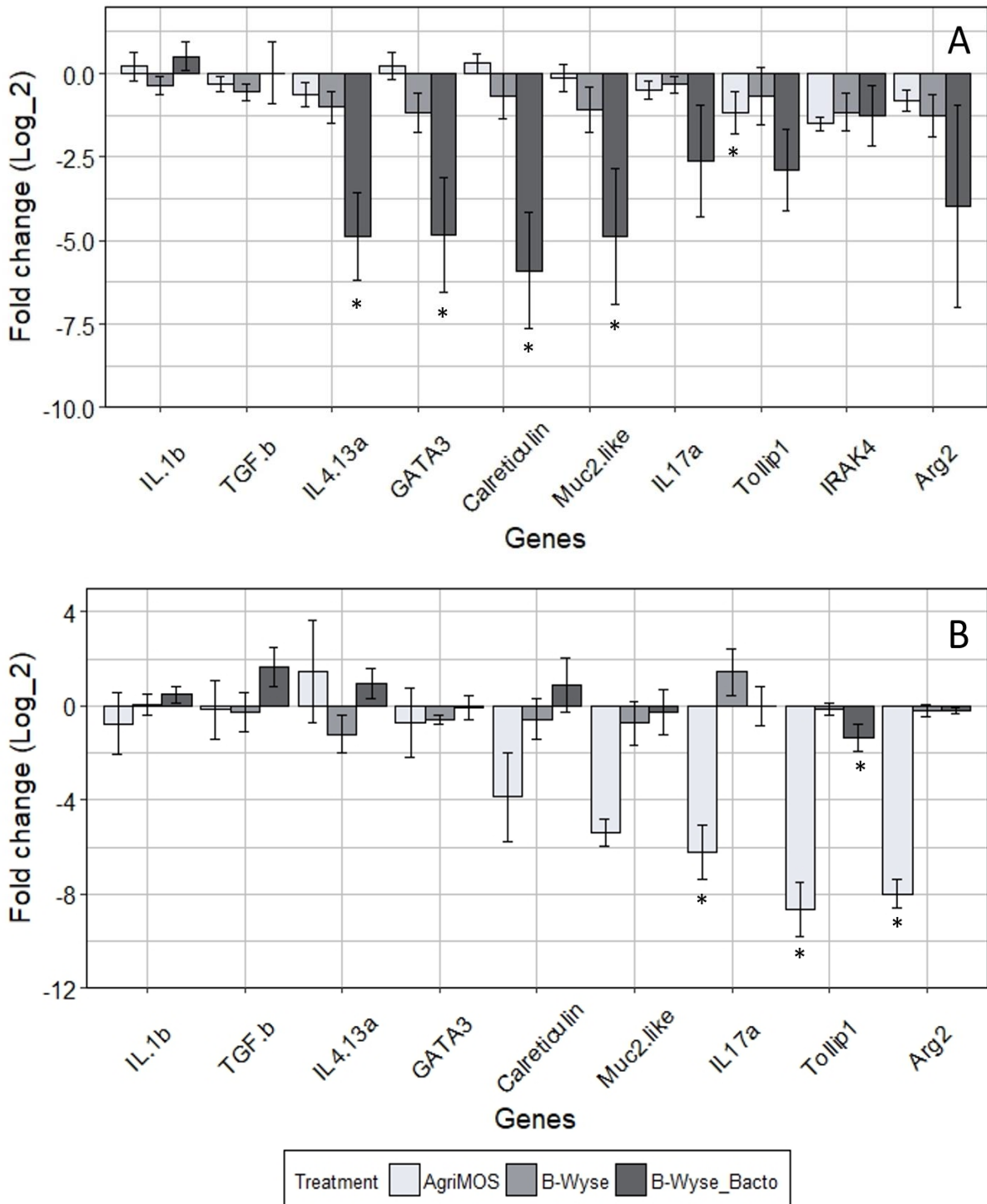


**Figure 4.11** Gene expression data (fold change (log<sub>2</sub>)) of 10 genes of interest in the SALT of fish fed four different dietary regimes A) prior to infection (T2) and B) 3 weeks post-infection (T4). Asterix denotes statistically significant difference to control,  $p < 0.05$ ; Data presented as mean  $\pm$  SE,  $n=6$ .

#### 4.3.5.3 RT-q-PCR analysis of the GALT

The relative fold change compared to the control of the 10 selected genes in the GALT after 6 weeks of feeding and prior to challenge (T2) and 3 weeks post challenge (T4) are presented in Figures 4.12 A) and B) respectively. In the distal intestine, fish fed the diets supplemented with B-Wyze + Bactocell® were observed as having significantly downregulated expression levels of IL4/13a ( $p = 0.008$ ), GATA3 ( $p = 0.024$ ), calreticulin ( $p = 0.008$ ), and Muc2-like ( $p = 0.032$ ) in their GALT, compared to the control-fed fish. Fish fed the AgriMOS and B-Wyze treatments demonstrated very little differences in the expression of the genes chosen for analysis following 6 weeks of feeding, compared to the control-fed fish.

Three weeks following infection however, fish fed the B-Wyze and B-Wyze + Bactocell®-supplemented dietary treatments exhibited very few differences in the expression of the genes analysed compared to the control-fed fish, while fish administered with the AgriMOS-supplemented treatment exhibited significant downregulation in the expression of IL-17a ( $p = 0.032$ ), Tollip1 ( $p = 0.040$ ) and Arg2 ( $p = 0.008$ ), compared to the control-fed fish.



**Figure 4.12** Gene expression data (fold change (log<sub>2</sub>)) of 10 genes of interest in the GALT of fish fed four different dietary regimes A) prior to infection (T2) and B) 3 weeks post-infection (T4). Asterix denotes statistically significant difference to control,  $p < 0.05$ ; data presented as mean  $\pm$  SE,  $n=5$ .

#### 4.4 Discussion

The present study is made up of two parts; the first comprised a 6-week feeding trial, while the second part introduced a controlled *L. salmonis* challenge. The first aim of this research was to investigate the effects of two commercially available prebiotics products (AgriMOS and B-Wyse), and a synbiotic (B-Wyse + Bactocell®) upon the growth performance indices of Atlantic salmon following 6 weeks of feeding and 1 and 3 weeks post challenge. The second aim of the research was to determine to what extent the dietary additives influenced the mucosal defences of the fish after six weeks, and also following sea lice challenge. The third aim was to further elucidate the underlying mechanisms responsible for alterations occurring at the MALT in response to the dietary additives, via the use of RT-q-PCR gene expression analysis. The fourth aim was to determine whether the dietary induced alterations to the mucosal defences improved the fish's resistance to sea lice settlement.

##### 4.4.1 Growth indices

Individual fish body weight, K-factor and SGR remained statistically unaffected by dietary treatment (T1 to T2) and pathogen challenge (T1 to T4). The overall weak growth of the fish (below 2-fold increase over the trial duration) should not be attributed to the nutritional value of the experimental diets as the Fulton's condition factor (K-factor) value fell between 1.05 and 1.15 for all treatments across all time-points, indicating the fish were in good nutritional condition. Instead, the low growth may be attributable to the repetitive interventions required to document the progress of the sea lice challenge. As in chapter three, under the excellent rearing conditions, indicated by the steady and optimal water quality, and in addition to the administration of high quality diets, there remained little scope for the

additives to exert a discernible influence upon the growth indices over the relatively short duration of the pre-challenge phase of the trial.

Concerning the B-Wyse supplemented diet, these results are in agreement with those of chapter three on rainbow trout and of that conducted on Caspian trout (Jami *et al.*, 2019), yet stand in contrast to those on Nile tilapia (Selim and Reda, 2015) and European sea bass (Rawling *et al.*, 2019). The results of the AgriMOS supplemented diets are also in agreement with previous studies. Torrecillas and colleagues reported no significant differences in K-factor or SGR between European sea bass that were administered the MOS treatment and those fed the control diet (Torrecillas *et al.*, 2011). Similarly, the SGR of Caspian trout remained unaffected by the dietary addition of 4g/kg of MOS (Jami *et al.*, 2019) and 5g/kg of MOS to Japanese flounder diets (Ye *et al.*, 2011). Interestingly, a significant increase in SGR was observed in European sea bass fed diets supplemented with a concentrated second-generation yeast outer cell wall compound (cMOS) compared to those fed the control diet (Torrecillas *et al.*, 2015), reiterating the importance of dosage when determining the efficacy and influence of dietary additives. The dietary supplementation of MOS + Bactocell® in European sea bass diets also did not stimulate significant alterations in fish growth and performance (Torrecillas *et al.*, 2018) nor did the inclusion of  $\beta$ -glucan + MOS + *L. plantarum* into a single diet for Caspian trout (Jami *et al.*, 2019). Overall, the effect of dietary additives on the growth and condition of a range of fish species, both carnivorous and omnivorous, remains varied and contentious, and serve to highlight discrepancies between investigations concerning dosage, basal formulation, fish age and maturation, and length of administration.

#### 4.4.2 Epidermal mucous quantity

Epidermal mucous represents a) the first physical barrier between the organism and the surrounding environmental water, inhibiting the entry of pathogens; b) a chemical barrier that contains enzymes and antibodies; and c) acts as a lubricant, aiding the movement of the fish through the water (Cerezuela *et al.*, 2011). Previous investigations have reported significant increases in epidermal mucous secretion in response to dietary additives. For example, epidermal mucous secretion was significantly enhanced by dietary intake of LAB in Atlantic salmon (Salinas *et al.*, 2008), MOS in European sea bass and rainbow trout (Torrecillas *et al.*, 2011; Rodriguez-Estrada *et al.*, 2013) and the synbiotic mixture of MOS and *E. faecalis* in rainbow trout (Rodriguez-Estrada *et al.*, 2013). Collectively, these results support those of the present study, in which fish fed all three of the experimental dietary regimes exhibited significantly increased secreted mucous quantities from the skin epidermis compared to those fed the control regime for 6 weeks. Concerning the prebiotic B-Wyse treatment, the results of this study differ to those of chapter three on rainbow trout in which fish fed the B-Wyse treatment did not exhibit significantly increased levels of secreted epidermal mucous. The inclusion levels of the additives were the same for both experiments, however these differences could be due to the length of administration as the rainbow trout were sampled after five weeks, while the Atlantic salmon were sampled after six weeks. Alternatively, this could be a species-specific response. In this experiment, the Atlantic salmon were post-smolts and therefore reared in seawater, while the rainbow trout were reared in fresh-water. Physiologically these species differ significantly; early work by Fast *et al.* (2002) compared the skin of different species of salmonid, reporting the outer epidermis of Atlantic salmon to be significantly thinner with fewer layers of epithelial cells than rainbow trout and



Coho salmon skin (Fast *et al.*, 2002). The thinner epidermis may necessitate the need for larger quantities of mucous in Atlantic salmon, serving to protect the thinner underlying epithelium from pathogenic invasion and attachment, and environmental fluctuations. Increased epidermal mucous quantities would decrease the number of successful pathogenic adhesions to the epithelium, reducing the exposure levels of the epithelium to eco-toxins produced by the pathogen, which hold the potential to disrupt the barrier function. Other endogenous and exogenous factors have also been shown to influence epidermal mucous secretions and can lead to significant changes in the composition, viscoelasticity, rheology and functionality of the mucous (Fæste *et al.*, 2019). Furthermore, while fish assigned to different treatments within the same experiment should be expected to experience near identical conditions, the same should not be assumed when comparing different investigations: factors such as feeding rate, water quality, and stocking density will differ between investigations, and affect mucosal secretions to varying degrees.

#### *4.4.3 L. salmonis challenge*

As previously discussed, increased mucous secretion has been proposed as an effective defence mechanism of host mucous-associated lymphoid tissues, which aid the “flushing” of pathogenic organisms out of the gut lumen or “sleuthing” and “shedding” away from the skin and gill tissues (Cone, 2009; Ángeles-Esteban, 2012). Considering the significantly increased epidermal mucous quantities produced by fish fed all three of the experimental treatments, this hypothesis is supported by the results of the present study, in which a significant reduction in the number of attached sea lice was observed in fish fed all three of the experimental diets 1 week post challenge also, and continued to be observed after three weeks for the B-Wyse and AgriMOS treatments. Although the mechanism by which dietary

additives contribute to the resistance of fish to external parasites remains to be fully elucidated, the results here would suggest that increased amounts of epidermal mucous aid in the prevention of sea lice settlement and attachment to the epidermal layers. After observing the ability of MOS-administration within their study to significantly reduce sea lice burdens, Dimitroglou and colleagues proposed the same reasoning as those above; that the quantity and possibly the quality of epidermal mucous influences sea lice attachment success (Dimitroglou *et al.*, 2011). These results stand in contrast to those of Refstie *et al* (2010) who did not observe any significant differences in lice abundance on fish fed a diet supplemented with MOS. However, the same study did report a significant 28% reduction in the number of attached sea lice following  $\beta$ -glucan administration (Refstie *et al.*, 2010). These results are again in contrast to those published by Covello and co-workers, who reported a lack of difference between the number of attached sea lice on fish fed the control diet compared to those fed a diet supplemented with  $\beta$ -glucan; the authors suggest that the inclusion level of the  $\beta$ -glucan was not potent enough to be used as an effective in-feed treatment for sea lice (Covello *et al.*, 2012). The present study demonstrated that the simultaneous inclusion of both  $\beta$ -glucans and a MOS product into a single diet had the most profound impact upon sea lice burdens of all the dietary treatments tested, significantly reducing the number of attached sea lice by 21.4% compared to the control regime. Even more so, this dietary addition conferred an extended level of protection onto the fish beyond those observed for the synbiotic treatment, which remained observable after three weeks of infection; future investigations should consider sampling beyond 3 weeks of infection in order to assess the longevity of this protection.

The potential mechanisms by which these additives act within the host fish will be discussed within the context of the results of this study, in relation to their ability to influence the humoral mucosal defences, the barrier defences of the MALT, and regulate the transcription of certain genes.

Although dietary treatment did not have any effect upon the settlement location of the lice, it is interesting to note that during the chalimus stage, only 5-10% of chalimus settled on the head and abdominal regions, while over 90% settled on fins with 50% settling on the dorsal fin alone. In contrast, when the lice reached the motile stage, approximately 70% of lice were recorded on the dorsal tail section of the abdomen alone, mostly at the base of the dorsal and adipose fins. This information may be of value to the development of the non-medicinal sea lice treatments, such as optical delousing technologies, that take advantage of underwater lasers to “shoot” lice off fish (Overton *et al.*, 2019).

#### 4.4.4 Quality of epidermal mucous

Following the examination of the proteomic profiles of epidermal skin and mucous, several studies have suggested that dietary additives are capable of modulating the mucous to aid in the prevention of sea lice attachment via a variety of different proteins associated with immune function, such as lysozyme and galectins (Martin and Król 2017). Lysozyme is one of the most important bactericidal enzymes of the innate immune system and constitutes an essential defence mechanism against pathogens in fish (Akhter *et al.*, 2015). Lysozyme lyses bacterial cells by degrading the peptidoglycan hetero-polymers found within the cell walls (Carbone and Faggio, 2016), yet despite the absence of peptidoglycan in parasitic envelopes, lysozyme has been previously reported to inhibit other parasites, such as amoeba (León-

Sicairos *et al.* 2006). Further studies to investigate whether the membranes of sea lice contain a similar component to bacterial peptidoglycan should therefore be considered.

While the response of lysozyme has been found to vary in its potency depending on fish species and the tissue location (Dash *et al.*, 2018), several studies have documented increased epidermal mucous lysozyme activity in response to feeding with dietary additives (Torrecillas *et al.*, 2011; Ye *et al.*, 2011; Sheikhzadeh *et al.*, 2012; Dawood *et al.*, 2015; 2016). In this investigation, lysozyme activity within the epidermal mucous of fish fed all three of the experimental regimes was significantly increased after six weeks of feeding and 1 week following exposure to sea lice. Contrasting results concerning the B-Wyse treatment are observed again here when comparing the results of chapter three. Although an increase in lysozyme activity was observed in rainbow trout fed the B-Wyse supplemented diet, the results were not found to be significantly different to those of the control. These results do not necessarily suggest greater susceptibility to particular pathogenic infections, but may simply result from lysozyme playing a lesser role in the innate immune responses of rainbow trout (Fast *et al.*, 2002). Alternatively, given the higher susceptibility of Atlantic salmon to sea lice infestations compared to rainbow trout (O'Donohoe *et al.*, 2016), and if lysozyme does indeed play a role in the degradation of sea lice membranes, the observed differences in lysozyme activity could indicate a potentially leading role for the enzyme in the defence of Atlantic salmon to parasitic invasions. In any case, based on the results of the present study, increased lysozyme activity within the mucosal layer would suggest a stimulation of the innate-immune defences within the epidermal mucous layer, in response to the dietary administration of the three of the experimental additives.

Proposed to have a role in the mucosal defences of fish, galectins are a member of the lectin family; carbohydrate-binding proteins that are able to agglutinate and engulf bacteria (Madusanka *et al.*, 2019). Provan and colleagues (2013) proposed that the upregulation of galectins in the epidermal mucous of fish suffering with even low levels of sea lice suggests that galectins may potentially be a part of the defence response to sea lice in Atlantic salmon, acting in a similar way as when encountering a bacterial pathogen (Provan *et al.*, 2013). While the abundance of galectins within the epidermal mucous was not specifically determined in the present study, no discernible differences in the protein content of the mucous were observed between fish fed any of the different dietary treatments, both pre- and post-challenge with *L. salmonis*. This is in contrast to previous studies in which a remarkable increase in skin mucous protein level was observed following administration of dietary xylo-oligosaccharide to Caspian white fish (*Rutilus frisii kutum*) (Hoseinifar *et al.*, 2014), and dietary pre-, pro- and synbiotic supplemented diets to rainbow trout (Hoseinifar *et al.*, 2015). These differing results may be due to the sensitivity of the protein assay employed: the use of more specialised and sensitive techniques such as proteomics would allow for more targeted results concerning the levels of galectins within the epidermal mucous. Alternatively, the method used to collect epidermal mucous from the fish has recently been demonstrated to affect the protein content of the mucous sample: a study confirmed qualitative differences present between skin mucous samples collected by three different methods, with a clear distinction between mucous absorbed onto medical wipes and samples collected via wiping or scraping (Fæste *et al.*, 2019). As expected, collection via scraping had the greatest impact on the epidermal surface while the observed difference in protein concentration within the mucous suggested a leakage of proteins from epithelial cells destroyed during collection (Fæste *et al.*, 2019). During this experiment, it is therefore possible that the underlying epidermal cells

were disrupted in equal proportion on each fish sampled. While this would indicate a standardised sampling methodology on the one hand, on the other it may also be disguising differences between the treatments that perhaps would have been distinguishable if a different, less invasive sampling method had been employed. Future studies would do well to consider the absorption method of sampling when it is important that the mucous be devoid of proteins from the underlying epithelium, and the wiping method when focussing on the amounts of secreted mucous so as not to compromise the underlying epithelium.

#### *4.4.5 Histological appraisal of the MALT tissues prior to challenge*

Contributing to the general enhancement of the mucosal barrier and conferring additional protection against pathogens, the mucous layer of the MALT is primarily produced by goblet cells present within the epidermis, the abundance of which has been suggested to reflect the health status of the mucosal tissues and as a possible stress index for fish (Salinas *et al.*, 2011; Sveen *et al* 2017; Reverter *et al.*, 2018). In the present study, the goblet cell abundance was measured in all three MALTs, in addition to the goblet cell area fraction in the SALT and GALT. Fish fed the AgriMOS treatment exhibited significantly increased goblet cell abundance in the SALT, GIALT, and GALT, in addition to significantly elevated CAF in the SALT and GALT, in comparison to those fed the control treatment following 6 weeks of feeding. As the primary organ in contact with the MOS additive, these results are not surprising within the intestine and are indicative of an enhanced mucosal response, as suggested by the presence of a greater number of larger mucous-producing cells, compared to the control. These results are in accordance with previous studies in which the abundance of goblet cells significantly increased in the anterior and posterior intestine of European sea bass fed a MOS-supplemented diet (Torrecillas *et al.*, 2011; 2013).

The increased goblet cell abundance in the GIALT and SALT, in addition to area fraction in the SALT of fish fed the AgriMOS-supplemented diet in the present study may be the result of the proposed interconnectivity between the MALT tissues. While it is unlikely that the skin and gill tissues come into direct contact with the dietary additives, the response invoked within the intestine could be transmitted further afield to the GIALT and SALT via the blood, potentially stimulating goblet cell production and enlargement within those tissues also.

Similarly, fish fed the B-Wyse treatment also demonstrated a significant increase in the number of goblet cells in all three MALT tissues, and the area fraction of goblet cells in the skin and gut after 6 weeks of feeding. These results are in accordance with those of previous investigations in which the addition of a  $\beta$ -glucan and MOS mixture significantly increased the number of goblet cells in the intestine of channel catfish (Zhu *et al.*, 2012) and Nile tilapia (Selim and Reda, 2015). However, these results stand in contrast to those of chapter three in which fish fed the B-Wyse-supplemented diet did not demonstrate significantly different goblet cell abundances compared to the control. As discussed in chapter three in relation to differences in claudin gene expression, the composition of the epidermal mucous produced by freshwater fish has been demonstrated to differ to that of sea water fish (Caipang *et al.*, 2011; Chasiotis *et al.*, 2012; Kolosov *et al.*, 2013). These potential compositional differences may in turn influence the mucous-producing goblet cells. Furthermore, the gills of fish have been described as having two distinct populations of mucous cell; one is located on the lamellar and one of the gill filament (Pittman *et al.*, 2018). While the discrete functions of the different goblet cell populations have not yet been definitively confirmed, it is possible that the dietary additives influence the population of goblet cells present upon the lamellae instead of those found on the gill filaments, the measurement of which were not conducted

within the present study. Future studies would do well to consider the influence of dietary additives upon both populations of goblet cell present within the GIALT.

Fish fed the synbiotic diet (B-Wyze + Bactocell®) also exhibited significantly elevated goblet cell abundance in all three MALT tissues, and significantly increased CAF in the SALT and GALT. Concerning the skin, these results are supported by those of chapter three in which fish fed the B-Wyze + TB supplemented diet exhibited significantly elevated goblet cell abundance and CAF in the skin of rainbow trout. However, results of the goblet cell abundance in the gill differ between the present study and those of chapter three in which no significant differences were observed in goblet cell abundance between fish fed the B-Wyze + TB-supplemented diet and the control fed fish. These results stand in contrast to previous investigations in which no significant increases in intestinal goblet cell abundance were observed from the combination of MOS + *P. acidilactici* in European seabass (Torrecillas *et al.*, 2018) and short chain fructo-oligosaccharides (FOS) + Bactocell® in Atlantic salmon (Abid *et al.*, 2013). These conflicting results are not surprising considering the amount of differences that exist between the trials described here and further highlight the discussions from chapter three concerning the difficulty of drawing conclusions about certain functional feed products from investigations that vary considerably. In the absence of a greater amount of trials investigating the effect of dietary additives upon the GIALT and SALT in particular, definitive conclusions will remain difficult.

