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# The impacts of wheat gluten products and short-chain fructooligosaccharides on the health and production of juvenile rainbow trout (*Oncorhynchus mykiss*)

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The impacts of wheat gluten products and short-chain  
fructooligosaccharides on the health and production of  
juvenile rainbow trout (*Oncorhynchus mykiss*)

by

Samuel W. Voller

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

Doctor of Philosophy

(September 2016)

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## Abstract:

### The impacts of wheat gluten products and short-chain fructooligosaccharides on the health and production of juvenile rainbow trout (*Oncorhynchus mykiss*)

Samuel W. Voller

Through the implementation of *in vivo* feeding trials, the efficacy of three wheat gluten (WG) products, vital (Amytex®), hydrolysed (Merripro®) and soluble hydrolysed (Solpro®) wheat gluten as replacement of soy protein concentrate, and scFOS prebiotic (Profeed®) supplementation were analysed to assess their impacts on intestinal health and production of juvenile rainbow trout.

Microbial community analysis in experiment one revealed a degree of diet based modulation with 7.5% and 15% inclusions of wheat gluten (WG) products. Bacterial species diversity was significantly reduced with 15% hydrolysed wheat gluten (HWG) inclusion compared to the plant protein control and 15% vital wheat gluten (VWG) treatments, with sequenced OTUs dominated by the phylum Firmicutes and possible promotion of probiotic species. No detrimental effects were observed on intestinal morphology. These findings led onto a longer duration feed trial with a more holistic, higher resolution approach.

Experiment two revealed modulation of the allochthonous intestinal microbiota, with increased proportions of *Enterococcus* and *Weissella* in the 10% and 20% VWG treatments. *Bacillus* and *Leuconostoc* relative abundances were significantly increased with 10% HWG and soluble hydrolysed (Sol) wheat gluten inclusions. HSP 70 transcripts were significantly down-regulated in all WG treatments compared to the basal soy protein concentrate treatment (SPC) and increased intraepithelial leukocyte counts were observed with 10% VWG inclusion. Growth performance was unaffected by 10% dietary inclusions of WG, however, FCR's were significantly improved in the 20% VWG treatment compared to the 10% HWG and Soluble treatments. This led to the investigation of increased inclusion levels of WG products in experiment three.

All WG treatments in experiment three yielded significantly improved growth performance. Somatic indices were significantly increased with 30% blended WG inclusion compared to the SPC treatment. Modulation of allochthonous intestinal microbiota was observed to a lower degree than the previous experiments, with a dose response observed with increasing blended WG inclusion.

In the final experiment two basal diets (SPC and 20% Blended) and two scFOS supplemented diets (SPC + FOS and 20% Blended + FOS) were investigated for the effect on growth performance, gut health and allochthonous microbial population. Growth performance was unaffected, however, modulation of the allochthonous microbial population was observed with an apparent synergistic effect of scFOS supplementation in WG diets. This synergistic trend was also observed in the transcription level expression of immune relevant genes. 20% WG inclusion with additional scFOS supplementation observed significant down regulation of the pro-inflammatory cytokine TNF- $\alpha$ , as well as HSP 70, CASP 3 and Glute ST compared to the 20% Blend treatment.

The present research demonstrates dietary inclusions of WG products, solely or blended, at the expense of soy protein concentrate to modulate the allochthonous microbial population, potentially promoting probiotic species, whilst reducing the levels of intestinal stress in juvenile rainbow trout. Supplementation of the prebiotic scFOS modulated the microbial populations, enhancing the proportion of potential probiotic species, and combined with WG inclusions, reduce intestinal and oxidative stress and inflammation biomarkers, with no observed deleterious effects.

## **Home Office statement**

All experimental work involving animals complied with the 1986 Animals Scientific Procedures Act, operating under Home Office project license PPL 30/2644 and personal license PIL 30/10401.

All experimental work involving animals further complied with the Plymouth University Animal Welfare and Ethical Review Committee.

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## 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.

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## **Presented Work**

### **Evaluation of dietary wheat gluten products and scFOS on gut health and growth performance of rainbow trout, *Oncorhynchus mykiss***

Voller S W., Rodiles A., Davies S J., Apper E., Merrifield D L.

Oral presentation

European Federation of Animal Science. Annual Meeting. Belfast, Ireland

September 2016

### **Evaluation of dietary wheat gluten products on gut health and growth performance of rainbow trout, *Oncorhynchus mykiss***

Voller S W., Rodiles A., Davies S J., Apper E., Merrifield D L.

Oral Presentation

World Aquaculture Society: Aquaculture America 2015. New Orleans, LA, U.S.A.

February 2015

### **The impact of wheat gluten as an alternative protein source and the prebiotic effects of short-chain fructooligosaccharides on health and production of salmonids.**

Voller S W., Davies S J., Apper E., Merrifield D L

Oral presentation

Tereos Syral. Aalst, Belgium

January 2015

### **Feed additives in salmonid aquaculture**

Voller S W.

Oral presentation

Interaction between GI tract microbe and piscine host Workshop. Chinese Academy of Agricultural Sciences. Beijing, China.

April 201

## Abbreviations

ABPs	Animal by-products
ADC	Apparent digestibility coefficients
ANFs	Anti-nutritional factors
ASAT	Aspartate aminotransferase
AU	Arbitrary units
Bp	base pairs
Casp 3	Caspase 3
CL	Crude lipid
CP	Crude protein
CT	Controlled temperature
DNA	Deoxyribonucleic acid
EF1- $\alpha$	Elongation factor 1-alpha
FCR	Feed conversion ratio
FM	Fish meal
FO	Fish oil
FOS	Fructooligosaccharide
GALT	Gut associated lymphoid tissue
GF	Germ-free
GI	Gastro intestinal
Glute ST	Glutathione S-transferase
HIS	Hepatosomatic index
Hp	Horse power
HSP 70	Heat shock protein 70
HWG	Hydrolysed wheat gluten

IL -10	Interleukin 10
IL-1 $\beta$	Interleukin 1-beta
IL-8	Interleukin 8
IP	Intraperitoneal
MOS	Mannan-oligosaccharide
NFE	Nitrogen-free extract
NSP	Non-starch polysaccharides
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PER	Protein efficiency ratio
PPC	Plant protein control
RNA	Ribonucleic acid
SCFA	Short chain fatty acids
scFOS	Short chain fructooligosaccharides
SEM	Scanning electron microscopy
SGR	Specific growth rate
SOD	Superoxide dismutase
Sol	Soluble hydrolysed wheat gluten
SPC	Soy protein control
SWG	Soluble wheat gluten
TAE	Tris-acetate-EDTA
TBE	Trisborate EDTA
TE	Tris and EDTA
TGF- $\beta$	Transforming growth factor-beta
TNF- $\alpha$	Tumour necrosis factor-alpha

Tris	Tris (hydroxymethyl) amino-methane
VWG	Vital wheat gluten
WG	Weight gain
XG	Times gravity
β-Actin	Beta-actin

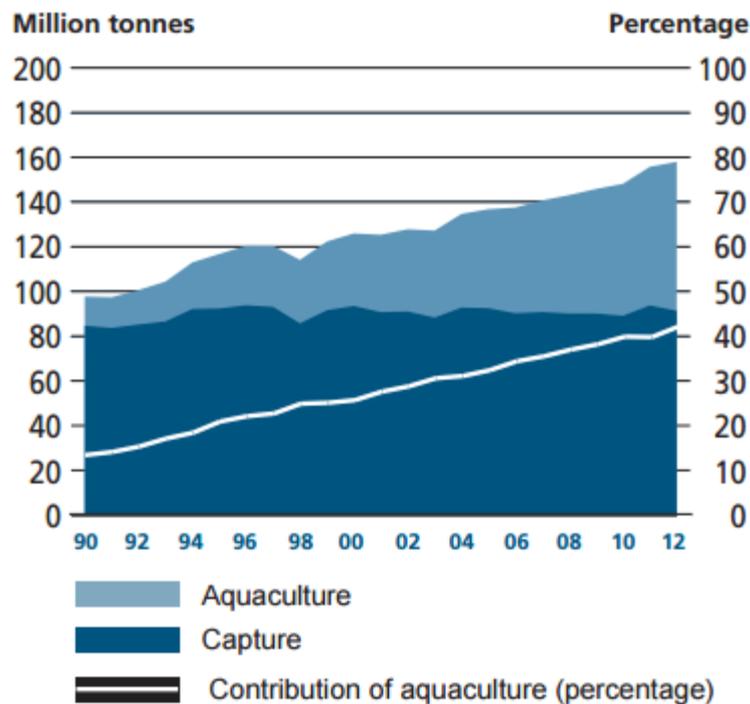
## **CHAPTER 1. Introduction**

### **1.1. Aquaculture; an overview**

Aquaculture, “the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants where some sort of intervention is made to enhance production” (FAO, 1995) is experiencing the fastest growth of any food producing sector. Annual global fish production averaged an 8.8% increase in the period from 1980 to 2010, and in 2013 expanded by 5.8% (FAO, 2012; FAO, 2014). Aquaculture production (excluding aquatic plants and algae) topped 73.78 million tonnes in 2014, estimated in value at US\$160.15 billion (FAO, 2016b). This increase in production has been seen at the same time as massive global population increase. The current global population is approx. 7.3 billion (2016), an increase in around one billion since 2003 (Nations, 2015). If current predictions are realised, the global population will reach 9.7 billion by 2050 and in turn will lead to an inevitable increase in the need and demand for high quality protein food sources over the coming years. This extra demand for protein will be compounded by the socio-economic rise in developing countries. As wealth increases the availability of new and more diverse food sources will bring an extra burden on agri-business to supply the demand. Fish forms an important source of animal protein and nutrients for a large proportion of the world's population, accounting for 16.9% of the world's animal protein intake or 6.5% of all protein consumed (FAO, 2012). The intensified farming of many aquaculture species will contribute to allowing currently seen levels of protein intake to be maintained through population growth and global demand.

Aquaculture provides jobs and income to millions of people worldwide, and with dwindling fish stocks in capture fisheries around the globe caused by years of over exploitation,

aquaculture has the scope to support the world’s fish requirements, providing an alternative means to seafood availability. In 2012, aquaculture accounted for 41% of global fishery production (Figure 1.1), with estimations of exceeding 50% by 2015.



**Figure 1.1.** Aquaculture and capture fisheries contribution to global fish production. Source: FAO (2014).

In 2014, 49.8 million tonnes of food fish was produced by the aquaculture industry. Fish production is only surpassed in the agriculture industry by the production of poultry and pigs, industries developed and honed over thousands of years.

### 1.1.1. Salmonids

The Salmonidae, comprising salmon, trout, grayling, char, and freshwater whitefish (of the subfamily - Coregoninae) are a family of ray finned, teleost fishes. These are characterised by an adipose fin, exclusively breed in fresh water, with members of the family presenting anadromous lifestyles, migrating to the sea to grow and mature before returning to fresh water rivers and streams to reproduce. Fresh water species account for 56.4% of the total

fin fish production, with diadromous fish species accounting for 6% of the industry. Despite the apparent small scale of salmonid production, the comparative high price of the end product gives salmonid aquaculture a disproportionately large share of the economic value of the industry. Finfish production from mariculture, which includes the salmon and trouts, represent only 12.6 percent of the total farmed finfish production by volume, whilst their value (US\$23.5 billion) represents 26.9% of the total value of all farmed finfish species (FAO, 2014).

Atlantic salmon (*Salmo salar*) constitute in excess of 90 percent of the global salmon culture market. Enjoyed throughout the major consumer markets of Europe, North America and Japan, production in 2014 reached 2.3 million tonnes (FAO, 2016a). Other farmed salmonid species produced (chinook salmon, *Oncorhynchus tshawytscha*, coho salmon, *Oncorhynchus kisutch*, rainbow trout/steelhead salmon, *Oncorhynchus mykiss*, brown trout/sea trout, *Salmo trutta* and Arctic char, *Salvelinus alpinus*) account for a further approx. 1 million tonnes, the majority of which is accounted for by the rainbow trout (812,939 tonnes in 2014) (FAO, 2016a; FAO, 2014).

The Atlantic salmon has long been a highly valued sport fish. The evolution of Atlantic salmon farming from the Victorian era of cultivation of eggs and juveniles for the restocking and enhancement of rivers for increased wild returns for anglers in the face of declining populations, to the full life cycle intensive aquaculture systems we see today, salmon farming is one of the greatest developments in the aquaculture industry. The production from 1 tonne in 1964 to 2.3 million tonnes in 2014 has seen the growth and progression of many economies (FAO, 2016a). Atlantic salmon are farmed around the globe, with the major producers situated in Norway, Chile and Scotland (Smaller production occurs in USA, Canada,

Ireland, Iceland, Tasmania and the Faroe islands). The farming of Atlantic salmon is without doubt one of the most technologically advanced aquaculture sectors. Driven by the high commodity price of the animal, not only has the physical engineering side of production advanced with intensification of production, countless scientific studies have been carried out into all areas of salmon production. This sustained progression and level of research resulted in a genetically modified (transgenic) strain (AquAdvantage Salmon, AquaBounty, MA, USA) which has been approved by the United States Food and Drug Administration (FDA) in 2015 for human consumption (FDA, 2015), closely followed by Health Canada. A world first for farmed animals and a precedent for global livestock producers.

Rainbow trout (*Oncorhynchus mykiss*), an excellent sport fish in its own right, has never commanded the same economic value or production levels of Atlantic salmon. Originally from North America, rainbow trout have been distributed to waters on every continent (bar Antarctica) for recreational angling and aquaculture purposes since 1987 (FAO, 2016a). Although production levels are much lower than Atlantic salmon, the rainbow trout industry has also enjoyed the boom seen in the last half of the 1900's. From meagre production of 4,400 tonnes in 1950, global production peaked in 2012 to in excess of 882,000 tonnes. Rainbow trout, although a nutritious and palatable fish with similar nutritional characteristics to salmon, has been burdened with a public perception of an inferior product to salmon. This has resulted in limiting sales and demand in the retail setting. Although the market for table fish could be stronger, rainbow trout are amongst the most popular sport fish for recreational anglers. More accessible than wild salmon, rainbow trout are stocked in many fresh water bodies around the world for the sole purpose of angling. This market demands enough fish to maintain many hatcheries and farms solely

for the purpose of restocking. The restocking market poses differing requirements on producers, striving for “fin perfect” palatable fish. The quality of the animal is of high importance with blemishes, shortened opercula, fin erosion or skeletal deformities rejected by paying anglers. As well as a sport and food fish, rainbow trout have played a key role in the scientific understanding and development of the salmonid industry, being utilised as a general salmonid model. Their comparatively simple life cycle, without the need for smoltification, allows animals to be kept in research environments more easily than salmon. Although production levels are significantly lower than Atlantic salmon, it can be seen that rainbow trout play an important role in the aquaculture and research industry.

### *1.1.2. Sustainability and aquafeed*

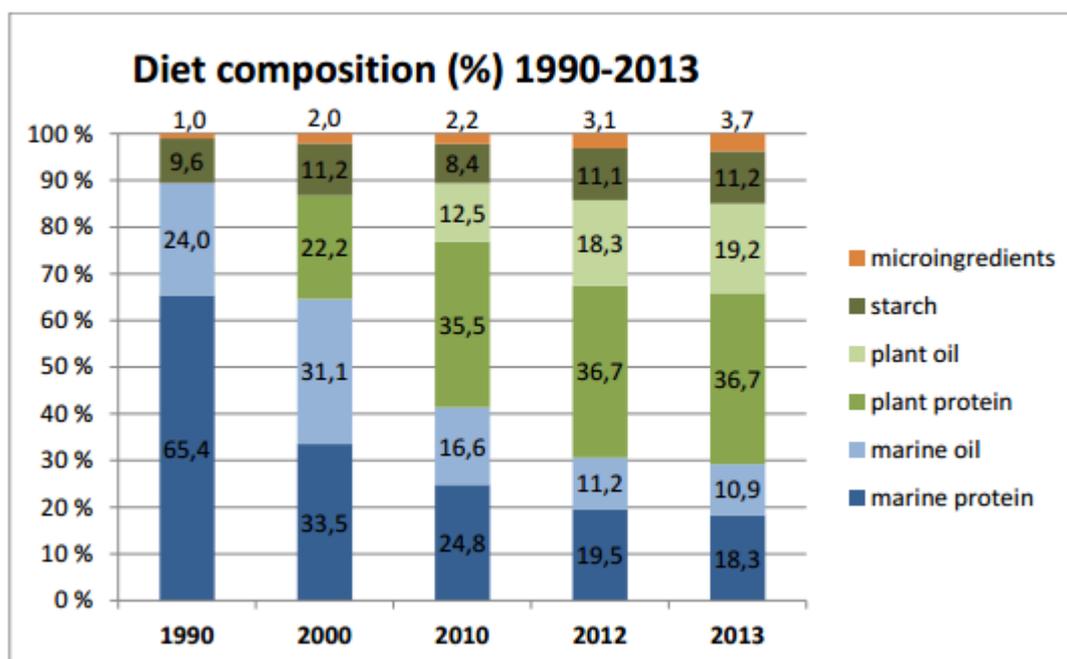
The aquaculture industry has long been under scrutiny over the sustainability of intensive production, be it disease, parasites, environmental impacts or fishmeal and fish oil inclusions in aquafeeds. Increasing the sustainability of aquafeeds is and will continue to be paramount in improving the long term sustainability and productivity of the aquaculture industry. The innately carnivorous nature of salmonids must be reflected in their feed. Salmon and trouts require energy rich diets containing high quality protein and lipid to perform optimally. This is reflected in the cost of aquafeeds for salmonids, where prices of 1000 to 1250 GBP per tonne are not uncommon for commercial grow-out diets, and can constitute 50% of operating expenses for farms (Shipton, 2013). The high quality protein and lipid components have historically been sourced from fishmeal and fish oil. Aquafeed producers have utilised fishmeal as the protein source of choice for marine fish and salmonids, due to its high protein content and exceptional amino acid profile. Fishmeal is also favourable due to its high nutrient digestibility, low antinutrient content, source of

essential fatty acids (EPA & DHA) and its previous availability and low cost (Gatlin et al., 2007; Tacon and Metian, 2008). Produced predominantly in Chile and Peru, fishmeal is produced from small bony pelagic fish species, with a lower economic value in their own right (FAO, 2015b). Typically species such anchovy (*Engraulidae* sp.), sardines (*Clupeidae* sp.) and jack mackerel (*Trachurus symmetricus*) are rendered by a process of steam cooking, pressing and milling to produce a meal. During the pressing stage the liquid fraction is removed, and further processing separates the Fish oil from the water. The fishmeal market is notoriously variable due to many external factors affecting production. Climate change, the cyclic phenomenon of El Niño, fishing quotas and natural disasters can all have a major impact on fishmeal and fish oil (FO) production (Oki and Kanae, 2006; FAO, 2015b; Tveterås and Tveterås, 2010). These variations in the market have seen prices vary greatly. In 2014 prices per tonne peaked at USD 2380, more than quadruple the price seen in April 2000 (USD 423)(Indexmundi, 2014). This huge increase in price and associated economic burden has also contributed to the effort of fishmeal and Fish oil replacement in formulations, as traditional high fishmeal based formulations are economically unsustainable. It is also becoming increasingly apparent how inherently unsustainable the fishmeal industry is in from an environmental standpoint. In the light of ever decreasing wild stocks, the removal of millions of tonnes (21.7 million tonnes in 2012 (FAO, 2014)) of small pelagic species, essentially the bottom of the food chain for many other marine species, is environmentally illogical. The industry is however improving, and the utilisation of fish remains and by-products constituted an estimated 35% of fishmeal production in 2012 (FAO, 2014; Olsen et al., 2014).

The replacement of fishmeal with alternative protein sources has enabled the expansions of aquaculture production despite the stagnated levels of fishmeal availability and elevations

in fishmeal cost. Annually approx. 6 million tonnes of fishmeal and 1 million tonnes of fish oil are produced globally. This finite resource is utilised in a vast number of applications, not just aquafeeds.

The replacement of fishmeal and fish oil in feeds for carnivorous species poses a significant challenge. To maintain growth and health parameters diets must have similar amino acid and essential fatty acid (EFA) profiles as fishmeal and fish oil. The possible use of carbohydrates in diets for salmonids is highly restricted and their over inclusion can result in the increased utilisation of fat as an energy source (Skiba-Cassy *et al.*, 2013). The inclusion levels of fishmeal and fish oil have been steadily decreasing with the progression and increased drive for sustainability of the industry. Figure 1.2 illustrates the reduction of fishmeal inclusion from 65.4% to 18.3% in Norwegian diets, the leading global producer over the course of 23 years. Fish oil also has seen massive reductions in utilisation over the same period, from 24% to 10.9%. This has only been achievable with vast research into alternative protein sources. Fishmeal inclusion in rainbow trout diets is now as low as 15% in many commercial feeds.



**Figure 1.2.** Percentage nutrient sources utilised in Norwegian aquaculture 1990 – 2013. Taken from Ytrestøyl *et al.* (2015).

Salmonid diets require high protein levels and the plant protein sources used, together with those with potential for use are coming under increasing scrutiny. Only eels require a higher fishmeal inclusion in feeds (up to 60% (Lucas, 2012)). The utilisation of plant proteins in aquafeeds is often limited by antinutritional factors (ANFs), and will be discussed in more depth in section 1.2.1. ANFs come in many different forms, some of the most important of these in aquafeeds include saponins, tannins, lectins, protease inhibitors, glucosinolates, phytates, non-starch polysaccharides, oligosaccharides, alkaloids, antigenic compounds, cyanogens and antivitamin (Francis *et al.*, 2001). Despite limiting ANFs present in plant proteins, they have become well-established as ingredients in commercial aquafeeds for both omnivorous and carnivorous species, allowing typical levels of fishmeal inclusion in salmonid feeds to be as low as 10-20%.

As aquafeeds evolve and develop there is a danger of crossing a critical physiological knife edge where boundaries in aquafeed formulation are pushed. Without rigorous research there is a danger of creating new problems which may need to be addressed.

### **1.2. Alternative protein sources**

As discussed previously, the requirement for energy rich diets with high quality protein sources is vital for salmonid aquaculture. There are many alternatives to fishmeal with the most abundantly utilised being plant derived protein sources. Alternative plant protein sources will be discussed further in section 1.2.1.