#### 4.4.6 Histological appraisal of the MALT tissues post-challenge

At the GIALT, the sea lice infection did not appear to have any significant effect upon goblet cell abundance. Following 1 and 3 weeks of sea lice infection, fish fed all three supplemented



diets continued to exhibit significantly increased numbers of goblet cells within the gills, compared to the control-fed fish. Chapter three discussed the drawbacks of using the numerical density of goblet cell observed within a tissue and further proposed the determination of goblet cell area fraction as a more informative measure. In order to determine the total area of goblet cell coverage within the SALT and GALT, scripts were written for use in Fiji software allowing for the differentiation between epidermal tissue and goblet cell, afforded by the different pigmentation as a result of histological staining. In the gill tissue however, this measurement was not possible due to the presence of similar coloured cells within the gill tissue that could not be definitively confirmed as goblet cells and that can be seen in Plate 4.1. The development of an alternative staining protocol that allows for the discrimination between these cells and the goblet cells would permit the use of scripts in R to calculate the CAF. Although no significant differences in goblet cell abundance was observed within the gills of fish fed the different dietary additives in the present study, it should not be assumed that the size of these cells remained unaffected.

Concerning the SALT post challenge, no significant differences in goblet cell abundance were observed between any of the treatments at 1 and 3 weeks post infection. Similarly, no significant differences in the area fraction of the goblet cells were observed between any of the treatments 1 week following challenge. At 3 weeks post challenge, however, fish fed all three of the supplemented diets presented with significantly increased CAF in the skin, compared to those fed the control diet. These results reiterate the goblet cell area fraction as a more informative measure. Additionally, while the mechanics of the host-parasite relationship between *L. salmonis* and Atlantic salmon are not completely understood, several investigations have reported on the ability of sea lice to immunomodulate their host by

secreting bioactive compounds (Holm *et al.*, 2015). Previously, zymography analysis has revealed a series of trypsin-like, low-molecular mass serine proteases in the mucous of sea lice infected Atlantic salmon; these molecules were not present within the mucous of uninfected fish, suggesting they originated from the sea lice (Firth *et al.*, 2000). The authors hypothesise that these trypsin-like molecules may aid in the digestion of the host tissue in a manner similar to that seen in other parasitic arthropods such as the cattle tick (*Boophilus micrplus*), the mosquito (*Aedes aegypti*), and the sheep blowfly (*Lucilia cuprina*). Alternatively, they may act to modulate the host immune response by inhibiting the activity of lysozyme in the mucous, as previously described in oysters (Garries *et al.*, 1996; Firth *et al.*, 2000). Prostaglandin E<sub>2</sub> is a potent vasodilator also found in *L. salmonis* secretions, and known to play a variety of roles in the feeding and avoidance of host immune responses in other arthropod parasites (Hamilton *et al.*, 2018). In addition to adversely affecting site-specific leucocyte recruitment and function, Prostaglandin E<sub>2</sub> is also proposed to stimulate the down-regulation of host inflammatory cytokines, the implications of which will be discussed later (Fast *et al.*, 2004). Cathepsin L is an alternative protease found within the secretory products of sea lice at all life stages, especially during the chalimus stage, which is of particular relevance in view of the results of the present chapter, and implicate its involvement in vital functions such as immunoevasion and establishment of the parasite on the host (McCarthy *et al.*, 2012). If these immunoevasive mechanisms were indeed being employed by the sea lice in this investigation, this may help to explain the reduction in CAF of goblet cells across all dietary treatments observed 1 week following challenge, when the lice were observed to be of the chalimus stage. After three weeks of infection, significant increases are again observed in CAF between control and experimental treatments, suggesting an advantageous dietary effect of the additives. It should be noted, however, that these differences were not

the result of goblet cell stimulation by the additives but rather due to the significant reduction in the area fraction of goblet cells observed within the SALT of fish fed the control treatment between 1 and 3 weeks of exposure to the sea lice. It may therefore be reasonable to propose that, in addition to their suppressive abilities of the humoral defences, these compounds are also able to orchestrate a negative effect upon the mucosal defences of the fish, of which the additives investigated here have shown some success at counteracting. Further research is warranted.

In the intestine, fish fed all three of the supplemented diets continue to demonstrate significantly increased goblet cell abundance and CAF compared to the control-fed fish at 1 and 3 weeks post challenge. These results are supported by those of studies concerned with goblet cell abundance in the intestine of European sea bass fed MOS-supplemented diets (Torrecillas *et al.*, 2011; Torrecillas *et al.*, 2013), and channel catfish (Zhu *et al.*, 2012) and Nile tilapia (Selim and Reda, 2015) fed diets supplemented with a mixture of MOS and  $\beta$ -glucan.

#### *4.4.7 Intestinal health measurements*

Further measurements were also taken to assess the overall health of the intestine after 6 weeks and at 1 and 3 weeks post challenge. While no significant differences were observed in lamina propria width between the different treatments, mucosal fold lengths were observed to be significantly increased in fish fed all three supplemented regimes, in accordance with results from previous investigations (Torrecillas *et al.*, 2011; Zhu *et al.*, 2012; Abid *et al.*, 2013). Considered in conjunction with the growth data, these are somewhat confounding results. It is well documented that the improvement of intestinal morphology induces increases in nutrient uptake, resulting in improved feed utilization and growth performance of the fish

(Zhu *et al.*, 201; Khodadadi *et al.*, 2018). Increases in goblet cell abundance and area fraction as observed in this study would also indicate improved intestinal health and mucosal defences. However, despite these morphological improvements, no growth increases were observed between fish fed supplemented experimental diets and the control treatment. This further supports the proposal that the high number and frequency of interventions necessary to monitor the progression of the sea lice challenge imposed a certain level of stress upon the fish, limiting growth. Short-term handling stress has been previously reported to have a detrimental impact upon fish growth (Portz *et al.*, 2006). It may therefore be advantageous for future studies to perform supplementary analysis on additional parameters of the intestinal tissues in order to provide a more holistic appraisal on the effect of the dietary additives upon the ultrastructure of the intestine. For example, the use of transmission electron microscopy to measure increases in microvilli length as reported by Torrecillas *et al.* (2013), scanning electron microscopy to measure improvements to microvilli density as observed by Rawling *et al.*, (2019), appraisal of Intraepithelial Leucocyte (IEL) abundance between the lamina propria and the microvilli brush border as observed by Abid *et al.* (2013), and evaluation of the tunica muscularis thickness to determine any increases as observed by Khodadadi *et al.* (2018).

#### *4.4.8 Expression of mucous-related genes*

During a genome-wide analysis of Atlantic salmon mucin genes, Sveen and colleagues were able to identify several mucin genes analogous to those found in mammals: two genes annotated as Muc2 were predominantly transcribed in the intestinal region, whereas the three Muc5 genes were mainly transcribed in the skin and gills (Sveen *et al.*, 2017). This tissue-specific distribution of mucin genes may be important to understanding host pathogen

interactions in mucosal tissues as previous studies have demonstrated the preferential binding of *Aeromonas salmonicida* to mucins isolated from the intestinal tract over mucins isolated from the skin due to the extent of mucin glycosylation (Padra *et al.*, 2014; Jin *et al.*, 2015).

Prior to infection, the expression of Muc5ac-like was significantly upregulated in the gills of fish fed the B-Wyse and B-Wyse + Bactocell®-supplemented diets, compared to those fed the control diet suggesting Muc5ac plays an important role in the GIALT of Atlantic salmon, the expression of which may be regulated in response to environmental stress or nutrition. Following 3 weeks of exposure to *L. salmonis*, the expression of the Muc5ac-like was significantly down regulated in the GIALT of fish administered diets containing all three of the dietary additives, in comparison to the control-fed fish. To the authors knowledge, there have been no previous studies concerned with the dietary modulation of mucin genes within the GIALT of Atlantic salmon, however, the significant down-regulation of Muc5b and Muc5ac expression has been previously reported in the skin of Atlantic salmon following acute handling stress (Sveen *et al.*, 2017). Within the present study, it is possible that *L. salmonis* exposure induces a similar stress response in the GIALT and that the significant downregulation of Muc5ac in fish fed the three experimental regimes could potentially be aiding the stress response. Recent molecular biology studies have indicated that certain mucins are additionally involved in signalling pathways that may lead to co-ordinated cellular responses such as cell proliferation, differentiation and adhesion, immune response, apoptosis, bacterial adhesion/ inhibition, and secretion of specialized cellular products (Perez-Sanchez *et al.* 2013), however further mechanistic studies are needed to confirm such involvements.

Although the mechanisms remain unclear, previous investigations in humans and rats have demonstrated the ability of dietary components, such as fibre, glucans and probiotics, to influence the mucin content of mucosal secretions (Lien *et al.*, 2001; Hino *et al.*, 2012), and the gene expression of several mucins in rats (Dykstra *et al.*, 2011) and pigs (Smith *et al.*, 2011). Furthermore, in a study on common carp, significant upregulation of Muc5B was observed in the skin of fish fed a  $\beta$ -glucan-supplemented diet (van der Marel *et al.*, 2012). Considering the transcriptional regulation of mucin genes demonstrated in the GIALT and SALT in the present study, it appears reasonable to suggest that it is not only the mucosal system of the intestine can be influenced by dietary additives, and underscores the interconnection of mucosal-associated lymphoid tissues within Atlantic salmon. However, future investigations would do well to include the analysis of a wider repertoire of mucin genes within the different MALT tissues in order to elucidate the tissue-specific transcription and potential transcriptional regulation of mucin genes in response to dietary additives.

#### 4.4.9 Expression of Tight Junction-associated genes

The altered expression of TJ proteins is hypothesized to be mediated by different mechanisms, occurring during various physiological or pathological states. The importance of these changes for disease development and progression, and the effects upon TJ-associated signalling mechanisms are still to be clarified in further detail (Balda and Matter, 2009). The significant down-regulation of the claudin and occludin genes in the gill tissue of fish fed all three of the supplemented diets following three weeks of sea lice exposure may be a further indication of the stress-regulation response discussed previously concerning Muc5ac expression, acting to influence TJ permeability and cell-to-cell communication in response to the pathogen infection, as has been demonstrated in carp skin during CyHV-3 infection (Adamek *et al.*,

2013). The role of the dietary additives under these circumstances may act to exacerbate the perceived stress response, affording a more rapid response of the host to the pathogen. Recently, the decreased transcription of claudins in Atlantic salmon skin was proposed as an energy-saving response by Sveen and colleagues, who further proposed that the observed differential expression patterns may demonstrate a differential coping mechanism, serving to balance energy demands in the different tissues (Sveen *et al.*, 2017). In the event of sea lice infection that primarily affects the skin, the downregulation of claudins within gill tissue may allow for the increased transcription of others in the skin, conferring increased protection on the SALT, where protection is needed the most.

However, in the SALT, the significant upregulation in the expression of claudin 6, claudin 7 and occludin was only observed in fish fed the synbiotic treatment. No other significant differences were observed between treatments for genes relating to TJs or mucins. Evidence to date suggests that the genomes of teleost fish possess large numbers of genes encoding for claudins (Kolosov *et al.*, 2013); it therefore follows logically that more than the three claudin genes examined in the present study are present within the MALT of teleosts, each with their own individual reactionary profile to external and internal stressors experienced by the fish. Furthermore, it should be remembered that claudins have been observed to perform different functions within different species. For example, claudin 28a is related to claudin 4 in mammals and can therefore be suspected to perform a role concerned with barrier function (Kolosov *et al.*, 2013). However, in salmon, this claudin does not respond to salinity as it does in tilapia, where increases in expression were observed after seawater to fresh-water transfer (Tipsmark *et al.*, 2008). At present, knowledge and understanding of the role of claudins in fish is

hampered by the lack of data concerned with their functionality, which is merely based upon their mammalian isoforms (Kolosov *et al.*, 2014) .

Future studies may benefit from sampling beyond three weeks to determine whether the observed significant differences between the control and experimental treatments persist. Additionally, the analysis of a wider range of TJ-associate and mucosal-related genes would provide a more holistic view of the changes occurring at the different MALT tissues in response to the additives and the sea lice infection.

#### *4.4.10 Immune-related gene expression*

Concerning the expression of immune-related genes in the gills following six weeks of feeding and prior to challenge, no discernible differences in the expression of the pro-inflammatory cytokine IL-1 $\beta$  were observed between any of the dietary treatments compared to the control. These results are in disagreement with previous studies in which the expression of IL-1 $\beta$  was significantly upregulated in the MALTs of Atlantic salmon fed a synbiotic mixture of short chain fructo-oligosaccharides + *P. acidilactici* (Abid *et al.*, 2013), and a multi-strain yeast fraction (MsYF) for 10 weeks (Rawling *et al.*, 2019). In fish, IL-1 $\beta$  is one of the first cytokines released during inflammation and is an important effector cytokine of the immune response (Rawling *et al.*, 2019). The absence of significant differences in the expression of IL-1 $\beta$  observed between fish fed the control and supplemented diets in the present study would suggest that an elevated inflammatory response has not been stimulated. Transforming Growth Factor (TGF)- $\beta$  is a pleiotropic, cytokine that plays several roles in the immune response due to its ability to exert both pro-inflammatory and immune-suppressive effects, in addition to regulating cellular proliferation and differentiation, remodelling and wound healing (Skugor



*et al.*, 2008; Wang and Secombes 2013; Holm *et al.*, 2015; Zou and Secombes, 2016). As discussed previously in chapter three, the lack of significant differences observed in the expression of IL-1 $\beta$  in the GALT may be due to the significantly increased expression of TGF- $\beta$ , as observed in fish fed the AgriMOS and B-Wyse + Bactocell<sup>®</sup> treatments, which may be suppressing the inflammatory response initiated by IL-1 $\beta$  (Wang and Secombes 2013).

IL-4 and IL-13 are closely related cytokines of the T helper (Th)-2 immune response which is especially important for defence against parasites; while IL-4 promotes Th2 development in a positive feedback loop, it also suppresses the development of Th1 and Th17 cells (Takizawa *et al.*, 2011). Although the exact mechanisms of activity are still to be definitively confirmed, in salmonids IL4/13 isoforms have been proposed to be capable of regulating the expression of immune related genes, such as antimicrobial peptides and acute phase proteins, increase the expression of cytokine receptors, and increase levels of IgM producing B cells (Sequeira *et al.*, 2020). GATA binding protein 3 (GATA3) is the transcription factor and master regulator of Th2 responses, enhancing the transcription of the genomically clustered Th2 cytokine genes IL-4 and IL-13 (Takizawa *et al.* 2011). Prior to challenge, fish fed diets with all three experimental additives expressed significantly elevated levels of IL-4/13a and GATA3 in the gills compared to those fed the control diet. These upregulations would suggest stimulation of the Th2 immune response.

After 3 weeks of infection, fish administered the B-Wyse-supplemented diet continued to demonstrate significant upregulation of both IL4/13a and GATA3 compared to the control fed fish. Taken together with the significant reduction of sea lice burdens following 1 and 3 weeks of exposure in fish fed the B-Wyse treatment, these results would suggest that the increased transcription of IL4-13a and the subsequent priming of the Th2 defences contribute toward

the defences of the fish to extracellular parasite. Furthermore, the continued significant upregulation of IL4/13a, GATA3 and TGF- $\beta$  may be an indication of a long-term benefit conferred upon the fish via the continued administration of the B-Wyse product.

Responses to parasites are often described in terms of Th1/Th2 dichotomy as above, yet recent studies have shown that host-pathogen interactions are more complex (Skugor *et al.*, 2008): the Th-17 subset has been identified as a distinct T helper lineage (Yang *et al.*, 2008). Induced by TGF- $\beta$ , Th17 cells orchestrate the mucosal defence against pathogens and mediate tissue inflammation by secreting the proinflammatory cytokines IL-17 and IL-22, which stimulate MALT tissues to secrete chemokines and an array of antimicrobial peptides which repel assault from diverse infectious agents (Zhang *et al.*, 2013). It is therefore possible that fish fed the AgriMOS- and B-Wyse + Bactocell<sup>®</sup>-supplemented diets that did not exhibit upregulation of IL-4/13a and GATA3 after three weeks of exposure but that continued to exhibit reductions in parasite load, are in fact switching from Th2 responses to that of Th17 cascade. This is further supported by the significant upregulation of IL-1 $\beta$  in the GIALT of fish fed all three of the supplemented diets after three weeks of exposure to the sea lice, as it is also involved in Th17 differentiation (Holm *et al.*, 2015).

In order to confirm the postulations outlined here, the expression analysis of certain other genes would be advantageous, providing further information as to the alterations occurring within the tissues and which immune cascades to target for a more effective immune response. For example, the analysed expression of IL-17a and IL-1R1 (a signal transducer and marker of the Th17 subset) would confirm Th17 activation and activity post challenge and in response to the dietary additives (Skugor *et al.*, 2008; Holm *et al.*, 2015). Matrix metalloproteinase (MMP)-13 is a collagenase that plays a key role in the MMP activation

cascade, remodels the extra-cellular matrix, and heavily contributes to wound repair in response to *L. salmonis* infection (Zhang *et al.*, 2013; Holm *et al.*, 2015). The expression of Inducible nitric oxide synthase (iNOS) by classically activated macrophages produces a cytotoxic environment and promotes vasodilation, which is important for early phases of wound healing (Skugor *et al.*, 2008). Increased expression of these genes would confirm the postulation above that dietary stimulation of TGF- $\beta$  aids the survival and resistance of fish to sea lice by stimulating wound healing and the differentiation of Th17 cells.

As in chapter 3, the expression of the target genes analysed in the SALT within the present study exhibited a wide level of variance within the treatment groups, with very little differences in expression compared to the control treatment pre and post challenge. This may suggest the efficacy of the dietary additives to influence the expression of genes within the SALT is lower compared to the demonstrated efficacy observed within the GIALT. Equally, these variations could be indicative of several highly activated individuals within the sample group, a result of advanced immunosuppression exerted by the sea lice, or indeed the development of mucosal tolerance. In light of the results from the present study and those of chapter three, future studies should therefore consider evaluating the effect of the dietary additives on the SALT during a much shorter exposure time in order to determine whether any immediate alterations occur in response to dietary supplementation. Fish fed the AgriMOS-supplemented diet did however exhibit significant increases in the expression of IL-1 $\beta$  at 3 weeks post challenge, and a significant downregulation in the expression of GATA3 in comparison to the control-fed fish, suggesting a stimulation of the inflammatory responses in conjunction with the suppression of the Th2 responses.

Concerning the expression of immune-related genes in the GALT, few differences in the expression of the 10 genes of interest were observed between fish fed the AgriMOS and B-Wyse supplemented diets compared to the control-fed fish. These results are rather unexpected, as previous studies have reported on the effects of many additives upon immune-related genes in intestinal tissue. However, considering the target genes assessed here, it proved difficult to find similar studies within the scientific literature that had examined the same target genes in response to functional dietary additive inclusion.

In the present study, each of the 10 target genes analysed within the different tissues were purposefully chosen based on their proposed function in order to provide specific insight into what may be occurring within the different MALT tissues in response to the dietary additives. IL-1 $\beta$  and TGF- $\beta$  are important pro- and anti-inflammatory cytokines that provide insight into the reaction of the tissue to the dietary ingredients and functional additives. IL4/13a is strongly expressed by Th2 cells, while the key transcription factor GATA3 and Arg2 are all involved in the differentiation and polarization of cells to the Th2 response, one that is of high importance during parasitic infection. IRAK4 modulates the functions of both the innate and adaptive immune systems and would provide insight as to whether the defences of the fish change in response to exposure to sea lice or long-term administration of the dietary additives. Calreticulin has been proposed as a biomarker for yeast-derived functional feeds such as those used in the current study and the tissue specific mucins and claudins, occludin and IL-17a are all involved in the mucosal and barrier function responses of the MALT tissues which constitutes the main focus of this thesis.

Evidently, drawing comparisons between the results of different investigations is challenging as a large number of factors and variables may differ between studies, including those of an

environmental, biological and nutritional nature, all of which may have an impact upon the observed results. Furthermore, when evaluating the influence of dietary additives upon the expression of immune-related genes, the analysis of so few target genes makes drawing definitive conclusions rather challenging. The use of high-throughput targeted gene expression technology such as Microfluidic qPCR to evaluate the expression of a much larger number of genes would prove to be very informative. Affording the examination of complete gene cascades and encompassing a much wider variety of target genes, the influence of dietary additives upon the MALTs of fish may be further elucidated. The use of such a methodological tool will be the focus of the next chapter, in conjunction with the final experiment of this research, conducted in open seawater pens.

### 4.5 Conclusions

In the present study, no significant differences were observed in individual body weight, K-factor or SGR between fish fed the different treatments pre- or post-challenge, in accordance with results of previous studies (Torrecillas *et al.*, 2011; Ye *et al.*, 2011; Torrecillas *et al.*, 2018; Jami *et al.*, 2019). The less than 2-fold increase in growth may be attributable to the repetitive interventions required to document the progress of the sea lice challenge, however Fulton's K-factor for all treatments fell within the range of 1.05 and 1.15 indicating good nutritional condition of the fish. As also observed in chapter three, under the excellent rearing conditions and in addition to the administration of high quality diets, little opportunity remained for the additives to exert an influence upon the growth indices of the fish over the relatively short duration of the pre-challenge phase of the trial.

Significant increases in the quantity of secreted epidermal mucous were observed in fish fed all treatments prior to challenge at 6 weeks, indicative of a dietary influence upon mucosal secretion. Additionally, significant elevations in lysozyme activity were observed in the mucous across all treatments after 6 weeks and 1-week post challenge compared to fish fed the control diet. While these results are supported by previous investigations (Sheikhzadeh *et al.*, 2012; Dawood *et al.*, 2015c; Dawood *et al.*, 2016) they stand in contrast to those of chapter three and may be due to the differences in the length of administration or as a function of species specificity. Significant decreases in attached sea lice abundance were observed on fish fed all three of the supplemented treatments after 1-week and at 3-weeks post challenge in fish fed the B-Wyse- and AgriMOS-supplemented diets, in comparison to lice burdens on the control fed-fish. Collectively these result support the proposition that the quantity and possibly the quality of epidermal mucous may influence sea lice attachment success (Dimitroglou *et al.*, 2011). Further, the continued significant decrease in sea lice abundance may be an indication of a long-term benefit conferred upon the fish via the continued administration of the B-Wyse and AgriMOS products.