Animal by-products (ABPs) are a highly regulated source of protein. European Union (EU) regulation (Regulation (EC) No 1069/2009) categorises “entire bodies or parts of animals, products of animal origin or other products obtained from animals that are not intended for human consumption” into 3 categories. Category 1 (Obtained from animals suspected of Transmissible Spongiform Encephalopathy infections) and Category 2 (obtained from dead stock or suspected of carrying infectious disease) are classified as high risk and cannot be utilised in the feed industry. Category 3 however is obtained from animals fit for human consumption, and is classified as low risk. Meat, fat trim, viscera, blood, bones and feathers are collected from the “waste” of the slaughtering process and utilised by the rendering industry to produce ABPs. A large proportion of animal carcasses are discarded (33 to 43% by weight) during the slaughter process, which has the potential to be re-purposed (FAO, 2002). Once rendered, bone meal, meat meal, blood meal (with the exception of ruminants), feather meal and poultry by-product meal (poultry meal) are all utilised in the animal feed industry. ABPs have a well-balanced amino acid profile encompassing all the essential amino acids including those often limited in plant derived proteins (especially lysine and

methionine) (Nunes et al., 2014). Currently under EU legislation, feather meal and poultry meat meal can be utilised in commercial formulations, yet consumer pressure prevents their utilisation due to perceived safety concerns following the BSE (Bovine Spongiform Encephalopathy) outbreak during the 1990's, restricting retailers acceptance of fish fed ABPs. Changes in consumer perception could significantly increase the use of ABPs in aquafeeds.

Krill meal is a relatively new protein source, expanding through recent developments in harvesting technology. Utilised in the human health market as well as animal feeds, krill meal and krill oil are excellent sources of protein and essential fatty acids (EPA and DHA), which are present in their more available phospholipid form (Ali-Nehari et al., 2012). The amino acid profile is suitable for maintaining fish health and growth and krill has the added benefit of being a natural source of carotenoids in the form of astaxanthin, which naturally pigments the flesh of fish. Astaxanthin is often added to feeds at extra expense to feed producers to create the pink flesh of salmonids expected by consumers, especially in Europe. Harvested in vast numbers, *Euphausia superba* (Antarctic krill) currently are the most exploited resource of the 80 species which are found throughout the oceans (Nicol et al., 2012). The ecological impact of the annual removal of 200,000 tonnes of krill in recent years (post 2009) is unknown, but krill like the fish utilised in the fishmeal and fish oil industry are at the bottom of the food chain for many marine species, including the many great cetaceans. Krill does however have two possible negatives for utilisation in aquafeeds. Firstly, Chitin, the main constituent of arthropod exoskeletons is inherently indigestible, and secondly, krill are bio-accumulators of fluoride, toxic to humans in high doses. The greatest tonnage of krill is harvested from the southern ocean (Nicol et al., 2012), and is processed

into krill meal and krill oil on board. This requires large investment in factory ships. The substantial harvesting effort and the relatively small production volumes of krill meal is likely going to limit its viability in the aquafeed market only to small inclusions, if any, due to the high price it demands (USD2.75 - 3.45 per kilo, (Katevas, 2014)). It is likely krill meal and oil will remain in the human health sector primarily and only be utilised as small volume aquaculture additives, rather than large scale inclusions.

Single cell proteins (SCP) are another explored means of alternative protein sources. Those explored in an aquafeed nature are algae, yeasts, fungi and other bacterial SCPs. Derived as by-products from other industries (such as algae from bio-fuel), or specific autotrophic growth, SCPs have been investigated in diets for numerous farmed species. SCP on average produce protein levels 45-50% CP and are reported to be abundant in important vitamin, minerals and lipids, with the protein and energy requirements to grow fish (Dallaire et al., 2007; Anupama and Ravindra, 2000).

### *1.2.1. Plant protein sources*

As the drive for increased sustainability and intensification has evolved the aquaculture industry, many plant proteins have undergone intensive investigation as potential alternatives to fishmeal, in a range of species. The aim of achieving fishmeal content below 10%, without impairing growth performance or organism health received much interest. Legumes, oilseeds and cereal grains are among some of the most investigated and commercially utilised protein sources. Candidate proteins must not only provide acceptable growth and health performance, they must be economically viable, readily available and practical to process, store and transport. Further beneficial characteristics sought after include low levels of ANFs, non-soluble carbohydrates and fibre. High protein content is

crucial, as well as favourable amino acid profile, palatability and nutrient digestibility (Gatlin et al., 2007). The replacement of fishmeal with plant proteins has seen inclusions fall greatly from the 1990's until now with reliance resting on certain plant protein sources, namely soya. Soya proteins have detrimental effects at high inclusion levels (discussed further later), as well as a high ecological/carbon footprint due to the growing locations and reliance on GM crops in the USA. This has led not only to a drive for fishmeal alternatives but also for plant proteins that can be utilised in a blend of plant proteins reducing the inclusion levels and reliance on single sources.

The annual production of plant derived proteins is vast. Approx. 230 million tonnes of plant based proteins are produced annually, and are competitively priced, generally between USD500-1800 per tonne (NOAA, 2011). Although not always having protein concentrations as high as fishmeal (table 1.1), developments in ingredient processing has enabled the concentration of protein in refined products, making their inclusion in aquafeeds more viable. With their wide availability and lower price point plant proteins are an obvious source of protein to be utilised in aquafeeds. However they do have problems. The palatability of plant proteins is of concern when utilised in extremely low fishmeal and fish oil formulations, digestibility and low levels of certain essential amino acids, namely lysine and methionine (table 1.1). The low levels of these amino acids often requires supplementation with free amino acids to meet the nutritional requirements of aquaculture species when plant proteins are incorporated at high inclusion levels (Davies et al., 1997; Nunes et al., 2014).

Ingredient	Dry matter	Protein	Lipid	Lysine	Methionine	Cystine
Fishmeal, herring*	92	72	8.4	5.57	2.08	0.74
Barley†	88	14.9	2.1	0.44	0.16	0.24
Canola*	93	38	3.8	2.27	0.7	0.47
Corn*	88	8.5	3.6	0.25	0.17	0.22
Corn gluten meal*	91	60.4	1.8	1.11	1.63	1.2
Cottonseed meal*	92	41.7	1.8	1.89	0.5	0.45
Lupin - <i>Lupinus angustifolius</i> ‡ (whole)	89	39.2	10.3	1.4	0.27	0.51
Field peas‡(whole)	89	25.6	1.3	1.5	0.21	0.31
Soybean meal, de- hulled*	90	48.5	0.9	3.08	0.68	0.75
Soy protein concentrate†	90	64	3	4.2	0.9	1
Wheat*	88	12.9	1.7	0.36	0.21	0.27

**Table 1.1.** Typical compositions (as-fed) of Fishmeal and alternative plant proteins. Adapted from (Gatlin et al., 2007).

\* Data from NRC (1993), † Data from NRC (1998), ‡ Data from Allan et al. (2000)

The main limiting factor of plant proteins in aquafeed are ANFs. ANFs are naturally occurring compounds, produced by plants, characteristically originating from nutrient stores and predator defence mechanisms. Once ingested, ANFs can affect nutrient acquisition and availability and nutritional pathology (Ferrando, 1983; Francis et al., 2001; Kumar et al., 2012). There are numerous ANFs present in plant derived proteins, with important antinutrients present in commonly utilised protein sources in aquafeeds as presented in table 1.2.

**Table 1.2.** ANFs found in commonly used alternative protein sources for aquaculture.Adapted from Francis *et al.* (2001).

Plant-derived nutrient source	Antinutrients present
Soybean meal	Protease inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins, allergens
Rapeseed meal	Protease inhibitors, glucosinolates, phytic acid, tannins
Lupin seed meal	Protease inhibitors, saponins, phytoestrogens, alkaloids
Pea seed meal	Protease inhibitors, lectins, tannins, cyanogens, phytic acid, saponins, antivitamins
Alfalfa leaf meal	Protease inhibitors, saponins, phytoestrogens, antivitamins

ANFs have become widely accepted to be detrimental in aquafeeds in high concentrations, causing adverse effects on the intestinal health and digestive function of fish (van den Ingh *et al.*, 1991). ANFs are nutritionally problematic when the animals that they are fed too lack the ability to catalyse their breakdown. There have been many potential routes explored in efforts to remove ANF content in protein sources for the livestock market, in turn increasing the nutritional functionality of the protein. Genetic selection of crops with desirable phenotypes has been suggested, such as a low-phytate soy line (Jervis *et al.*, 2015). However, this is a long term solution and the advancement of GM organisms to reduce ANF's crops, although accepted in legislation, still carries a stigma with consumers. It does however carry potential for the future (Lucht, 2015). The most common form of ingredient modification prior to inclusions in agrifeeds is refinement. A common first step in the refinement process is dehulling, the process of removing non-starch polysaccharide (NSP) rich husks, which are of no nutritional value and indigestible in monogastric animals (Bowyer, 2016). Further refinement can then be carried out in order to remove or degrade ANFs to insure ensure

loss of function and therefore any detriment to consuming fish. ANF's can be categorised into two types, heat-labile and heat-stable (Greathead, 2003). Heat-stable ANFs such as NSPs, soluble fibre, carbohydrates, saponins and allergens are more difficult to remove than heat-labile ANFs, which can be destroyed or denatured by heat. Heat-labile ANF's are responsible for impairments such as gut enteritis and comprise lectins, antivitamin, phytates and trypsin inhibitors (Greathead, 2003; Francis et al., 2001). The significant increase in the production of glutes, protein concentrates and isolates which increase protein concentrations from standard cereal grains and legumes also inherently reduces many heat-stable ANFs through the removal of fractions with little to no nutritional value, such as starch.

The removal of all ANFs from plant protein sources is an unlikely proposition. Significant economic investment is demanded by each new removal technique, and adds an increased cost to the end product. The physical state of ANFs within the nutrient matrices of plants also adds additional dimensions to the challenge of removal. Saponins are often found bound to amino acids which are crucial for animal nutrition (Potter et al., 1993). The intensive effort in trying to remove such bound complexes risks denaturing the fraction that is trying to be protected. The addition of exogenous enzymes (such as amylase, pepsin and trypsin), to diets to combat ANFs within the digestive system of the animal has been shown to improve nutrient utilisation, reduce environmental impacts and reduce feeding costs, and could be a future method of further ANF mitigation (Castillo and Gatlin Iii, 2015).

### **1.3. Wheat gluten**

Wheat is the third most produced cereal worldwide, only surpassed by rice and maize. Global production of wheat reached in excess of 728 million tonnes in 2014, produced

predominantly in Asia, Europe and the Americas (FAO, 2015a). Europe is the second largest producer of wheat, producing over 237.5 million tonnes in 2014, 33% of the global production, with only Asia producing more. The use of wheat derived proteins could be considered more sustainable than many other alternative plant protein sources. The ecological footprint of wheat derived proteins utilised in European feed formulations is consequently much lower than other plant protein sources, such as soya, which is inevitably shipped from the Americas who produce 87.7% of the global volumes.

Gluten meals, high-protein co-products from the removal of starch from certain cereal grains, are highly digestible proteins, rich in glutamine which are considered to be potentially beneficial plant protein sources. Wieser (2007) simply defined gluten as “the rubbery mass that remains when wheat dough is washed to remove starch granules and water-soluble constituents”, containing 75-85% protein and 5-10% lipid (depending of the extensiveness of the washing process). The remainder consisting of starch and non-starch carbohydrates. Gluten is comprised of hundreds of polypeptides, half being monomeric gliadins, containing intrachain di-sulfide bonds, and polymeric glutenin, di-sulfide-cross linked polypeptides (Day et al., 2006). Gliadins and glutenins give gluten its distinctive properties. Gliadins (30-100 kDa) provide viscosity and extensibility, whilst glutenin (100-10,000 kDa) provides gluten's elasticity and tenacity (Apper-Bossard et al., 2013). Wheat gluten has become a commodity in its own right over the course of the past 50 years. Through the increased industrial removal of wheat starch from gluten and the associated drying technologies to retain functional properties, wheat gluten has become increasingly more economically viable as a feed ingredient. Transported around the world in its dry state (vital wheat gluten), rehydration regenerates the gliadin and glutenin functional properties

of the wheat gluten (Day et al., 2006). Not only is wheat gluten beneficial as a nutrient source, the wheat gluten's visco-elastic and cohesive properties act as a pellet binder. Extruded aquafeeds have traditionally used starches as pellet binders. Wheat gluten's binding properties negate the need for this added starch, which, without heat treatment, carnivorous species such as Atlantic salmon and rainbow trout have a limited ability to digest and utilize (Krogdahl et al., 2004). High dietary inclusions of digestible carbohydrates also inhibits the control of blood glucose concentration in salmon (Storebakken et al., 2000; Hemre et al., 1995; Krogdahl et al., 2004) and rainbow trout (Bergot, 1979; Krogdahl et al., 2004). Glycaemia regulation is also inhibited in other species including catfish, cyprinids and red sea bream when administered glucose (Wilson, 1994). This inability to digest and utilise starch forces inclusions to be minimal and prevents extensive use as a binder. Wheat gluten inclusion in extruded diets also generates greater radial expansion, specific density and peak breaking forces compared with fishmeal and soy protein concentrate based diets, whilst prolonging pellet breakdown times due to the associated insolubility of wheat gluten (Draganovic et al., 2011; Day et al., 2006).

As well as having a low carbohydrate content, detrimental in salmonid diets (Skiba-Cassy *et al.*, 2013), wheat gluten is also low in phytic acid, an ANF. Phytic acid binds to phosphorous in turn making it unavailable for utilisation by fish. Phosphorous is vital in aquafeeds due to the numerous pivotal roles it plays. Production of bones and scales, ATP and nucleic acid synthesis, membranes and hormones all require phosphorous (McDaniel et al., 2005). The phosphorous level of wheat gluten however is relatively low, yet highly available due to the absence of phytic acid. This in turn results in wheat gluten having a higher availability of phosphorous for salmonids than soy bean meal, soy protein concentrate, corn gluten meal

and even fishmeal (Sugiura et al., 1998). Phytic acid can also have the effect of limiting the bio-availability of other cations (calcium, magnesium and zinc) and protein (Storebakken et al., 1998; Denstadli et al., 2006; Fredlund et al., 2006). Phosphorous levels in diets are carefully calculated not only due to these reasons but also due to downstream effects of high effluent levels of phosphorous on the environment, including premature eutrophication of waterways.

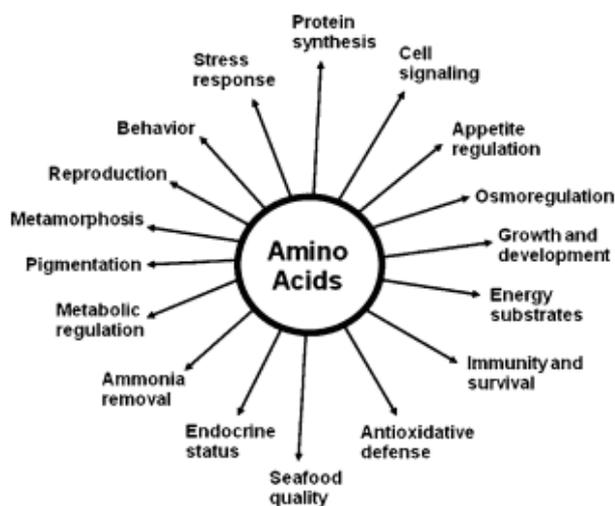
A major concern in the replacement of fishmeal in aquafeeds is dietary fibre content, which must remain low, especially in carnivorous species which have a limited ability to digest fibre. Hilton et al. (1983) demonstrated reduced growth rates and dry matter digestibility coefficients in juvenile rainbow trout fed diets of 10% fibre. Due to these effects, dietary fibre is often considered to be an ANF. These detrimental effects of dietary fibre are not exclusive to rainbow trout, they have been shown to occur in Atlantic salmon and in omnivorous cyprinids (Hossain et al., 2001; Refstie et al., 1999). Vital wheat gluten is very low in fibre, typically <1%, much lower than many other plant protein sources. As a result such effects have not been documented with wheat gluten inclusion in aquafeeds. As well as being low in fibre, wheat gluten is also extremely low in other antinutritional factors, which plague other plant protein sources and restrict levels of inclusion. Vital wheat gluten has had no association with intestinal enteritis, which is often a detrimental consequence of high plant protein inclusion, especially soybean meal, in salmonids (Bakke-McKellep et al., 2007; Refstie et al., 2000; UrÁN et al., 2008).

### *1.3.1. Amino acid profile*

The energy requirements of fish are much lower than other farmed terrestrial animals due to their ammonotelic, poikilothermic and water supported aquatic lifestyle. However, fish

require a much higher protein content in their diet to achieve maximum growth rate and these proteins/amino acids provide much of their energy needs (Kaushik and Seiliez, 2010). Alternative protein sources must not only be high in crude protein (CP), but also have a beneficial/suitable amino acid profile. Vital wheat gluten as a protein source is ~80% CP, higher than many other protein sources utilised in aquafeeds, including fishmeal (70-75% CP) and other plant proteins, soy protein concentrate (~75% CP), soy bean meal (48% CP), pea protein concentrate (~50% CP), distillers dried grains (28-32% CP) and corn gluten meal (~62% CP) (Hardy, 2010; Tibbetts et al., 2006).

The amino acid profile of wheat gluten is interesting for applications for fish but not ideal. An imbalance in the amino acid profile can lead to decreased protein utilisation and associated growth rates essential for intensive farming. Amino acids, being the building blocks for protein, are greatly associated with growth performance. Amino acids also play key roles in development and health (Figure 1).



**Figure 1.3.** Roles of amino acids in growth, development and health of fish. Taken from Li *et al.*(2009)

Wheat gluten is low in three essential amino acids, arginine, threonine and most importantly lysine. The levels of lysine, the first limiting amino acid in wheat gluten, only comprise 1.5-1.7g/100g of wheat gluten, and in turn diets with high inclusion levels (>30%) require additional supplementation with free lysine to ensure adequate lysine levels for specific species (1.9% of the diet for rainbow trout, 4.3% CP, and 1.6-1.8% of the dry diet for Atlantic salmon) (Wilson and Cowey, 1985; Walton et al., 1986; Berge et al., 1998). This addition of free amino acids in an aquafeed results in increased costs which can limit inclusions of wheat gluten to maintain economic viability. The addition of industrially produced free amino acids is not now uncommon in formulations. At the first time of production (Japan 1960's for L-Lysine) the price of synthetic amino acids was prohibitive, yet with the advances in modern biotechnology the costs have dramatically fallen. If purchased in bulk (USD1500 per metric ton, (FAO, 2004)) free amino acids can readily be supplemented in deficient formulations.

Wheat gluten is a highly digestible protein source (Davies et al., 1997), and with lysine and methionine supplementation the digestibility coefficient for protein and energy can reach 97 and 84%, respectively, when utilised as the sole protein source (45% CP) for rainbow trout. 50% CP replacement with additional lysine supplementation has been shown to achieve growth performances superior to those of a control diet containing 25% full fat soya bean meal (Gomes et al., 1995; Davies et al., 1997). It has been established there is no requirement for supplementation of threonine for rainbow trout, however, arginine, the second limiting amino acid, has generated contradicting opinions with wheat gluten inclusions (Davies et al., 1997; Pfeffer et al., 1992). Wheat gluten despite being low in some amino acids has a high proportion of others. Glutamine is one such amino acid, with

reported health benefits. Amino acids with reported benefits, be it growth performance, health or even fillet palatability are often referred to as “functional amino acids”. Glutamine is one of the most abundant free amino acids in fish plasma, and is vital for the synthesis of purine and pyrimidine nucleotides in every cell, acid-base balance and many other key roles in fish (Li et al., 2009). The reported beneficial effects of glutamine, 35 to 40% CP of wheat gluten, is well summarised by the review of Apper-Bossard et al. (2013). For all rapidly proliferating cells, glutamine is a major substrate, playing a key role in maintaining intestinal morphology. This includes the enterocyte cells that line the gastrointestinal tract and are responsible for the absorption of nutrients in the digestive system (Trichet, 2010). This has been demonstrated by various studies in various species. In juvenile channel catfish (*Ictalurus punctatus*) high level free glutamine inclusion (20-30g kg<sup>-1</sup>) significantly increased micro villi and enterocyte height and enterocyte migration rate in the posterior, mid and anterior intestine. Increased numbers of proliferating cells were also observed in the posterior intestine (Pohlenz et al., 2012a). Morphometric analysis of enteric structures were improved in both juvenile hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) fed diets with both 1 and 2% free glutamine (Cheng et al., 2012), and juvenile hybrid sturgeon (*Acipenser schrenckii*♀ × *Huso dauricus*♂) fed diets containing up to 5% wheat gluten with additional glutamine supplementation (Qiyu et al., 2011). There is additional evidence for glutamine being a substrate for immune cells, such as leukocytes, thereby aiding and modulating the immune response (Trichet, 2010; Pohlenz et al., 2012b). Not only does glutamine aid the immune system, it also has a function in providing protection from free radicals and other reactive oxygen species. Glutamine can act as a precursor in the synthesis of glutathione, a key antioxidant in animals, plants and fungi, protecting against cellular damage and nitric oxide production (Wu, 1998; Li et al., 2009). Wheat gluten also contains

glutamate, although at a lower level. Glutamate or glutamic acid, is a precursor for glutamine via ATP-dependant glutamine synthetase, as well as another key energy source for fish (Li et al., 2009).

Leucine, an amino acid known for triggering muscle protein synthesis and proteolysis inhibition in mammals, is also abundant in wheat gluten, approximately 7.9g/100g CP (Li et al., 2009). In mammals, for cell growth, autophagy and regulation of protein translation as well as activation of the mammalian target of rapamycin (mTOR) is required (Nicklin et al.). The Akt-TOR pathway is thought to play a key role in both muscle growth and cellular metabolism in fish. The pathway regulates these through gene expression and protein synthesis or degradation. The Akt signalling pathway promotes cell survival. It has been shown that the Akt-TOR-signalling pathway is unaffected by fish oil or protein replacement (Lansard et al., 2009), and that it can be stimulated by leucine (Nicklin et al.).

### **1.3.2. Growth performance**

These functional properties of wheat gluten, combined with its high digestibility and low levels of ANFs have resulted in promising applications in aquafeeds. There have been interesting results showing that dietary inclusion of wheat gluten's can maintain growth performance and health including immunological status at varying inclusion levels, both with and without lysine supplementation, for a number of commercially relevant species. The high digestibility of wheat glutes leads to increased apparent crude protein digestibility coefficients in salmonids for aquafeeds increasing in vital wheat gluten inclusion (Storebakken et al., 2000; Davies et al., 1997). Storebakken et al. (2000) reported apparent digestibility coefficients (ADC) of CP in Atlantic salmon increasing from 86.6% to 93.6% as wheat gluten inclusion in a fishmeal based diet rose from 0 to 50% respectively. ADCs for fat

and energy were also reported to be elevated in 25% wheat gluten diet, compared to the fishmeal control. These effects translate into maintained growth performance in feeds containing even high levels of wheat gluten compared to fishmeal based control formulations. Davies et al (1997) showed that with 0.58% lysine supplementation, a diet with 28.65% wheat gluten had significantly better growth performance than the fishmeal control and soy bean meal diet. Storebakken et al. (2000) reported comparable growth performance and final body weight of Atlantic salmon fed 35% vital wheat gluten without additional free amino acid supplementation compared with the reference diet, and there were no differences observed for rainbow trout when wheat gluten was included at 19.4% of a diet containing other plant protein sources (Tusche et al., 2012). Rodehutscord et al. (Rodehutscord et al., 1995) reported no influence on growth performance when 100% fishmeal was replaced by wheat gluten and crystalline amino acids. These results are not exclusive to carnivorous salmonids. Juvenile European sea bass (*Dicentrarchus labrax*) maintained growth performance, palatability, energy and nitrogen retention even with 50% fishmeal replacement (Tibaldi et al., 2011), similar results to that which had been seen in 2003 with 29% wheat gluten inclusion. However, reduced weight gain and final fish weight were observed with 41% wheat gluten inclusion by Tibaldi *et al.* (2003). In sea bream (*Sparus aurata L.*), protein and energy efficiencies were significantly increased with a decreased feed conversion ratio in a fishmeal devoid, 51% wheat gluten diet (Kissil and Lupatsch, 2004). There is no literature presenting detrimental effects of wheat gluten inclusions at commercially relevant levels for carnivorous species, generally less than 30% of a single plant protein.