Prior to challenge, significant increases in goblet cell abundance were observed in the GIALT, SALT, and GALT across all treatments, compared to the control-fed fish, in addition to significant increases in goblet cell area fraction in the SALT and GALT across all treatments. At both 1 and 3 weeks post challenge, significant increases in goblet cell abundance were observed in the GIALT and GALT across all treatments compared to the control-fed fish, in addition to significantly increased CAF in the GALT. However, no significant differences were observed in goblet cell abundance or CAF in the SALT of fish fed all three supplemented treatments compared to the control, 1 week post challenge. This may be a result of the

immunomodulation exerted upon the fish by the sea lice via the bioactive compounds that they reportedly secrete as a form of immunoevasion during the chalimus life stage (Holm *et al.*, 2015). At 3 weeks post infection, the CAF of the goblet cells within the SALT of fish fed the dietary additives were observed to be significantly greater than that of the control fed fish. However, this was not due to a stimulatory effect of the additives upon the goblet cells but rather the result of a significant decrease in the CAF of the control fish between 1 and 3 weeks post-challenge. It may therefore be reasonable to propose that the bioactive compounds secreted by the sea lice are able to orchestrate a negative effect upon the mucosal defences of the fish, of which the additives have shown some success at counteracting.

Prior to challenge, a significant increase in the expression of Muc5ac-like was significantly upregulated in the GIALT of fish fed the synbiotic treatment compared to the control, however after three weeks of infection, significant downregulations in the expression of Muc5ac-like and claudins 27a, 28a and 28b were observed in the GIALT of fish fed all three of the additive treatments. This may be indicative of an energy saving response as previously postulated by Sveen *et al.* (2017), conserving energy for the upregulation of alternative genes, such as claudin 6 and 12 and occludin that were observed to be significantly upregulated in the SALT of fish that were administered the synbiotic treatment 3 weeks post infection. However, considering the large size of the claudin family it is certainly possible that many more claudins are expressed within the MALT tissues. The further analysis of a wider range of claudins would contribute to our understanding of claudin function and tissue-specificity in the mucosal-associated tissues.

Concerning the immune-related gene expression, evidence of Th1 and Th2 immune response stimulation was observed in fish fed all three of the experimental regimes in the GIALT as

demonstrated by the significantly elevated expression of TGF- $\beta$ , IL4/13a and GATA3, compared to the control-fed fish. In the GALT, and SALT however transcription regulation in response to the dietary additives was low, with large variations in expression observed within treatments. The analysis of a much larger range of genes would provide a much more holistic view of the alterations occurring at the MALT tissues in response to dietary additive supplementation and pathogen challenge investigated in the present study. Recent advances in technology have resulted in the emergence of Microfluidic qPCR (MF-q-PCR) as an attractive high-throughput targeted gene expression alternative to microarray and RNA-seq technologies: a single chip run allows for the simultaneous screening of 96 arrays against 96 samples. MF-q-PCR analysis will be one of the main focusses of the next chapter.



**The effect of prebiotic and synbiotic dietary supplementations on the growth and mucosal defences of Atlantic salmon reared in open sea pens, with particular focus on high throughput real-time qPCR**



## 5.1 Introduction

The environmental conditions and stress events experienced by an individual fish during *in vivo* laboratory-based investigations (such as those conducted in chapters three and four), will differ to those experienced by a fish during open sea pen aquaculture. Throughout lab-based experiments, water quality parameters are monitored closely and maintained to optimal levels where required, pathogen exposure is minimised except where intended during strict challenge experiments, and handling events are kept to a minimum. However, in the course of commercial Atlantic salmon aquaculture farming in open sea pens, environmental water parameters, such as water temperature, salinity, and dissolved oxygen fluctuate daily; pathogens are ubiquitous within the surrounding water; and handling events are much more frequent due to the necessity for net changes, grading during grow out periods, and vaccination events (Ostrander, 2000; Ángeles-Esteban, 2012; Oliva-Teles, 2012). Additionally, considerably higher stocking densities, social interactions, competition for food, and the potential exposure to predators are experienced by the fish (Zwollo 2018). As discussed in previous chapters, these stressful events may influence the performance of the fish in terms of growth (Bernier and Peter, 2001; Aluru and Vijayan, 2009), immunity and disease resistance (Engelsma *et al.*, 2003; Costello, 2009), morphology (Gauberg *et al.*, 2017) and behaviour (Barton, 2002). The degree of stress elicited by the aforementioned factors will vary depending upon the number, type, severity, and length of exposure to the experienced stressor(s): the consequential impact of the imposed stress upon the welfare of the fish will also vary (Sundh, 2009). As such, a dietary supplement that influences favourable changes in fish within the context of a laboratory-based experiment may not influence similar beneficial results within fish experiencing a more changeable and stressful environment, such as those experienced in open sea pen aquaculture. However, the inverse may also be true; the

controlled or pristine conditions of laboratory-based experiments may mask conditions that manifest under more challenging conditions. To this end, it is prudent to investigate experimental feed additives in both controlled environments, such as those experienced in laboratory-based experiments, and in less controlled environments, that reflect the conditions experienced by the fish in aquaculture, such as open sea pens.

Large scale gene expression analysis has been an essential tool for many biological and medical investigations for over a decade (Spurgeon *et al.*, 2008). High-throughput technologies such as microarray and RNA-sequencing allow for the simultaneous measurement of thousands of transcripts, with the ultimate aim of understanding responses via differentially expressed genes i.e. those that exhibit transcription regulation in response to an environmental condition or treatment (Martin *et al.*, 2016). As a hybridisation-based approach, DNA microarrays are extremely powerful tools that allow one to probe virtually the entire transcriptome to produce an overall picture of gene expression behaviour (Spurgeon *et al.*, 2008). Microarrays were first used in fish studies in the late 1990s (Martin *et al.*, 2016), while salmonid cDNA microarrays were constructed shortly after the large-scale sequencing of salmon and trout cDNA libraries by several research institutes (Krasnov *et al.*, 2011). Genome-wide and specialised platforms have been applied to a wide range of tasks in diverse research areas including fish nutrition (Król *et al.*, 2016), stress physiology (Akbarzadeh *et al.*, 2018) and toxicology (Martyniuk *et al.*, 2020), while studies of fish diseases and immunity have addressed responses to bacteria (Eslamloo *et al.*, 2020), viruses (Dalmo, 2018), fungi (Roberge *et al.*, 2007), parasites (Sutherland *et al.*, 2017), vaccines (Lund *et al.*, 2019), and inflammatory stimulators (Perez-Sanchez *et al.*, 2015).

Despite the impressive achievements with this technology, microarray platforms suffer certain restrictions and disadvantages (Krasnov *et al.*, 2011). One of the intrinsic limitations of micro-array technology is that it relies on the existing knowledge of genomic sequences and therefore only pre-determined genes can be analysed (Martin *et al.*, 2016). As most fish possess approximately 20,000 - 30,000 genes, a well-designed oligo microarray should be able to assess >90% of the transcriptome, however microarrays do not allow for the clear identification between paralogs of duplicated genes or the analysis of genetic differential splicing which results in high background noise and ambiguity due to non-specific hybridisation (Qian *et al.*, 2014; Martin *et al.*, 2016).

RNA-sequencing, also known as whole transcriptome shotgun sequencing, is a relatively new concept that employs next-generation sequencing (NGS) technologies to sequence cDNA directly from an RNA sample of interest via a three-step method; library construction, sequencing on a specific NGS platform, and bioinformatics analysis (Qian *et al.*, 2014). The power of RNA-seq lies in the detection and quantification of all transcripts, including previously unknown ones, within a single method, while the strategy applied to analyse the produced data varies based upon the organisms studied and the research goals (Sundaram *et al.*, 2017). For higher vertebrates with well-annotated genomes, RNA-seq analyses are straightforward due to the relative ease of linking differentially expressed genes to a specific functional pathway. In species with less complete gene annotation and functional knowledge, data analysis becomes much more complicated due to lower sequence identity and species-specific evolution of gene families (Sundaram *et al.*, 2017).

To date, RNA-sequencing has been utilised in a substantial number of fish biology studies, including not only model species such as zebrafish (Hu *et al.*, 2019), but also commercially

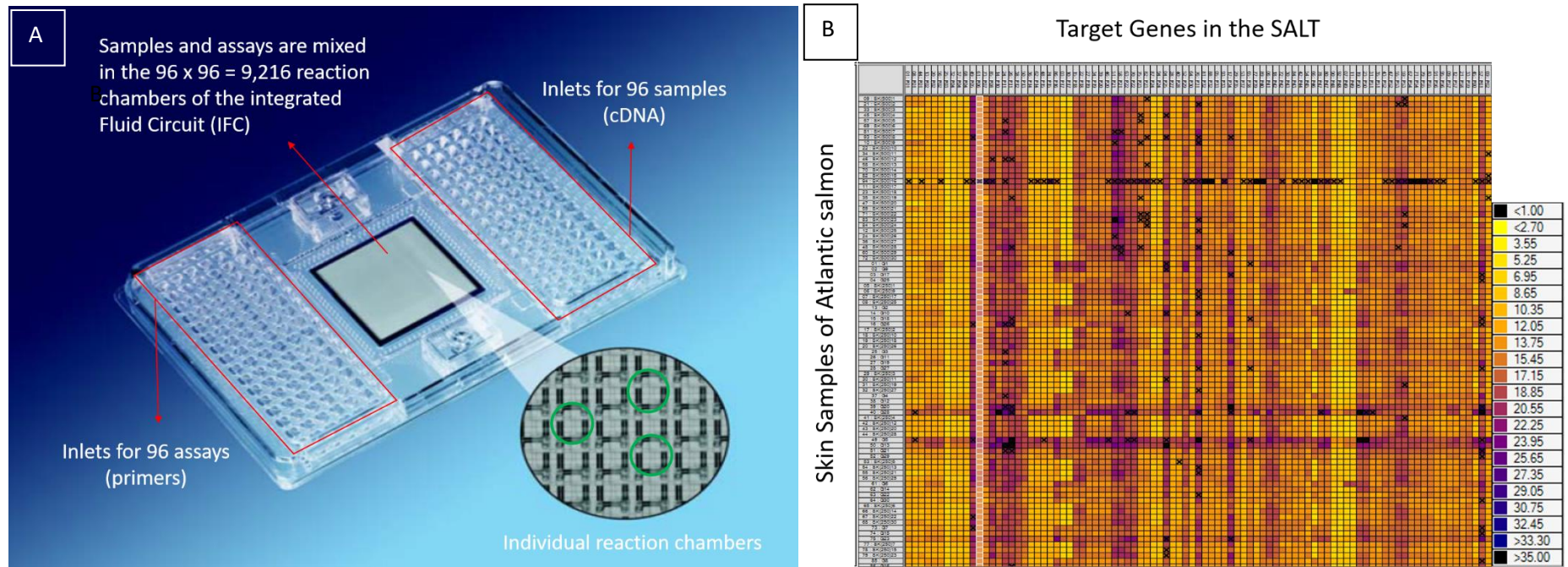
important and eco-environmentally relevant fish species also. For example, RNA-seq investigations have contributed to our knowledge of proliferative gill disease in Atlantic salmon (Król *et al.*, 2019), the role of certain genes in transgenic rainbow trout (Han *et al.*, 2018), the generation of skin and scale transcriptome data for European sea bass (Pinto *et al.*, 2017), and the identification and annotation of mucin genes in channel catfish (Liu *et al.*, 2020).

In recent years, there has been an increasing shift away from the use of DNA microarrays, towards RNA-sequencing technology. Notwithstanding the success of these technologies, it has become common practice to validate the results of these platforms with real time PCR (RT-q-PCR) which has excellent sensitivity, dynamic range and reproducibility, and is widely regarded as the “gold-standard” for data validation measurements (Spurgeon *et al.*, 2008, Gorgannezhad *et al.*, 2019). Unfortunately, however, qPCR is a low throughput technique, limiting the number of genes that can be verified.

In the early 2000s, advancements in the field of microfluidics have since allowed the arbitrary fluidic manipulations at the nanolitre scale, and have led to the development of a number of new tools and technologies including single cell gene expression (Warren *et al.*, 2006), cell culture (El-Ali *et al.*, 2006) and protein crystallization (Anderson *et al.*, 2007). One particular development of note is the creation of microfluidic matrixes, or “dynamic arrays”, that allow all possible combinational assays to be performed on a set of reagents, while realising significant reductions in pipetting effort, labour and reagent consumption (Spurgeon *et al.*, 2008). Recently, microfluidic dynamic arrays have been used to perform high throughput gene expression measurements with RT-q-PCR, as demonstrated in previous studies by Crane *et al.* (2018) and Gorgannezhad, Stratton and Nguyen (2019).

As presented in Figure 5.1A, the 96.96 dynamic array allows for the combination of 96 assays with 96 samples, resulting in 9,216 individual PCR reactions. Utilising the same chemistry as traditional qPCR, these reactions are controlled by the Integrated Fluidic Circuit (IFC) that consists of a network of fluid lines and NanoFlex™ valves made from an elastomeric material, which deflect under pressure to create a tight seal, and are used to regulate the flow of liquids in the IFC (Spurgeon *et al.*, 2008). Imaging occurs at the end of each thermal cycle and analysis software generates data from each of the reaction wells: data is displayed as a heat map in Figure 5.1B, where each square represents an individual reaction well and the colour indicates the  $C_t$  value according to the legend shown on the right of the figure.

Recently, Microfluidic-qPCR (MF-q-PCR) has emerged as an attractive high-throughput targeted gene expression alternative to microarray and RNA-seq technologies: cost-effective with low operational costs, easily customisable and theoretically producing directly comparable results across investigations (Miller *et al.*, 2016). In studies such as this one, the simultaneous screening of 96 assays allows for the inclusion of a wide range of target genes of interest, from the more generalist down to specific receptors and complete immune cascades, providing a holistic view of the gene expression profile and immune-competence of the tested samples.



**Figure 5.1.** A) Image of a 96.96 dynamic array chip, indicating the location of the sample and detector inlets in which the gene expression assay reagents are added. The insert shows an enlarged section of the IFC in which individual reaction chambers can be seen, highlighted by the green circles. Modified from “Fluidigm User Guide” (2018) B) Adapted screenshot of the resulting, software-generated heat map of Atlantic salmon skin samples processed on a 96.96 dynamic array chip at INRA. Each square represents one reaction chamber of the chip. The colour indicates the  $C_T$  value according to the legend shown.

In light of the observed benefits resulting from the dietary supplementation of the products B-Wyse and B-Wyse + Bactocell® in the previous chapters, these treatments were further investigated in the current experiment, aiming to further confirm their potential suitability and applicability for *in situ* use in aquaculture. The products will be supplemented into the diets of Atlantic salmon held in open sea pens and administered for a total of 14 weeks. As in the previous trials, the influence of these additives upon the growth indices, mucosal barrier defences and pathogen resistance will be investigated. Additionally, building upon the information already accumulated from previous chapters, investigations of this chapter will include the gene expression analysis of a wide range of immune-relevant and mucosal-associated target genes: using MF-q-PCR, the expression of 59 target genes in the SALT, GIALT and GALT of Atlantic salmon will be analysed. The aim of these analyses is to provide a more holistic view of the alterations and changes observed in the mucosal tissues in response to the dietary feed additives and to provide additional insights and information concerning feed additive interactions with the immune system of the fish.

### 5.2. Materials and methodologies

All experimental work involving fish was conducted in accordance with the UK Animals Scientific Procedures Act 1986 and the Animal Welfare and Ethical Review Body of Marine Harvest, UK.

#### 5.2.1 Experimental design

The trial was set-up and conducted by the Norwegian seafood company Marine Harvest ASA, in their open sea research pens, based in Loch Ailhort on the Ardnish peninsular, Scotland.



During the 14-week trial, seawater pens of 125m<sup>3</sup> volume were employed. Water quality parameters were measured daily at a depth of 5m and were typical of spring conditions: temperature ranged from 6.6°C at the start of the trial (March) to 13.9°C (June), with an average of 9.3°C; salinity ranged from 17‰ to 35‰; and oxygen saturation was consistently greater than 80%.

### *5.2.2 Experimental fish and feeding rates*

Atlantic salmon post-smolts (approx. 120g) of the Aquagen strain were sourced from Loch Arkaig Smolt Unit (RAS) and randomly distributed into each of nine sea pens at a stocking density of 150 fish per pen. Each of the dietary regimes was randomly assigned to each of the pens in triplicate. Fish were fed to excess twice daily either by hand or by automatic feeder, and unconsumed food removed using a lift-up system.

### *5.2.3 Sampling schedule and specifics*

Sampling was conducted by Marine Harvest employees. Fifteen fish were randomly selected from each pen after 8 and 14 weeks of feeding the experimental dietary regimes. Due to the location of the pens in open water, a latent sea lice infection beyond the control of the investigation was present at the time of sampling: following humane sacrifice, fish were inspected for sea lice settlement. Based on external appearance, sea lice were identified as *Caligus elongatus* or as one of the 5 life-stages of *Lepeophtheirus salmonis* (chalimus, pre-adult, male, female, gravid female). Epidermal skin mucous was collected from all fish in addition to morphometric measurements. For gene expression analysis, a subpopulation of 5 fish per pen (15 per treatment) were randomly selected, from which skin (8 and 14 weeks) and gill (14 weeks only) tissue samples were excised, as described in chapter 2, section 2.2.

### 5.2.4 Diet preparation

Three iso-nitrogenous and iso-lipidic dietary regimes were formulated to meet the known nutritional requirements of Atlantic salmon. Each diet was manufactured by BioMar into two pellet sizes (3mm and 4.5mm) in order to accommodate the growth and pellet preference of the fish throughout the duration of the trial. The 3mm pellet diets were administered from the start of the trial up to and including the first sampling at 8 weeks, at which point a mixture of the 3mm and the 4.5 mm pellets was offered, leading to the gradual reduction of the 3mm pellet. All six diets were delivered to the research facilities of Marine Harvest at Loch Ailhort prior to the start of the trial, following BioMar in-house proximate analysis (presented in Table 5.1) and quality control checks.

**Table 5.1** Ingredient inclusion levels (%) of the experimental diets formulated for Atlantic salmon in addition to their target composition (%)

Ingredient (%)	Control		B-Wyse		B-Wyse + Bactocell®	
	3mm	4.5mm	3mm	4.5mm	3mm	4.5mm
Marine origin ingredients	41.30	44.16	41.30	44.16	41.30	44.16
Land origin ingredients	55.27	54.08	55.13	53.93	55.10	53.90
Other micronutrients	3.39	1.72	3.39	1.72	3.39	1.72
Yttrium	0.00	0.05	0.00	0.05	0.00	0.05
Bactocell®*	0.00	0.00	0.00	0.00	0.03	0.03
B-Wyse*	0.0	0.0	0.15	0.15	0.15	0.15
<b>Target composition of feed (%)</b>						
Moisture	7.20	7.30	7.20	7.30	7.20	7.30
Fat	23.90	24.10	23.90	24.10	23.90	24.10
Protein	44.80	43.60	44.80	43.60	44.80	43.60
Ash	8.30	7.60	8.50	7.60	8.50	7.60

\*Dietary additives provided by Lallemand SAS

### 5.2.5 Fish growth and condition

Calculations to determine feed efficiency, growth performance and fish condition were performed as follows:

- Weight gain (WG) = final weight (g) – initial weight (g)
- Feed Conversion Ratio (FCR) = feed intake (g) / weight gain (g)
- Specific Growth Rate (SGR) =  $((LnW2 - LnW1) / T) \times 100$ : where  $LnW1$  and  $LnW2$  are the initial and final natural logarithmic weights, respectively, and  $T$  is the number of days in the feeding period.
- Survival Rate (SR) (%) = (final number of fish / initial number of fish) x 100.

### 5.2.6 Epidermal mucous

Mucous quantity was determined from individual fish (n=45 per treatment) upon sampling: refer to chapter 2, sections 2.2 for methodology.

### 5.2.7 Sea lice observations

In order to assess the extent of the latent sea lice infection in the different treatment pens at the trials end-point, three different measurements were calculated as follows:

- Abundance = mean lice count / number of fish
- Intensity = mean lice count / number of infected fish
- Prevalence (%) = (number of infected fish / number of uninfected fish) x 100

### 5.2.8 Gene expression

Gill and skin samples for gene expression analysis were sent to the University of Plymouth following conclusion of the trial.

#### 5.2.8.1 RNA extraction and cDNA synthesis

RNA extraction from tissues and cDNA synthesis of all samples was carried out as described in chapter 2, section 2.6.1 by the PhD candidate on 9 fish per treatment. Individual samples were checked for quality control purposes at multiple stages and were removed from analysis if considered above or below acceptable limits.

#### 5.2.8.2 Reference genes, genes of interest and analyses

$\beta$ -actin and Elf1 $\alpha$  were used as reference genes for each sample in order to standardise the results by eliminating variation in mRNA and cDNA quantity and quality (Bustin *et al.*, 2009). The stability and suitability of  $\beta$ -actin and Elf1 $\alpha$  as reference genes were confirmed by generating an expression stability measure “M” for each reference gene using the calculations outlined in Vandesompele *et al.* (2002). No amplification product was observed in negative controls and no primer–dimer formations were observed in the control templates. Modification of gene expression was represented with respect to the control group being sampled at the same time as the treatment group. Primer sets for a further 57 genes of interest were designed and their efficiency determined by the PhD candidate, including those associated with:

- pro- and anti-inflammation ;

- immune-regulation;
- Th2- and Th17-associated cytokines;
- oxidative stress;
- toll-like receptor (TLR) signalling;
- tight-junction-associated claudins;
- and genes associated with mucosal composition.

Important characteristics pertaining to the primers are presented in Table 5.2 in the Appendix: Atlantic salmon primer pair sequences, annealing temperature (in °C), amplicon size (bp), and primer efficiency (E-value) designed for the gill and skin, for use on the microfluidic dynamic array at INRA.