### *1.3.3. Health*

The health benefits to fish, especially salmonids, of wheat gluten inclusion have not been extensively studied and what is available is often contradictory. Plant protein inclusions are considered to affect the immune response, intestinal morphology, and even the activity of enzymes operating in the gastrointestinal tract. Rainbow trout protease activity in the proximal intestine was reduced as a result of increasing plant protein replacement of fishmeal (Santigosa et al., 2008), whilst antioxidant status was improved through increased glutathione redox status in the blood and liver of gilthead sea bream (Sitjà-Bobadilla et al., 2005). Protease inhibitors are common in plant protein sources, and are another ANF which must be considered when utilising plant protein sources. Wheat gluten's functional properties may help to promote the immune system and not cause the effects seen with large inclusions of soy derived proteins. Soy-containing diets above 60% have been shown to cause reduced integrity of the enterocytes and villi, increased mucosal vacuoles and an inflammatory response in the lamina propria and a reduced immune capacity only deteriorating further with increased inclusion. Macrophage respiratory burst activity, an important part of the innate immune system, was also reported to be detrimentally affected at inclusion levels above 60% (Burrells et al., 1999). Rumsey et al. (1994) however reported increased macrophage, neutrophil and monocyte activity in high inclusion soy fed rainbow trout, although an inflammatory response or hypersensitivity may have caused these results. Data for the effect of wheat gluten on these parameters is not widely available although it has been shown in Atlantic salmon that a 35% replacement of fishmeal with vital wheat gluten did not result in pathological changes in the distal intestine observed with a 15% soy bean meal inclusion (Storebakken et al., 2000). Low level inclusion of hydrolysed wheat gluten, a 5% replacement of fishmeal, increased growth performance,

immunity and gut morphology in juvenile hybrid sturgeon (Qiyou et al., 2011; Zhu et al., 2011). These results were more attributed to an increase in dietary glutamine which was shown with inclusion levels of 1% achieving similar results. These effects have also been seen in weaning piglets, susceptible to intestinal disorders during this stage of their life, including increased microvilli length (Blasco et al., 2005).

### *1.3.4. Lipid metabolism*

Lipid metabolism is another important factor in farmed species due to its ability to affect the quality and taste of flesh and provide the essential fatty acids to consumers. The benefits of PUFA n-3 (omega 3 fatty acids) in the human diet is considered common knowledge and emphasis must be encouraged to maintain levels in fish flesh due to consumer pressure. These essential fatty acids originate from marine protein and oil sources and salmonids are efficient converters of fed omega 3 fatty acids into fatty acids in their flesh. Salmonids retain 30–75 percent of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) depending on the level of fish oil in feed (FAO, 2014). From the period of 2006 to 2015, a significant increase in terrestrial fatty acids has been observed in the flesh of Scottish farmed salmon, with decreasing levels of omega 3 fatty acids (EPA and DHA) as the utilisation of plant proteins and oils has increased in salmonid diets (Sprague et al., 2016).

It has been well established that high inclusion levels of plant proteins in aquafeeds has the effect of modulating the lipid metabolism pathways. High levels of a blend of plant proteins has been shown to reduce the plasma cholesterol levels in gilthead sea bream, European sea bass and in fillet composition of rainbow trout (Sitjà-Bobadilla et al., 2005; Kaushik et al., 2004; de Francesco et al., 2004). PUFA acid (PUFA n-3 and n-6) ratios in the muscle of European sea bass were affected by blended plant protein inclusion (increased PUFA n-6,

decrease PUFA n-3), with similar results observed in rainbow trout (Messina et al., 2013; de Francesco et al., 2004). The mechanisms of this change in lipid composition are not fully understood but is thought to be a combination of different amino acid profiles of the plant protein ingredients, increased starch content of plant protein diets and the presence of antinutritional factors such as isoflavones affecting the lipogenic enzymes (Kaushik et al., 1995). Wheat gluten based diets did not however affect postprandial plasma cholesterol levels in European sea bass and it was concluded that soy proteins played a key role in the reduction of these parameters (Robaina et al., 1999).

### **1.4. Intestinal microbiota of fish**

The gastrointestinal (GI) tract plays a key role in digestive function and the immune system (Gómez and Balcázar, 2008). The intestinal brush border and the associated endogenous and exogenous microbiota play a key role in the defence against pathogenic bacterial species (Gómez and Balcázar, 2008), have an effect on organism development and tissue physiology (Bates et al., 2006). Maintaining gut health, growth performance and disease resistance are of significant importance when considering feed ingredients.

The aquatic nature of fish exposes these animals to the ever changing surrounding environment and microbial composition of the water they live in. The GI tract is a primary site of entry for pathogens, many being opportunistic and capable of proliferation without host interaction (Moriarty, 1998; Ringø et al., 2007). Affected by external factors, such as diet, from first feeding (Ingerslev et al., 2014a) the role of a stable balanced intestinal microbiota is vast, associated with epithelial integrity, nutrient digestion and absorption, assembly of gut associated lymphoid tissue (GALT), angiogenesis and pathogen resistance (Gómez and Balcázar, 2008; Husebye et al., 1994; Rawls et al., 2004; Stappenbeck et al.,

2002). The use of germ-free (GF) animals has advanced our knowledge of the bacterial communities present within the GI tract, allowing a higher level of variable control, reproducibility and precise experimental design (Coates, 1975). Rawls *et al.* (2006) suggested a core microbiome affected by selection pressures of the hosts intestinal conditions, after observing transplanted microbiota from normal type zebrafish and mice to GF animals. Community lineages stayed similar to the donor organisms, however relative abundance of lineages changed to resemble the normal gut microbiota of the recipient animal. Investigations by Roeselers *et al.* (2011) and Wong *et al.* (Wong et al., 2013) add further weight to a proposed core microbiome in zebrafish and rainbow trout respectively. However if the conclusions of Rawls *et al.* (2006) regarding microbiota modification due to intestinal habitat change within the organism, feed formulations will be sure to affect intestinal conditions, and in turn the intestinal microbiota (Jacobs and Lupton, 1986).

### *1.4.1. Indigenous intestinal microbiota of rainbow trout.*

Currently the vast majority of our knowledge on the intestinal microbiota is obtained from farmed fish. Current farming husbandry practices such as the disinfection of eggs, treatment of pathogens with salt, formalin, chloramine T or the use of antibiotics will have undoubtedly affected the composition of their intestinal microbiota at some point in their lives. The high stocking densities and high food ration in many species will also have an effect on farmed species compared with their wild counterparts. However, the high turnover and colonisation rates of intestinal tracts, observed as 25 days for Chinook salmon (*Oncorhynchus tshawytscha*) after erythromycin administration (Moffitt and Mobin, 2006), should return gut microfloras to “normal” for their environments. The preliminary colonisation of intestinal tracts occurs in the post-hatching period of swim-up and first

feeding, where water is consumed to maintain osmotic balance and the first food particles are eaten and enter the gut (Ingerslev et al., 2014b; Olafsen, 2001; Reitan et al., 1998). Once established and with age the microbiota becomes more stable and can be differentiated into two groups; The allochthonous microbiota, present in the digesta of the animal, and the autochthonous, associated with the brush border and epithelial mucosa in the intestine and stomach and are more likely to be resident.

Numerous authors and studies have investigated the gut microbiology of rainbow trout. Now outdated culture-dependent methods have reported cultivable bacterial levels between  $8 \times 10^2 - 7 \times 10^8$  CFU g<sup>-1</sup> and variable dominant species between sample locations (Spanggaard et al., 2000). Utilising physiologic and biochemical criteria *Enterobacteriaceae*, *Vibrionaceae* and pseudomonads were identified as predominant species, with other Gram-positive and Gram-negative species present. These culture-dependent methods have now been far outclassed in coverage and resolution by culture-independent high throughput methods. The utilisation of 16S rRNA gene sequencing has enabled far greater depth in microbiome research. High throughput sequencing of the 16S rRNA gene started the movement towards “next generation sequencing” and show amazing levels of sequence depth. The most prominent investigations into the bacterial microbiota of rainbow trout utilising these techniques are by Wong *et al.*(2013) and Desai *et al.*(2012). Atlantic salmon intestinal microbiota have also been analysed utilising 16S rRNA gene sequencing by Dehler *et al.* (2016) and Gajardo *et al.* (2016).

Wong *et al.* (2013) identified 3,376 OTUs from two dietary treatments, a fishmeal based diet and a grain based diet. The authors go on to suggest a core microbiome in rainbow trout with many classes strikingly similar in abundance and diversity across the dietary treatments

and rearing conditions. Dominant classes present across all treatments were *Bacilli* (48.6% ± 9.3% of sequences per sample), *Alphaproteobacteria* (21.8% ± 5.8%), *Gammaproteobacteria* (17.1% ± 7.6%), *Betaproteobacteria* (3.8% ± 2.0%), and *Clostridia* (2.2% ± 1.3%) (Wong et al., 2013). Mansfield et al. (2010) observed dominance of *Gammaproteobacteria* and *Firmicutes* (41% and 59% of clones, respectively) in fishmeal based diets, with the dominance of *Firmicutes* increasing to 97% in soybean based diets. This trend was also observed by Desai et al. (2012), who also noted the dominant phyla to be the *Firmicutes* (197 OTUs identified) and *Proteobacteria* (609 OTUs identified). Both Desai et al. (2012) and Wong et al. (2013) observed many of the similar genera in the intestines of rainbow trout. Both sets of experimental fish contained the genera *Weissella*, *Aeromonas*, *Enterococcus*, *Lactococcus*, *Pseudomonas*, *Leuconostoc*, *Escherichia/Shigella*, *Streptococcus*, *Bacillus*, *Sphingomonas*, *Veillonella*, *Acinetobacter*, *Chryseobacterium*, *Pantoea*, *Acidovorax*, *Klebsiella* and *Citrobacter* despite experimental animals being completely independent and unrelated. These observed genera have also been identified in the GI tract of wild rainbow trout (*Aeromonas*, *Acinetobacter*, *Enterococcus*, *Pseudomonas*, *Escherichia* and *Streptococcus*) (Trust and Sparrow, 1974). Grain based diets in the Wong et al. (2013) investigation enriched *Lactobacillus* and *Streptococcus* genera content in the intestine tract.

The high resolution of high throughput methodologies has allowed massive improvements in the analysis of the microbiome and changes within it. The so called “core” microbiome outlined by Wong et al. (2013) cannot be fully accepted. Despite numerous genera being present in both studies by Wong et al. (2013) and Desai et al. (2012), no species or genera were present in all replicates across both studies (Wong et al., 2013). The change in microbial populations of fishmeal based diets over time was observed by Desai et al. (2012),

decreasing in richness and diversity showing how variable and changing the microbiome can be. This, teamed with the lack of consistency of genera between two independent investigations, also contributes weight to an argument that microbiomes adapt to the environment their host lives in, yet when established can maintain remarkable consistency within an aquaculture facility (Wong et al., 2013). It should be noted that bacteria are not the only constituent of the intestinal tract microflora. Yeasts, viruses and Archaea are also found in the intestine of fishes. Yeasts, a much larger cell than bacterial cells, can surpass bacteria with the greater proportion of cultivable microbiota in extreme environments, such as the deep sea (Ohwada et al., 1980) and occur in farmed rainbow trout at levels observed at  $3.0 \times 10^3$  and  $0.5 \times 10^2$  cells per gram of intestinal tissue for white and red-pigmented yeasts respectively (Andlid et al., 1995).

### *1.4.2. Intestinal microbiota and nutrition*

The microbiota of the GI tract is associated with a range of nutritional functions, from nutrient utilisation and digestion to the production of short chain fatty acids (SCFAs), vitamins and minerals and amino acids (Nayak, 2010). The primary mechanism of bacterial aid in the digestion of feed nutrients is that of enzyme excretion. In an extensive review by Ray *et al.* (2012), amylase, cellulase, lipase, proteases, chitinase and phytase are all reportedly produced by GI microbiota, aiding in the digestive process. The bacterial species and genera responsible for enzyme production are also extensively reviewed revealing contributions from *Enterobacter*, *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Pseudomonas*, *Flavobacterium*, *Bacillus*, *Pseudoalteromonas*, *Micrococcus*, *Brochothrix*, *Staphylococcus*, *Agrobacterium*, *Brevibacterium*, *Rhodococcus*, *Photobacterium* and yeasts amongst others. The production of digestion aiding enzymes has been observed in

numerous species, farmed and wild, from Indian major carps (*Catla catla*) (Mukherjee and Ghosh, 2016) to coral reef species (Smriga et al., 2010). The contribution of these enzyme-producing bacteria however is highlighted as an unknown, due to the different intestinal morphology between species (e.g., presence of stomachs, pyloric caeca and intestinal length) and complexity of the intestinal microbiota, however the modulation of the intestinal microflora to enrich bacterial species producing specific valuable enzymes, such as cellulase for carnivorous species could have beneficial consequences. Carnivorous species inherently have difficulties digesting carbohydrates, and the enhancement of beneficial bacterial species producing specific enzymes could increase nutrient digestibility and in turn growth performance when utilising plant protein sources in aquafeeds.

Another area of bacterial enhancement in the GI tract is the production of short chain fatty acids (SCFAs). Predominantly researched in herbivorous and omnivorous species (Kihara and Sakata, 1997; Mountfort et al., 2002; Kihara and Sakata, 2002; Clements et al., 1994), little information is available for production in carnivorous teleosts. Produced via the fermentation of carbohydrates in the alimentary canal, the primary end products formed are acetate, propionate and butyrate (Wong et al., 2006) and are comparable in teleosts to carnivorous and herbivorous mammals and herbivorous reptiles (Bugaut, 1987; Mountfort et al., 2002), an interesting observation due to fish's ectothermic nature. SCFAs are readily absorbed and utilised heavily by the GI tract enterocytes (predominantly butyrate) as energy sources as well being utilised by peripheral tissue (acetate) and the liver (propionate) (Wong et al., 2006). Levels of production are mediated by the microflora present in the GI tract and their abundance. SCFAs not only play a nutritional role in the GI tract, their presence is suggested to protect against GI disorder development and in humans

has been shown to lower cholesterol and inhibit certain cancers and cardiovascular disease (Wong et al., 2006). High SCFA concentrations in the intestine may also decrease the intestinal pH in turn making the GI tract more inhospitable to opportunistic pathogenic species.

SCFAs levels observed in the hind gut of temperate marine herbivorous fishes (*Kyphosussydneyanus*, *Odaxpullus*, and *Aplodactylusarctidens*) showed the most abundant SCFA in the GI tract to be acetate followed by propionate and butyrate. Both investigations by Mountfort *et al.*, (2002) and Clements *et al.*(2007) utilised the same marine herbivorous species and observed high presence of polymer degrading Clostridia bacteria in the hind gut, most likely responsible for the degradation/fermentation of polysaccharides to SCFAs.

### 1.4.3. Intestinal microbiota and immunity

The aquatic nature of fish exposes the intestinal tract to a vast variety of pathogenic and non-pathogenic organisms that must be recognised and reacted to. The GI tract and associated microbiota is often the first line of defence to pathogens entering the animal through ingestion. The GI microbiota of fish play a key role in both immunity and barrier function. The immune system of teleosts can be categorised into two systems, the innate and the adaptive. The gut associated lymphoid tissue (GALT) of the intestine must utilise both immunological and non-immunological defences to prevent pathogenic invasion whilst tolerating the normal gut microbiota.

The innate immune system plays a proportionally larger role in immunity in fish than observed in terrestrial mammalian species (Ellis, 2001; Whyte, 2007). The innate immune system is classified as non-specific and as such responds to a vast array of pathogenic occurrences, but has no ability to acquire memory. The adaptive immune system has the

ability to acquire a memory, however due to the poikilothermic nature of fish this is often slow to respond to pathogenic insults. As such, the adaptive immune system of salmonids may take as long as 4-6 weeks to produce antibodies whilst the innate immune defences can respond in a matter of hours or days (Ellis, 2001).

Once a pathogen gains entry to the GI tract it will encounter the first lines of defence. In finfish this is the physical intestinal barrier, the indigenous microbiota and mucus. The intestinal microbiota combat pathogenic species in two main ways; competitive exclusion of pathogenic cells competing for binding sites, nutrients and energy and through the production of bacteriocins (ribosomally synthesised anti-microbial peptides (AMPs) which have the ability to kill or inhibit other bacterial strains (Yang et al., 2014)). Goblet cells, distributed throughout the enterocyte cells of the GI tract, produce mucus that is vital to the digestive barrier, trapping and translocating pathogens before excretion. The commensal bacteria have been highlighted by Deplancke and Gaskins (2001) to be able to effect mucin gene expression and bioactive compound release and in turn the localised mucus layer. The intestinal microflora and the mucus trapping pathogens and containing other secretory factors, capable of pathogen protection, make the mucus lining of the GI tract an important part in the first line of defence against bacterial pathogens.

The gut microbiota can also affect the inflammation and chemotaxis of immune relevant cells in the intestinal tissue. Through the expression of microbe associated molecular patterns (MAMPs) recognised by pattern recognition receptors (PRRs), signalling cascades can be activated. Toll-like receptors (TLRs) are one of the most well documented PRR's in fish, initialising cascades resulting in the translation of cytokine, defensin and chemokine proteins. The MAMPs recognised by TLRs can be categorised into two groups, PAMPs

(pathogen associated molecular patterns) and CAMPs (commensal-associated molecular patterns) and will result in different outcomes. Cytokine expression is often analysed as a function of physiological and immunological competence (Fast et al., 2007), with those commonly studied in fish include Interleukins 1 $\beta$ , 8 and 10 as well as tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ). Further information on inflammatory pathways can be found in an extensive review by Foey & Picchiatti (2014).

Rawls et al. (2004) also observed the microbial effect on innate immunity genes. GF zebra fish genes coding for complement component 3, glutathione peroxidase, serum amyloid A1, C-reactive protein, and myeloperoxidase were all down-regulated in GF fish compared to conventional fish.

### **1.5. Prebiotics**

Prebiotics, described as 'A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon (Ringø et al., 2010) and are an area of increasing interest in aquaculture research. In humans prebiotics escape digestion in the stomach and small intestine and are fermented in the large intestine and colon into mainly lactate and SCFAs, including propionate, acetate and butyrate by bacteria such as *Bacillus* sp. and *Lactobacillus* sp. (Bornet et al., 2002). Butyrate is an essential energy source for the epithelial cells lining the mammalian colon (and in fish), and aid against autophagy. Increasing energy supply for these cells may help promote cell proliferation and defence against disease (Donohoe et al., 2011; Lupton, 2004). As well as increased energy availability, modulation of specific microflora to promote characteristics is another mode of action associated with prebiotics. It has been shown that modulation of the intestinal microflora in chickens through a 0.75%

inclusion level of fructooligosaccharide (FOS) reduced the level of *Salmonella tryphimurium* present in the ceca post challenge, reducing their susceptibility of colonisation (Bailey et al., 1991). This effect was attributed to the promotion of bacterial species in the intestine that could competitively exclude the pathogen from binding sites, reducing their risk of infection. The small inclusion of prebiotics may also have applications in aquafeeds to aid the proliferation of probiotics fed to fish species, and an alternative method to increase the level of beneficial commensal bacteria in the intestine, without attempting to add live bacteria to feed which can complicate feed preparation and handling (Merrifield et al., 2010).

There are many potential prebiotics utilised in a huge array of commercially important species. Reviewed extensively by Ringø *et al.* (2010), specific prebiotics of note are Inulin, fructooligosaccharides (FOS), mannanoligosaccharides (MOS), galactooligosaccharides (GOS), arabinoxyloligosaccharides (AXOS) (Ringø et al., 2010) and importantly for this thesis short chain Fructooligosaccharides (scFOS).

### **1.6. short-chain fructo-oligosaccharides**

Fructooligosaccharides naturally occur in a number of edible plants such as onions, wheat and asparagus. They are produced commercially in one of two ways, either from sucrose, utilising fungal fructosyltransferase, or the partial hydrolysis of inulin (Bornet et al., 2002). Described simply, FOS are inulin-type oligosaccharide, consisting of  $\beta$ -(2-1) linkages of D-fructose, with a terminal D-glucosyl (Yun, 1996). Derived from sucrose, FOS is usually represented by  $GF_n$ , with  $n = 1-5$  in short-chain fructo-oligosaccharides, as linear fructose oligomers polymerise (Bornet et al., 2002). There is some evidence, limited in salmonids, that scFOS can act as a prebiotic and provide health and growth benefits when

supplemented in diets at very low inclusion levels, typically 0.1-1% commercially. The utilisations and observations of FOS and scFOS in investigations is presented in table 1.3.

**Table 1.3.** Investigations and observations of the utilisation of FOS and scFOS in aquatic species. Adapted from Ringø et al. (2010).