#### *5.2.8.3 Microfluidic q-PCR dynamic array*

cDNA samples and primer stocks were shipped to the research facilities of INRA in Toulouse, France, where Microfluidic RT-q-PCR reactions were performed using a 96.96 dynamic array on the BioMark HD™ platform (Fluidigm®). Pre-mixes for each sample were made by mixing 3.0 µl of 2X SsoFast EvaGreen Supermix with low ROX (BioRad PN172-5211), 0.3µl of 20x DNA Binding Dye (Fluidigm PN 100-7609), and 2.7µl Pre-amplified and Exo 1-treated sample. Assay mixes contained 3.0µl 2x Assay Loading Reagent (Fluidigm PN100-7611), 2.7µl 1x DNA Suspension Buffer (TEKnova PN T0221), and 0.3µl of 100µM combined forward and reverse primers [the final concentration of each primer in the final reaction was 500Nm]. After priming the Integrated Fluidic Circuit (IFC), 5µl of each assay and 5µl of each sample premix was pipetted into the respective inlets on the IFC. Once in the wells, the components were pressurized into the chip using a NanoFlex™ IFC controller. The components were then

systematically combined into 5,310 parallel reactions. During the cycling program, the chip was imaged at the end of each cycle and analysis software generated PCR curves for each reaction. Data was viewed using the Real-Time PCR Analysis software, version 4.3.1 (Fluidigm®), using default quality threshold of 0.65 and linear baseline correction. Peak sensitivity was set at 7, the peak ratio threshold was set at 0.80, while the melt temperature  $T_m$  ranges were set individually based on the peaks observed in standards, as per the manufacturers recommendations. Individual reactions were excluded from analysis if they failed any of the melt curve quality parameters or had a peak outside the  $T_m$  range.  $C_t$  values were exported as a Comma Separated Values file into Microsoft Excel for analysis.

#### *5.2.9 Statistical analyses*

Raw data concerning the growth, mucous quantity and sea lice counts were sent to the PhD candidate following the conclusion of the trial. All statistical analyses and data manipulation was performed by the PhD candidate as described below.

Statistical analysis of the sea lice infection was conducted in Quantitative Parasitology software, version 3.0 after Reiczigel *et al.* (2019). A Bootstrap 2-sample t-test was used for the comparison of mean abundances and mean intensities, and Fisher's exact test was used for comparing prevalence's.

All other statistical analysis were performed using R Studio version 1.2.5042 (RStudio PBC, Boston USA). Statistical analysis for all qPCR data were carried out using the permutation tests in R, following Röhmel (1996). Analysis of growth performance and mucous quantity were assessed by one-way ANOVA tests in R following Shapiro-Wilk test for normality, and with

Tukey high significant difference (HSD) post-hoc tests where differences occurred. The level of significance was accepted at  $p < 0.05$ . All gene expression data is presented as mean  $\pm$  standard error (SE), while all other data is presented as mean  $\pm$  standard deviation (SD).

### 5.3. Results

#### 5.3.1 *Fish growth indices*

The growth performance data is presented in Table 5.3. Over the trial's 14-week duration, individual mean body weight increased 3.5-fold and mortality was consistently low with survival >99% across all pens. During the first 8 weeks of the trial, prior to the first sampling and during which time the 3mm pellet feed was administered, no statistically significant differences were observed between the experimental treatments and the control treatment with regard to FCR or SGR. During the final 6 weeks of the trial in which the 4.5mm pellet was administered, fish fed the B-Wyze diet exhibited an increased SGR (+3.6%) compared to those fed the control, however this increase was not significant and no significant differences were observed between any of the treatment groups for SGR or FCR.

#### 5.3.2 *Epidermal mucous quantities*

After 8 weeks, fish fed both the B-Wyze ( $0.28 \pm 0.12$ ,  $p = 0.001$ ) and B-Wyze & Bactocell<sup>®</sup>-supplemented ( $0.28 \pm 0.10$ ,  $p = 0.001$ ) diets presented with significantly more epidermal skin mucous, compared to fish fed the control diet ( $0.20g \pm 0.08$ ). After 14 weeks of feeding the experimental diets, fish fed the B-Wyze ( $0.35 \pm 0.1$ ,  $p = 0.003$ ) treatment continued to exhibit significantly elevated quantities of epidermal mucous, compared to those fed the control diet. Results are presented in Table 5.4.

**Table 5.3** Growth indices of Atlantic salmon over the duration of the 14-week trial. Data are presented as mean  $\pm$  SD; no significant differences,  $p < 0.05$ ;  $n = 3$ .

Time-point	Parameter	Control	B-Wyse	B-Wyse + Bactocell®
Initial	Body weight (g)	117.4 $\pm$ 0.80	117.1 $\pm$ 2.90	120.0 $\pm$ 0.80
8 weeks	Body weight (g)	264.4 $\pm$ 2.70	267.0 $\pm$ 7.00	267.0 $\pm$ 7.10
	Weight gain (g)	153.4 $\pm$ 9.40	149.4 $\pm$ 11.90	145.4 $\pm$ 9.30
Final	Body weight (g)	406.9 $\pm$ 4.00	424.3 $\pm$ 7.50	416.7 $\pm$ 4.30
	Weight gain (g)	140.3 $\pm$ 4.50	159.4 $\pm$ 4.00	154.1 $\pm$ 4.40
Overall performance	Total feed intake (kg)	38.3 $\pm$ 0.70	38.2 $\pm$ 0.40	37.0 $\pm$ 0.50
	SGR: 3mm pellet (%/day)	1.5 $\pm$ 0.01	1.5 $\pm$ 0.01	1.4 $\pm$ 0.01
	SGR: 4.5mm pellet (%/day)	0.9 $\pm$ 0.01	1.0 $\pm$ 0.01	0.9 $\pm$ 0.01
	FCR: 3mm pellet (au)	0.8 $\pm$ 0.02	0.8 $\pm$ 0.06	0.8 $\pm$ 0.03
	FCR: 4.5 mm pellet (au)	1.2 $\pm$ 0.01	1.0 $\pm$ 0.06	1.0 $\pm$ 0.01
	Survival (%)	99.8	99.8	99.6

**Table 5.4** Skin mucous and sea lice measurements for fish fed the different experimental dietary regimes. Data is presented as mean  $\pm$  SD;  $n = 45$ . Columns with letters denote significant difference between those of a different letter on the same row, where  $p < 0.05$ .

Parameter	Time-point	Experimental diet		
		Control	B-Wyse	B-Wyse + Bactocell®
Mucous quantity (g)	8 weeks	0.20 $\pm$ 0.08 <b>a</b>	0.28 $\pm$ 0.12 <b>b</b>	0.28 $\pm$ 0.10 <b>b</b>
	14 weeks	0.28 $\pm$ 0.12 <b>a</b>	0.35 $\pm$ 0.10 <b>b</b>	0.32 $\pm$ 0.12 <b>ab</b>
Sea lice abundance (n/fish)	8 weeks	0.2 $\pm$ 0.2	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1
	14 weeks	1.0 $\pm$ 0.3 <b>ab</b>	1.1 $\pm$ 0.1 <b>a</b>	0.7 $\pm$ 0.1 <b>b</b>
Sea lice prevalence (%)	8 weeks	17.3 $\pm$ 16.7	21.3 $\pm$ 10.1	17.3 $\pm$ 9.2
	14 weeks	69.3 $\pm$ 14.0 <b>a</b>	64.0 $\pm$ 0.0 <b>ab</b>	49.3 $\pm$ 2.3 <b>b</b>
Sea lice intensity (n/infected fish)	8 weeks	1.0 $\pm$ 0.0	1.2 $\pm$ 0.1	1.0 $\pm$ 0.0
	14 weeks	1.4 $\pm$ 0.3	1.7 $\pm$ 0.3	1.5 $\pm$ 0.2



### 5.3.3 Sea lice observations

At the first sampling point, 8 weeks after commencement, the prevalence, intensity and abundance of sea lice was low across all pens with no significant differences observed between treatments. Upon termination of the trial, after 14 weeks, sea lice observed on sampled fish comprised 73% *Lepeophtheirus* spp. and 27% *Caligus* spp.

No significant differences were observed in sea lice abundance between fish fed the control and the supplemented diets after 14 weeks, however fish fed the B-Wyse treatment ( $n/\text{fish} = 1.1 \pm 0.1$ ) had significantly more attached sea lice compared to the B-Wyse + Bactocell<sup>®</sup> treatment ( $n/\text{fish} = 0.7 \pm 0.1$ ,  $p = 0.023$ ). The prevalence of sea lice was observed to be significantly lower in fish fed the B-Wyse + Bactocell<sup>®</sup>-supplemented ( $49.3\% \pm 2.3$ ) diet compared to those fed the control treatment ( $69.3\% \pm 14.0$ ,  $p = 0.013$ ). No significant differences were detected in the intensity of the sea lice infection between fish fed any of the experimental diets.

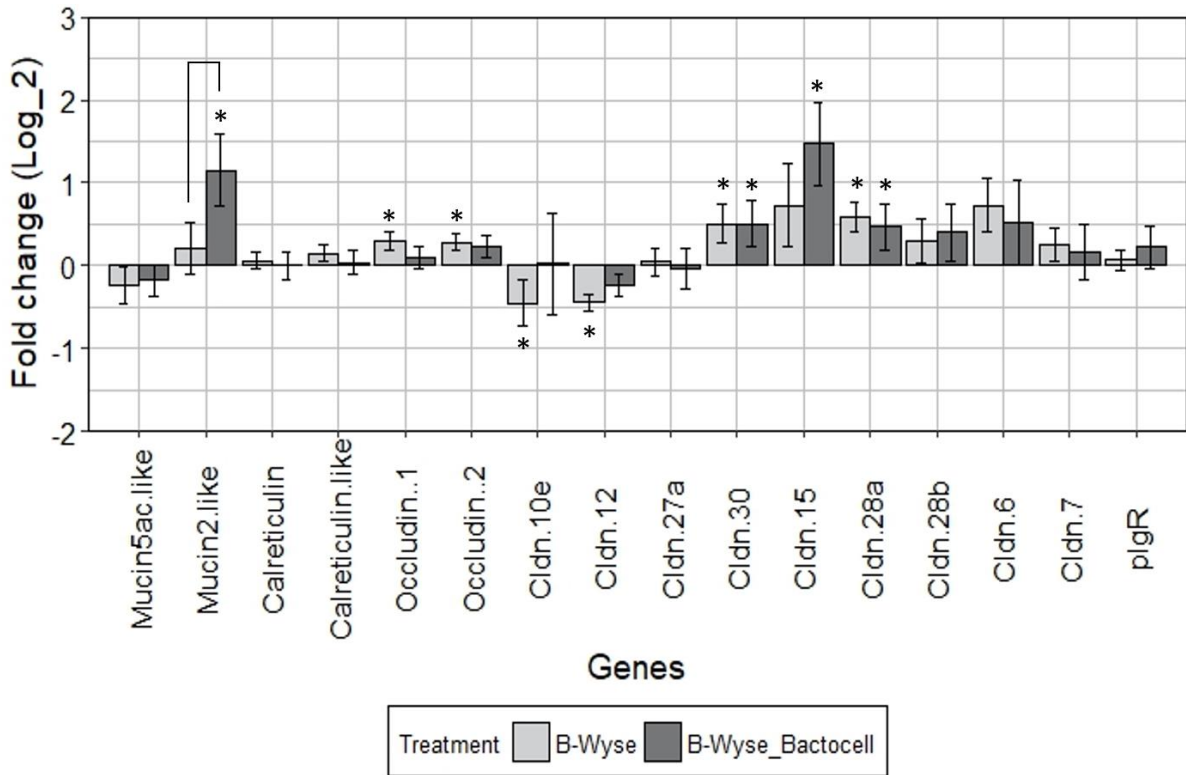
### 5.3.4 Gene expression

#### 5.3.4.1 Gene expression analysis of the SALT after 8 weeks

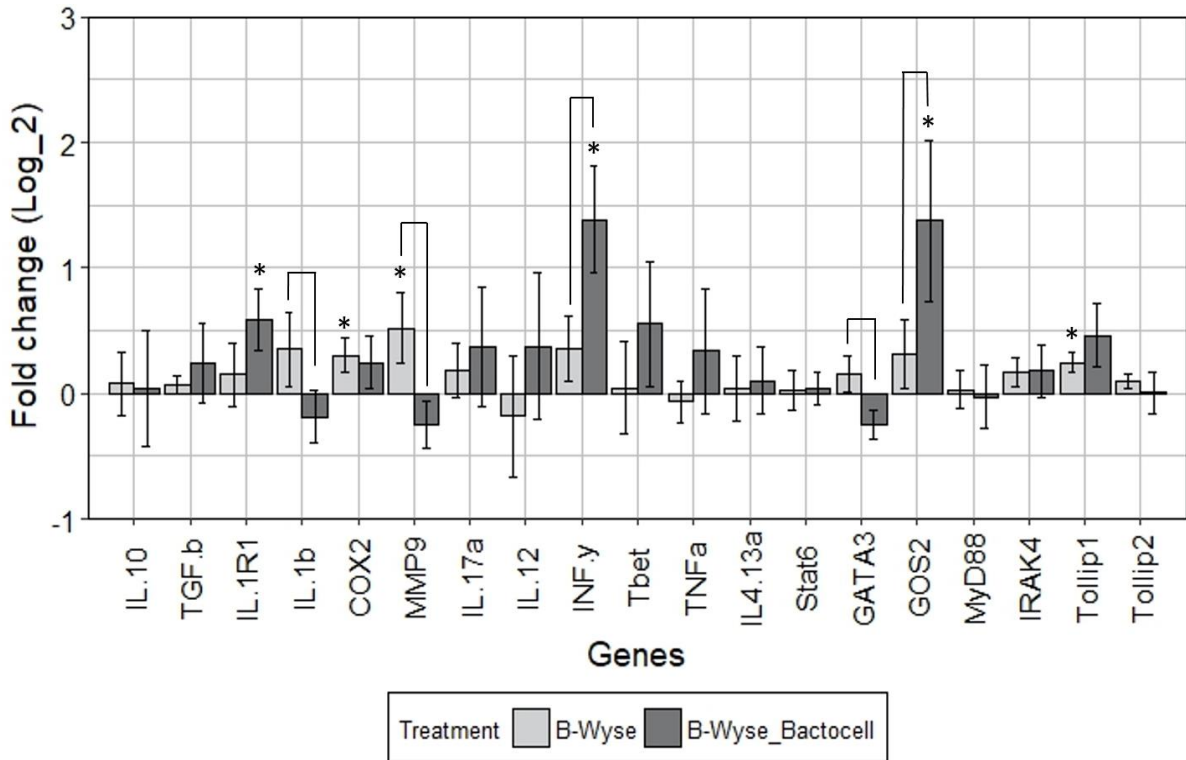
The relative fold change compared to the control of the 57 genes analysed by MF-q-PCR in the SALT after 8 weeks are presented in Figures 5.2, 5.3 and 5.4. Fish fed the B-Wyse+ Bactocell<sup>®</sup>-supplemented diet exhibited significantly increased expression of Muc2-like compared to fish fed the control ( $p = 0.010$ ) and B-Wyse-supplemented ( $p = 0.026$ ) diets. The expression of claudin 15 was also observed to be significantly upregulated in the skin of fish fed the B-Wyse + Bactocell<sup>®</sup>-supplemented ( $p = 0.0135$ ) diet in comparison to the control fed fish. Fish fed the B-Wyse treatment exhibited significantly elevated expression levels of

occludin-1 and occludin-2 compared to the control ( $p = 0.020$  and  $0.009$  respectively), in addition to the significantly downregulated expression of claudin 10e ( $p = 0.037$ ) and claudin 12 ( $p = 0.001$ ), compared to fish fed the control. Additionally, fish fed both the B-Wyse and B-Wyse + Bactocell<sup>®</sup> treatments demonstrated significantly elevated expression of claudin 30 and claudin 28b, in comparison to the control-fed fish.

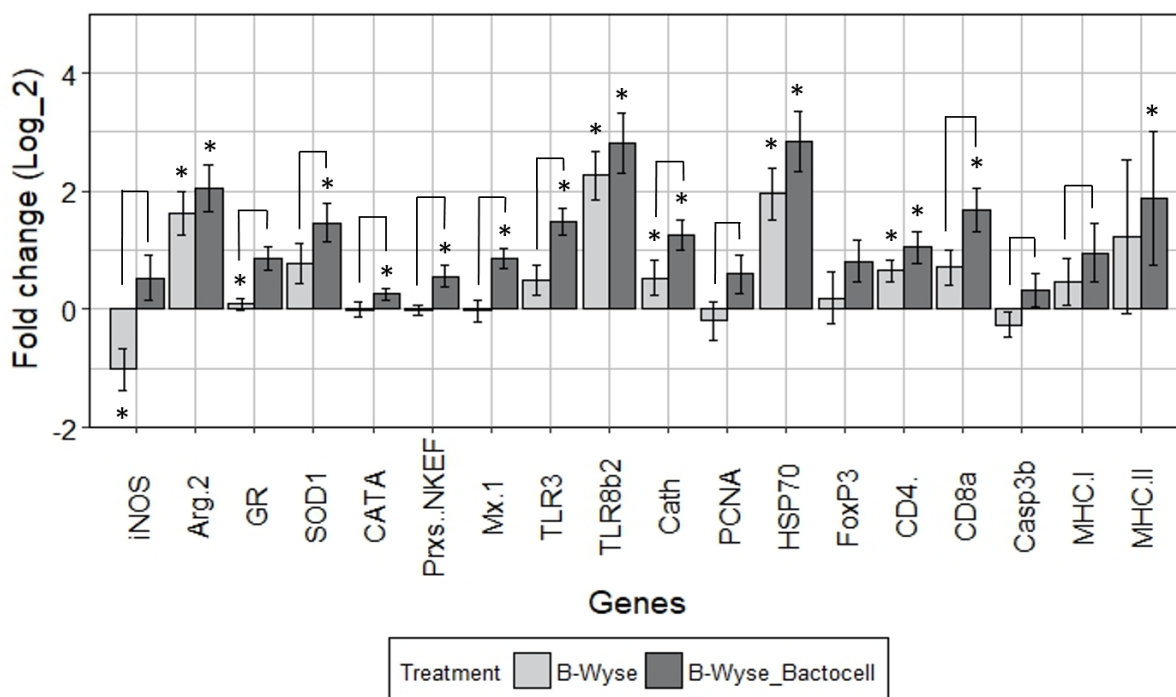
Fish fed the B-Wyse + Bactocell<sup>®</sup> treatment demonstrated significant upregulation of IL-1R1 ( $p = 0.027$ ) compared to the control fish and significantly elevated expression of INF- $\gamma$ , SOD1, MHC-II and GOS2 in comparison to the control ( $p = 0.005$ ,  $p = 0.049$ ,  $p = 0.045$  and  $p = 0.014$ , respectively) and B-Wyse-fed fish ( $p = 0.030$ ,  $p = 0.030$ ,  $p = 0.021$  and  $p = 0.023$ , respectively). Conversely, fish fed the B-Wyse-supplemented diet exhibited significantly increased expression of MMP9 in comparison to both the control ( $p = 0.033$ ) and B-Wyse + Bactocell<sup>®</sup>-supplemented ( $p = 0.012$ ) fish, in addition to significantly elevated COX2 ( $p = 0.039$ ), Casp3b ( $p = 0.033$ ) and Arg2 ( $p = 0.010$ ) expression in comparison to the control-fed fish. Conversely, the expression of transferrin was demonstrated to be significantly downregulated in the skin of fish fed the B-Wyse treatment in comparison to fish fed the control ( $p = 0.048$ ) and B-Wyse + Bactocell<sup>®</sup>-supplemented ( $p = 0.005$ ) diets.



**Figure 5.2** Gene expression data (fold change (log<sub>2</sub>)) of target genes analysed in the SALT of Atlantic salmon fed experimental diets supplemented with B-Wyse and B-Wyse + Bactocell® for 8 weeks; n=9. Data presented as mean ± SEM. Asterix denote a significant difference between the supplemented diet and the control diet; lines connecting bars denote significant difference between the supplemented diets ( $p < 0.05$ ).



**Figure 5.3** Gene expression data (fold change (log<sub>2</sub>)) of target genes analysed in the SALT of Atlantic salmon fed experimental diets supplemented with B-Wyse and B-Wyse + Bactocell® for 8 weeks; n=9. Data presented as mean ± SEM. Asterisk denote a significant difference between the supplemented diet and the control diet; lines connecting bars denote significant difference between the supplemented diets ( $p < 0.05$ ).

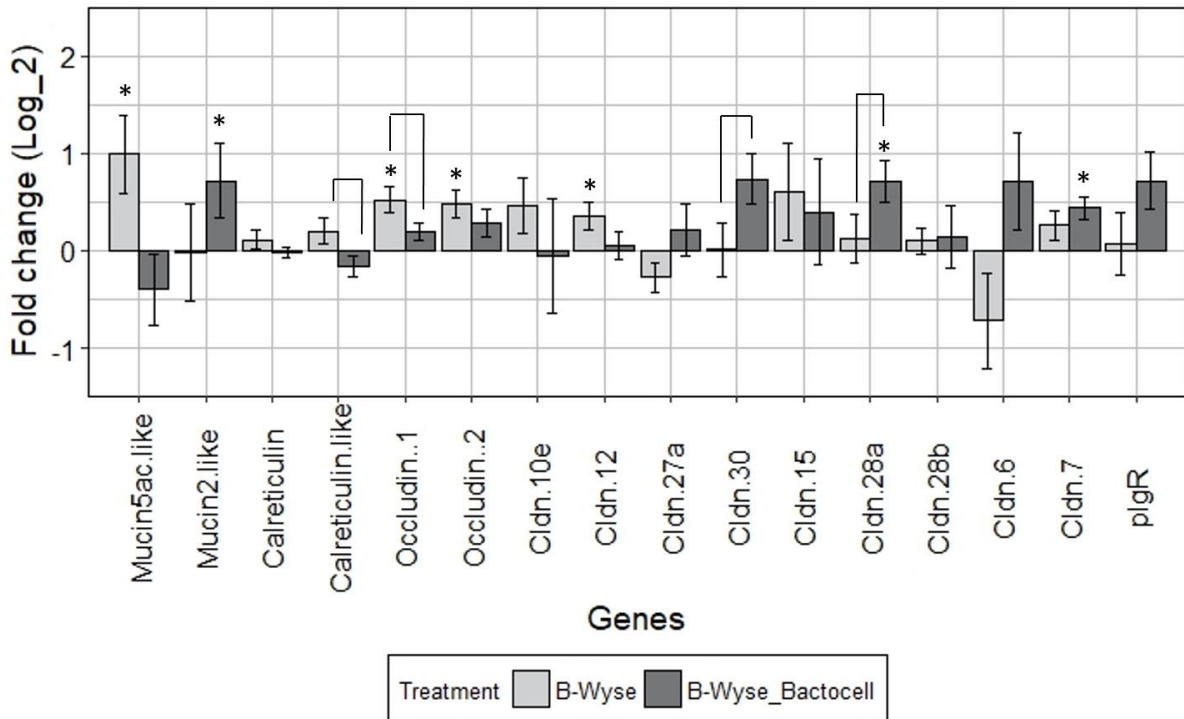


**Figure 5.4** Gene expression data (fold change (log<sub>2</sub>)) of target genes analysed in the SALT of Atlantic salmon fed experimental diets supplemented with B-Wyse and B-Wyse + Bactocell® for 8 weeks; n=9. Data presented as mean ± SEM. Asterix denote a significant difference between the supplemented diet and the control diet; lines connecting bars denote significant difference between the supplemented diets ( $p < 0.05$ ).

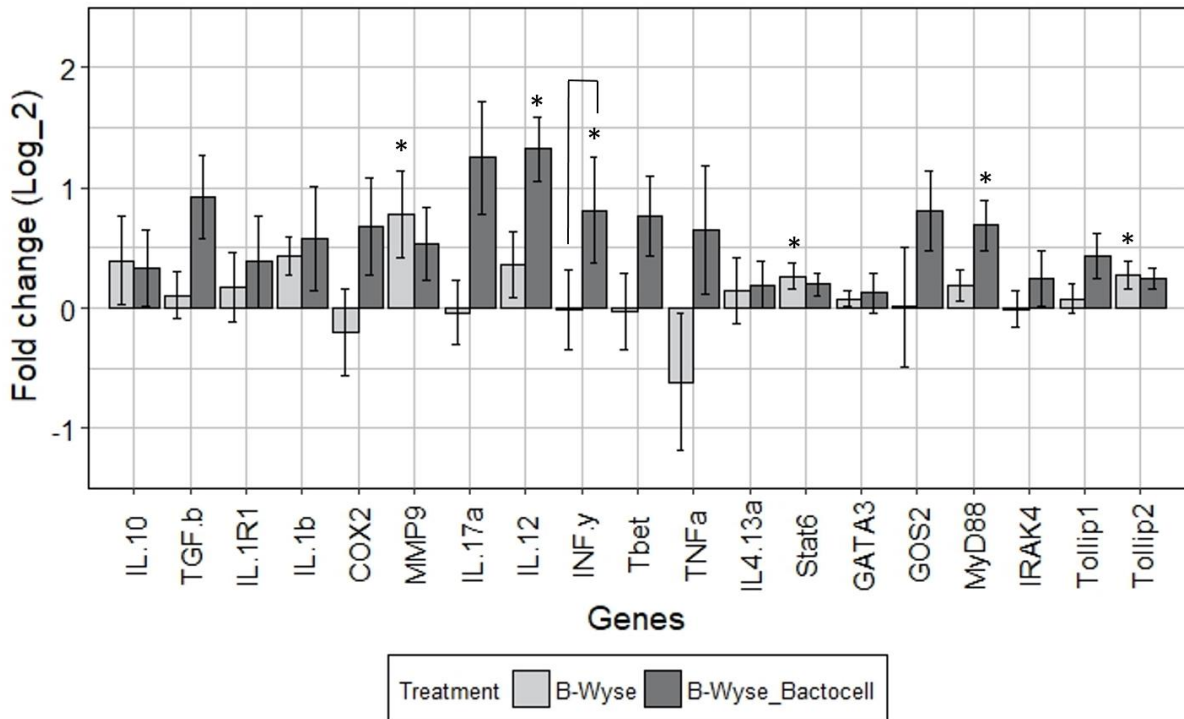
#### 5.3.4.2 Gene expression analysis of the SALT after 14 weeks

The relative fold change compared to the control of the 57 genes analysed by MF-q-PCR in the SALT after 14 weeks are presented in Figures 5.5, 5.6 and 5.7. Fish fed the B-Wyse treatment exhibited significantly increased expression of Muc5ac ( $p = 0.011$ ), occludin 2 ( $p = 0.003$ ), claudin 12 ( $p = 0.015$ ), MMP9 ( $p = 0.021$ ), Tollip2 ( $p = 0.012$ ), Mx1 ( $p = 0.004$ ), TLR3 ( $p = 0.011$ ) and MHC-I ( $p = 0.022$ ) compared to the control- fed fish and significantly increased expression of occludin1, in comparison to fish fed the control ( $p = 0.001$ ) and B-Wyse + Bactocell<sup>®</sup>-supplemented ( $p = 0.042$ ) diets.

Significant elevations in the expression of Muc2-like ( $p = 0.030$ ), claudin 7 ( $p = 0.008$ ), IL-12 ( $p = 0.02$ ), MyD88 ( $p = 0.034$ ), Prxs/NKEF ( $p = 0.017$ ) and PCNA ( $p = 0.013$ ) were observed between fish fed the B-Wyse + Bactocell<sup>®</sup>-supplemented diet and the control-fed fish. Additionally, fish fed the B-Wyse + Bactocell<sup>®</sup>-supplemented diet exhibited significantly increased expression of claudin 28a, IFN- $\gamma$  and CD8 $\alpha$  in comparison to fish fed the control ( $p = 0.049$ ;  $p = 0.050$ ; and  $0.006$ , respectively) and B-Wyse-supplemented ( $p = 0.023$ ;  $0.040$ ; and  $0.005$ , respectively) diets.

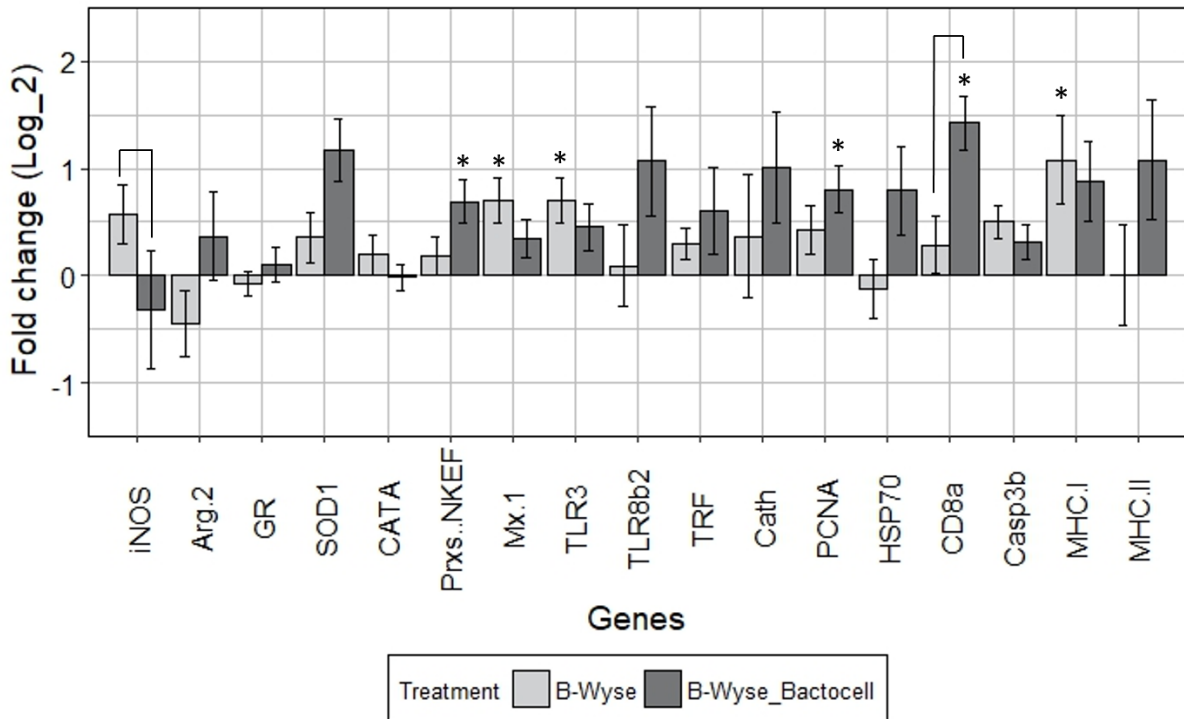


**Figure 5.5** Gene expression data (fold change ( $\log_2$ )) of target genes analysed in the SALT of Atlantic salmon fed experimental diets supplemented with B-Wyse and B-Wyse + Bactocell® for 14 weeks;  $n=9$ . Data presented as mean  $\pm$  SEM. Asterix denote a significant difference between the supplemented diet and the control diet; lines connecting bars denote significant difference between the supplemented diets ( $p < 0.05$ ).



**Figure 5.6** Gene expression data (fold change ( $\log_2$ )) of target genes analysed in the SALT of Atlantic salmon fed experimental diets supplemented with B-Wyse and B-Wyse + Bactocell<sup>®</sup> for 14 weeks;  $n=9$ . Data presented as mean  $\pm$  SEM. Asterix denote a significant difference between the supplemented diet and the control diet; lines connecting bars denote significant difference between the supplemented diets ( $p < 0.05$ ).





**Figure 5.7** Gene expression data (fold change ( $\log_2$ )) of target genes analysed in the SALT of Atlantic salmon fed experimental diets supplemented with B-Wyze and B-Wyze + Bactocell® for 14 weeks;  $n=9$ . Data presented as mean  $\pm$  SEM. Asterisk denote a significant difference between the supplemented diet and the control diet; lines connecting bars denote significant difference between the supplemented diets ( $p < 0.05$ ).

### 5.3.4.3 Gene expression analysis of the GIALT after 14 weeks

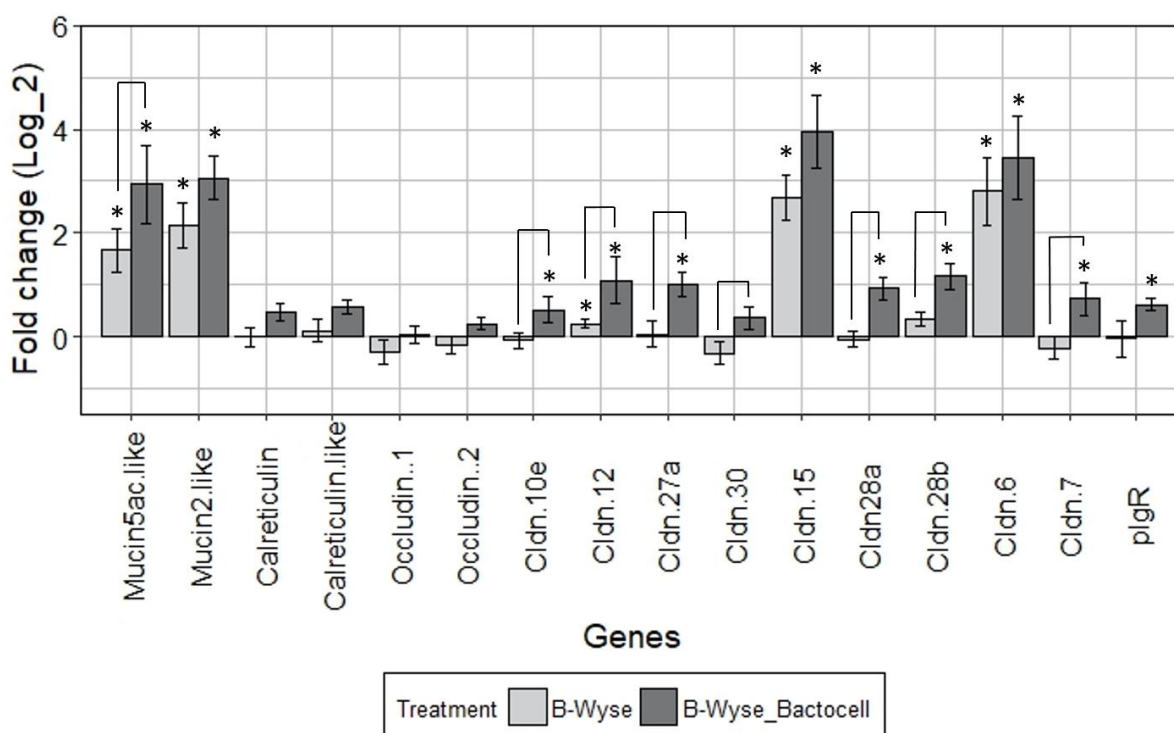
The relative fold change compared to the control of the 57 genes analysed by MF-q-PCR in the GIALT after 14 weeks are presented in Figures 5.8, 5.9 and 5.10. Fish fed the B-Wyse and B-Wyse + Bactocell® treatments exhibited significantly increases in the expression of Muc2-like ( $p = 0.005$  and  $0.001$ ), claudin 15 ( $p = 0.001$  and  $0.004$ ), claudin 6 ( $p = 0.001$ ;  $0.011$ ), TGF- $\beta$  ( $p = 0.001$  and  $0.001$ ), COX2 ( $p = 0.012$  and  $0.003$ ), IL17a ( $p = 0.004$  and  $0.002$ ), IL-12 ( $p = 0.046$  and  $0.002$ ), TNF- $\alpha$  ( $p = 0.001$  and  $0.001$ ), GOS2 ( $p = 0.001$  and  $0.001$ ), IRAK4 ( $p = 0.007$  and  $0.004$ ), Tollip1 ( $p = 0.007$  and  $0.001$ ), Arg2 ( $p = 0.001$  and  $0.001$ ), TLR8b2 ( $p = 0.001$  and  $0.001$ ), HSP70 ( $p = 0.003$  and  $0.001$ ), CD4+ ( $p = 0.028$  and  $0.004$ ), in comparison to the control-fed fish.

Concerning the mucous-associated genes, fish fed the B-Wyse + Bactocell®-supplemented diet demonstrated significant elevation in the expression of Muc5ac-like ( $p = 0.005$  and  $0.016$ ), claudin 10e ( $p = 0.018$  and  $0.005$ ), claudin 12 ( $p = 0.017$  and  $0.032$ ), claudin 27a ( $p = 0.002$  and  $0.003$ ), claudin 28a ( $p = 0.001$  and  $0.001$ ), claudin 28b ( $p = 0.001$  and  $0.007$ ), claudin 7 ( $p = 0.028$  and  $0.007$ ) in comparison to fish fed the control and B-Wyse-supplemented diets, respectively.

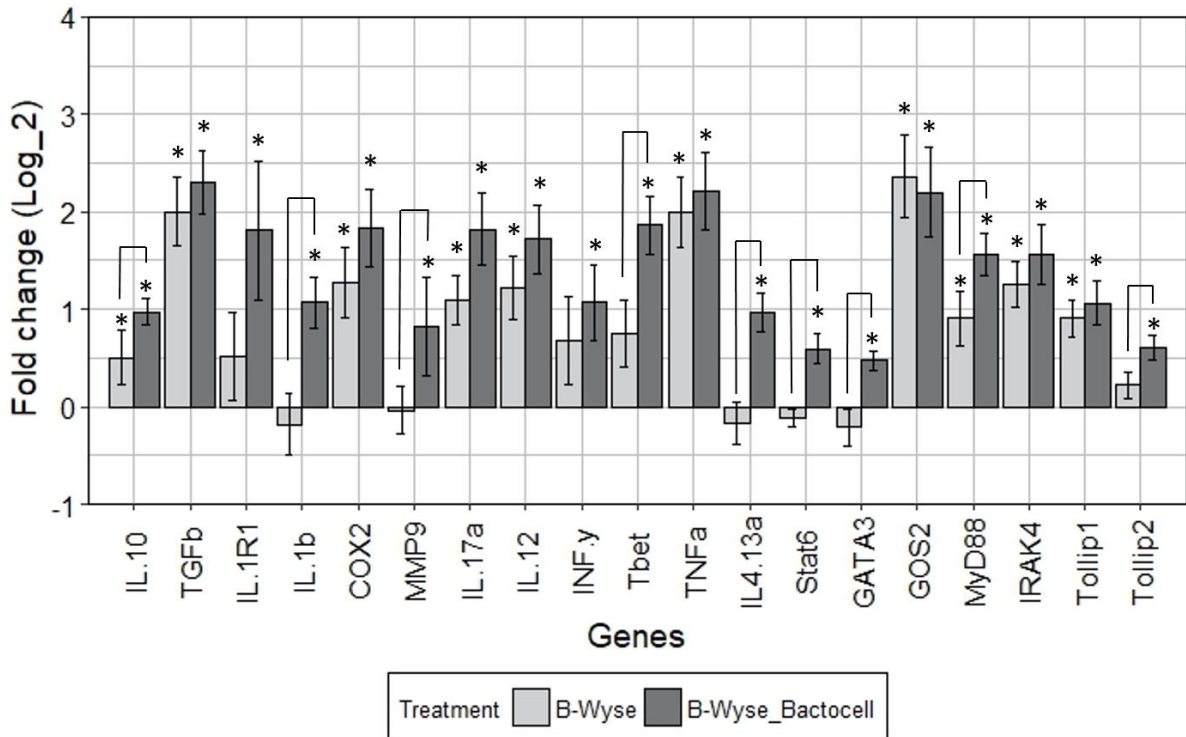
Differences were also observed in the expression of the immune-related genes; fish fed the B-Wyse + Bactocell®-supplemented diet exhibited significantly increased expression of IL-10 ( $p = 0.02$  and  $0.039$ ), IL-1 $\beta$  ( $p = 0.002$  and  $0.002$ ), MMP9 ( $p = 0.032$  and  $0.031$ ), Tbet ( $p = 0.001$  and  $0.005$ ), IL4/13a ( $p = 0.003$  and  $0.001$ ), Stat6 ( $p = 0.010$  and  $0.003$ ), GATA3 ( $p = 0.025$  and  $0.001$ ), MyD88 ( $0.001$  and  $0.023$ ), and Tollip2 ( $p = 0.001$  and  $0.035$ ) compared to fish fed the control and B-Wyse treatments, respectively. Additional significant increases in the expression of GR ( $p = 0.001$  and  $0.001$ ), SOD1 ( $p = 0.007$  and  $0.035$ ), CATA ( $p = 0.011$  and

0.043), Prxs/NKEF ( $p = 0.003$  and  $0.003$ ), Mx1 ( $p = 0.002$  and  $0.003$ ), TLR3 ( $p = 0.001$  and  $0.001$ ), Cath ( $p = 0.001$  and  $0.005$ ), and CD8 $\alpha$  ( $p = 0.005$  and  $0.011$ ) in comparison to fish fed the control and B-Wyсе-supplemented diets, respectively.

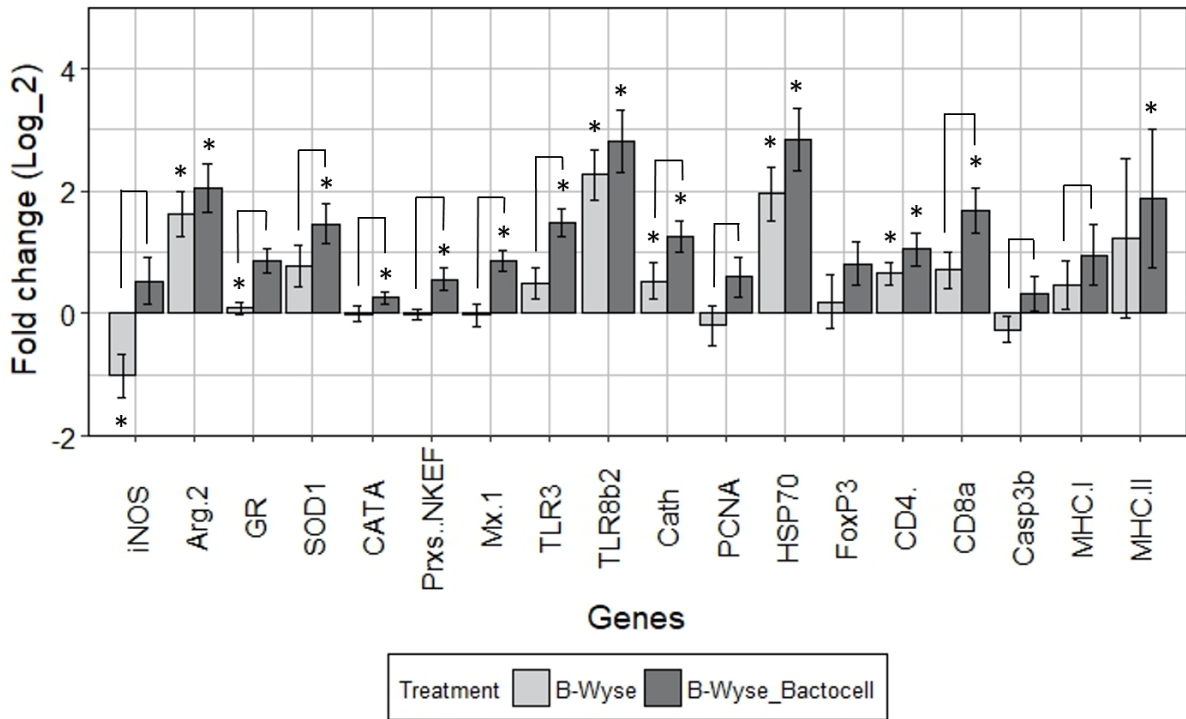
In comparison to the control-fed fish, additional significant increases in the expression of plgR ( $p = 0.036$ ), IL-1R1 ( $p = 0.022$ ), IFN- $\gamma$  ( $p = 0.017$ ), and MHC-II ( $p = 0.043$ ) were observed in the B-Wyсе + Bactocell<sup>®</sup>-supplemented diet. Also in comparison to the control-fed fish, fish fed the B-Wyсе-supplemented diet demonstrated significant elevations in the expression of Muc5ac-like ( $p = 0.001$ ), IL-10 ( $p = 0.044$ ), MyD88 ( $p = 0.003$ ), and Cath ( $p = 0.032$ ).



**Figure 5.8** Gene expression data (fold change ( $\log_2$ )) of target genes analysed in the GIALT of Atlantic salmon fed experimental diets supplemented with B-Wyсе and B-Wyсе + Bactocell<sup>®</sup> for 14 weeks;  $n=9$ . Data presented as mean  $\pm$  SEM. Asterix denote a significant difference between the supplemented diet and the control diet; lines connecting bars denote significant difference between the supplemented diets ( $p < 0.05$ ).



**Figure 5.9** Gene expression data (fold change ( $\log_2$ )) of target genes analysed in the GIALT of Atlantic salmon fed experimental diets supplemented with B-Wysey and B-Wysey + Bactocell® for 14 weeks;  $n=9$ . Data presented as mean  $\pm$  SEM. Asterix denote a significant difference between the supplemented diet and the control diet; lines connecting bars denote significant difference between the supplemented diets ( $p < 0.05$ ).



**Figure 5.10** Gene expression data (fold change (log<sub>2</sub>)) of target genes analysed in the GIALT of Atlantic salmon fed experimental diets supplemented with B-Wyse and B-Wyse + Bactocell® for 14 weeks; n=9 . Data presented as mean ± SEM. Asterix denote a significant difference between the supplemented diet and the control diet; lines connecting bars denote significant difference between the supplemented diets ( $p < 0.05$ ).