Prebiotic	Dose	Fish species	Fish weight (g)	Results	References
FOS	10 g kg <sup>-1</sup>	Atlantic salmon	200 ± 0.6	→ Feed intake, growth and digestibility.	Grisdale-Helland et al. (2008)
FOS	5 and 10 g kg <sup>-1</sup>	Rainbow trout	150	↑ weight gain, Ca + energy content ↓ carcass crude protein	Ortiz et al. (2013)
FOS	10, 20 and 30 g kg <sup>-1</sup>	Caspian roach ( <i>Rutilus rutilus</i> )	0.67 ± 0.03	↑ Ig levels, lysozyme activity, ACH50, digestive enzyme activities, growth performance	Soleimani et al. (2012)
FOS	0, 2 and 6 g kg <sup>-1</sup>	Hybrid tilapia (Oreochromis niloticus ♀ × Oreochromis aureus ♂)	57	→ Growth rate ↑ Survival ↑ Non-specific immunity.	He et al. (2003)
FOS	20 g kg <sup>-1</sup>	Turbot larvae ( <i>Scophthalmus maximus</i> )	N/A	↑ Growth rate Effects on gut microbiota (Bacillus and Vibrio).	Mahious et al. (2005)
FOS	0, 1.5 and 2.5 g kg <sup>-1</sup>	Soft-shell turtle ( <i>Triortyx sinensis</i> )	N/A	↑ Growth rate at 0.25% inclusion ↑ SOD activity at 0.25% inclusion ↓ Lysozyme activity.	Ji et al. (2004)
FOS	10 g Kg <sup>-1</sup>	Red drum ( <i>Sciaenops ocellatus</i> )	7	→ Growth rate ↓ neutrophil oxidative radical production ↑ serum lysozyme	Zhou et al (2010)
scFOS	0.8 or 1.2 g kg <sup>-1</sup>	Hybrid tilapia	5.6 ± 0.02	↑ Growth rate, feed intake, feed conversion → Survival and condition factor ↑ <i>Vibrioparahemolyticus</i> , <i>Aeromonashydrophila</i> , <i>Lactobacillus spp.</i> , <i>Streptococcus faecalis</i> .	Lv et al. (2007)
scFOS	1 g kg <sup>-1</sup>	Hybrid tilapia	1.24 ± 0.01	↑ Uncultured bacterium clones and <i>Thiothrix eikelboomii</i> .	Zhou et al. (2009)

scFOS	0.25, 0.5, 0.75, 1, 2, 4 and 8 g kg <sup>-1</sup>	White shrimp ( <i>Litopenaeus vannamei</i> )	75.4 ± 0.8	→ Weight gain, feed conversion and survival scFOS affected gut microbiota.	Li <i>et al.</i> (2007)
scFOS	0, 0.4, 0.8, 1.2 and 1.6 g kg <sup>-1</sup>	White shrimp	0.17	↑ Growth rate, feed intake, feed conversion scFOS affected gut microbiota.	Zhou <i>et al.</i> (2007)
scFOS	5, 10 and 20 g kg <sup>-1</sup>	Turbot	31.6 ± 0.02	→carcass composition, growth performance ↓Malic enzyme activity	Guerreiro <i>et al.</i> (2015a)
scFOS	5, 10 and 20 g kg <sup>-1</sup>	Turbot	31.6 ± 0.02	→ Innate immunology and haematology	Guerreiro <i>et al.</i> (2014)
scFOS	10g kg <sup>-1</sup>	European sea bass ( <i>Dicentrarchus labrax</i> )	60 ± 0.01	→Growth performance, ↑Glucokinase /glycolytic activity.	Guerreiro <i>et al.</i> (2015c)
scFOS	10, 25, 50 g kg <sup>-1</sup>	gilthead sea bream ( <i>Sparus aurata</i> )	32 ± 0.01	↑ Growth rate, liver glycogen, plasma glucose ↓plasma cholesterol, ASAT activities.	Guerreiro <i>et al.</i> (2015b)

SOD = superoxide dismutase, ASAT = aspartate aminotransferase. symbols represent an increase (↑), decrease (↓) or no effect (→) on the specified response.

### 1.6.1. Growth performance

The only literature for the use of FOS as a prebiotic in salmonids reported no beneficial effect on feed intake, survivability, digestibility or growth performance in on-growing Atlantic salmon, post 16 week feed trial (Grisdale-Helland et al., 2008). Feed efficiency however was improved in the diets containing FOS (1%), yet carcass proximate composition analysis and apparent nutrient digestibility were unaffected. Promising results have been seen regarding growth performance in turbot larvae (2% FOS) (Mahious et al., 2006), and the modulation of the autochthonous bacterial microflora, specific growth rate (SGR), feed conversion ratio (FCR) and daily feed intake of hybrid tilapia post 8 week feeding trial of diets containing 1% scFOS. Hepatopancreasomatic index was also reduced (Zhou et al., 2009; Lv et al., 2007). The stimulated bacterial growth however were not the traditional bacteria associated with beneficial effects such as *Bacillus* sp. and *Lactobacillus* sp., but non-traditional soil and water associated bacteria and uncultured species. Little information is available on the effects of scFOS on the growth performance of salmonids.

### 1.6.3. Health and immunology

Relatively few studies have investigated prebiotic effects on the modulation of the immune system in salmonids (Sealey et al., 2007; Staykov et al., 2007; Rodriguez-Estrada et al., 2013). The only immunological parameters Grisdale-Helland *et al.* (2008) investigated post 16 week trial was neutrophil oxidative radical production and serum lysozyme activity. Both parameters were unaffected by FOS inclusion. Similar results have been seen in red drum (*Sciaenops ocellatus*) post 8 week feeding trial. Reduced neutrophil oxidative radical production, utilised in the respiratory burst process by neutrophils and monocytes was reduced, a key aspect of the non-specific immune system. Lysozyme serum activity however

significantly increased. Lysozyme which acts on peptidoglycans in the cell wall of gram positive bacteria is another key component of the innate immune system. As well as these effects, increased microvilli height was observed in the pyloric caeca and the proximal and mid intestine post FOS supplementation, showing FOS's ability to modulate the intestinal morphology in certain instances (Zhou et al., 2010). Guerreiro *et al.* (2015c) observed increased nutrient utilisation. Increased enzyme activity and glucose and lipid metabolism was seen with 1% scFOS inclusions in juvenile European sea bass. The available information on the use of scFOS as a prebiotic in salmonids is lacking, the investigations in this thesis will hopefully add a body of work to the effects of scFOS on the health and performance of rainbow trout.

### **1.7. Conclusions**

As the aquaculture industry grows annually, research must continue into alternatives enabling the volume of aquafeed produced to increase, with the finite fishmeal available and without the dependence on a sole alternative protein source. Identification of plant protein products offering additional health benefits and fewer antinutritional factors is of high priority for the continued drive for sustainability in aquafeeds. Producing feeds that grow fish rapidly with low feed conversion ratios is no longer sufficient. It is vital we are aware of how nutrition integrates with disease status of fish, stress physiology, and the potential challenge of fish from pathogens from the external environment. Insuring inclusion levels shown to be detrimental to species are not exceed, and protein sources with functional characteristics beneficial to the health and physiology of fish are incorporated will allow aquafeed to achieve the needs of the industry. It is vital research continues in this field, and novel ingredients and additives be explored fully for a variety of species. The

modulation of the innate immune response and intestinal microbiota whilst improving the gut morphology, antioxidant state and reducing the inclusion levels of other detrimental plant proteins is a real possibility for wheat glutens and scFOS in aquafeeds.

### **1.8 Thesis objectives and aims**

The aim of this research is to assess the potential role of wheat glutens and scFOS in aquafeeds for the salmonid industry via the analysis of gut health and growth performance. Inclusion rates, mechanisms of action, and novel insights into effects on the intestinal microbiota and health status of the intestine will be assessed through a series of three feeding trials.

**Preliminary trial.** Initial assessment of the impact of wheat glutens on the intestinal microbiota and gross intestinal structure of juvenile rainbow trout.

**Trial 1.** Assessing the effects of three wheat gluten sources, as replacement of soy protein concentrate, on the intestinal microbiota, health and localised immune and stress response of juvenile rainbow trout.

**Trial 2.** Assessing commercially relevant blended wheat glutens and inclusion levels on the growth performance, condition, and intestinal health of juvenile rainbow trout, when included at the expense of soy protein concentrate.

**Trial 3.** Assessing the effect of scFOS supplementation on the intestinal microbiota, localised immune and stress response and SCFA production in soya based and wheat gluten inclusion diets.

## **CHAPTER 2. General methodologies**

### **2.1. Overview**

Methods specific to individual trials, including feed formulations, can be found in their relevant experimental chapters. Unless otherwise indicated, all methodologies were carried out at the University of Plymouth, UK, under the approval of the institutional Animal Ethics Committee.

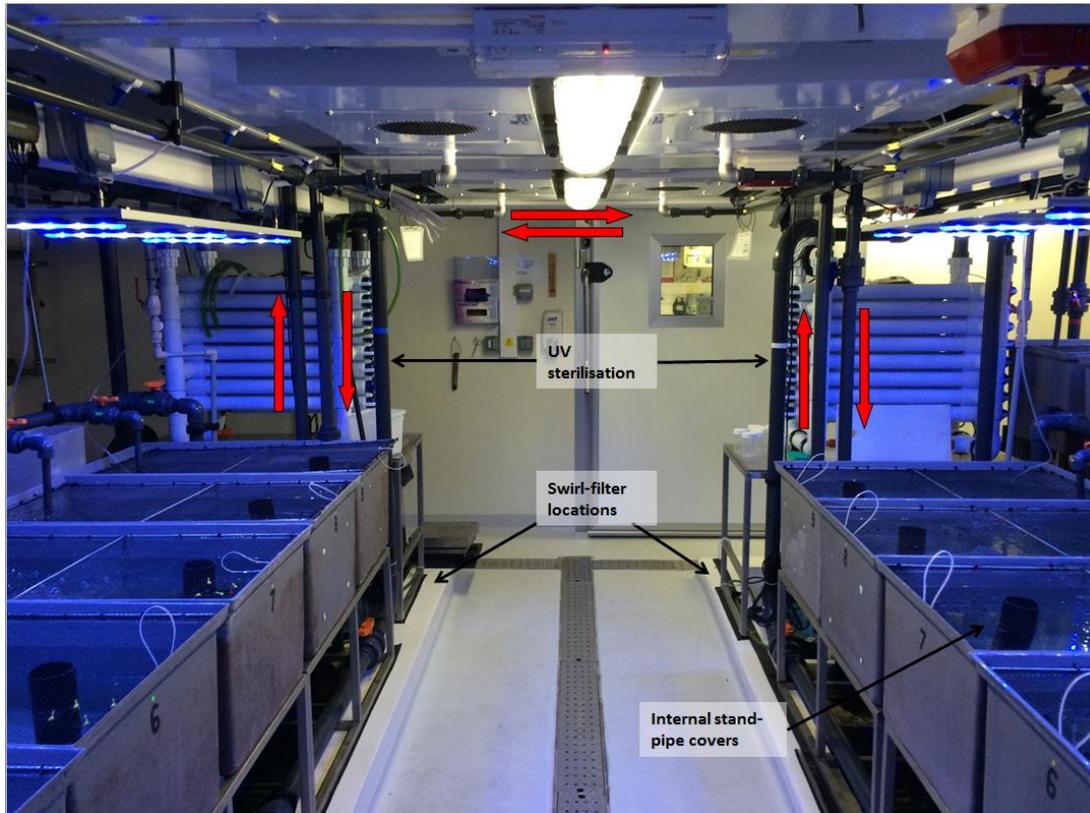
### **2.2. Experimental animals and housing**

Over the course of the experimental trials rainbow trout (*Oncorhynchus mykiss*) were utilised as a model for the salmonid aquaculture industry. For investigations carried out at the University of Plymouth, XXX triploid rainbow trout were sourced and delivered from a commercial fish farm (Exmoor fisheries, Somerset, UK) utilising their standard procedure. On arrival at the University of Plymouth, fish were acclimated for 2 hours with the addition of quarantine system water to a holding vessel (Rubbermaid tilt truck) before being introduced to a flow through quarantine system. All fish entering the aquaria underwent a ten day prophylactic treatment with a proprietary solution (FMC mixture, NT Labs, Wateringbury, Kent) before being graded by size into tanks of experimental systems 1 and 2. After a further 2 weeks acclimation and feeding between 1% and 2% body weight daily to obtain a uniform stock size, experimental animals were distributed evenly into the 110 litre system tanks for the start of feeding trials (average tank biomass  $\pm$  1.5% overall mean). Over the course of the quarantine and conditioning period, fish were fed a commercially available trout diet (BioMar Efico Enviro, BioMar; DK) at approximately 2% body weight (BW) per day.

Further information on numbers of fish per tank and stocking densities can be found in specific experimental chapters.

Two parallel systems (each with 9 tanks) had water exchanged between the two for minimum of 2 hours per day utilising a Sicce 14000 pump (Pozzoleone, VI – Italy) in a sump in each system to exchange water between the two, maintaining experimental conditions across both (Image 2.1). Water chemistry was maintained at appropriate levels for rainbow trout: total free ammonia was maintained below  $0.1 \text{ mg L}^{-1}$ , nitrite below  $1.0 \text{ mg L}^{-1}$  and nitrate below  $50 \text{ mg L}^{-1}$ . Water temperature was maintained at  $15.5 \pm 1^\circ\text{C}$  through ambient air temperature of the controlled temperature (CT) system room, cooled by air-conditioning units. Mechanical filtration was achieved through bespoke swirl filters, with coarse nylon filter media. Biological filtration was achieved through fluidised beds of plastic extruded biomedica (K1 Kladness media) in each system. UV treatment of water was achieved through a P8-Twin 880W UV steriliser (Tropical Marine Centre, Bristol, UK) supplied by an Argonaut-AV100-2DN-S 0.75Hp pump (Hydroair International, Varde, Denmark) on each system. System water was supplied to the tanks by a Sicce 14000 pump circulating approximately 1,440 litres per tank per hour. Biomedica movement was achieved through water circulation powered by an Argonaut-AV100-2DN-S. Aeration to the system, air stones in tanks and perforated pipework ladders in biomedica sumps, was supplied via side channel air blowers (Rietschle Ltd.; Hampshire, UK). Photo period was set to 12 hours light and 12 hours dark throughout housing, and daily dissolved  $\text{O}_2$ , temperature and pH was monitored daily using a Hach HQ 40d probe (Hach Lange GmbH, Düsseldorf, Germany). Nitrogenous waste water chemistry was monitored weekly using a Hach Lange DR 2800 spectrophotometer utilising

cuvette tests for ammonia (LCK304), nitrite (LCK341) and nitrate (LCK340) (Hach Lange GmbH, Düsseldorf, Germany).



**Figure 2.1.** System design highlighting UV water treatment and mechanical swirl-filters. Red arrows indicate direction of water travel utilised for the mixing of the 2 otherwise independent systems.

### 2.3. Experimental diets and formulation

All diets were designed and manufactured at the University of Plymouth, utilising ingredients approved for animal consumption. Experimental diets were formulated with Feedsoft pro™ feed formulation software (Version 3.1, Texas, USA) and were designed to enable the optimisation of inclusion levels of target ingredients and additives, through the

ability to influence health and performance. Feed formulations were designed to achieve the minimum known nutritional requirements of rainbow trout (NRC 2011). Feed ingredients were mixed using a Hobart food mixer (Hobart Food Equipment, Sydney, Australia, model no: HL1400–10STDA). Warm water and oil were then added before cold press extrusion (PTM P6 extruder, Plymouth, UK) through an appropriate size die to produce a pellet of the correct diameter. Diets were air dried (air convection oven) at 45°C before being broken up by hand to achieve the required size.

### **2.4. Experimental feeding**

Each tank of fish was randomly allocated an experimental dietary formulation. Fish were fed 1.5-2.4% of tank biomass over the course of three feeds daily (09:00, 13:00 and 17:00). Feed ration was calculated from weekly or bi-weekly weighing and increased daily based on an assumed FCR of 1, unless otherwise stated.

### **2.5. Growth performance and feed utilisation**

Growth performance parameters were based on net biomass (weight) gain (WG). Experimental animals were weighed in bulk, by tank. Tared tubs of system water received aeration or a constant flow of system water during the weighing procedure to minimise the risk of oxygen deprivation whilst in the tubs. Biomass was sampled on a weekly or bi-weekly basis to the accuracy of 1 gram. Further details can be found within respective experimental chapters.

Utilising the recorded tank biomass and animal numbers, calculations could be carried out to assess feed conversion ratio (FCR), specific growth rate (SGR) and protein efficiency ratio (PER). Calculations were made as follows:

$$WG (g/fish) = Final\ wt.\ (g) - Initial\ wt.\ (g)$$

$$FCR = Feed\ intake\ (g) / Weight\ gain\ (g)$$

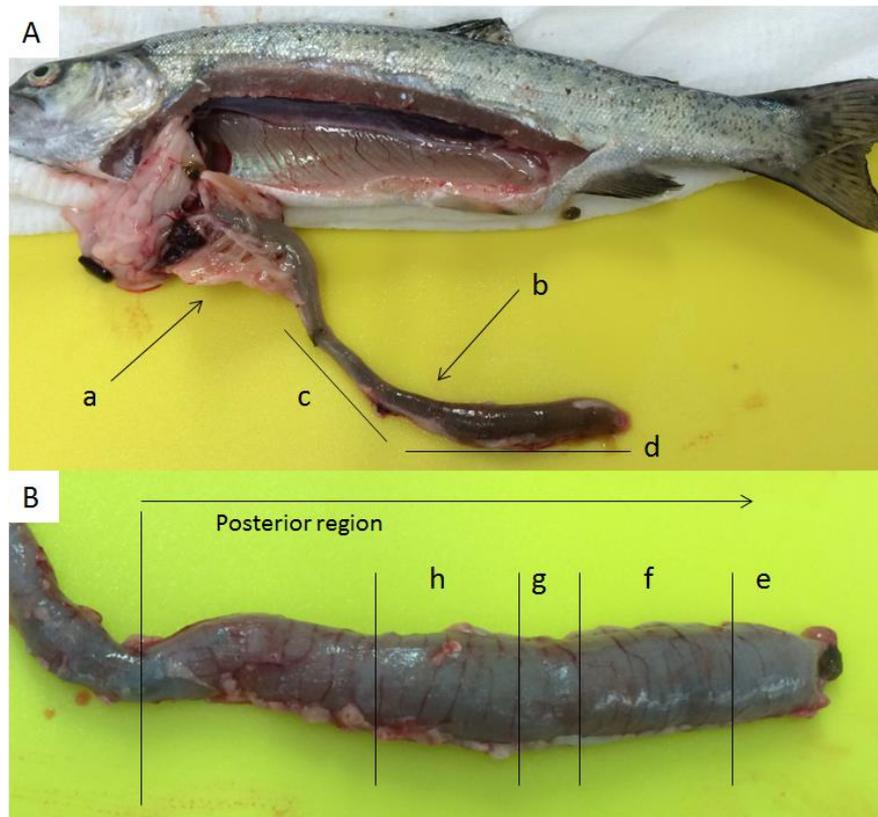
$$SGR = 100 \times ((Ln\ final\ wt.\ (g) - Ln\ Initial\ wt.\ (g)) / (days\ fed))$$

$$PER = Weight\ gain\ (g) / Protein\ intake\ (g)$$

## 2.6. Sampling protocol and fish dissection

A minimum of two fish per tank were sampled during the sampling process, ensuring an  $n \geq 6$  was achieved for all samples per dietary treatment. Euthanasia was achieved in accordance with the schedule one procedure of the Animals (Scientific Procedures) Act 1986. Aseptic conditions were used for microbiological sampling. Dissection occurred to remove specific organs/tissues for analysis. Once an incision into the Intraperitoneal (IP) cavity had been made from the anal vent to the pectoral fins, the intestine was cut just inside the anal vent. The intestine was then gently removed from the fish, removing visceral fat attached in the process. Once the intestine was cleared of fat and extended from the fish, the anterior end of the intestine was cut just below the pyloric caeca enabling the intestine to be excised from the fish. The intestine of trout can easily be identified into anterior and posterior regions, at the thickening of the gut (Figure 2.2a). All samples were taken from the posterior intestine. Once removed from the fish, the posterior portion (5 mm) was discarded to avoid

artefacts from the dissection process. The next 10 mm was removed for light microscopy. This was fixed in a 10% formalin saline solution for 48 hours at 4 °C before being transferred to 70% ethanol and stored at 4 °C. The next 3 mm of posterior intestine was removed and cut longitudinally into two pieces, to be utilised for scanning electron microscopy (SEM). Immediately after extraction, SEM samples were washed in 1 % S-methyl-L-cysteine, phosphate buffered saline (PBS) solution for a minimum of 20 seconds to remove mucus, before fixation in 2.5 % glutaraldehyde in pH 7.2, 0.1 M sodium cacodylate buffer. Samples were then stored at 4 °C. The next 5mm were excised for gene expression analysis and stored in RNA later® (ThermoFisher Scientific) at -20 °C. Figure 2.2b shows sampling regions and sections. Digesta samples for microbiological analysis were taken from the posterior intestine. Once the intestine had been excised, digesta was gently eased out with the aid of forceps into sterile 1.5 ml micro centrifuge tubes. Intestine that had digesta removed were not sampled for any other analysis to prevent the identification of artefacts from the digesta removal process in subsequent analysis.



**Figure 2.2.** Illustration of sampling processes. A) Removal of the intestinal tract from sampled animal. a; Pyloric ceca. b; Thickening of intestinal tract identifying change from anterior to posterior regions. c; Anterior intestinal region. d; Posterior intestinal region. B) Excised sample locations. e; Area discarded. f; Light microscopy. g; Scanning electron microscopy. h; Gene expression.

## 2.7. Somatic indices

### 2.7.1. Condition factor (*K-factor*)

Fulton's *K-factor* was utilised as an indicator of fish condition. Briefly, euthanised fish were weighed to 1 mg and measured from tip of the snout to fork in the tail.

*K-factor* was calculated utilising the formulae:

$$K\text{-Factor (AU)} = 100 \times (FW / FL^3)$$

Where; FW = Fish weight (g) and FL = fork length (cm)

### *2.7.2. Hepatosomatic index*

The hepatosomatic index (HSI) was calculated as in index of health. Briefly, post euthanasia, fish weight (to 1 mg) was taken before dissection occurred. Whole livers were removed and weighed (to 0.1mg) and HSI was calculated as follows:

$$\text{Hepatosomatic index (AU)} = 100 \times (\text{LW} / \text{FW})$$

Where; LW = liver weight (g) and FW = pre-dissected fish weight (g)

### *2.7.3 Viscerosomatic index*

The viscerosomatic index (VSI) was calculated as in index of health. Briefly, post euthanasia, fish weight (to 1 mg) was taken before dissection occurred. Intier viscera, from oesophagus to anus with visceral fat attached as well as associated organs, were removed and weighed (to 0.1mg) VSI was calculated as follows:

$$\text{Viscerosomatic index (AU)} = 100 \times (\text{VW} / \text{FW})$$

Where; VW = Viscera weight (g) and FW = pre-dissected fish weight (g)

## 2.8. Proximate analysis

Diet, carcass and feed ingredient moisture, crude protein, lipid, ash and gross energy levels were analysed in duplicate or triplicate in accordance with the protocols of the AOAC (2016) as described below. Prior to analysis all samples were milled into a homogenous powder/substance. Diets were analysed on an as fed basis. Carcass composition data were analysed on a dry weight basis, as milling of carcasses occurred post drying.