## 5.4 Discussion

To the author's knowledge, very few nutrition trials have been performed on Atlantic salmon in open sea pens to date, such as that performed by Dimitroglou *et al.*, (2011) and in the present study. In the course of commercial Atlantic salmon aquaculture farming in open sea pens, fish experience many stressful situations including fluctuations in water quality, high stocking density, pathogenic assaults, and handling during vaccination events, which may influence the performance of the fish (Ángeles-Esteban 2012, Oliva-Teles 2012). As such, it is prudent to investigate the influence of feed additives in different environments that expose the fish to different challenges. In the present study, the prebiotic B-Wyse was supplemented into the diets of Atlantic salmon, both alone and in combination with the commercial probiotic Bactocell® (derived from *P. acidilactici*) and administered to Atlantic salmon for 14 weeks in open sea pens.

The influence of these additives upon the growth indices, mucosal barrier defences and sea lice resistance were evaluated. Additionally, the expression levels of 59 genes were analysed by use of MF-q-PCR with the aim of providing a more holistic view of the alterations occurring in response to the administration of the dietary additives at the SALT and GIALT.

### 5.4.1 Growth indices

Concerning the growth indices of the fish, no significant differences in individual body weight, FCR or SGR were observed between fish fed the experimental dietary regimes following 8 and 14 weeks of feeding. These results support those observed in chapters three and four, in which rainbow trout and Atlantic salmon fed a B-Wyse-supplemented diet exhibited no significant differences in growth indices compared to those fed the control regime. Daily

measurements of water quality parameters surrounding the pens were optimal and typical of spring conditions, with no fluctuations beyond those deemed acceptable for Atlantic salmon growth, as described in Ostrander (2000). The experimental diets used in this trial were optimally designed and formulated to meet the known nutritional requirements of Atlantic salmon. Taken together, in light of the FCR and SGR values observed across all treatments (8 weeks = <0.8 FCR and >1.5 SGR; 14 weeks = <1.1 FCR and >0.95 SGR) and in addition to the high survival rates (>98%), it can be concluded that the nutritional availability and rearing conditions experienced by the fish were adequate for their survival during the experimental period. However, as a result, there appeared to be little scope for the experimental dietary additives to induce any positive benefits on the growth metrics. These results are additionally supported by those of previous investigations in which no significant differences in growth indices were observed in response to prebiotic (Torrecillas *et al.*, 2011; Ye *et al.*, 2011; Jami *et al.*, 2019) and synbiotic supplementation (Torrecillas *et al.*, 2018; Jami *et al.*, 2019).

#### 5.4.2 Epidermal mucous analysis and sea lice observations

The epidermal skin mucous is an essential barrier, serving to protect the fish from environmental biotic and abiotic factors, and pathogenic insults (Dash *et al.*, 2018). A wide range of dietary additives have been previously documented to increase epidermal mucous secretion in fish, including probiotics (Salinas *et al.*, 2008), prebiotics (Torrecillas *et al.*, 2011), paraprobiotics (Dawood *et al.*, 2016), and synbiotics (Rodriguez-Estrada *et al.*, 2013; Dawood *et al.*, 2015b). It has been proposed that, as part of the Th2 response, the hyper-secretion of epidermal mucous further assists in dislodging and removing parasitic pathogens away from the epidermis in fish (Holm *et al.*, 2015). Indeed, increased mucous secretion has previously been described in Atlantic salmon following infection with the sea lice *L. salmonis* (Fast *et al.*,

2002). On the contrary, it has also been hypothesised that increased mucous production may be important for parasite survival, serving as a nutritional component of the diet of developing lice, as has been discussed for *Gyrodactylus* infection, and based upon the assertion that sea lice feed upon the mucous, skin and blood of their host (Kania *et al.*, 2010; Hamilton *et al.*, 2018).

In support of the studies mentioned above, both the prebiotic and synbiotic-fed fish of this study demonstrated significantly elevated quantities of secreted epidermal mucous compared to those fed the control treatment, following eight weeks of feeding. Considering the very low infection level of sea lice at this point, these observations are also conducive with those of chapter four in which Atlantic salmon fed the prebiotic and synbiotic diets for six weeks also exhibited significant increases in the amount of secreted epidermal mucous compared to fish fed the control treatment. These results are suggestive of a dietary influence upon epidermal mucous production in Atlantic salmon.

After 14 weeks, the sea lice infection was more pronounced with a higher abundance, intensity and prevalence across all pens. Fish fed the B-Wyse treatment continued to exhibit significantly increased secreted epidermal mucous quantities than those fed the control diet, yet presented no significant reduction in sea lice abundance compared to the control-fed fish. In contrast, fish fed the synbiotic diet exhibited no differences in the amount of secreted mucous compared to the control fish, yet significantly fewer sea lice were recorded on the synbiotic-fed fish compared to the control-fed fish. These results add to the confounding evidence already reported concerning the relationship between epidermal mucous secretions and the intensity of sea lice infections.



Within the scientific literature, several studies have reported on the ability of dietary feed additives to modulate proteins within the mucous, such as lysozyme and galectins that are associated with immune function and that aid in the prevention of lice invasion. (León-Sicairos *et al.*, 2006; Provan *et al.*, 2013; Akhter *et al.*, 2015; Carbone and Faggio, 2016; Martin and Król, 2017). As discussed in chapter 4, host immunomodulation by parasitic arthropods has also been described: Fast *et al.* (2002 and 2007) previously reported that secretions from *L. salmonis* pre-adults and adults include trypsins and prostaglandins (PGs), which have profound effects upon the host inflammatory and immune responses both directly (i.e. anti coagulation, vasodilation, and necrosis) and indirectly through stress induced immunosuppression (Tully and Nolan, 2002; Fast, 2014). As such, gene expression analysis may provide further insight into the changes and alterations that occur within the host fish in response to sea lice infection.

The results of the present study differ from those of the controlled sea lice challenge performed in chapter four in terms of dietary influence upon mucous production and resistance to sea lice settlement. Aside from the additional challenges of *Caligus* spp. in the present investigation, one major difference is the intensity of the sea lice infection. In the open sea pens, the sea lice infection was very mild with infected fish carrying an average of 2 sea lice, while in chapter four fish were infected with an average of 23 sea lice per fish. While it has been reported that the attachment of as few as 6 sea lice to a single fish can have a detrimental effect on the welfare of the fish, infections on wild and farmed populations very rarely reach the levels observed in chapter four (Núñez-Acuña *et al.*, 2015). The aquaculture industry is very proactive in monitoring sea lice infections and intervening where required in order to protect wild salmon stocks in addition to avoiding costly lice infections on

neighbouring farms (Kragesteen *et al.*, 2019). In Norway regulations for allowable sea lice limits were introduced in early 2009 which have since been readjusted to the lower limits of 0.5 gravid lice per salmon in general and 0.2 during wild migration periods, while the newly introduced National Operational Salmon Lice Monitoring System essentially estimates the infection pressure on wild salmonids (Myksvoll *et al.*, 2018). In Scotland, the “Code of Good Practice” recommends slightly less stringent threshold levels: 0.5 gravid lice per fish during migratory periods, otherwise 1 gravid lice per fish is acceptable (Marine Scotland 2017). When trialling compounds for their beneficial disease resistance attributes, it is first necessary to test their potency against intense infection pressures such as those in chapter four, but equally importantly in non-laboratory, less controlled situations such as those of the present study.

#### 5.4.3 MF-q-PCR analysis

##### 5.4.3.1 Expression in the SALT: mucosal- and TJ-associated genes

Certain dietary additives such as fibre, glucans and probiotics have previously been reported to influence the expression of mucin-encoding genes and mucin content of mucosal secretions in terrestrial animals (Dykstra *et al.*, 2011; Smith *et al.*, 2011; Hino *et al.*, 2012). A study of note by van der Marel *et al.* (2012) has demonstrated the regulation of mucins genes by dietary additives is possible in the various MALT tissues of fish, of which the results of chapters three and four also support.

In the present study, fish fed the B-Wyse treatment exhibited significant upregulation of Muc5ac-like, compared to fish fed the control treatment after 14 weeks, while expression of

Muc2-like was significantly upregulated in the SALT of fish fed the B-Wyse + Bactocell® treatment at both 8 and 14 weeks, compared to fish fed the control diet. Previous work has suggested a tissues-specific expression of Muc2 in the intestine and pyloric caeca of fish, however the results of this study would suggest an important role for Muc2 in the skin of fish also (Sveen *et al.*, 2017). These results are in support of previous work that has demonstrated the influence of dietary additives upon the expression of mucous-associated genes (van der Marel *et al.*, 2012) and further underscores the interconnectivity of the mucosal tissues.

Similarly, claudin 28a expression was significantly upregulated within the SALT of fish fed the synbiotic and B-Wyse treatments following 8 weeks of feeding and continued in fish fed the synbiotic diet following 14 weeks, compared to those fed the control diet. Claudins constitute a major component of the barrier defences, playing important role in the creation of TJ complexes and the regulation of mucosa permeability in both mammals and fish (Adamek *et al.*, 2013). This significant upregulation in both experimental treatments at 8 weeks and the long-term upregulation in response to the synbiotic diet after 14 weeks would suggest that claudin 28a has a prominent and advantageous role in the barrier defences of fish. Kolosov *et al.* (2013) previously proposed the same hypothesis due to the close relation of piscine claudin 28a with mammalian claudin 4, which is prominently expressed throughout the lung epithelium (Koval, 2017). The results of this study would support this hypothesis.

After eight weeks of feeding, the significant upregulation of claudin 30 expression in the skin of fish fed both the prebiotic and synbiotic diet, compared to the control-fed fish was observed, indicative of a dietary response to the feed additives and suggesting an important role for claudin 30 in the SALT. These results are in agreement with those of chapter three, in which rainbow trout fed the synbiotic-supplemented diet also exhibited significantly elevated

expression levels of claudin 30 in the skin following 8 weeks of feeding. Previous studies have suggested claudin 30 is a gill specific claudin, however the results of the present study support accumulating evidence to suggest claudin 30 is not gill-specific (Engelund *et al.*, 2012; Kolosov *et al.*, 2013). Further investigations into the role claudin 30 plays in establishing cell-to-cell contact and epithelial integrity is warranted.

Interestingly, claudin 15 has been previously reported to be highly abundant in the intestinal tissue of salmon, while claudin 12 was considered to be highly specific to gill tissue in rainbow trout (Tipsmark *et al.*, 2010; Chasiotis and Kelly, 2011). In the present study, both claudin 12 and claudin 15 were significantly upregulated in the SALT of fish fed the supplemented diets, compared to the control-fed fish. A benefit of MF-q-PCR is that it allows for the analysis of a wide range of genes in a variety of tissues that may not necessarily be analysed using standard RT-q-PCR techniques due to the associated cost of analysis and based on previous reports of tissue specificity (Loh *et al.*, 2004).

Occludin is a tetraspan protein that localises exclusively to TJ fibrils at sites of cell-to-cell contact (González-Mariscal *et al.*, 2003). In mammals, evidence suggests occludin plays an important role in the formation and enhancement of the TJ barrier via its extracellular domains, which “occlude” the intracellular space and therefore restrict the paracellular movement of solutes (González-Mariscal *et al.*, 2003; Chasiotis *et al.*, 2012; Cummins, 2012). In fish, the gill tissue has been shown to exhibit the highest levels of occludin transcript abundance, while mechanistic studies have confirmed the barrier-forming role of occludin in the gill and its regulation in response to a number of environmental and systemic variables, such as low pH, following food deprivation, and when circulating levels of cortisol are chronically elevated (Cummins 2012; Kolosov *et al.*, 2014). In the present study, both occludin

isoforms were observed to be significantly upregulated in the SALT of fish fed the B-Wyse treatment following 8 and 14 weeks of feeding, compared to the control-fed fish. Similar to the expression of claudin 28a discussed above, the significant upregulation of occludin at 8 and 14 weeks would be suggestive of an important role in epithelial integrity and that the expression of occludin may be regulated by the dietary supplementation of the prebiotic. The importance of TJs in barrier function and epithelial integrity has long been accepted within the scientific community, yet given the considerable number of components involved in the formation of the complex, our understanding of TJs in the MALT tissues of fish remains to be fully elucidated, especially with regard to the multiple factors and stimulus that may influence its function. MF-q-PCR is a technique that may aid in the rapid accumulation of data pertaining to the interaction of claudins at TJs via the simultaneous analysis of a large range of target genes of interest.

#### *5.4.3.2 Expression in the SALT: immune-related genes*

In fish, the initiation of the immune response within a host exposed to a pathogen depends on the recognition of pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular pattern (MAMPs) present on the pathogen by specific pattern recognition receptors (PRRs) present on host innate cells such as macrophages and dendritic cells (Chettri *et al.*, 2011). Serving as the primary sentinels in the host, macrophages detect danger signals using a series of receptors including Toll-like receptors (TLR), intracellular PRRs and the interleukin-1 receptor (IL-1R) (Chettri *et al.*, 2011). PAMPs, such as flagellin, lipopolysaccharide (LPS),  $\beta$ -glucan and mannans, presented on the surface of products used as immunostimulants initiate the immune response of the host in the same manner,

instigating distinctive reactions and the production of specific effector molecules targeting different pathogen types, depending on PAMP presentation (Álvarez-Rodríguez *et al.*, 2018).

In mammals, COX-2 is an inflammatory-related enzyme that plays a central role in the initiation of inflammatory reactions and also catalyses the reaction in which arachidonic acid is converted into PGs, however in fish, the regulation and molecular mechanisms of COX-2 remain largely unexplored (Wang *et al.*, 2016). In the present study, fish fed the B-Wyze supplemented diet exhibited significantly elevated transcription of COX-2 following 8 weeks of feeding compared to the control-fed fish. These results are supported by those of an *in vitro* study in which rainbow trout head kidney leucocytes were exposed to different PAMPs mimicking viral, bacterial and fungal infections:  $\beta$ -glucan (from the baker's yeast, *S. cerevisiae*) exposure at doses of 0.05 and 0.5mg/mL stimulated the expression of COX-2 after 4 and 12 hours, yet expression was diminished at a higher dose of 5mg/mL (Chettri *et al.*, 2011). This suggests that immunostimulant dosage may be an important parameter influencing the host immune response, while the significantly elevated expression of COX-2 in the skin of B-Wyze fed fish in the present study would suggest that the dosage levels were adequate to provoke such a response. The lack of significant difference in the expression of COX-2 in the SALT of fish fed the synbiotic diet compared to the control fish may be suggestive of too high dosage: the interaction of the probiotic and the prebiotic may be resulting in a diminished response as previously demonstrated *in vitro* by Chettri *et al.* (2011).

The elevated expression of COX-2 would serve to benefit the fish via the increased production of PGs due to their previously reported involvement in gastrointestinal cytoprotection (Oxley *et al.* 2010); inducing the closure of TJs in order to reduce disruption of epithelial barrier function (Blikslager *et al.* 1997); stimulating mucin synthesis and release rates; and

influencing their associated physiochemical properties (Enss *et al.*, 1995, Plaisancié *et al.*, 1998). The increased production of PGs in the posterior gut of European seabass has been reported in response to dietary prebiotic administration for 9 weeks (Torrecillas *et al.* 2014). It may therefore be possible that the dietary additives investigated in the present study are able to regulate the transcription of COX2 in the SALT, which may prove to be beneficial during incidence of sea lice infection, as previously discussed in chapter four.

As a consequence of its ability to cleave structural extracellular matrix molecules, mammalian matrix metalloproteinase-9 (MMP-9) is associated with vital inflammatory processes such as leucocyte migration and tissue remodelling and regeneration (Chadzinska *et al.*, 2008). Although knowledge of the biological function of piscine MMP-9 is still limited, it is known to be able to regulate the cell matrix composition, aiding in the cell proliferation and migration, and also cell-to-cell communication, and as such is evaluated as a marker of inflammation (Zhang *et al.*, 2013, Rajaram *et al.*, 2016). Previous studies have demonstrated the upregulation of MMP-9 in response to LPS in an *in vitro* study on trout macrophages (MacKenzie *et al.*, 2006), the upregulation of MMP-9 in trout head kidney leucocytes simultaneously treated with TNF $\alpha$  and LPS *in vitro* (Johnson *et al.*, 2004) and upregulation in response to bath-vaccination with live attenuated *Vibrio anguillarum* in zebrafish (Zhang *et al.* 2013). In the present study, MMP-9 was observed to be significantly upregulated in the SALT of fish fed the B-Wyse treatment after 8 and 14 weeks of feeding, compared to the control-fed fish, suggesting dietary stimulation of the inflammatory response has been achieved.

Macrophages possess a key function in the immune response against parasites; they are not only able to phagocytose parasites but they are also involved in complement activation,

chemotaxis, cytokine-associated intracellular communication, and antigen-presentation (Allen and Sutherland, 2014). Macrophages can be subdivided into classical and alternative states; classically activated macrophages play important roles in Th1 immune responses against intracellular pathogens by the production of reactive oxygen species (ROS) and nitric oxide (NO), while alternatively activated macrophages play important roles in Th2 immune responses against extracellular pathogens by exhibiting increased phagocytic activity and enhanced gene expression of the major histocompatibility complex (MHC)-II (Skugor *et al.*, 2008; Severin *et al.*, 2010). In fish, Arginase-2 (Arg2) has been demonstrated to be differentially regulated and involved in the alternative activation of macrophages, suggesting it plays a leading role in Th2 immune responses which is considered to be particularly important in relation to defence against parasites (Skugor *et al.*, 2008; Severin *et al.*, 2010). A number of studies have measured the expression of Arg2 during bacterial and parasitic infection, while the significant upregulation of Arg2 in sea lice infected Atlantic salmon has also been reported (Forlenza *et al.*, 2011; Fast, 2014). In the present study, fish fed the B-Wyse-supplemented diet exhibited significantly elevated expression of Arg2 in the skin after 8 weeks compared to the control-fed fish. Strong mucosal responses such as the results observed in the present study in relation to secreted mucous quantities, coupled with the significantly elevated expression of Arg2 are consistent with Th2 immune responses, which may be attributed to dietary B-Wyse administration.

The Th1 immune response is focussed towards the protection against viral infections and other intracellular pathogens (Skugor *et al.*, 2008). Type II interferon (IFN)- $\gamma$  is produced by Th1 cytokines and Natural killer (NK) cells to promote Th1 responses (Castro and Tafalla, 2015) and is involved in several aspects of immunity, including activation of macrophages and T-



helper lymphocytes, stimulation of antigen presentation, control of cell proliferation and apoptosis, induction of antiviral state, immunomodulation, inflammation and leukocyte trafficking, among other functions (Pereiro *et al.*, 2019). Following the first identification of IFN- $\gamma$  in Fugu in 2004 (Zou, 2004), it has been described in a variety of other species, including rainbow trout (Zou *et al.*, 2005), zebrafish (Igawa *et al.*, 2006), and Atlantic salmon (Robertsen, 2006). IL-12 is also involved in the development and effector functions of Th1 cells that drive cellular mediated immunity (CMI) responses (Castro and Tafalla, 2015). The production and activity of IFN- $\gamma$  is regulated by different molecules which are critical for the effects mediated by this cytokine; after infection, antigen-presenting cells (APCs) secrete IL-12, IL-8 and IL-1 $\beta$  which are the main promoters of IFN- $\gamma$  synthesis (Pereiro *et al.* 2019). In the present study, fish fed the synbiotic diet exhibited significantly increased expression of IFN- $\gamma$  compared to the control and prebiotic fed fish after 8 and 14 weeks, while the expression of IL-12 was significantly elevated in the synbiotic fed fish following 14 weeks of feeding in comparison to the control and prebiotic fed fish also. These results further infer the stimulation of the Th1 immune repertoire in responses to the dietary additives.

#### *5.4.3.3 Expression in the GIALT: mucous- and TJ-associated genes*

The pattern and distribution of mucins in human tissues is well known (Pérez-Sánchez *et al.*, 2013). At present, gene expression studies concerned with fish mucins remain scarce yet information is beginning to accumulate concerning the regulatory effect of nutrition and pathogen exposure. In humans, Muc2 is a secreted gel forming mucin found primarily in the gastrointestinal and bronchial tissues (Kim, 2012). Similarly, in fish Muc2 has been described as a prominent mucin within the gastrointestinal tract of Gilthead sea bream (Pérez-Sánchez *et al.* 2013), Atlantic salmon (Sveen *et al.*, 2017), common carp (van der Marel *et al.*, 2012),

and zebrafish (Jevtov *et al.*, 2015). Sveen *et al* (2017) recently proposed that Muc2 expression is inducible in the gills: following an experiment in which the transcription of Muc2 was significantly increased in the gills of Atlantic salmon in response to handling stress, the authors further suggested a role for Muc2 during stress-induced responses in the gill. Furthermore, human-MUC2 expression was observed to be significantly upregulated in the lungs in response to *Pseudomonas aeruginosa* LPS in both the surface epithelium and submucosal glands of human bronchial explants (Li *et al.*, 1997) and in response to Gram-positive and Gram-negative bacteria (Dohrman *et al.*, 1998). In the present study, fish fed both the prebiotic and synbiotic diet exhibited significantly increased expression of Muc2-like, compared to the control-fed fish, following 14 weeks of feeding. However, in contrast, no significant differences were observed in the expression of Muc2-like in the GIALT of common carp fed a  $\beta$ -glucan supplemented diet, compared to those fed the control diet (van der Marel, 2012). As discussed previously, dosage may be an important factor influencing the regulatory ability of dietary additives. Unfortunately, the  $\beta$ -glucan inclusion levels were not disclosed for the aforementioned study, yet it would appear that the inclusion levels of the present study are sufficient for the induction of Muc2 in the gills. Alternatively, these differing results may be due to a species-specific response.

Although the exact roles remain to be fully elucidated, human-MUC5ac is a major gel forming mucin of the airway and thus believed to contribute to both the defensive barrier function and the rheology of airway mucous, while it has been further suggested that MUC5ac expression is inducible during airway inflammation (Evans *et al.*, 2009; Kim, 2012). Previous investigations carried out in humans and various animal models have reported the stimulated gene expression of several mucins as a result of the influence of short-chain fatty acids

(Gaudier *et al.*, 2004; Burger-van Paassen *et al.*, 2009), certain probiotics (Dykstra *et al.*, 2011), glucans (Smith *et al.*, 2011), and food-derived peptides (Martínez-Maqueda *et al.*, 2012). In contrast, administration of phytochemicals such as resveratrol (Lee *et al.*, 2012) and quercetin (Li *et al.*, 2012) were observed to down regulate the expression of Muc5ac in rats and MUC5ac in humans, respectively. In fish, Muc5ac is primarily described in the skin and gill. A recent study conducted on Atlantic salmon observed the significantly increased transcription of Muc5ac in the gills in response to handling stress (Sveen *et al.*, 2017). Additionally, significant differential regulation of Muc5 genes was observed in the gill of Atlantic salmon suffering from Amoebic Gill Disease caused by the parasite *Neoparamoeba perurans*: the authors propose that these results indicate a key role for Muc5-type mucins in the respiratory organs of fish during parasitic infection (Marcos-López *et al.*, 2018). In the present study, fish fed both the synbiotic and prebiotic experimental diets demonstrated significantly increased expression of Muc5ac following 14 weeks of feeding in comparison to the control-fed fish. As discussed for Muc2 above, these results may suggest a role for Muc5ac during stress-induced responses in the GIALT, aiding in the defence of the epithelial barrier and in maintaining epithelial integrity.