### 2.8.1. Moisture content

Percentage moisture was calculated by the drying of a known weight of sample for a period of time until a constant weight was achieved. Drying occurred at 105°C in a fan assisted oven (Genlab Ltd, UK). Percentage moisture was calculated following the formulae:

$$\text{Moisture (\%)} = ((\text{wet wt. (g)} - \text{dry wt (g)}) / (\text{wet wt. (g)})) \times 100$$

### 2.8.2. Crude protein

The kjeldahl method was utilised to assess crude protein (CP) in diet, carcass and faeces through the determination of nitrogen content and a subsequent conversion factor. The resulting total nitrogen content is multiplied by 5.95 for plant derived proteins (6.25 for animal derived proteins) to calculate crude protein. Milled homogenous sample was weighed (100 – 150 mg) into micro Kjeldahl tubes with the addition of a catalyst tablet (3 g K<sub>2</sub>SO<sub>4</sub>, 105 mg CuSO<sub>4</sub> and 105 mg TiO<sub>2</sub>) (DBH Chemicals Ltd, Dorset, UK). To the tubes, 10 ml concentrated (>95 %) low nitrogen sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) (Fisher Scientific, Leicester, UK) was added and digestion carried out in a Gerhardt Kjeldatherm 40 tube digestion block

(Gerhardt Laboratory Instruments, DE). The digestion process follows 100°C for 30 minutes, 225°C for 1 hour then 380°C for 1 hour. Post digestion samples were left to cool overnight prior to distillation in an automated Vapodest 40 unit (Gerhardt Laboratory Instruments, DE). During the distillation phase ammonium (NH<sub>4</sub>) is converted into ammonia (NH<sub>3</sub>) through the addition of excess base which is then boiled into a receiving solution containing boric acid, forming an ammonium borate complex. Quantification of nitrogen content is achieved through exact neutralisation of the receiving solution with concentrated H<sub>2</sub>SO<sub>4</sub>. Triplicate acetanilide standards and duplicate concentrated casein standards with known nitrogen content allowed the quantification of nitrogen recovery efficiency. Nitrogen content was calculated following the formulae:

$$\% \text{ Nitrogen} = ((TV_s - TV_b) \times AN \times MW_N) / \text{sample weight}$$

Where; TV<sub>s</sub> = Titration volume of sample (ml). TV<sub>b</sub> = titration volume of the blank (ml). AN = acid normality of H<sub>2</sub>SO<sub>4</sub> (0.02). MW<sub>N</sub> = molecular weight of nitrogen (14.0067 u)

Efficiency of nitrogen recovery was calculated with the formulae:

$$\% \text{ Efficiency} = (100 / KAC) \times \text{acetanilide sample result}$$

Where; KAC = known acetanilide nitrogen content (10.36)

Correction of nitrogen content results to 100% efficiency was conducted with the formulae:

$$\text{Corrected value} = (\% \text{ Nitrogen} / \% \text{ efficiency of run}) \times 100$$

Conversion of % nitrogen to % crude protein was calculated as follows:

$$\text{Crude protein (\%)} = \text{Nitrogen \%} \times \text{cf}$$

Where; cf = Conversion factor (5.95 or 6.25 dependent on sample type)

### 2.8.3. Crude lipid

Crude lipid (CL) content of samples was determined in triplicate through hot solvent extraction. A known weight of sample (approx. 3 g weighed to 0.1mg) was added to a cellulose thimble and plugged loosely with a cotton wool bung. Thimbles in thimble holders were then inserted into pre-weighed extraction beakers containing anti-bumping granules then flooded with 40ml petroleum ether. The extraction beakers were inserted into a 6 place Soxtherm unit (C. Gerhardt; DE) with additional multistate automated control system (Multistat, C. Gerhardt; DE) and run at 200°C. Post extraction, extraction beakers were removed from the Soxtherm unit and placed in a fume cupboard for any remaining petroleum ether to evaporate. The extraction beaker containing the extracted lipid was then re-weighed to 1 mg. Crude lipid was calculated with the following formulae:

$$\text{Crude lipid (\%)} = ((\text{FBW} - \text{IBW}) / \text{SW}) \times 100$$

Where; FBW = final beaker weight (g), IBE = Initial beaker weight (g), SW = sample weight (g).

### 2.8.4. Ash

Percentage ash was calculated by incineration of a known sample weight in a muffle furnace (Carbolite ELF; Derbs, UK). Samples of 400 - 600 mg (to 0.1mg) were incinerated in ceramic

crucibles of known weight (to 0.1mg) at 550°C for 12 hours. After cooling in a desiccation chamber, percentage ash was calculated as follows:

$$\text{Ash (\%)} = \text{FW} / \text{IW} \times 100$$

Where; FW = final weight of sample after incineration (g) and IW = Initial sample weight.

### 2.8.5. Gross energy

Gross energy determination was conducted in duplicate utilising a Parr Adiabatic Bomb Calorimeter, model 1356 (Parr Instrument Company, IL, USA). Approximately 1 g of sample was formed into a pellet with the use of a pellet press and weighed to 0.1mg. The formed and weighed pellet was placed into a nickel crucible and a ~10cm fuse wire shaped into a 'U' shape to touch the top of the pellet. The pellet and fuse were inserted into the bomb chamber which was then filled with pure oxygen and lowered into the instrument bucket containing 2kg of water. Sample weight was entered into the computer control system which then combusts the sample and records the temperature change in the water jacket formed around the bomb in the instrument bucket. The instrument logarithm determines a MJ gross energy per Kg value.

## 2.9. Haematological and serological analysis

Blood was taken from fish immediately after euthanasia (schedule 1), from the caudal vein that runs along the underside of the vertebrae, between the anal vent and caudal fin. Blood was extracted utilising a 25 gauge needle and 1 ml syringe, and transferred to a 1.5ml micro centrifuge tube. From this stage, blood was taken for the following applications.

### 2.9.1. Haematocrit

Quantified by Brown (1988), the haematocrit is a measure of the erythrocyte fractions in the blood. Freshly extracted blood was drawn into heparinised capillary tubes (75 µl) and sealed with a plasticine bung (Cristaseal, Hawksley; West Sussex, UK). Capillary tubes were centrifuged at 10,500 XG for 5 minutes to separate the fractions of blood, before being read with a Microhaematocrit Tube Reader (Hawksley; W Sussex, UK), measuring packed cell volume proportion (%) of the samples.

### 2.9.2. Haemoglobin

Haemoglobin was analysed as a measure of health status. Analysis was carried out in accordance with Rawling *et al.* (2012) utilising a turbidimetric method. To 1 ml of Drabkin's alkaline ferricyanide-cyanide solution (D5941, Sigma-Aldrich Co.; Dorset, UK), 4 µl of freshly drawn blood was added, gently mixed by pipette, and left to incubate at room temperature (~20 °C) for 4 hours. Post incubation, absorbance was read at 540 nm in a spectrophotometer (Helios Epsilon, Thermo Scientific; MA, USA). Haemoglobin was calculated utilising the following:

$$\text{Haemoglobin (g/dl)} = (\text{Abs}_{sa} / \text{Abs}_{st}) \times \text{DF}$$

Where;  $\text{Abs}_{sa}$  = sample absorbance,  $\text{Abs}_{st}$  = standard absorbance. DF = dilution factor (200).

### 2.9.3 Serum lysozyme analysis

Post removal of small quantities of freshly drawn blood for prior analysis (sections 2.9.1, 2.9.2), microcentrifuge tubes with the remaining blood was allowed to clot on ice for 4

hours, then stored for 12 hours at 4 °C. After incubation at 4 °C, samples were centrifuged at 2,500 XG for 5 minutes. Serum was then removed by pipette and stored at -80 °C until analysed.

Serum lysozyme activity is commonly utilised as an indicator of innate immune status. Serum lysozyme activity was quantified utilising a kinetic turbidimetric assay adapted from Demers and Bayne (1997). *Micrococcus lysodeikticus* (4698, Sigma-Aldrich Co.; Dorset, UK) was suspended in 0.05 M Na<sub>2</sub>HPO<sub>4</sub> (pH 6.2) at a concentration of 200 mg/ml, and utilised as the substrate. 25 µl of serum was added to microplate wells, followed by 175 µl of substrate solution, using a multi-channel pipette. Mechanical agitation started immediately and absorbance read at 530 nm every 30 seconds for 5 minutes (OPTImax microplate reader, Molecular Devices LLC; CA, USA). Samples were run in quadruplet. Lysozyme units were calculated as follows:

$$1 \text{ U of Lysozyme} = \Delta 0.001 \text{ Abs/min}$$

## **2.10. Molecular microbial analysis**

### *2.10.1. DNA Extraction*

DNA extraction occurred utilising the PowerFecal™ DNA isolation kit (Cambio, Cambridge, UK) with the addition of a lysis step prior to the manufacturers protocol. Briefly, 500 µl of lysozyme (50 mg / ml in TE buffer) was added to 100mg of sample weighed into PCR clean (RNAse, DNAse free) micro centrifuge tubes. Samples were then homogenised in a vortex mixer, and incubated for 30 minutes at 37 °C. Post incubation, samples were centrifuged at 13,000 XG for 2minutes and the supernatant discarded. The remaining sample was re-

suspended in 750 µl bead solution and added to a bead tube with 60 µl Solution C1 and vortexed briefly before incubation at 60 °C for 10 minutes. Post incubation, samples were placed into clips on a flat-bed vortex pad and vortexed at maximum speed for ten minutes. Samples were then centrifuged at 13,000 XG for 1 minute and 400-500 µl of supernatant added to new PCR clean micro centrifuge tubes. To the new tube, 250 µl solution C2 was added, vortexed briefly and incubated at 4 °C for 5 minutes. Post incubation, samples were centrifuged at 13,000 XG for 1 minute and 600 µl supernatant transferred to another new PCR clean micro centrifuge tube prior to the addition of 200 µl solution C3. The supernatant and C3 solution were briefly vortexed and incubated at 4 °C for 5 minutes. Post incubation, centrifugation at 13,000 XG occurred again and 750 µl of supernatant was removed to a new PCR clean micro centrifuge tube and 1200 µl solution C4 added. Six hundred and fifty µl of this supernatant was then added to a spin filter column and centrifuged at 13,000 XG for 1 minute. The flow through was discarded, and this step repeated for all the supernatant. Five hundred µl of solution C5 was then added to the column and spun at 13,000XG for one minute, flow through was discarded and the column spun again to dry the filter membrane. The filter column was then removed from its collection tube and placed into a new PCR clean micro centrifuge tube. To the filter, 40 µl solution C6 was added to elute the DNA. The column in the micro centrifuge tube was then spun at 13,000 XG for one minute, the filter was discarded, leaving the extracted DNA in the micro centrifuge tube. The whole extraction procedure was carried out under aseptic technique to minimise the risk of sample contamination.

### 2.10.2. PCR-denaturing gradient gel electrophoresis (PCR-DGGE)

PCR amplification of the V3 region of 16S rRNA gene was carried out using the reverse primer P2 (5'- ATT ACC GCG GCT GCT GG -3') and the forward primer P3 (5'- CC TAC GGG AGG CAG CAG -3'), with a GC clamp added at the 5' end (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G -3') after Muyzer *et al.* (1993). Thirty  $\mu$ l PCR reactions were carried out with the following reagents utilising 0.5  $\mu$ l P1 and 0.5  $\mu$ l P2 primers (50 pmol  $\mu$ l<sup>-1</sup>), 15  $\mu$ l RedTaq™ (Bioline, London, UK), 12  $\mu$ l molecular grade water and 2  $\mu$ l DNA template. Thermal cycling was conducted using a Techne TC-512 (Thermal Cycler; Staffordshire, UK) set to 95 °C for 5 minutes, followed by two cycles of 1 minute at 95 °C, 2 minutes at 65 °C and 3 minutes at 72 °C. This cycle was repeated with a 1 °C decrease in annealing temperature every second cycle until a final temperature of 55 °C. Once 55 °C annealing temperature is reached, a further 10 cycles were run. Post PCR, PCR amplicon size and quality was analysed by running the samples through a 1.5% agarose gel at 80 volts for 45 minutes as described in section 2.10.5.

Denaturing gradient gel electrophoresis (DGGE) was performed using a DCode mutation system (Bio-Rad, CA, USA). PCR products were run on an 8% polyacrylamide gel (160 mm x 161 mm) containing 40%–60% denaturing gradient (where 100% denaturant is 7 M urea and 40% formamide). The gel was run at 65 V for 17 h at 65°C in Tris-acetate-EDTA (TAE) buffer and stained for 30 min in 100 ml 1xTAE buffer containing 10 ml of SYBR Gold nucleic acid gel stain (Molecular Probes, UK). Visualization was carried out in a Bio-Rad universal hood II (BioRad laboratories, Italy). The resulting gel was transformed into operational taxonomic units (OTUs) presence/absence and band intensities using Quantity One™ software (BioRad laboratories, CA, USA).

### 2.10.3. Sanger sequencing

DGGE band selected for sequencing were isolated from the gel utilising a pipette tip and re-suspended in molecular grade water before a further PCR using P2 and P1 (5' – CCT ACG GGA GGC AGG AG- 3'). A further 1.5% agarose gel was run under the same conditions as previously described to assess amplicon size and quality. The PCR product was then cleaned using a QIAquick PCR Purification Kit (Qiagen, Germany), and sent for sequencing at GATC laboratories, Germany. Received sequences were subsequently BLAST searched in GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to achieve a level of identification. Genus and species were accepted at 95% and 97% respectively.

### 2.10.4. High throughput sequencing

High throughput analysis focused on the 16S rRNA V1-V2 region. PCR amplification was achieved utilising the reverse 338R (5' - GCW GCC WCC CGT AGG WGT – 3') and forward 27F (5' - AGA GTT TGA TCM TGG CTC AG – 3') primers, diluted to 50 pmol  $\mu\text{l}^{-1}$  (Eurofins MWG, Ebersberg, Germany). Thirty  $\mu\text{l}$  reactions were carried out utilising the following reagents. 15  $\mu\text{l}$  MyTaq™ (Bioline, London, UK), 1  $\mu\text{l}$  338R and 1  $\mu\text{l}$  27F primer, 9  $\mu\text{l}$  molecular grade water and 4  $\mu\text{l}$  DNA template. The PCR conditions comprised an initial denaturing period of 7 minutes at 94°C, followed by 10 touchdown cycles of 30 s at 94 °C, 30 s at 62 °C (reducing by 1 °C per cycle) and 30 s at 72 °C. This was then followed by a further 25 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30s and a final extension of 72 °C for 7 minutes.

PCR products were purified using Agencourt AMPure XP (Beckman Coulter, Ca, USA) using the manufacturer's standard protocol and quantified with a Qubit® 2.0 Fluorometer (Invitrogen, Ca, USA). Amplicon fragment concentrations were then assessed using an Ion

Library Quantitation Kit (Life Technologies™, USA) and then adjusted to 26 pM. Amplicons were attached to Ion Sphere Particles using Ion PGM Template OT2 200 kits (Life Technologies™, USA) according to the manufacturer's standard protocol. Multiplex sequencing was carried out with Ion Xpress Barcode Adapters (1-16 Kit; Life Technologies™) on a 316™ chip (Life Technologies™) on an Ion Torrent Personal Genome Machine (Life Technologies™). Sequences were binned by sample and filtered to remove low quality reads within the PGM software. Data were exported as FastQ files.

Taxonomic analysis of sequence reads was conducted with FASTX-Toolkit (Hannon Lab, USA) after the removal of low quality scores (Q score < 20). De-noising and analysis of sequences was conducted with QIIME (Caporaso *et al.*, 2010a). OTU mapping was performed utilising the default pipeline of QIIME with USEARCH (Edgar, 2010) removing chimeras (putative erroneous reads). Greengenes database (DeSantis *et al.*, 2006) was used for the assignment of taxonomic classification of OTUs utilising the RDP classifier (Wang *et al.*, 2007), which clustered the sequences at 97% similarity with a 0.80 confidence threshold. Multiple alignment of the representative sequences for each OTU was created using PyNAST (Caporaso *et al.*, 2010b) with a minimum sequence length of 150 base pairs (bp) and 97% identification. Utilising the 16S microbial Nucleotide BLAST-NCBI database, highest homologous species or genera were identified (>98% similarity at 150 bp).

### 2.10.5. Gel electrophoresis

Agarose gel electrophoresis was carried out in a pharmacia electrophoresis tank with 1 x Trisborate EDTA (TBE) buffer. Gels were formed from 1.5% agarose with additional GelRed™ nucleic acid dye (Biotium Inc, Fremont, CA, USA). Wells were created with a comb allowing

the loading of 6-8 µl of sample and loading buffer (Bioline). Five µl of Hyper ladder IV (Bioline) as well as positive and negative controls were included in each gel run.

### *2.10.5. RNA extraction and cDNA synthesis*

Total RNA extraction from the posterior intestine was conducted using TRIzol (Invitrogen, Carlsbad, CA, USA) as carried out by (Pérez-Sánchez *et al.* 2011). RNA purity and concentration was assessed using a NanoDrop™ spectrophotometer (NanoDrop Technologies, Wilmington, USA) and stored at -20 °C prior to use. Total RNA was treated with TURBO DNA-free™ (Thermo Fisher Scientific, MA, USA) to remove any DNA contamination. cDNA synthesis was carried out utilising iScript cDNA Synthesis Kit (Bio-Rad CA, USA), with 1 mg RNA template in a 20 µl reaction. cDNA was stored at -20 °C until usage.

### *2.10.6. Quantitative real time PCR (gene expression analysis)*

PCRs were performed in an iQ5 iCycler thermal cycler (Bio-Rad) following the SYBR green methodologies. Two µl of each samples cDNA was pooled to create a standard for primer efficiency determination. This was carried out on 1/10 dilutions of the pooled cDNA and the resulting plots of Ct values versus the logarithmic cDNA input, using the equation;

$$E = 10(-1/slope)$$

QPCR reactions were carried out on either 96 or 384 well plates (Thermo Scientific; MA, USA) utilising 7.5 µl reactions. The reagents used in triplicate reactions per dilution were as follows: 2 µl of diluted (1/10) cDNA, 3.75 µl 2x concentrated iQ™ SYBR Green Supermix (Bio-Rad), (SYBR Green was the fluorescent intercalating agent), 0.225 µl of forward and reverse

primers (0.45 µl total at 0.3 µM concentration) and 1.3 µl of DEPC treated H<sub>2</sub>O (Thermo fisher scientific). Thermal cycling conditions were as follows: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 60 s at 60 °C (58°C for primers with 58°C annealing temperatures) with fluorescence recorded at the end of each cycle. Reactions and quality control measures were carried out in accordance with the MIQE guidelines (Bustin *et al.* 2009). Additional melt curve (dissociation curve) analysis was carried out to ensure single peaks in all cases. Reaction volumes and conditions were the same for sample analysis, and carried out in duplicate per sample. β actin and elongation factor 1α were utilised as housekeeping genes. Primers for genes were designed utilising Primer3web v.4.0.0 ([www.Primer3.ut.ee](http://www.Primer3.ut.ee)) and ordered from Eurofins MWG Operon's oligo synthesis service (Ebersberg, Germany). Specific genes of interest and primers utilised can be found in respective experimental chapters.

Data were analysed utilising the iQ5 optical system software version 2.0 (Bio- Rad) containing Genex Macro iQ5 Conversion and genex Macro iQ5 files. The spreadsheet calculations are based on the algorithms of Vandesompele *et al.* (2002) and the GeNorm manual (<http://medgen.ugent.be/~jvdesomp/genorm/>). Delta CT levels were normalised with a normalisation factor (NF), generated from the two housekeeping genes in GeNorm, to produce a normalised expression level (NEL) per gene. Formulae utilised were as follows.

$$\Delta CT = \text{primer efficiency}^{(CT \text{ value} - \text{minimum CT value observed in gene or interest})}$$

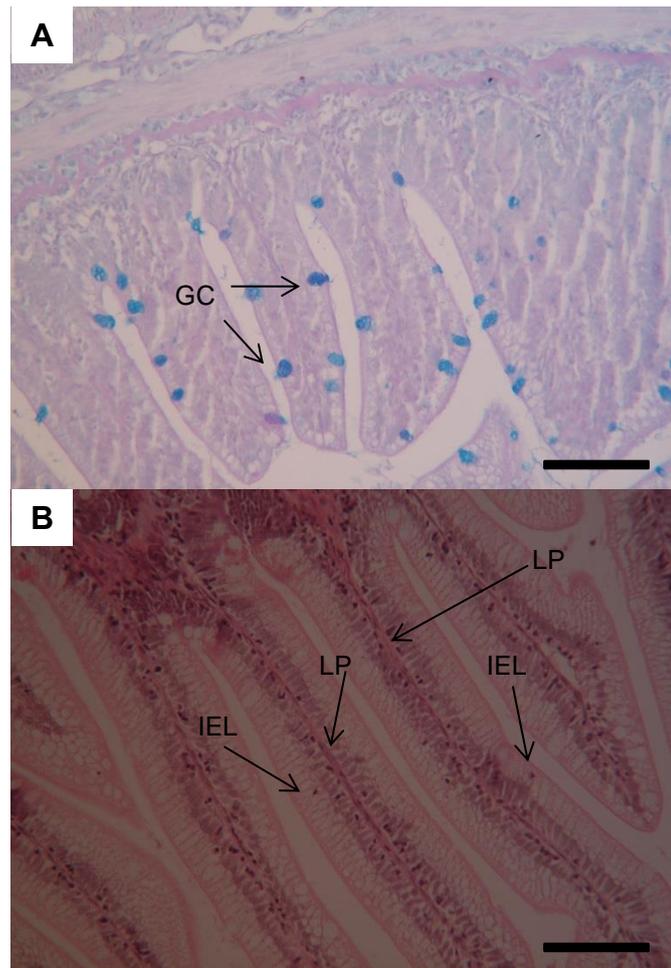
$$NEL = \Delta CT / NF$$

Descriptive statistics and NELs per treatment per gene were then analysed utilising RStudio (available at <https://www.rstudio.com/products/rstudio/download2/>), and pairwise

comparisons were carried out utilising permutation tests. Significance was accepted as  $P < 0.05$ .

### **2.11. Light microscopy**

One centimetre sections of the posterior intestine were sampled, fixed and stored as described in section 2.8. After fixing and storing in 70%, samples were dehydrated through a graded ethanol series. Once dehydrated, samples were embedded in paraffin wax utilising a Leica EG1150H tissue processor, in plastic cassettes. Sectioning of samples was carried out utilising a microtome (Leica RM2235) and subsequently, ultrathin sections (5  $\mu\text{m}$ ) were mounted onto microscope slides. Once mounted onto slides, wax sections were dried for a minimum of 24hrs at 30 °C prior to staining. Multiple slides per sample were stained with both haematoxylin and eosin (H&E) (Figure 2.3b) and periodic acid-Schiff with Alcian Blue (PAS) (Figure 2.3a) with the aid of a Leica 371 Autostainer XL (Leica; Bucks, UK). Post staining, slides were cover slipped with DPX and left to dry again for minimum of 24 hrs at 30 °C. A Leica DMIRB microscope mounted with an Olympus E410 digital SLR camera was used to capture micrographs at varying magnifications. Image analysis was carried out with the aid of Image J 1.45 (National Institutes of Health, USA). Lamina propria widths were analysed as an average of 3 measurements per fold (top middle and bottom), in ten folds per sample. Goblet cells and intraepithelial leukocytes (IELs) were counted along measured entire intestinal folds (Figure 2.3b), and calculated as cells per 100  $\mu\text{m}$ . Analysis of internal perimeter ratio was unachievable due to the high degree of folding present in the intestine or rainbow trout, often resulting in large sections of irregular 2D section morphology.



**Figure 2.3.** A) Example of PAS stain with visible goblet cells. B) Example of H&E staining, identifying IELs and lamina propria for analysis. Scale bars = 100  $\mu\text{m}$ .

### 2.12. Statistical analysis

Data are presented as means  $\pm$  standard deviation (SD), unless otherwise stated. Statistical analyses were carried out using Minitab 16 (Minitab 16 statistical software, Minitab Inc. State college Pennsylvania, USA). Data were tested for normality using a Kolmogorov–Smirnov test, a one-way ANOVA was carried out thereafter if data were normally distributed. Significant differences between treatments were determined by Tukey’s *post hoc* test. Non-normally distributed data were subjected to Kruskal-Wallis tests and subsequent independent U-tests. Percentage and ratio data were log transformed prior to statistical analysis. Significance was accepted at  $P < 0.05$ .

### 2.12.1. PCR-DGGE

PCR-DGGE banding patterns were transformed into intensity matrices using Quantity One software, version 4.6.3 (Bio-Rad Laboratories), after Schauer *et al.* (2000), in order to evaluate similarities between treatments. Primer V6 software (Clarke & Gorley 2006) was utilised to determine similarity percentages (SIMPER) and ANOSIM (one-way analysis of similarity) which was used to determine pairwise comparisons between PCR-DGGE fingerprint profiles (Abell and Bowman, 2005).

Primer V6 software was also utilised for ecological calculations. The total number of operational taxonomical units (OTU's) ( $S$ ) was calculated from the sum of distinct PCR-DGGE bands per sample. Margalef species richness was calculated utilising the formula:

$$(d = (S - 1)/\ln (N))$$

Where;  $S$  = number of species,  $N$  = total number of individuals (unit = total intensity units).

Shannon diversity index was calculated using the formula:

$$(H' = 9 \sum (p_i (\ln p_i)))$$

Where;  $p_i$  = proportion of the total number of individuals in the  $i$ th species.

After calculation per sample, data were subjected to a one-way ANOVA.

High-throughput sequencing data analysis was carried out utilising QIIME, Good's estimator of coverage was calculated using the formula:

$$(1 - (\text{singletons}/\text{individuals})) \times 100.$$

Chao 1 index was calculated using the formula:

$$\text{Schao1} = S + (n1-1) / (n2+1)$$

Where; Schao1 = estimated richness,  $S$  = number of observed species,  $n1$  = number of OTU's singletons,  $n2$  = number of OTU's doubletons.

Bray-Curtis was calculated using the formula:

$$Cn = 2jn / (na + nb)$$

Where;  $na$  = the total number of individuals in treatment A,  $nb$  = the total number of individuals in treatment B,  $2jn$  = sum of the lower of the two abundances for species found in both sites.

The phylogenetic distance metric (PD) analyses minimum total branch length covering all taxa within a sample on the phylogenetic tree.

### 2.12.2. High throughput sequencing

Alpha diversity matrices, Chao1 and Shannon's diversity index for high throughput sequencing results were calculated through QIIME, and rarefied OTU tables to calculate Good's coverage, assessing sampling depth coverage via observed genera. Inter-sample beta diversity analysis (metrics) were calculated using weighted unique fraction metric (UniFrac) distances (Lozupone *et al.*, 2007) and Bray-Curtis similarity (Bray and Curtis, 1957).

Statistical analysis was applied to sequences representing >0.1 % of any treatment. Non parametric Kruskal-Wallis was followed with the post-hoc Tukey-Kramer test, performed using STAMP v2 0.8. Significance was accepted at  $P \leq 0.05$ .

**CHAPTER 3a. The short term impacts of wheat gluten products on the intestinal microbiota and gross intestinal structure of juvenile rainbow trout (*Oncorhynchus mykiss*): A preliminary investigation.**

**3.1a. Introduction**

As the ever increasing drive for sustainability in the aquaculture industry continues, there is now greater scrutiny on alternative protein sources that have the potential to raise the standard of the industry by reducing fishmeal and soybean inclusion. As the global production volume of fishmeal has plateaued at around 6 million tonnes (IFFO.com, 2015), and with ever increasing cost, research efforts into alternative protein sources for inclusion into aquafeeds has greatly increased over the past two decades (Alexis, 1997; Espe et al., 2006; Carter and Hauler, 2000; Gomes et al., 1995; Watanabe et al., 1997; El-Sayed, 1999; Hardy, 2010; Burr et al., 2012). Advances in aquafeed formulations have been driven in part by the aquafeed producers, striving for maximum benefits from a least cost formulation strategies. Consequently feed ingredient producers are now actively seeking economically viable and suitable protein sources to meet public perception for ethical animal production (Tacon and Metian, 2008).

Wheat gluten, a high protein (75-85%) by-product from the removal of starch from cereal grains (wheat), is an interesting and promising protein, receiving some attention as an alternative protein source in the early to mid-1990's (Pfeffer and Henrichfreise, 1994; Rodehutscord et al., 1994; Rodehutscord et al., 1995; Rodehutscord et al., 1997). More recently wheat gluten has received attention as a partial replacer for fishmeal and soya

products (Davies et al., 1997; Hansen et al., 2006; Helland and Grisdale-Helland, 2006; Tusche et al., 2012; Messina et al., 2013; Apper et al., 2016). Such studies have revealed no detrimental effects of the protein source on growth performance with inclusion levels up to 30%. It must be noted, the amino acid profile of wheat gluten is comparatively low in arginine, threonine and most importantly lysine as a first limiting amino acid and may require additional supplementation in diets. However, with additional lysine supplementation, wheat gluten has displayed digestibility coefficients of 97% and 84% for protein and energy respectively in rainbow trout compared to the basal formulation (Pfeffer and Henrichfreise, 1994), and protein and lysine coefficients of 90-95% for Atlantic salmon and Atlantic cod (Storebakken et al., 2000; Tibbetts et al., 2006). Despite lacking in lysine, wheat gluten is rich in other amino acids. Glutamine, a conditionally essential amino acid, is a major energy source for rapidly proliferating cells as well as many other vital cellular functions, comprising 35-40% of the CP of wheat gluten. Glutamine is often considered a functional amino acid and supplementation is reported to have both morphometric effects, observed in hybrid striped bass (*Morone chrysops x Morone saxatilis*) (Cheng et al., 2012) and modulatory effects on the immune response within the intestine of channel catfish (*Ictalurus punctatus*) (Trichet, 2010; Pohlenz et al., 2012).

The health and structure of the intestine of fish is of high importance as the GI tract plays a key role in digestive function and the immune system, acting as a first line of defence to many water borne fish pathogens. Morphological effects of wheat gluten on the intestine of fishes has been investigated, however, the specific effect of wheat gluten on gut microflora have yet to be fully explored. The intestinal microbiota are recognised to contribute to mucosal barrier function, producing antimicrobial substances as well as providing physical

site competition and protecting the host from potential pathogens (Salinas and Parra, 2015). Throughout the literature there is a paucity of information on the effect of wheat gluten on gut health and the intestinal microbiota, except for a more recent study utilising a hydrolysed wheat gluten that has observed modulation of the allochthonous microbiota (Apper et al., 2016). To the author's knowledge there is no available data on the comparison of differing wheat gluten sources (differing levels of refinement / hydrolysis).

Due to the lack of published information on the dietary effects of wheat gluten on the intestinal microflora of rainbow trout, a preliminary 2 week trial was carried out to assess any early modulation of the gut microbiota and to ascertain if such potential changes may affect the intestinal tract.

### **3.2a. Materials and methods**

All experimental work involving fish was conducted under the UK Home Office project licence PPL 30/2644 and was in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Plymouth University Ethical Committee.

#### *3.2.1a. Experimental design*

Three hundred and seventy five rainbow trout (XXX triploid genotype and wild phenotype) were obtained from Exmoor Fisheries (Somerset, UK). After a two week acclimation period, at the Aquatic Animal Nutrition and Health Research Facility at the University of Plymouth, the fish were graded and randomly distributed into 15, 120L fibreglass tanks (25 fish per tank; average weight =  $48.49 \pm 0.58\text{g}$ ) in a 7,000 litre closed recirculation system. Over the course of a two week short exposure trial, varying levels of vital (comprising 7.5% and 15% of the

diet) and hydrolysed (7.5% and 15%) wheat gluten meals were fed to rainbow trout at the expense of soy protein concentrate, along with a plant protein control. Dietary treatments were randomly attributed to triplicate tanks, and fed at a rate of 1.5 - 2.5% of biomass per day in equal rations at 09:00, 13:00 and 17:00 daily. Feed was adjusted daily on a predicted FCR of 1.2, based on initial biomass weights. Rainbow trout were maintained at  $15 \pm 1^{\circ}\text{C}$  with a 12:12 light dark photoperiod. pH was maintained at  $7.0 \pm 0.5$  and >85% dissolved oxygen. Temperature, pH and dissolved oxygen were monitored daily. Water ammonia, nitrite and nitrate were monitored weekly and maintained within the acceptable range for the species, and managed with water changes to negate any detrimental build-up of compounds.

### *3.2.2a. Experimental diets*

All experimental diets were formulated and manufactured at the University of Plymouth as described in section 2.3. Experimental wheat gluten products, vital wheat gluten (Amytex<sup>®</sup>) and hydrolysed wheat gluten (Merripro<sup>®</sup>), were supplied by Tereos Syral (Marckolsheim, France). Two inclusion levels of vital wheat gluten, 7.5% and 15% (diet VWG 7.5 and VWG 15 respectively) and two inclusion levels of hydrolysed wheat gluten, 7.5% and 15% (diet HWG 7.5 and HWG 15) were included in expense of soy protein concentrate, in the same formulation as a plant protein control diet (diet PPC). Proximate composition of all diets was carried out prior to the start of experimental feeding as described in section 2.8. Diet formulation and proximate composition are displayed in in Table 3.1a.

**Table 3.1a.** Dietary formulation and proximate composition (%).

	Diets				
	PPC	VWG 7.5	VWG 15	HWG 7.5	HWG 15
<b>Ingredient (g / Kg)</b>					
Herring Meal <sup>5</sup>	10	10	10	10	10
Soya protein concentrate <sup>1</sup>	51.86	42.68	33.5	42.04	32.22
Soyabean meal <sup>7</sup>	10	10	10	10	10
Hydrolysed wheat gluten <sup>3</sup>	-	-	-	7.5	15
Vital wheat gluten <sup>4</sup>	-	7.5	15	-	-
Corn Starch <sup>6</sup>	7.76	9.92	12.08	10.11	12.45
Fish oil <sup>2</sup>	16.49	16.11	15.72	16.54	16.58
L-Lysine HCL <sup>6</sup>	1.85	1.76	1.66	1.78	1.71
Calcium carbonate <sup>8</sup>	1	1	1	1	1
Vitamin mineral Premix <sup>9</sup>	0.5	0.5	0.5	0.5	0.5
CMC-binder <sup>6</sup>	0.5	0.5	0.5	0.5	0.5
Antioxidant Mix <sup>10</sup>	0.03	0.03	0.03	0.03	0.03
<b>Proximate composition (%)</b>					
Moisture	3.98	4.25	4.19	4.00	3.99
Protein	48.00	48.51	48.81	48.21	49.03
Lipid	19.21	19.32	19.11	19.5	19.52
Ash	6.11	5.39	4.96	4.39	4.44
Energy (MJ Kg <sup>-1</sup> )	22.59	22.19	22.49	22.30	22.78

<sup>1</sup> SPC 60 (BioMar, DK); <sup>2</sup> Epanoil (Seven Seas, UK); <sup>3</sup> Merripro® (Tereos Syral, FR); <sup>4</sup> Amytex® (Tereos Syral, FR) <sup>5</sup> LT94 Herring meal (CC Moore, UK); <sup>6</sup> (sigma Aldrich, UK); <sup>7</sup> HP 100 (Hamlet, DK); <sup>8</sup> (Fisher Scientific, USA); <sup>9</sup> PNP Fish: Ash 78.7%, Ca 12.1%, Mg 1.56%, P 0.52%, Cu 0.25 g/kg, Vit. A 1.0 µg/kg, Vit D3 0.1 µg/kg, Vit. E 7 g/kg (Premier Nutrition, UK); <sup>10</sup> Ethoxyquin 0.075 gKg<sup>-1</sup>, BHT 0.05 gKg<sup>-1</sup>, Natural tocopherols 0.2 gKg<sup>-1</sup> (Premier Pet Nutrition, UK).

### *3.2.3a. Sampling*

Two fish per tank were sampled at the end point of the trial for microbiology, and a further two for electron microscopy ( $n = 6$  per analysis). Fish were euthanised with an overdose of MS-222 ( $200 \text{ mgL}^{-1}$ , Pharmaq, UK) for 5 minutes, followed by destruction of the brain. Fish for microbiological analysis were dissected and samples obtained under aseptic conditions. The intestine of the fish was excised post pyloric caeca to the anal vent, visceral fat removed and samples taken from identical areas of the posterior region as described in section 2.6. Digesta for microbiological analysis of allochthonous bacterial community was collected from the entire posterior intestine utilising sterile forceps and collected in PCR clean / sterile micro-centrifuge tubes, before storage at  $-20^{\circ}\text{C}$ .

### *3.2.4a. Microbiological analysis*

DNA extraction and Denaturing gel gradient electrophoresis was carried out on digesta samples as described in sections 2.10.1. and 2.10.2. respectively, on five of the six samples taken per treatment. The sample omitted was selected at random providing a final  $n = 5$  per treatment, allowing all samples to be run on a single DGGE gel, allowing cross treatment comparison.

### *3.2.5a. Scanning electron microscopy*

The posterior intestines of two fish per tank ( $n=6$ ) were sampled for scanning electron microscopy as described in section 2.6. After washing in 1% S-methyl-L-cysteine, phosphate

buffered saline and fixation in 2.5% glutaraldehyde in pH 7.2, 0.1 M sodium cacodylate buffer samples were removed from the fixative and rinsed twice in 0.1 M sodium cacodylate buffer by immersion for 15 minutes. Post washing, samples were alcohol dehydrated through graded ethanol (30%, 50%, 70% and 90%) immersion for 15 minutes in each, followed by 100% ethanol twice for a further 15 minutes each. Samples were then critical point dried (Emitech K850, Kent, UK) utilising ethanol as the intermediate fluid and CO<sub>2</sub> as the transitional fluid. Dried samples were gold sputter coated (Emitech K550, Kent, UK) after being mounted on aluminium stubs using fine silver paint (Ag in methyl isobutylketone). Electron micrographs were taken with a JSM 6610 LV (Jeol, Tokyo, Japan) SEM, and analysed for qualitative gross structure damage and tissue necrosis.

### **3.3a. Results**

#### *3.3.1a. Gross observations*

Over the course of the two weeks the fish accepted the diets well, indicating no detrimental effect of wheat gluten sources on palatability. Survivability was unaffected by dietary treatment over the course of the short feeding trial.

#### *3.3.2a. Intestinal microbiology*

##### *3.3.2.1a. PCR-DGGE*

Amplified V3 16S rRNA PCR-DGGE fingerprints and associated dendrograms from the allochthonous (digesta) microbiota extracted from the posterior intestine are presented in Figure 3.1a. Ecological parameters and pairwise comparisons are presented in Table 3.2a.

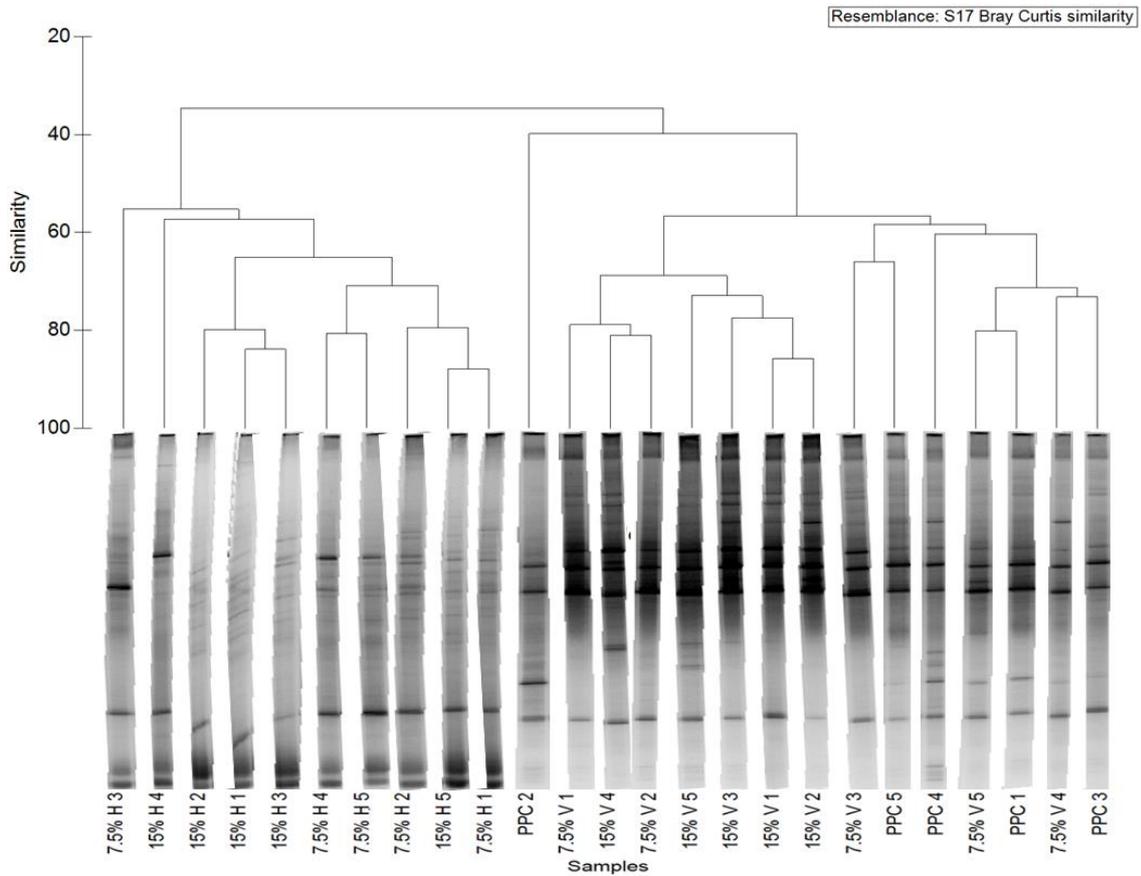
The denaturing gradient gel revealed a total of 48 distinct OTU's within the digesta of the sampled fish. Comparison of the bacterial communities from the fish fed the experimental diets against those of the plant protein control revealed a relatively low level of similarity (29.98 – 55.83%) (Permanova), decreasing with increasing wheat gluten inclusion levels in each treatment as well as the type of wheat gluten. The communities from fish fed the vital wheat gluten diets were more similar to those of the PPC diets than the hydrolysed wheat gluten diets, as can be seen with clustering in Figure 3.1a. Two distinct clusters can be observed from the cluster analysis (Figure 3.1a). Firstly a cluster of approximately 55% similarity containing the sample replicates from fish fed the HWG 7.5 and HWG 15 diets. The second cluster contained the sample replicates from the VWG 7.5 and VWG 15 diets and the PPC diet, clustering with a similarity of approximately 40%. The vital wheat gluten treatments clustered with approximately 60% similarity within the second cluster. All treatments, bar 7.5% vital wheat gluten and PPC treatments were significantly different from one another ( $P < 0.05$ ). Intra-treatment similarity (SIMPER) revealed 15% vital wheat gluten treatment samples to have significantly higher level of similarity ( $76.21 \pm 4.84$ ) than the 7.5% vital wheat gluten and PPC treatments ( $66.93 \pm 11.26$  and  $69.12 \pm 11.75$ , respectively) ( $P < 0.05$ ).

Compared to the PPC, the ecological parameters and OTU number were unaffected by the inclusion of wheat gluten. Bacterial diversity significantly decreased from  $2.84 \pm 0.19$  in the PPC diet to  $2.61 \pm 0.13$  in the 15% hydrolysed post two week feeding ( $P < 0.05$ ). Further significance were observed between wheat gluten treatments with diversity increasing with increasing vital wheat gluten inclusion from  $2.64 \pm 0.12$  in the 7.5% wheat gluten treatment to  $2.88 \pm 0.19$  in the 15% vital wheat gluten treatment ( $P < 0.05$ ). Diversity was significantly

reduced in the 15% hydrolysed treatment compared to the 15% vital wheat gluten treatment ( $2.61 \pm 0.13$  and  $2.88 \pm 0.19$ , respectively) ( $P < 0.05$ ).

### 3.3.2.2a. DGGE sequence analysis

A total of 24 OTU's were excised from the PCR-DGGE gel for sequence analysis (Figure 3.2a, Table 3.3a). Not all excised bands produced sequences of sufficient quality for taxonomic identification. The soy protein control and vital wheat gluten diets visually show more resemblance to each other than to the hydrolysed diet fingerprint. OTU's 2 (*Enterococcus faecium*), 4 (unidentified bacterial with 86% similarity to *Macrococcus caseolyticus*), 10 (unidentified bacterium with 86% similarity to *E. faecium*) 12 (*Enterococcus durans*), 20 (uncultured bacterium) and 24 (uncultured bacterium) show more prevalence in the SPC and vital wheat gluten diets than in the hydrolysed treatments. OTU's 7 (Uncultured *Clostridiales* bacterium) and 13 (*Lactobacillus mali*) were observed across all treatments. OTU's 1 (uncultured bacterium), 21 (uncultured bacterium) and 8 (unidentified bacterium with 89% similarity to *B. coagulans*) were predominantly detected in the hydrolysed wheat gluten treatments. The presence / absence of the sequenced OTU's in each replicate of each of the dietary treatment is presented in Table 3.3a.



**Figure 3.1a** PCR–DGGE fingerprint profiles with cluster analysis dendrogram representing relatedness of microbial communities of the posterior intestinal digesta of rainbow trout fed experimental diets for 2 weeks. DGGE fingerprints represent amplified V3 region of the corresponding samples used in the dendrogram. Sample codes are PPC = PPC treatment, 7.5% V = VWG 7.5 treatment, 15% V = VWG 15 treatment, 7.5% H = HWG 7.5 treatment and 15% H = HWG 15 treatment. Numbers 1-5 post sample code indicate treatment replicate number.

## Chapter 3

**Table 3.2a.** Allochthonous microbial community analysis from the PCR-DGGE of the bacterial communities in the posterior intestine of Rainbow trout fed experimental diets for 2 weeks. ANOVA + *post hoc* Tukey's, superscripts denote significance. Significance accepted at  $P < 0.05$ . Values expressed as means  $\pm$  standard deviation.