In light of the tremendous molecular plasticity of the vertebrate TJ complex, it is becoming increasingly more apparent that the maintenance of a seemingly static adult gill and skin barrier may involve intricate and highly specific molecular adjustments of the TJ complex under a range of environmental and physiological conditions (Gauberg *et al.*, 2017). To date, very few functional studies have been conducted into a large number of the claudins found in teleost fishes, and even fewer have investigated the potential of feed additives to influence their transcriptional expression. As one of the major TJ components, claudins define paracellular

permeability to small ions by sealing the paracellular space and by forming ion channels that provide a selective pathway for the passage of ions and water molecules (Samanta *et al.*, 2018). Despite the lack of definitive knowledge on claudin function and description in Atlantic salmon tissues, the results of the present investigation in which a significant elevation in the expression of claudins 15 and 6 were observed in the GIALT of fish fed the synbiotic and prebiotic diet after 14 weeks, would suggest not only the potential of the dietary additives to influence claudin expression but also the important and potentially beneficial contributions of these specific claudins to the TJ complex in the GIALT. Furthermore, fish fed the synbiotic diet also exhibited significant increases in the expression of claudins 7, 10e, 12, 25a, 27a, and 28a in the GIALT, compared to fish fed both the control and B-Wyse diet. This may be indicative of the synergistic effect of the synbiotic treatment, providing further stimulation of claudin gene expression beyond that of the individual dietary products. It is presumed that highly expressed claudins are likely to act as important regulators of gill permeability (Chasiotis *et al.*, 2012), while alterations in the expression of proteins from the paracellular junction complex may result in an opening of the mucosa for pathogens, toxins and ions and subsequently affect homeostasis (Adamek *et al.*, 2013). For example, the down-regulation of certain claudins has been observed prior to psoriatic lesion development in mammals, while the infection of Rhinovirus reportedly down-regulates claudin 1 mRNA expression in human nasal epithelial cells (Watson *et al.*, 2007; Yeo and Jang, 2009). It would therefore appear reasonable to consider the up regulation of so many claudins in the gill in response to the synbiotic diet as being beneficial to the host.

The plgR is an essential component of the mucosal immune system in teleosts, mediating the trans-epithelial transport of immunoglobulin molecules into the mucosal layer (Yu *et al.*,

2018a). In the GIALT of fish fed the synbiotic diet, significant increases in the expression of pIgR were observed in comparison to the expression levels observed in the control-fed fish. In previous studies, IgT and IgM expression in the nasal mucous of rainbow trout was significantly upregulated in response to bath infection with the parasite *Ichthyophthirius multifiliis* (Yu *et al.*, 2018b), while the expression of pIgR was significantly upregulated in the skin and gills of the dojo loach (*Misgurnus anguillicaudatus*) following bacterial challenge with *Aeromonas hydrophila* (Yu *et al.*, 2018a). Increased pIgR expression would serve to benefit the fish by way of increased transport of immunoglobulins into the mucous to defend the host from pathogenic insults. This would be especially advantageous in open sea cage culture where pathogens are ubiquitous within the environment, and also especially significant for the gills which are considered major portals of entry for various pathogens (Foey and Picchiatti, 2014). These results represent an advantage of dietary synbiotic administration: enhancement of the mucosal barrier defences.

#### 5.4.3.4 Expression in the GIALT: immune-related genes

Significantly elevated expression levels of COX-2 were observed in the GIALT of fish fed both the prebiotic and synbiotic diet in comparison to the control-fed fish. This is in accordance with the increased expression of mucin and TJ-related genes also observed in the GIALT as COX-2-induced PG production is known to stimulate mucin secretion and TJ closure, as discussed previously (Enss *et al.*, 1995; Blikslager *et al.*, 1997). The additional significant upregulation of IL-1 $\beta$  and MMP-9 expression in the GIALT of fish fed the synbiotic treatment could be indicative of a synergistic response, stimulating a more intense inflammatory response within the gills of the fish in comparison to those fed the control and prebiotic

treatments. This induced inflammatory response will aid in the protection of the fish by being “primed” for future pathogenic insults.

The interaction of IL-1 $\beta$  with its heterodimeric receptor (IL-1R1) is pivotal to downstream signalling and dictates the outcome of the cellular responses: IL-1R1 may be induced by high concentrations of IL-1 $\beta$  and is suggested to be a potential negative regulator to dampen excessive IL-1 $\beta$  effects (Foey and Picchiatti, 2014). IL-10 is also an anti-inflammatory cytokine, acting as a suppressor and exerting a conserved role in dampening inflammatory responses, while TGF- $\beta$  is reportedly involved in the regulation of the immune response, cellular proliferation and differentiation, and tissue remodelling and wound healing (Holm *et al.*, 2015). The significantly elevated expression of IL-10, TGF- $\beta$  and IL-1R1 in the synbiotic fed fish compared to the control suggest toward activation of the anti-inflammatory response. IL-10 has been proposed as an important factor maintaining the balance between an intense and rapid immune response against invading pathogens and the regulation of potential pathological injury (Rawling *et al.*, 2019).

A number of studies in teleosts have also focussed on the function of TGF- $\beta$  in driving Th17 differentiation in combination with other cytokines, such as IL-6. Responses to parasites are often described in terms of the Th1/Th2 dichotomy, however a novel effector subset known as Th17 has been described in teleosts (Skugor *et al.*, 2008). In mammals, Th1, Th2 and Th17 reciprocally regulate the development and function of each other, while regulatory T-cell subset (T<sub>reg</sub>) suppress all three subsets: the regulatory cytokines control inflammation in an effort to prevent immunopathology but in doing so reduce the effectiveness of immune mechanisms responsible for the expulsion of the parasites, such as those associated with Th2 responses (Skugor *et al.*, 2008). Cells of the subset Th17 produce both IL-17 and IL-22 that

induce pro-inflammatory cytokine production by epithelial cells, therefore affecting the epithelial cell turnover and mucosal barrier integrity (Foey and Picchiatti, 2014). In the present study, significant upregulations in the expression of IL-17a were observed in the GALT of fish fed both the synbiotic and prebiotic treatment, compared to those fed the control diet. This increased expression of IL-17a would therefore infer enhanced barrier integrity of the gill epithelium. Moreover, recent research in humans and mice have proposed the role of IL-17 in muco-cutaneous immunity against *Candida albicans*, an opportunistic yeast pathogen, frequently found within the human microflora, supporting the proposal that the Th17 subset play a role in antifungal immunity in mammals. (Sparber and LeibundGut-Landmann, 2015). If this is also the case for piscine Th17 cells, within the context of the present study, it is possible that induced upregulation of IL-17a is a result of the recognition and interaction of host PRRs with the fungal PRRs presented by the yeast components of the dietary supplement within the experimental diets. This dietary interaction would prove most beneficial for the host fish resulting in the enhancement of the mucosal defences and barrier integrity.

Expressed by multiple cell types, including those of Th1, Th2 and Th17 polarisation, TNF $\alpha$  is a critical cytokine that induces cell survival, apoptosis, and necrosis and contributes to both physiological and pathological processes: acting to potentiate the ongoing immune response by increasing the transcription of critical Th1 or Th2 cytokines, TNF $\alpha$  may have proinflammatory or immunosuppressive effects depending on the context, duration of exposure and disease state (Wang and Secombes, 2013). In addition to TNF- $\alpha$ , INF $\gamma$ , IL-12 and the transcription factor T-bet are all involved in the development and effector functions of the Th1 cells that drive CMI responses to intracellular-resident pathogens (Foey and Picchiatti,

2014). In the present study, fish fed the synbiotic diet exhibited a significant upregulation of INF $\gamma$ , while fish fed both the prebiotic and synbiotic diets demonstrated significantly increased expression of IL-12 and TNF $\alpha$ , in comparison to the control-fed fish. This increased expression in response to the dietary additives would stimulate the immune defences of the fish for a rapid and potent immune response in the event of an intracellular invasion.

As discussed in previous chapters, IL4/13a are signature cytokines produced by Th2 cells that mediate immunity against multicellular pathogens. The Th2 cytokine-induced responses are mediated by signal transducer and activator of transcription (Stat)-6 and the master regulator GATA3 (Takizawa *et al.*, 2011; Wang and Secombes, 2013). The binding of IL4/13a to their receptors results in the phosphorylation of Stat6, which dimerizes, translocates to the nucleus and induces GATA3 expression, thereby creating a positive feedback loop to maintain IL4/13a production in Th2 cells (Wang, Johansson, *et al.* 2016). In the present study, the significant upregulation in the expression of IL4/13a, Stat6 and GATA3 in the gills of fish fed diets supplemented with B-Wyse + Bactocell<sup>®</sup> compared to fish fed both the control and B-Wyse-supplemented diets is indicative of a synergistic Th2 response to the dietary additives. Previously, the expression of IL4/13a and GATA3 have been observed to be highly expressed in the skin and gills of Atlantic salmon, supporting the hypothesis that these tissues are “Th2-skewed” (Takizawa *et al.*, 2011). Furthermore, Th2 cytokines in mammals are known to stimulate goblet cell hyperplasia and overexpression of Muc5ac and or Muc5B, while distinct patterns of Muc5 and IL4/13a expression have been observed during infections of Amoebic Gill Disease (Marcos-López *et al.* 2018). The result of this study would also indicate a skewedness of the GIALT to Th2 responses considering the increased expression of the Th2



associated genes discussed here and the significantly elevated expression of Muc5ac-like and Muc2-like in the gill discussed previously.

### 5.5 Conclusions

In the present study, a 14-week trial was conducted in order to assess the effect of prebiotic and synbiotic dietary inclusion on the health of Atlantic salmon reared in open sea pens: an environment more indicative of those found in commercial aquaculture practices than those of laboratory-based investigations. Although no significant differences in growth indices were observed between the different treatments for either pellet size administered, it can be reasonably assumed that the nutritional availability and rearing conditions experienced by the fish were optimal and conducive to growth, as indicated by the SGR and FCR values, the high survival rates and daily water quality measurements. These results are supported by those of the previous chapters, and of previous studies (Torrecillas *et al.*, 2018; Jami *et al.*, 2019).

Significant increases in mucous quantity were also observed in fish fed the prebiotic and synbiotic-supplemented diets in comparison to those fed the control treatment. Due to the location of the trial, a latent sea lice infection was present during the investigation, the prevalence of which was observed to be significantly reduced on fish fed the synbiotic-supplemented diets after 14 weeks, compared to the numbers observed on the control-fed fish. These results differ to those of chapter four (Leclercq *et al.*, 2020) and stand in contrast to those of a previous investigations in which prebiotic-fed Atlantic salmon exhibited a significant reduction on sea lice reared in open sea pens (Dimitroglou *et al.*, 2011).

Within the scientific literature, evidence is accumulating on the transcriptional regulation of certain genes in the SALT, GALT and GIALT of teleosts in response to nutrition, pathogens and stress. The results presented here support the proposition of an interconnectivity between teleost MALT tissues, resulting in common or shared protection at the mucosal barriers (Ángeles-Esteban 2012; Salinas, 2015). In mammals, this concept is largely discredited due to the apparent compartmentalisation of the MALT tissues, the extent of which is not considered to be as pronounced in fish (Salinas, 2015). Significant increases in the expression of mucous-associated and TJ-related genes were observed in response to both experimental diets in both MALT tissues. The expression of certain claudins further highlighted the advantages of MF-q-PCR analysis, as some claudins, (such as claudins 12, 15 and 30), previously thought to be tissue-specific, exhibited significantly increased expression beyond levels observed in the control-fed fish in alternative tissues. Concerning the immune-related genes, significant elevations in the expression of genes relating to the inflammatory response (COX2 and MMP9), Th2 polarisation (Arg2, IL4/13a, GATA3), the Th17 subset (IL-17a) and many others, especially within the GIALT, were observed in the mucosal tissues of fish fed the prebiotic and synbiotic treatments, in comparison to the control-fed fish. The results of the Microfluidic q-PCR analysis presented in this chapter demonstrate the transcriptional regulation of a number of genes in response to dietary-supplement administration in both the GIALT and SALT of Atlantic salmon.

## General discussion

The mucosal immune system of vertebrates has been described as one of the most sophisticated examples of evolution found in nature (Gomez *et al.*, 2013). Consisting of both bioactive compounds and physical barriers, our knowledge of the complex nature of mucosal protection in fish is only just beginning to be elucidated (Sveen *et al.*, 2017). Comprising three *in vivo* feeding trials, the body of research presented within this thesis investigated the influence of a range of dietary additives upon the health and growth of salmonids, with the aim of contributing to the growing body of research focussing on the underlying mechanisms governing the mucosal barrier defences of teleost mucosal-associated lymphoid tissues. Accumulating evidence to date advocates the potential of feed additives, such as probiotics, prebiotics, parabiotics and synbiotics, to positively influence the integrity of the barrier defences of the GALT, SALT and GIALT in both mammals and fish (for review see Caipang and Lazado, 2015; Peatman and Beck, 2015; Peterson 2015; Trushenski 2015). Via the regulation of the associated TJs and the mucosal layer, these observations support the proposition of an interconnectivity between teleost MALTs that are considered to exhibit a lesser degree of compartmentalisation than mammalian counterparts (Ángeles-Esteban, 2012; Salinas 2015). Observations of growth performance, epidermal mucous quality and quantity, and histological appraisal of the MALT tissues with particular focus upon the mucous-producing goblet cells were conducted, in addition to the gene expression analysis of a range of mucosal-associated and immune-related targets. Collectively, this research provides additional insight into the response of the mucosal barrier defences of teleost MALTs to dietary additives.

The first experiment (chapter three), supplemented a prebiotic (B-Wyse), and a parabiotic (TB), alone and in combination, into a nutritionally appropriate and optimally designed basal

diet. Following 8 weeks of administration to rainbow trout, no observable significant differences were detected in individual body weight, SGR or FCR between fish fed the experimental and control regimes. Although these results differed to those of previous studies (Ji *et al.*, 2017; Khodadadi *et al.*, 2018; Jami *et al.*, 2019), the overall performance of the fish was excellent. In addition to the optimal rearing conditions experienced by the fish and the administration of high quality diets that induce good animal performance, there appeared to be little scope for the investigated dietary additives to induce any positive benefits upon the growth metrics.

Epidermal mucous observations revealed no significant influence of the dietary additives upon the quality or quantity of mucosal secretions. Concerning histological appraisal of the MALT tissues, goblet cell area fraction (CAF) proved to be the most informative and representative measure, taking the area of the cell into consideration within the context of the epidermal tissue. Significant increases in CAF were observed in the SALT of fish fed all three supplemented regimes compared to the control fed fish, supporting the proposal that dietary inclusion of prebiotics, parabiotics and a combination of the two, may act in a stimulatory capacity upon goblet cell morphology within the skin tissue of rainbow trout. This results stands in contrast to those reported previously on European seabass (Torrecillas *et al.*, 2015). However, this may prove to be a function of species-specific physiology rather than efficacy of the functional additives, considering that both the number of goblet cells and the chemical composition of the mucous they produce has been reported to vary greatly among fish genera (Peterson, 2015). Further demonstration of the influence of the dietary additive supplementation upon the mucosal defences of rainbow trout was concluded from the observations of significantly increased Muc17-like transcription in the GIALT, SALT and GALT, in addition to the significantly elevated expression of calreticulin in the SALT. Furthermore, a

positive influence upon the mucosal epithelial integrity was observed via the significantly increased expression of claudins 10d and 10e in the GALT, and claudin 30 in the SALT.

The transcriptional regulation of a selection of immune-relevant genes was also investigated within the MALT tissues of rainbow trout fed the differing experimental regimes by use of real time q-PCR. In the GALT, significant elevations in the expression of the pro-inflammatory cytokine IL-1 $\beta$  after 5 weeks were observed in fish fed the prebiotic, parabiatic and synbiotic fed fish compared to fish fed the control diet. Additionally, fish fed the parabiatic and prebiotic treatments demonstrated significantly increased expression of TGF- $\beta$  after 5 weeks, while fish fed all three of the supplemented diets exhibited significantly increased expression levels of TGF- $\beta$  compared to the control-fed fish after 8 weeks. Taken together these results would suggest stimulation of the inflammatory responses of the fish as a result of continued exposure to the supplemented dietary regimes. Furthermore, considering the immunosuppressive potential of TGF- $\beta$  and its de-limiting effect on IL-1 $\beta$ , the absence of significant differences in the expression of IL-1 $\beta$  after 8 weeks may be attributable to the significant increases in the expression of TGF- $\beta$ , suggesting a degree of immunomodulation is also occurring within the MALT in order to prevent the detrimental effects of immunopathology.

Th17 cells are considered orchestrators of the mucosal defences, secreting the pro-inflammatory cytokines IL-17 and IL-22 (Zhang *et al.*, 2013). Fish fed the prebiotic treatment demonstrated significantly increased expression of IL-17a after 5 weeks, which would be indicative of the stimulation of Th17 defences within the GALT. However in contrast, fish fed the prebiotic, parabiatic and synbiotic treatments all presented significant downregulation in the expression of IL-17a after 8 weeks which may result from the generation of tolerance

towards the supplemented dietary additives or possibly from a lack of antagonism (Gomez *et al.* 2013). It would be interesting for future studies to include a pathogen challenge in order to determine whether the increased expression of IL-17a may be maintained in response to the dietary additives and in the presence of an antagonist. Dietary stimulation of the Th2 immune responses are also suggested by the significantly increased expression of IL4/13a and the master regulator GATA3 in the GALT of fish fed the prebiotic treatment for 5 weeks. An interesting inclusion for future studies would be to include histological examination of the intestinal mucosa to assess whether the mucous-producing cells were also influenced by the dietary additive supplementation in the GALT.

Very few studies have considered the stimulation of immune-related genes by feed additives within the GALT, yet considering it is a portal of entry for several pathogens, the GALT is an immunologically active tissue. In chapter three, transcription regulation was observed in the GALT of fish fed the additive supplemented diets, further supporting the proposed interconnectivity of the MALT tissues and the efficacy of the additives at the inclusion levels tested here. After 5 weeks, significant increases in the expression of IL4/13a, GATA3 and TGF- $\beta$  were observed in fish fed the prebiotic, parabiocotic and synbiocotic treatments compared to the control-fed fish. After 8 weeks, significantly increased expression of IL-1 $\beta$  and TGF- $\beta$  were also observed in the GALT of fish fed the prebiotic, parabiocotic, and synbiocotic supplemented diets. Concerning the SALT, the expression of immune-related genes exhibited a wide level of variance within the treatment groups with very little difference in comparison to the control treatment. These results are somewhat surprising considering the close interaction of the SALT with the external environment. Conversely, these results may suggest a higher level of mucosal tolerance within the SALT as a result of the close interaction with the surrounding water and the inherent and continual bombardment by stressors and potential pathogens.

Future studies would do well to include the collection of skin samples in the immediate interim following commencement of the trial and exposure to additives.

Within the scientific literature, contrasting responses to the same dietary additives have been reported when administered to different fish species. To this end and in light of the positive influence of the additives upon the mucosal barrier defences of freshwater rainbow trout observed in chapter three, chapter four evaluated the effect of the B-Wyse product, an additional prebiotic (AgriMOS) and a synbiotic (B-Wyse + Bactocell®) on the growth indices, mucosal responses and barrier integrity of Atlantic salmon (*Salmo salar*) post-smolts. After 6 weeks, a controlled sea lice (*L. salmonis*) challenge was introduced in order to determine whether the dietary additives were able to confer additional protection on the fish in defence of the pathogen.

Concerning the growth indices, no significant differences were observed in individual body weight, SGR or K-factor between fish fed the different treatments after 6 weeks and post challenge. These results are supported by those of studies conducted previously (Torrecillas *et al.*, 2011; Ye *et al.*, 2011; Torrecillas *et al.*, 2018; Jami *et al.*, 2019). Despite the overall weak growth of the fish (<2-fold increase over the trial duration), the K-factor value for all treatments fell between 1.05-1.15 across the duration of the trial, indicating good nutritional condition. As observed in chapter three, the administration of high quality diets in addition to the excellent rearing conditions experienced by the fish, there remained little scope for the additives to exert any additional influence upon the growth indices of the Atlantic salmon.

Prior to pathogen challenge, significant increases in the quantity of epidermal mucous were observed in fish fed all of the supplemented diets, compared to the control fed-fish. Similarly, the levels of lysozyme activity within the mucous were significantly elevated in fish fed all

three of the supplemented treatments, pre-challenge and 1 week post challenge. While these results are supported by other investigations within the literature (Ye *et al.*, 2011; Sheikhzadeh *et al.*, 2012; Dawood *et al.*, 2015c; Dawood *et al.*, 2016), they stand in contrast to those of chapter three concerning the B-Wyse supplement. This may be due to the differing lengths of administration between the two trials or may be indicative of a species-specific response to the prebiotic. The thinner epidermis of Atlantic salmon compared to rainbow trout has been previously reported (Fast *et al.* 2002) which may necessitate the need for larger quantities of secreted mucous, serving to protect the thinner underlying epithelium from pathogenic invasion and environmental fluctuations. Stimulated increases in epidermal mucous quantity in response to the dietary additives would decrease the number of successful pathogenic adhesions to the epithelium, reducing the exposure levels of the epithelium to eco-toxins produced by pathogens, and the subsequent potential disruption of the barrier functions.

One week following challenge with *L. salmonis* the abundance of attached sea lice was significantly reduced across all treatments compared to the control-fed fish, while fish fed the B-Wyse and AgriMOS treatments continued to exhibit significantly reduce sea lice abundances 3 weeks post challenge in comparison to the control-fed fish. The results are in accordance with those concerning mucous quantity and lysozyme activity observed within the present study and in addition to the previous postulation that the quality and quantity of epidermal mucous may be an influencing factor determining the extent of sea lice settlement (Dimitroglou *et al.*, 2011). Further, the continued significant decrease in sea lice abundance after 3 weeks may be an indication of a long-term benefit conferred upon the fish via the continued administration of the prebiotic products; further studies should consider a longer challenge experiment in order to determine whether this level of protection is maintained



beyond three weeks. As the mucous layer provides the initial interface between attaching copepodid stages and the host fish, Jensen (2015) proposes that alterations to the properties and composition of the mucous layer through the use of functional diets provides a promising route for improving control of this parasite.