	Ecological parameters			PERMANOVA				
	OTUs	Richness†	Diversity‡	SIMPER (Similarity%)	df	f	$p$ (perm)	Similarity (%)
PPC	18.4 $\pm$ 3.78	1.65 $\pm$ 0.33	2.84 $\pm$ 0.19 <sup>ab</sup>	69.12 $\pm$ 11.75 <sup>b</sup>				
VWG 7.5	15.0 $\pm$ 2.00	1.33 $\pm$ 0.18	2.64 $\pm$ 0.12 <sup>bc</sup>	66.93 $\pm$ 11.26 <sup>b</sup>				
VWG 15	18.8 $\pm$ 3.63	1.63 $\pm$ 0.31	2.88 $\pm$ 0.19 <sup>a</sup>	76.21 $\pm$ 4.84 <sup>a</sup>				
HWG 7.5	15.8 $\pm$ 2.17	1.43 $\pm$ 0.19	2.72 $\pm$ 0.14 <sup>abc</sup>	61.67 $\pm$ 11.09 <sup>ab</sup>				
HWG 15	14.6 $\pm$ 1.82	1.35 $\pm$ 0.16	2.61 $\pm$ 0.13 <sup>c</sup>	57.62 $\pm$ 13.27 <sup>ab</sup>				
Treatment					4	9.187	0.001	
Residuals					20			
Total					24			
Pairwise comparisons						t		
PPC vs VWG 7.5					8	1.403	0.09	55.83
PPC vs VWG 15					8	2.383	0.01	51.11*
PPC vs HWG 7.5					8	2.788	0.01	40.21*
PPC vs HWG 15					8	3.445	0.01	29.98*
VWG 7.5 vs VWG 15					8	1.718	0.02	62.27*
VWG 7.5 vs HWG 7.5					8	3.094	0.01	40.57*
VWG 7.5 vs HWG 15					8	3.709	0.01	31.3*
VWG 15 vs HWG 7.5					8	4.409	0.01	36.89*
VWG 15 vs HWG 15					8	5.010	0.01	28.87*
HWG 7.5 vs HWG 15					8	1.581	0.03	62.59*

SIMPER = similarity percentage within replicates of each treatment; PERMANOVA = analysis of similarities between treatments.

†Margalef species richness:  $d = (S - 1)/\log(n)$ .

‡Shannon's diversity index:  $H' = -\text{SUM}(p_i \cdot \log(p_i))$ .

<sup>abc</sup> Superscript letters denote significant differences accepted at  $P < 0.05$

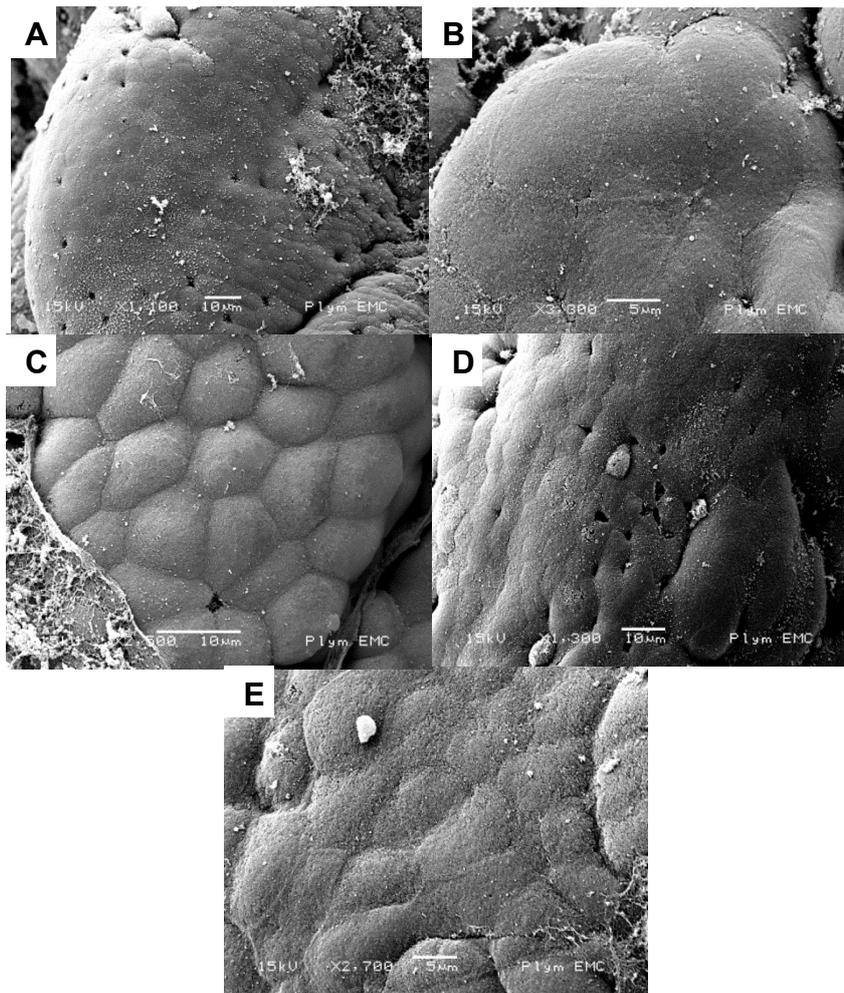
\* Indicates significance between individual pairwise comparisons

Table 3.3a. Closest bacterial relatives (% similarity) of excised and sequenced bands from the PCR-DGGE of rainbow trout digesta samples from the posterior intestine post 2 week feeding of experimental diets. Presence absence of bands within treatment replicates is indicated in column 2-6. Numbers represent bands present in number of replicates. 0 = not present in any replicate, 5 = present in all five treatment replicates.

Band ID	Band presence					Phylum	Nearest neighbour	Alignment similarity (%)	Accession number
	Replicates								
	PPC	VWG 7.5	VWH 15	HWG 7.5	HWG 15				
1	1	0	2	5	5	-	Uncultured bacterium	100	HM216400.1
2	5	5	5	5	5	Firmicutes	<i>Enterococcus faecium</i>	100	HG798652.1
4	5	5	5	5	2	Firmicutes	<i>Macrococcus caseolyticus</i>	86	KC969082.1
7	5	5	5	5	5	Firmicutes	<i>Uncultured Clostridiales bacterium</i>	100	KF506177.1
8	0	0	0	5	5	Firmicutes	<i>Bacillus coagulans</i>	89	KF611995.1
10	4	3	5	0	0	Firmicutes	<i>Enterococcus faecium</i>	86	GU200735.1
11	4	4	5	0	1	Firmicutes	<i>Uncultured Enterococcus sp.</i>	86	HM053734.1
12	4	3	5	0	4	Firmicutes	<i>Enterococcus durans</i>	100	JN815032.1
13	2	4	4	4	5	Firmicutes	<i>Lactobacillus mali</i>	100	AB690200.1
14	0	2	3	3	2	-	Uncultured bacterium	100	EU661258.1
15	1	2	1	3	1	-	Uncultured bacterium	100	JQ191795.1
19	5	3	0	0	0	Firmicutes	<i>Bacillus subtilis</i>	90	HQ236077.1
20	2	1	1	5	5	-	Uncultured bacterium	100	HQ395801.1
21	4	1	3	2	0	-	Uncultured bacterium	100	AB366781.1
24	2	1	1	0	0	-	Uncultured bacterium	73	FJ162911.1

### *3.3.3a. Electron microscopy*

Analysis of the gross structure of the posterior intestine utilising qualitative low magnification scanning electron microscopy revealed the intestine of SPC fed fish to look healthy with uniform enterocytes and densely packed microvilli (Figure 3.3a). Vital wheat gluten treatments also resulted in a healthy ultra-structure with no observable areas of necrosis or signs of enteritis. Hydrolysed wheat gluten treatments showed similar uniform ultra-structure, with no signs of necrosis, malformed or less dense microvilli or increased intercellular spaces between enterocytes.



**Figure 3.2a.** SEM images of posterior intestine post two week short exposure to experimental diets. A. SPC, scale bar represents 10  $\mu\text{m}$ . B. VWG 7.5, scale bar represents 5  $\mu\text{m}$ . C. VWG 15, scale bar represents 10  $\mu\text{m}$ . D. HWG 7.5, scale bar represents 10  $\mu\text{m}$ . E. HWG 15, scale bar represents 5  $\mu\text{m}$ .

### 3.4a. Discussion

The present study utilised microbiological and scanning electron microscopy to assess the initial impacts of vital and hydrolysed wheat gluten on the gross structure and intestinal microbiota of juvenile rainbow trout fed experimental diets for two weeks.

It has long been established that the addition of ingredients into aquafeeds has the ability to modulate the gut microbiota. Soya based proteins have been utilised heavily as alternative proteins for fishmeal replacement and the decreased reliance of the aquaculture industry on wild caught fishmeal. As such, soy proteins and protein concentrates have been the focus of many previous investigations into their effect and modulation of the intestinal microbiota of salmonids (Heikkinen et al., 2006; Merrifield et al., 2009; Green et al., 2013; Reveco et al., 2014). The current literature on the effects of wheat gluten inclusion on the gut microbiota is very limited, with no investigations into the effects on rainbow trout. The quantification of the change in intestinal microbiota caused by dietary treatments is complicated by individual animal variation. Despite genetically similar animals being reared in production environments, with the same external environments and dietary regime, considerable variation is observed on an animal-to-animal basis in both aquatic and terrestrial species (Hill et al., 2005; Heikkinen et al., 2006; Mansfield et al., 2010). The increased use of culture independent analysis methods and individual fish as sample replicates, instead of historical pooling of samples, is creating a more robust picture of the intestinal microbiology.

The PCR-DGGE analysis of the allochthonous bacterial community of the posterior intestine reveal that the inclusion of both vital and hydrolysed wheat glutens have a modulatory effect in juvenile rainbow trout, when compared to the plant protein control treatment.

Two distinct clusters can be observed from the cluster analysis (Figure 3.1a), Firstly a cluster of sample replicates from fish fed 7.5% and 15% hydrolysed wheat gluten diets. The second cluster contained the sample replicates from the 7.5% and 15% vital wheat gluten diets and the PPC. The clustering of replicates from similarly formulated dietary treatments indicates a dietary effect on the microbiota. The clustering of similar dietary regimes is not uncommon in fish fed plant based diets and has been reported by Desai *et al.* (2012), Green *et al.* (2013) and Apper *et al.* (2016). The ecological parameters revealed the number of OTU's present and the richness in the allochthonous bacteria was not affected by dietary treatment. Species diversity was, however, elevated in the 15% vital wheat gluten treatment compared to the 7.5% vital and 15% hydrolysed wheat gluten treatments. The 15% hydrolysed treatment community diversity was also significantly reduced compared to the plant protein control. This elevation in species diversity is contradictory to ecological parameters seen in Asian sea bass fed a 6% HWG diet compared to a plant protein control (Apper *et al.*, 2016). However, the increase in bacterial diversity could be considered advantageous, providing more competition and resistance to opportunistic or invading pathogens entering the GI tract through feed or the environmental water (Apper *et al.*, 2016).

Replicate similarity within treatments (SIMPER) was also significantly elevated in the 15% vital wheat gluten treatment compared to the 7.5% vital and PPC treatments, and numerically higher than the hydrolysed wheat gluten treatments, suggesting reduced intra-individual dietary based variation. The pairwise comparisons of the banding profiles and intensities of treatments reveal only the 7.5% vital what gluten and the PPC treatments to

have similar PCR-DGGE fingerprints. All other treatments showed significant differences between one another (Table 3.2a).

PCR-DGGE band sanger sequencing from this preliminary investigation revealed a dominance of Firmicutes in the samples which returned sequences with sufficient quality for taxonomic identification (Table 3.3a). This is not unexpected and prior investigations have reported Firmicutes as a major contributor to the intestinal microbiota, as well as Proteobacteria (Huber et al., 2004; Heikkinen et al., 2006; Pond et al., 2006; Mansfield et al., 2010; Navarrete et al., 2010a). Although not conclusive or representative of the microbial population of the allochthonous bacteria, the presence/absence of species within treatment replicates can be assumed a dietary effect. Sequences aligned to the *Enterococcus*, *Macrococcus*, *Lactobacillus* genera and family Clostridiales were present in the current investigation across all treatments (Table 3.3a), and have been previously described in the normal intestinal microflora of salmonids (Wong et al., 2013; Al-Hisnawi et al., 2015; Askarian et al., 2012; Ringø and Gatesoupe, 1998; Navarrete et al., 2010a). Sequences aligned (alignment similarity 89%) to *Bacillus coagulans* were present in 100% of the hydrolysed wheat gluten dietary treatments, and none of the PPC or VWG treatment groups. *Bacillus coagulans*, is currently being utilised in the health food and prophylactic market as a probiotic for humans (Hong et al., 2005), as well as showing growth and health benefits in common carp (*Cyprinus carpio*) when fed as a probiotic in concentrations of  $1 \times 10^7$  to  $4 \times 10^7$  cfu/g (Xu et al., 2014). Improved growth performance was also observed by Lin *et al.* (2012) when using *B. coagulans* as a probiotic for koi carp (*Cyprinus carpio koi*). A sequence with 86% similarity to *Enterococcus faecium* and another uncultured *Enterococcus* species (alignment similarity 86%) were predominantly present in the PPC and vital wheat gluten

treatments (Table 3.3a). *Enterococcus* spp., Gram positive lactic acid bacteria, have been well established as part of the commensal intestinal bacteria of rainbow trout, and members of *Enterococcus* genera are considered potential probiotics in a range of fish species (Del'Duca et al., 2013; Swain et al., 2009; Wong et al., 2013; Merrifield, 2014; Ringø and Gatesoupe, 1998; Mansfield et al., 2010).

The inclusion of wheat gluten into experimental diets in this study revealed no detrimental effects on the intestinal morphology at a qualitative level. These findings are not surprising due to the low ANF content compared to soy proteins which it is replacing (Apper-Bossard et al., 2013). The high glutamine content of wheat gluten may also contribute to this effect. Glutamine, used as a major substrate by highly proliferating enterocytes of the intestine (Trichet, 2010), has been shown to improve the intestinal morphology of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) and channel catfish (*Ictalurus punctatus*) (Pohlenz et al., 2012; Cheng et al., 2012). This effect has also been observed in broilers fed hydrolysed wheat gluten as a partial soy protein replacement (van Leeuwen et al., 2004).

### **3.5a. Conclusion**

The inclusion of wheat gluten in aquafeeds at the expense of soy protein for rainbow trout had no obvious adverse effects on qualitative observations of the intestinal morphology at the end of a two week feeding period, likely due to high levels of glutamine and low levels of ANFs. Further quantitative analysis of the intestinal histology and morphology is required to assess the effects of dietary wheat gluten inclusions.

The allochthonous microbial community OTU numbers or richness were unaffected by dietary inclusions of wheat gluten. Microbial diversity was only significantly decreased in the 15% hydrolysed treatment compared to the PPC diet. Observations of species differences between experimental treatments, and the clustering of the hydrolysed treatments apart from the PPC and VWG treatments would suggest a degree of diet caused modulation. All treatments maintained an approximately 35% similarity to one another, and with the relatively low resolution of the intestinal microbiota analysis achievable with PCR-DGGE compared with high throughput sequencing, it cannot be disregarded that the overall allochthonous community are resistant to diet based variation as observed by Wong *et al.* (2013). Indeed, all sequenced OTUs were members of the Firmicutes, regardless of treatment abundances. Further work utilising higher resolution microbial analysis techniques must be carried out to give a more in depth view of the effect of wheat gluten on the allochthonous bacterial community of rainbow trout.

From the results observed in this preliminary experiment, further, longer term trials utilising a multi-disciplinary and higher resolution approach will enable further insight into the potential role of wheat glutens in the replacement of fishmeal and soy proteins in aquafeeds.

**Chapter 3b: The effect of dietary wheat gluten products on gut health, allochthonous intestinal microbial population and growth performance of juvenile rainbow trout (*Oncorhynchus mykiss*).**

**3.1b. Introduction**

As discussed in section 3.5a, diets containing wheat gluten up to inclusion levels of 15% caused significant, but non detrimental, modifications in the intestinal microflora of rainbow trout. There were no observed detrimental impacts on the gross structure or morphology of the intestinal enterocytes at a qualitative level, and palatability of the diets was good. It is not however, clear if the microbial modulations persist for longer periods, nor is it clear of the lack of detrimental impacts on intestinal health continue after longer exposure, or if inflammatory responses are manifested at the molecular level. Therefore, a second, full scale, feed trial was conducted to investigate the gastrointestinal tract health and growth performance utilising a multidisciplinary approach.

The expression of genes in the intestine can be utilised as an indicator of gut health and immunological status (Mulder et al., 2007). To the authors knowledge, the effect of wheat gluten on the expression of inflammatory cytokines, stress biomarkers and cell proliferation associated genes have not yet been reported for juvenile rainbow trout fed dietary wheat glutes. These analyses have the capability to reveal the effect of wheat gluten, and their metabolites, on intestinal health. Specific beneficial amino acids and their metabolites have considerable potential for health benefits via a number of mechanisms (Li et al., 2009). High throughput sequence analysis will build on the PCR-DGGE observations in Chapter 3a,

providing a higher resolution and thus further insight into the effects of wheat glutes on the gut microbiota of rainbow trout.

The effect of the type of wheat gluten will also be more intensively analysed. As observed in chapter 3a, hydrolysed and vital wheat glutes can have differing effects on the intestinal microbiota. Building on this, the current chapter will include the analysis of three types of wheat gluten: an un-processed (vital) wheat gluten and two variations of hydrolysed wheat gluten. A hydrolysed wheat gluten with the soluble portion of the protein removed (hydrolysed) and a soluble hydrolysed wheat gluten, where the soluble portion of the protein remains (soluble). The use of proteolytic enzymes is a well known and efficient method of protein modification (Adler-Nissen, 1986). Through controlling reaction conditions during the hydrolysis process, specific hydrolysate characteristics can be achieved. Vital wheat gluten, insoluble at neutral pH, can be hydrolysed to enhance foaming and emulsifying properties and importantly its solubility at varying pH's (pH 2 to pH 12) (Kong et al., 2007; Popineau et al., 2002; Mimouni et al., 1994). The hydrolysis process will not only alter the properties of the wheat gluten as a pellet binder, it is hypothesised that the resulting low molecular weight peptides produced are more readily absorbed in the intestine, without the need for digestion in the stomach, in turn making the protein more available to the fish (Tello et al., 1994).

The objectives of this investigation are to assess the impacts of varying types of wheat gluten on the growth performance and intestinal health of juvenile rainbow trout. A multidisciplinary approach will achieve a holistic view of the promising alternative protein sources. High throughput sequence analysis will enable high resolution observations of the

potential impacts of wheat gluten on the allochthonous bacterial population. This study also aims to assess the impact of dietary wheat gluten on the intestinal immune status through the analysis of the relative gene expression of the anti-inflammatory cytokines interleukin 10 (IL 10) and transforming growth factor beta (TGF  $\beta$ ) and the pro-inflammatory cytokines interleukin 8 (IL 8) and tumour necrosis factor alpha (TNF  $\alpha$ ). As a measure of antioxidant status and cellular stress level, the gene expression of Glutathione S-transferase (Glute ST) and heat shock protein 70 (HSP 70) were also assessed. Microscopic and histological techniques were utilised to assess potential impacts on intestinal morphology.

### **3.2b. Materials and methods**

All experimental work involving fish was conducted under the UK Home Office project licence PPL 30/2644 and was in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Plymouth University Ethical Committee.

#### *3.2.1b. Experimental design*

Four hundred and sixty five rainbow trout (XXX triploid genotype and wild phenotype) were obtained from Exmoor Fisheries (Somerset, UK). After a two week acclimation period, at the Aquatic Animal Nutrition and Health Research Facility at the university of Plymouth, the fish were graded and randomly distribute into 15, 120L fibreglass tanks (31 fish per tank; average weight =  $24.80 \pm 0.31\text{g}$ ) in a 7,000 litre closed recirculation system. Over the course of a 66 day nutritional feed trial, varying levels of vital (comprising 10% and 20% of the diet), hydrolysed (10%) and soluble (10%) wheat gluten meals were fed to rainbow trout at the expense of soy protein concentrate, along with a soy protein control. Dietary treatments

were randomly attributed to triplicate tanks, and fed at a rate of 1.5 - 2.5% of biomass per day in equal rations at 09:00, 13:00 and 17:00 daily. Feed was adjusted daily on a predicted FCR of 1.2, based on initial biomass weights, and subsequent bi-weekly tank biomass weighing data. Rainbow trout were maintained at  $15 \pm 1^{\circ}\text{C}$  with a 12:12 light dark photoperiod. pH was maintained at  $7.0 \pm 0.5$  and  $>85\%$  dissolved oxygen. Temperature, pH and dissolved oxygen were monitored daily. Water ammonia, nitrite and nitrate were monitored weekly and maintained within the acceptable range for the species, and managed with water changes to negate any detrimental build-up of compounds. At the trial end point, samples were taken for carcass composition, haemato-immunology, histology, molecular gene expression and microbiological analysis.

### *3.2.2b. Experimental diets*

Five experimental diets were formulated and manufactured at the University of Plymouth as described in section 2.3. The experimental wheat gluten products, vital wheat gluten (Amytex<sup>®</sup>), hydrolysed wheat gluten (Merripro<sup>®</sup>) and hydrolysed soluble wheat gluten (Solpro<sup>®</sup>) were supplied by Tereos Syral (Marckolsheim, France). Two inclusion levels of vital wheat gluten, 10% and 20% (diet 10% Vital and 20% Vital, respectively) and two inclusion levels of hydrolysed wheat glutens at 10% (diets 10% Hydro and 10% Sol, respectively) were formulated with wheat gluten incorporated at the expense of soy protein concentrate, in the same formulation as a soya protein control diet (diet SPC). Proximate composition of all diets was carried out prior to the start of experimental feeding as described in section 2.8. All diets were iso-nitrogenous and iso-lipidic. Diet formulation and proximate composition can be seen in Table 3.1b.

**Table 3.1b.** Dietary formulation and proximate composition (%).

	Diets				
	SPC	10% Vital	20% Vital	10% Hydro	10% Sol
<b>Ingredient (g / Kg)</b>					
Herring meal <sup>7</sup>	10	10	10	10	10
Soya protein concentrate <sup>1</sup>	52.00	39.62	27.38	38.77	38.49
Soyabean meal <sup>8</sup>	10	10	10	10	10
Hydrolysed wheat gluten <sup>3</sup>	-	-	-	10	-
Vital wheat gluten <sup>4</sup>	-	10	20	-	-
Soluble wheat gluten <sup>5</sup>	-	-	-	-	10
Corn starch <sup>6</sup>	7.63	10.65	13.53	10.89	11.33
Fish Oil <sup>2</sup>	16.49	15.98	15.47	16.55	16.46
L-Lysine HCl <sup>6</sup>	1.85	1.72	1.59	1.76	1.69
Calcium carbonate <sup>9</sup>	1	1	1	1	1
Vitamin mineral premix <sup>10</sup>	0.5	0.5	0.5	0.5	0.5
CMC-Binder <sup>6</sup>	0.5	0.5	0.5	0.5	0.5
Antioxidant mix <sup>11</sup>	0.03	0.03	0.03	0.03	0.03
<b>Proximate composition (%)</b>					
Moisture	4.36	4.33	4.2	4.14	3.96
Protein	48.04	48.51	48.97	48.41	49.08
Lipid interluken	19.56	19.47	19.09	19.51	19.55
Ash	6.11	5.45	4.89	4.49	4.38
Energy (MJ/Kg)	22.55	22.06	22.52	22.7	22.81

<sup>1</sup> SPC 60 (BioMar, DK); <sup>2</sup> Epanoil (Seven Seas, UK); <sup>3</sup> Merripro® (Tereos Syral, FR); <sup>4</sup> Amytex®(Tereos Syral, FR) <sup>5</sup> Solpro®(Tereos Syral, FR) <sup>7</sup> LT94 Herring meal (CC Moore, UK); <sup>6</sup> (sigma Aldrich, UK); <sup>8</sup> HP 100 (Hamlet, DK); <sup>9</sup> (Fisher Scientific, USA); <sup>10</sup> PNP Fish: Ash 78.7%, Ca 12.1%, Mg 1.56%, P 0.52%, Cu 0.25 g/kg, Vit. A 1.0 µg/kg, Vit D3 0.1 µg/kg, Vit. E 7 g/kg (Premier Nutrition, UK); <sup>11</sup> Ethoxyquin 0.075 gKg<sup>-1</sup>, BHT 0.05 gKg<sup>-1</sup>, Natural tocopherols 0.2 gKg<sup>-1</sup> (Premier Pet Nutrition, UK).