Histological examinations conducted in chapter four revealed the potential of the dietary additives to influence goblet cell proliferation and physiology; significantly increased goblet cell abundances were observed in the GIALT, SALT and GALT of fish fed all three of the supplemented regimes prior to challenge, in addition to the significantly increased CAF in the SALT and GALT. These results are in accordance with those of the mucous quantity of the SALT and of studies in different species, yet continue to stand in contrast to those of the chapter three concerning the B-Wyse treatment. At 1 and 3 weeks post challenge, goblet cell abundance remained statistically elevated across treatments within the GIALT and GALT, in addition to the CAF in the GALT across all treatments.

In the SALT 1 week post challenge, no significant differences were observed in goblet cell abundance between fish fed any of the dietary treatments. This may be an indication of immunomodulation exerted upon the fish via the bioactive compounds that sea lice reportedly secrete: trypsin-like serine proteases, Prostaglandin E<sub>2</sub> and Cathepsin L have all been proposed to play important roles in the establishment of the sea lice to the host via immunoevasive and immunoregulatory mechanisms (Fast *et al.*, 2004; McCarthy *et al.*, 2012; Hamilton *et al.*, 2018). The activity of these compounds may explain the observations in the SALT 1 week post challenge when the lice were observed to be at the chalimus stage of development. At three weeks post challenge, significant increases in CAF were observed in the SALT of fish fed the supplemented regimes compared to the control-fed fish, while no

significant differences were observed in goblet cell abundance. These differences did not result from influence of the dietary additives upon the size morphology of the goblet cells but rather due to a significant reduction in the CAF of goblet cells between 1 and 3 weeks post challenge within the SALT of fish fed the control treatment. It may therefore be reasonable to propose that, in addition to their suppressive abilities of the humoral defences, these compounds produced by chalimus-stage sea lice are also able to orchestrate a negative effect upon the mucosal defences of fish, of which the dietary supplements investigated here have shown some success at counteracting.

In terms of intestinal health, significant increases in the fold length between fish fed the control and supplemented treatments were consistently observed prior to challenge and, 1 week and 3 weeks following challenge. These results are somewhat contentious as increased fold length is usually associated with increased growth of the fish, which was not observed over the course of the trial. These results support the proposition that the overall weak growth of the fish is a result of repetitive interventions necessary to monitor the pathogen challenge, which may have imposed a certain level of stress upon the fish throughout the trial, inhibiting their growth potential.

Results of this research also revealed the regulatory influence of the dietary additives upon mucin and TJ-associated gene transcription. Three weeks following challenge, significantly increased expression of Muc5ac-like, claudin 6 and claudin 12 were observed in the SALT of fish administered the synbiotic treatment, yet a significant downregulation in the expression of Muc5ac-like and claudins 27a, 28a and 28b were observed in the GIALT of fish fed all three of the supplemented diets compared to the control. This may be indicative of an energy-saving response as proposed by Sveen *et al.* (2017) following observations of decreased

claudin transcription in Atlantic salmon in response to acute handling stress. It has been further proposed that the observed differential expression patterns may demonstrate a differential coping mechanism, serving to balance energy demands in the different tissues (Sveen *et al.*, 2017): the dietary additives may serve to exacerbate this stress response by stimulating a more rapid conservation of energy.

In the GALT in particular, evidence of Th1 and Th2 cascade stimulation was observed in fish fed all three of the supplemented treatments as demonstrated by the significantly elevated expression of TGF- $\beta$ , IL4/13a and GATA3, compared to the control-fed fish. In the GALT and SALT however, transcription modulation in response to the dietary additives was low, with large variations in expression observed within treatments. These results were somewhat surprising, especially in comparison to the gene expression results of chapter three. However, considering the genes chosen for analysis here, it proved difficult to find alternative studies that were relevant and comparable. Evidently, drawing comparisons between the results of different investigations is challenging as a large number of factors and variables may differ between studies, including those of an environmental, biological and nutritional nature, all of which may have an impact upon the observed results. Furthermore, when evaluating the influence of dietary additives upon the expression of immune-related genes, the analysis of so few target genes makes drawing definitive conclusions rather challenging. Recent advances in technology have resulted in the emergence of microfluidic qPCR (MF-q-PCR) as an attractive high-throughput targeted gene expression alternative to microarray and RNA-seq technologies: a single chip run allows for the simultaneous screening of 96 arrays against 96 samples. MF-q-PCR was employed in chapter five with the aim of providing a more holistic view of the alterations and changes in expression occurring in the various MALTs in response

to dietary additives, and to provide additional insights concerning interactions between dietary additives and the immune system of the fish.

Building on the favourable results of chapter four, the B-Wyse and B-Wyse+ Bactocell® additives were additionally investigated in chapter five. To the authors knowledge, very few studies have been conducted to evaluate the effect of dietary additives upon the growth, mucosal barrier defences and immune stimulation of Atlantic salmon in a realistic commercial environment such as the one conducted in chapter five in open sea pens. As in the previous chapters, no significant differences in individual body weight, FCR or SGR were observed between the different dietary regimes. The nutritional availability and rearing conditions experienced by the fish were adequate for the survival of the fish during the experimental period yet little opportunity for the improvement of the growth indices was afforded by the nutritional supplements. These results are additionally supported by those of previous studies concerning the dietary supplementation of prebiotics (Ye *et al.*, 2011; Jami *et al.*, 2019) and synbiotics (Torrecillas *et al.*, 2018; Jami *et al.*, 2019) on growth performance.

Epidermal mucous quantities were significantly increased in fish fed the supplemented experimental diets, compared to the control-fed fish following 8 weeks of feeding, which is suggestive of a dietary-induced response. At the mucosal barriers, upon recognition of PAMPs and MAMPs, Toll like receptor (TLR) and NOD-like receptor (NLR) signalling pathways are activated, causing alterations in the expression of mucins (van der Marel, 2012). The increased mucous secretion observed in the present study and those of the previous chapters suggest the activation of the signalling cascades in response to the dietary prebiotic and synbiotic supplementation.

Due to the nature of the open sea pens, a latent sea lice infection was present for the duration of the trial. At 8 weeks, the sea lice infection was very mild with very few fish presenting with sea lice. However at 14 weeks, the intensity had increased to an average of 1.0-1.2 lice per infected fish. No significant differences were observed in sea lice abundance between the control-fed fish and those fed any of the supplemented diets after 8 or 14 weeks of feeding. However, a significant difference was observed between the prebiotic and synbiotic treatments; the synbiotic-fed fish presented with significantly fewer sea lice than those fed the prebiotic diet. Additionally, after 14 weeks, the synbiotic-fed fish exhibited a significantly lower prevalence than the control fed fish, while no significant differences were observed between the supplemented treatments. These results differ to those of chapter four in which fish fed the prebiotic and synbiotic treatments demonstrated significantly reduced sea lice abundances 1 week following challenge, compared to the control-fed fish and those of previous studies in which dietary additives significantly reduced sea lice loads (Dimitroglou *et al.*, 2011; Torrecillas *et al.*, 2011; Rodriguez-Estrada *et al.*, 2013). A noticeable and perhaps important difference between the sea lice infection of this study and that of chapter four is the intensity of the infection. In the open sea cage trial, the infection was very mild with infected fish carrying an average of 2 sea lice, while in chapter four individual fish were infected with an average of 23 sea lice each. It is possible that the at such low infection levels, the Atlantic salmon in chapter five are tolerating the sea lice loads instead of mounting an energy-demanding immune response to remove the parasite(s). During intense infections such as those observed in chapter four, the detrimental impact of so many lice will almost certainly be beyond tolerable levels, necessitating the activation of defence mechanisms.

Furthermore, the gene expression results of chapter five revealed a modulating effect of B-Wyse and B-Wyse + Bactocell® dietary inclusion on mucin-related and TJ-associated gene,

significantly effecting their expression in the GIALT and SALT, and further underscoring the interconnectivity of the MALT tissues. While mucin expression has been previously demonstrated to vary between fish species and MALT tissues within the scientific literature (Pérez-Sánchez *et al.* 2013, Sveen *et al.* 2017), the differences in supplement-induced mucin expression between those of chapter five and others in chapter four may be a result of the intensity of the sea lice infection experienced by the fish. In chapter five, Muc5ac expression was significantly increased in the GIALT of fish fed the prebiotic and synbiotic treatments after 14 weeks in comparison to the control-fed fish, however in chapter four, 3 weeks post challenge, Mu5ac-like was significantly downregulated in the GIALT of fish fed the same dietary additives at the same dosage as in chapter five. This may be a further indication of the immunosuppressive activity of the sea lice upon the host, favouring their establishment on the epithelia.

By employing MF-q-PCR in chapter five, 59 target genes of interest were simultaneously analysed in three tissues: SALT at 8 weeks, SALT at 14 weeks and GIALT at 14 weeks, returning a very large amount of data. The transcriptional regulation of a large number of genes in both the GIALT and SALT in response to the dietary additives was evident from the results. Both the prebiotic and the synbiotic investigated demonstrated a profound modulatory ability, influencing the expression of a large number of genes associated with the mucosal barrier protection properties in the SALT and GIALT of Atlantic salmon. The results concerning claudin expression are of particular interest and serve to highlight the advantages of MF-q-PCR technology, allowing for the simultaneous analysis of a large variety of claudins and TJ-associated genes. The significant upregulation of so many claudin genes in the gill indicates their importance in maintaining the integrity of the epithelial layer as the downregulation of claudin genes in MALT tissues has been demonstrated to result in an opening of the mucosa

for pathogens, toxins and ions and, as a result, also affect homeostasis (Adamek *et al.*, 2013). These results may further indicate the highly specific role claudins have been reported to play in terms of functionality within different tissues.

Future work on claudins employing immunohistochemistry techniques would be interesting and assist in the confirmation of gene expression results currently being reported within the field. The further use and development of this technique would add to our knowledge concerning the tissue specificity of certain claudins in salmonids and would provide information concerning the interaction of claudins to form specialised tight junction complexes, to which gene expression analysis do not lend.

The differential expression of a variety of immune related genes including those involved in the inflammatory response, Th2 polarisation and the Th17 subset further supports the concept of an interconnectivity between teleost MALT tissues, leading towards a degree of common or shared protection at the mucosal barriers. As can be inferred from the results of chapter five, the dietary additives investigated here may be considered health promoting, significantly bolstering the immune defences of the fish beyond those of control-fed fish.

However, given certain discrepancies between the results of chapter four and five, it would appear prudent for future work to consider corroborating lab based trials with subsequent *in situ* investigations that are more reflective of commercial aquaculture practices and the environmental stressors experienced by the fish. Additionally, considering the encouraging results of the sea lice challenge in chapter four, future work would do well to consider a continuation of the trial duration beyond three weeks following challenge to determine the longevity of the protection conferred upon fish fed the B-Wyse and AgriMOS products, by way of significantly reduced sea lice counts. Furthermore, controlled pathogen challenges

may consider the use of alternative species of sea lice, such as *Caligus spp.* either alone or in conjunction with *L. salmonis* as were experienced by fish in chapter 5.

Functional feed supplement investigations remain a vital and interesting area of research, one that has presented the potential to improve the health of fish and, by extension, the sustainability of the aquaculture industry. However, more research is still required, particularly concerning the influence of dietary additives on the mucosal-associated lymphoid tissues.

## 6.2 Conclusions

The research present within this thesis provides novel information concerning the effect of certain dietary additives on the health of rainbow trout and Atlantic salmon. Investigations of chapter three and chapter four revealed the potential of dietary additives to influence the MALT tissues, directly via the quantity and quality of secreted epidermal mucous, and indirectly by influencing the abundance and morphology of goblet cells. Results of chapters four and five further demonstrated the health promoting benefits of the dietary additives as evidenced by the significant decreases in sea lice burdens. Perhaps most importantly, the use of Microfluidic qPCR technology in chapter five provided a holistic view of the extent of transcription modulation occurring within the MALTs in response to the dietary additives. Collectively, the research of this thesis has demonstrated the interconnectivity of the MALTs within salmonids and revealed the regulatory ability of certain dietary additives to enhance the mucosal barrier defences of the SALT, GIALT and GALT. The progressive expansion of this area of research is vital for the continued advancement of the aquaculture industry.



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## Appendix

**Table 5.2** Atlantic salmon primer pair sequences, annealing temperature (in °C), amplicon size (bp), and primer efficiency (E-value) designed for the gill and skin, for use on the micro-fluidic dynamic array at INRA.

Gene Name	Accession Number	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Annealing Temp	Amplicon size	E-value	
						Gill	Skin
β-actin	NM_001123525.1	ACGGCATCGTCACCAACTG	CTCCTCTGGTGCCACTCTCA	60	83	2.05	1.97
Ef1α	NM_001141909	GGCTGATTGTGCTGTGCTTAT	CACGAGTCTGCCCGTTCTTT	60	80	2.05	2.15
IL-10	EF165028.1	ACGAAGGCATTCTACACCACTT	CACCGTGTGCGAGGTAGAACT	60	83	2.10	2.10
TGF – β	EU082211	AAGGACCTGGGCTGGAAATG	CCTGGGAGTACTTGTCTCTGT	60	106	2.05	2.05
IL-1R1	XM_014173634.1	GGGTCAGGACGTAGGGTTTC	CACATCGCTACTCCAAGAATGA	60	111	1.97	1.93
IL-1β	AY617117	AGGAGGGAAGCAGGGTTCA	CATCAGGACCCAGCACTTGT	60	81	2.01	2.05
COX2	AY848944	TGTTGAGGAAGCGGTTTATTCC	TGAAGAACTGGTGGGTGAAGT	58	87	2.05	1.90
MMP13	NM_001123522.1	TGAGCCTGAACAAACCCTCAT	TGTAACCATTGAGAGCCCACAT	60	134	2.05	1.97
MMP9	NM_001140457.1	AGAAGGTGGAGGGAGCAATG	TCCACATCCAGCCTCCAGTA	60	82	1.97	1.97
IL-17α	KJ921972	CCACCAACAGCAGCCAGTA	CCTGGTGGCTGAAGTGTAGA	60	80	2.05	2.10
MyD88	NM_001136545.1	GGAGCTGTTTGACGCCTTCA	TGTGGTCCGTCTGCTCTAAC	60	89	2.05	2.10
Mucin 5ac-like	XM_014181327.1	TCCGCAACTGGTCATGTTGTA	GCGGCTGGAGAGACTATC	60	83	2.05	2.10

## Appendix

Table 5.2 continued (1)

Gene Name	Accession Number	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Annealing Temp	Amplicon size	E-value	
						Gill	Skin
Mucin 5b-like	XM_014175874.1	CGACTCAACGTGGATGTAGGA	GCGACCACTAGCCAGAAAGA	60	81	2.01	2.01
Muc 2-like	XM_029770456.1	CGACTCAACGTGGATGTAGGA	GCGACCACTAGCCAGAAAGA	60	81	2.10	2.10
Calreticulin	BT058985	AGGCAAGAACCACCTCATCAA	GTGCCTGACTCCACCTTCTC	60	132	1.97	2.05
Calreticulin- like	XM_014139671.1	ACAAGGGCAAGAATCACCTCAT	TCTGGTTTGGGTTTCAGGATCAG	60	93	2.21	2.05
Occludin #1	XM_014143411.1	CCAGGGCATCTTCATCAACCA	GCAGGATGATCAGACCCACAA	60	110	1.90	2.01
Occludin #2	XM_014143412	CCAGGGCATCTTCATCAACCA	GCAGGATGATCAGACCCACAA	60	110	1.93	2.05
Claudin 10e	BK006391.1	TTCTGTCACCAACTGCCAAGA	ACAGACAGAGCACCCATCAG	60	93	2.01	2.01
Claudin 12	BK006392	TGGCTACTCTGGCTGCTACT	GCAGAACTGGAGCAGTCTCA	60	82	2.01	1.97
Claudin 27a	BK006400	CCAGATGCAGTGTAAGGTCTAC	AGGAAGATGCCGACGATACC	60	111	2.01	2.01
Claudin 30	BK006405.1	GCCTTCATCGGCGAGAACAT	ACCTGTACTCTGGACCACACA	60	81	1.97	2.01
Claudin 15	XM_014206890.1	GTCGGGATGCAGTGTTCTAAAG	TGGGTGATGTTGAAAGCATAACC	60	140	2.01	2.01
Claudin 25b	BK006399.1	TGCTCTGCTGTTCTGTAAGA	ACACATGTTCTGCCCTGTAGAT	60	111	1.93	1.93

## Appendix

Table 5.2 continued (2)

Gene Name	Accession Number	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Annealing Temp	Amplicon size	E-value	
						Gill	Skin
Claudin 28a	BK006401.1	CTGGCTATCATCGGGTTCCT	ACGATGTTGGCTCCGATGAA	60	86	2.10	2.05
Claudin 28b	BK006402.1	GCCATCGCTAGTGGAGTCAT	AGAAGTCTCGGATGATGGTGT	60	91	1.86	2.15
Claudin 6	BK006386	TCATCGTCATCAGCATGGTACT	GGCTCAGGCAGTTGGTACAC	60	81	2.05	2.05
Claudin 7	BK006387	ACTATTGCCTGCTCGTGGTA	AGGCAATGAAGATGGCTGAAC	60	106	2.05	1.83
pIgR	GQ892057.1	ACAGCCTCCCTTCACATCAA	CCTGTATCCCTGGCTGTAGAG	60	114	1.90	2.10
MHC Class I	AF508864.2	CTGCTCGTCGTTGCTGTTGTT	GAAGTGCTGGCCGGAACAAA	58	80	1.93	1.93
MHC Class II	AM259954.1	CGTTTCCCTGACCCTGGTTT	GGAGTATCGGCACTGTCTCA	60	82	1.97	2.01
CD4+	NM_001146408.1	TGTGGTGGGTGAGGTGATCT	TCACTTCCACCCACAGCTTTA	60	114	1.90	2.05
CD8 $\alpha$	AY693393.1	GCAAGACAACGCTGGAATGG	ATCTGCTCCTCGCTGAAGAC	58	99	2.01	2.15
FoxP3	NM_001198847.1	TCCATCATCACCAGGCATCAT	GCAGCAGAGTCCGTTGAGAA	60	81	2.01	2.01
IL-4/13a	AB574339	AGCATGGCGGAACAATACAC	GGTCCCAGTCAAATGTGTAACA	58	84	2.10	2.21
Stat6	NM_001123654.1	GCCAGAAAGCACAGAGGAATC	CTTCGATCCACAGCCAGAAG	60	99	2.01	2.10

## Appendix

Table 5.2 continued (3)

Gene Name	Accession Number	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Annealing Temp	Amplicon size	E-value	
						Gill	Skin
GATA3	NM_001171800	CAGCAACAGTCGCCAACAG	CGGCTGTGAGACTCCAGTTT	60	87	2.05	2.15
GOS2	XM_014146969.1	ATCACTGACGAGCCTGATGT	TGATAGACGCCCTCCTCTGA	60	91	2.01	2.15
iNOS	XM_014214976.1	ACAGGGAAATCACAGACATTGG	CTCTCCTGTTCCATGTCACTGA	60	113	1.97	2.10
Arg2	XM_014190234	TGTGGGCAGAGCTAACAACT	TCCTCCAAGCATGATCGTAGTG	60	82	2.10	2.05
GSH-Px	NM_001146603.1	GTCCGTAGATACAGCCCTAACA	TCAGCGGCAGTCTTTGAAAT	60	80	2.01	2.10
SOD1	XM_014198383.1	GCTGACAGTGTGGCTAAGATCA	ATGGTCCTGCCGATGATAGAG	60	80	2.10	2.15
CATA	XM_014123794.1	GCCATGCTGTTCCCATCCTT	CCAGAAGTCCCACACCATGT	60	87	2.01	2.10
Prxs/ NKEF	XM_014179684.1	TGGTGGATGGACAGTTCAAAGA	AGTGAAGTCCAGCGGGTAGA	58	86	2.01	1.93
Casp3b	DQ008069.1	CCAATGACCAGACTGTGCAA	ATGGCTCAGCATCACACACA	58	101	1.97	2.05
IRAK-4	NM_001141766	TCTTTCCACGAGCTGAAGAAGA	CAAAGCCTCCCTACCAAGT	60	88	2.05	2.05
Tollip1	XM_014175875.1	TGAGAGAGGGCAGTGTGGTT	CAGGTTGATCATGCCCTCCTT	60	80	1.93	2.01
Tollip2	AM691831.1	GGCACTGTAGGACGGCTTAG	GTAAGGGTCCATGCGTGTC	60	81	2.05	2.05



## Appendix

Table 5.2 continued (4)

Gene Name	Accession Number	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Annealing Temp	Amplicon size	E-value	
						Gill	Skin
IL-12B	XM_014205516.1	ATGTGGTTACGGGAGGCAAA	CAGTGAATGCAGATCTCGACTT	60	123	1.97	2.05
INF- $\gamma$	NM_001171804.1	AGGACACGTTTGAGGACAGT	CCCGTCTGGTTCAGCATCTG	60	94	2.01	2.05
Tbet	GU979861.1	CCCTTCCTGAGTTTCAACATCA	TGGTGCTGGTCGGATAGGA	58	86	2.05	2.28
TNF $\alpha$	NM_001123617.1	GCACCGAAGACAACAAGGTTTA	GCTGAACACTGCTCCCACATA	60	131	2.01	2.05
Cathelicidin	NM_001123573.1	CTGTGGAAGAGCAGGCTTTC	CTCTGACTGGTCCACATCCT	60	83	2.10	2.01
Transferrin	NM_001123655.1	GCCGTGGGAGATGAGTACAG	GGCAACATCTCCAGCATCTTC	60	102	2.15	2.01
PCNA	XM_014161524.1	CCCTGGTGGTGGAGTACAA	TGAAGCCTCCTCGTCAATCT	60	80	2.15	2.15
HSP70	KU885451.1	TCCTGGTGAAGATGAGGGAGAT	TAGTGGCCTGTCTCTGTGAATC	60	108	2.10	2.10
Mx-1	XM_014133087.1	CCCTCAAGGTGGAAGGACATT	ATGTCCACCACCATCTCTTCT	60	81	1.97	2.10
TLR3	KP231342.1	ACCTCAAGCACCTGACTAAGA	TGGCTGGAAGGAGAAGTCATC	60	128	2.10	2.10
TLR82b	HF970588.1	CGTCCCTGGATCTGTCATACA	TGAGCCTCGTCCAGTTAAAGT	60	129	2.05	2.01