### *3.2.3b. Sampling*

Throughout the course of the feeding trial, tank biomass was in bulk weighed bi-weekly, and prior to end point sampling to allow the calculation of growth performance as described in section 2.5.

At the end point of the 66 day feed trial, two fish per tank were euthanised and samples taken for intestinal microbiota analysis. A further two fish per tank were euthanised and samples taken for haematology, microscopy and gene expression ( $n = 6$  per treatment, per analyses). Two fish per tank were also euthanised for analysis of carcass composition. Fish were euthanised via concussion followed by destruction of the brain, in accordance with the schedule one procedure of the Animals (Scientific Procedures) Act 1986. Fish for microbiological analysis were dissected and samples obtained under aseptic conditions. The intestine of the fish were excised post pyloric caeca to the anal vent, visceral fat removed and samples taken from identical areas of the posterior region as described in section 2.6.. Digesta for microbiological analysis of allochthonous bacterial community was collected from the entire posterior intestine under aseptic conditions utilising sterile forceps and collected in PCR clean / sterile microcentrifuge tubes, before storage at  $-20^{\circ}\text{C}$ .

### *3.2.4b. Proximate composition*

Proximate composition of diets and carcasses was carried out as described in section 2.8.

### *3.2.5b. Haematological and serological analysis*

Blood was taken from the caudal vein of three fish per tank at the conclusion of the feed trial, achieving an  $n = 6$  per treatment. Hematological and serological analysis was carried out as described in section 2.9.

### *3.2.6b. High throughput sequencing*

Samples of posterior intestine digesta for high throughput sequence analysis were taken aseptically from 2 fish per tank, providing  $n=6$  per treatment. DNA was extracted from 100 mg of sample as described in section 2.10.1. High throughput sequencing was carried out as described in section 2.10.4.

### *3.2.7.b Scanning electron microscopy*

The posterior intestine of 2 fish per tank ( $n=6$ ) were sampled for scanning electron microscopy as described in section 2.6. Sample preparation and imaging was conducted as described in section 3.2.5a. for qualitative gross structure damage, tissue necrosis and quantitative microvilli density.

Microvilli density (arbitrary units; AU) was calculated utilising ImageJ 1.45. Electron micrographs of microvilli taken at x20, 000 magnifications were threshold adjusted to a consistent point, and a ratio of background (gaps between microvilli) and foreground (microvilli) was calculated, achieving a density unit.

### *3.2.8b. Light microscopy*

Light microscopy of the posterior intestine was carried out on two fish per tank (n = 6) as described in section 2.11.

### *3.2.9b. Gene expression*

#### *3.2.9.1b. RNA extraction, cDNA synthesis and real-time PCR*

Two fish per tank were sampled for gene expression analysis. RNA extraction, cDNA synthesis and real-time PCR were carried out as described in sections 2.10.5 and 2.10.6.

#### *3.2.9.2b. Reference genes, genes of interest and analysis.*

Beta-actin ( $\beta$ -actin) and elongation factor 1-alpha (EF1- $\alpha$ ) were utilised as reference genes, as described in section 2.10.6. The absence of primer-dimers, amplification products in negative controls and acceptable primer efficiencies were insured prior to and whilst running samples.

Genes of interest, Primers and their sequences can be found in Table 3.2b. Expression analysis was carried out as described in section 2.10.6.

**Table 3.2b.** Primer information used for real-time PCR analysis.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Amplicon size (bp)	Acc. No	E-value	Annealing temp
$\beta$ actin	ACTGGGACGACATGGAGAAG	CCACCCTCAGCTCGTTGTAG	57	AJ438158.1	2.0	60
EF1- $\alpha$	AGGCTCCATCTTGGCTTCTC	GGGACCAGACTCGTCGTACT	76	AF498320.1	2.1	60
IL-8	CGGAGAGCAGACGTATTGGTAA	GAGCTGGGAGGGAACATCTC	58	HG917307.1	1.9	60
TNF- $\alpha$	TGGGTGTGAGTGACATCGTTAT	AGACCCTCAGCATCTGGTACT	87	HE717002.1	2.1	60
Glute-ST	CCTTCTCATTGGCTGACGTTAT	GCCGTAGACAGCCCAAAG	70	NM_001160559.1	2.0	58
IL-10	GCTGGACGAAGGGATTCTACA	GCACCGTGTGAGATAGAACTT	89	NM_001245099.1	2.1	60
TGF- $\beta$	TGCCTTGTGATTGTGGGAAAC	CCTCAGCTTGTTTCATCCCTGAT	68	AJ007836.1	2.1	60
HSP70	TTGGCCGCAGGTTTGATGAT	CTTCAAAGGGCCAATGCTTCAT	60	K02550.1	2.1	60

### 3.2.10b. Statistical analysis

Statistical analysis was carried out as described in section 2.12.

### **3.3b. Results**

#### *3.3.1b. Gross observations*

Over the course of the 66 day feed trial fish accepted the experimental diets well and grew consistently throughout the trial. Survivability was unaffected by dietary treatment.

#### *3.3.2b. Growth performance and carcass composition*

Growth performance was assessed at the end point of the 66 day feed trial, based on the final tank biomass weights. Assessment of growth performance was achieved through FCR, SGR, mean fish weight, PER, K factor and survivability (Table 3.3b). Rainbow trout in all treatments performed well throughout the trial with good appetites, achieving good growth performance with FCR's ranging from  $0.92 \pm 0.01$  in the 20% vital treatment to  $1.10 \pm 0.08$  in the 10% Hydro diet.

FCR was significantly improved ( $P < 0.05$ ) in the 20% Vital treatment compared to the 10% Hydro and 10% Sol diets. The FCRs of the other treatments were not significantly different from one another. Protein efficiency ratio (PER) showed the same trend with a significantly improved PER ( $P < 0.05$ ) in the 20% Vital treatment compared to the 10% Hydro and 10% Sol diets. All other treatment PER's were not significantly different from one another. There was no significant difference between treatments in regards to SGR or mean fish weight at the end point of the trial, despite numerical trends. K-factor and survivability were also unaffected by dietary treatment.

Carcass moisture and protein were unaffected by dietary treatment (table 3.4b). Carcass lipid was significantly decreased in the 10% and 20% vital wheat gluten treatments ( $11.19 \pm 0.47\%$  and  $11.6 \pm 0.11\%$  respectively) compared to the SPC and 10% Hydrolysed treatments ( $12.3 \pm 0.16\%$  and  $13.00 \pm 0.19\%$  respectively). The 10% Vital treatment was also significantly reduced compared to the 10% Soluble treatment ( $12.08 \pm 0.02\%$ ). Carcass ash was not significantly affected by wheat gluten inclusions compared to the SPC treatment, yet the 10% Hydrolysed treatment was significantly greater than the two vital treatments. Carcass energy was significantly elevated in the 10% hydrolysed treatment ( $27.95 \pm 0.19 \text{ MJ kg}^{-1}$ ) compared to all other treatments, whilst the 20% Vital treatment ( $26.68 \pm 0.01 \text{ MJ Kg}^{-1}$ ) was significantly reduced compared to all other treatments (table 3.4b).

### *3.3.3b. Haematology*

Analysis of blood taken for the assessment of haemato-immunological parameters at the conclusion of the feed trial showed no effect of dietary treatment on lysozyme activity or haematocrit (Table 3.5b.). Haemoglobin levels were significantly higher in the 10% Vital treatment compared to the 10% Sol treatment ( $41.186 \pm 5.52 \text{ mg } 100\text{ml}^{-1}$  and  $32.174 \pm 5.58 \text{ mg } 100\text{ml}^{-1}$ , respectively) ( $P < 0.05$ ).

**Table 3.3b.** Growth performance of rainbow trout post 66 day feed trial.  $n = 3$ . Superscripts denote significance. Significance accepted at  $P < 0.05$ .

	SPC	10% Vital	20% Vital	10% Hydro	10% Sol
FCR	0.99±0.03 <sup>ab</sup>	0.97±0.07 <sup>ab</sup>	0.92±0.01 <sup>a</sup>	1.10±0.08 <sup>b</sup>	1.07±0.06 <sup>b</sup>
SGR (%)	1.65±0.08	1.69±0.11	1.76±0.01	1.60±0.06	1.59±0.04
Mean fish weight (end point) (g)	70.24± 3.05	72.89±6.62	78.29±1.91	68.10±1.77	70.50±2.61
Protein efficiency ratio	2.10±0.05 <sup>ab</sup>	2.13±0.14 <sup>ab</sup>	2.22±0.03 <sup>a</sup>	1.89±0.14 <sup>b</sup>	1.91±0.10 <sup>b</sup>
K factor	1.409±0.06	1.421±0.05	1.433±0.10	1.387±0.07	1.406±0.12
survivability (%)	95.83±1.80	95.83±1.80	91.67±1.80	88.54±11.83	89.58±7.22

**Table 3.4b.** Carcass composition of rainbow trout post 66 day feed trial.  $n = 3$ . Superscripts denote significance. Significance accepted at  $P < 0.05$ .

	SPC	10% Vital	20% Vital	10% Hydro	10% Sol
Moisture (%)	69.730±0.22	70.860±0.83	70.281±1.14	69.330±0.50	70.860±0.83
Protein (%)	16.7±0.6	16.81±0.55	16.38±0.43	16.42±0.29	15.59±0.38
Lipid (%)	12.3±0.16 <sup>ab</sup>	11.19±0.47 <sup>d</sup>	11.6±0.11 <sup>cd</sup>	13±0.19 <sup>a</sup>	12.08±0.02 <sup>bc</sup>
Ash (%)	1.61±0.01 <sup>ab</sup>	1.57±0.01 <sup>b</sup>	1.57±0.01 <sup>b</sup>	1.63±0.03 <sup>a</sup>	1.59±0.01 <sup>ab</sup>
Energy (MJ Kg <sup>-1</sup> dry weight)	27.45±0.07 <sup>b</sup>	27.25±0.12 <sup>b</sup>	26.68±0.01 <sup>c</sup>	27.95±0.19 <sup>a</sup>	27.41±0.13 <sup>b</sup>

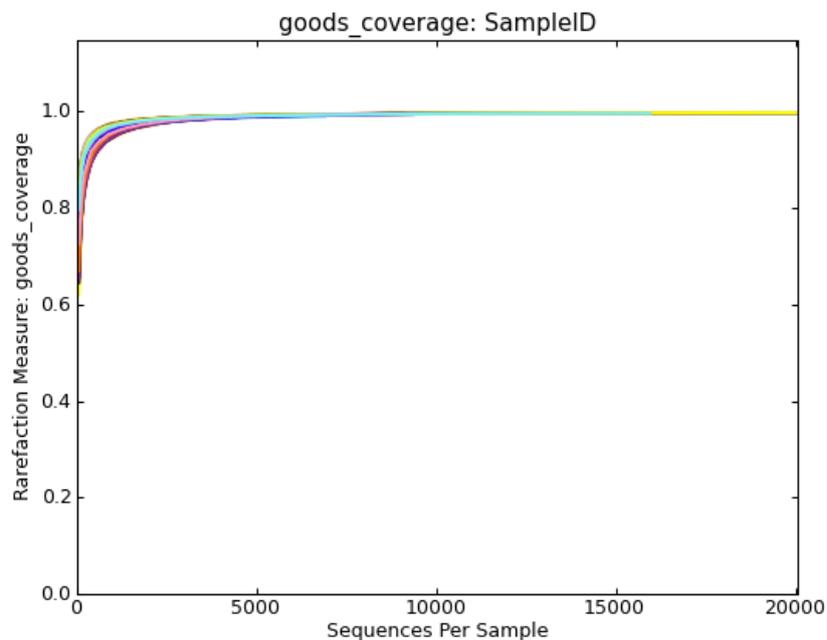
**Table 3.5b.** Haematological and serological parameters of rainbow trout post 66 day feed trial.  $n = 15$ . Superscripts denote significance. Significance accepted at  $P < 0.05$ .

	SPC	10% Vital	20% Vital	10% Hydro	10% Sol
Haemoglobin (mg/100ml)	36.829±8.25 <sup>ab</sup>	41.186±5.52 <sup>a</sup>	37.852±5.15 <sup>ab</sup>	34.007±8.41 <sup>ab</sup>	32.174±5.58 <sup>b</sup>
Haematocrit	44.364±8.88	46.200±5.75	43.333±4.60	41.363±9.45	40.091±7.54
Lysosyme activity	911.9±316.2	843.0±227.1	1026.1±398.6	854.2±221.8	1129.2±595.7

3.3.2b. High-throughput sequencing

Post trimming and quality control, 1,156,465 reads were retained for downstream analysis, identifying 1,064 distinct OTU's. Alpha rarefaction analysis of goods coverage reveals estimations of >0.989 for the total species present per sample. Refraction of Goods coverage plateaued after approx. 5,000 reads per sample (Figure 3.1b.), suggesting that the bacterial communities were fully sampled and data are representative of the population. Alpha diversity parameters can be found in Table 3.6b.

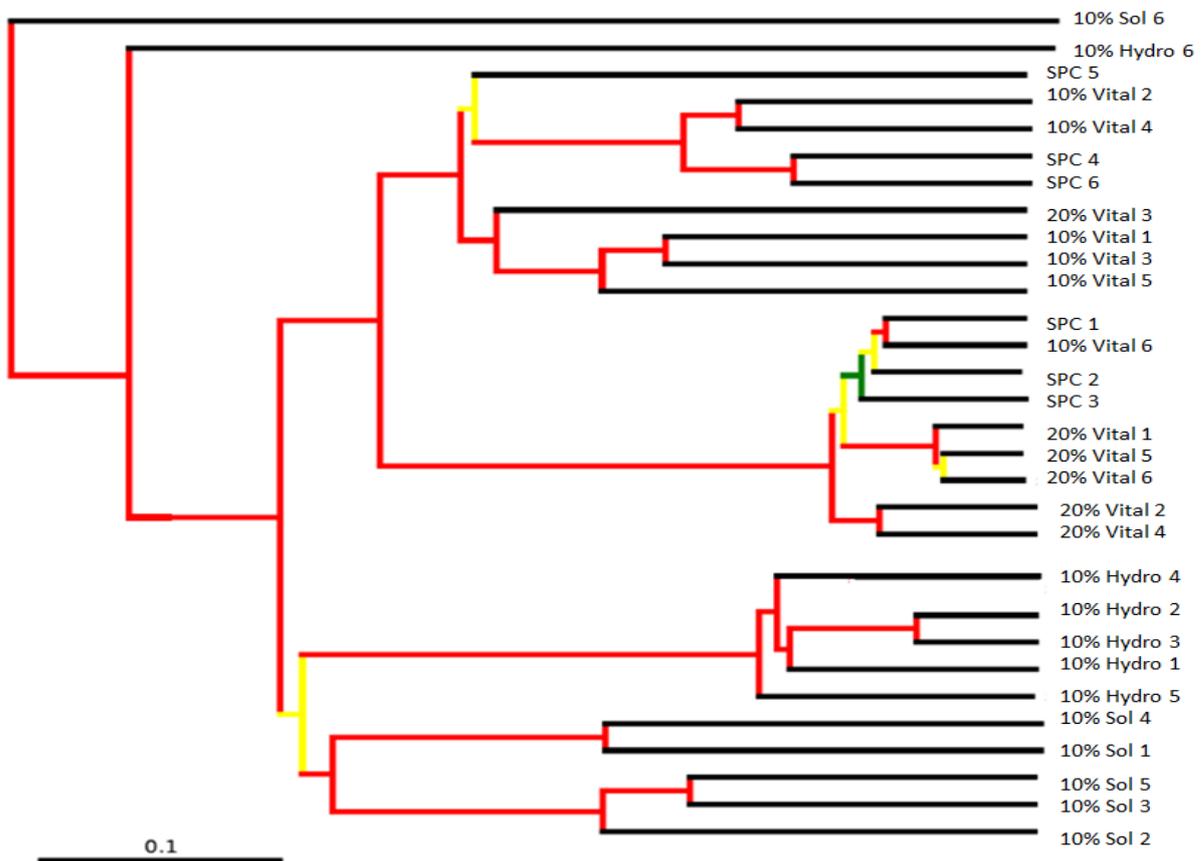
Bray Curtis analysis (figure 3.2b) revealed two main clusters. The first cluster consisting of the hydrolysed treatments (10% Hydro and 10% soluble) and the second cluster of the vital (10% vital and 20% vital) and the SPC treatment. Two samples , 10% soluble replicate #6 and 10% hydro replicate #6, are distinct from both clusters.



**Figure 3.1b.** Alpha rarefaction curves of Goods coverage representing % of total species present within a sample as a function of the sequencing effort.

**Table 3.6b.** High throughput sequencing alpha diversity parameters, goods coverage estimations by treatment and phylogenetic distance of the allochthonous bacterial communities in the posterior intestine of rainbow trout post 66 day feeding trial.

treatment	Goods coverage	Observed species	Chao1 index	Shannon index	Phylogenetic distance
SPC	0.993±0.001 <sup>ab</sup>	627.33±93.87	811.84±71.64	5.24±0.78	22.78±3.11 <sup>ab</sup>
10% Vital	0.993±0.002 <sup>ab</sup>	688.66±93.87	844.53±81.86	5.82±0.66	24.42±3.22 <sup>a</sup>
20% Vital	0.995±0.003 <sup>a</sup>	668±93.87	835±82.97	4.8±0.97	24.02±3 <sup>ab</sup>
10% Hydro	0.99±0.002 <sup>ab</sup>	555.16±93.87	747.06±62.01	5.54±0.67	19.67±1.19 <sup>b</sup>
10% Sol	0.989±0.004 <sup>b</sup>	567.16±93.87	742.67±88.7	5.64±1.26	20.7±2.91 <sup>ab</sup>



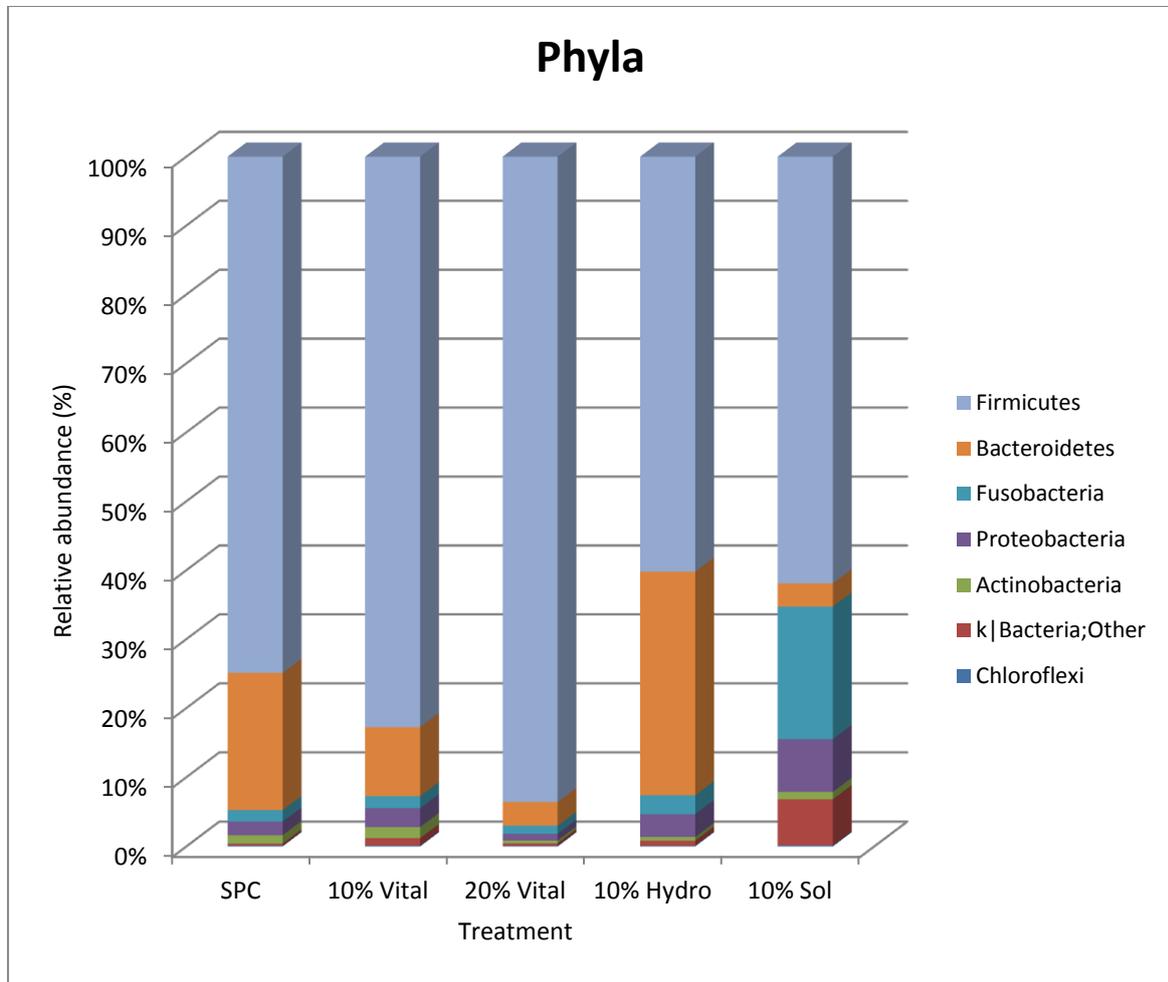
**Figure 3.2b.** Bray-Curtis UPGMA UniFrac clustering of reads from treatment replicates of the allochthonous bacterial communities from the posterior intestine of rainbow trout, post 66 day feeding trial. Jackknife support is: Red (75-100%), yellow (50-75%) and green (25-50%). Scale bar indicates 10% divergence.

The sequence distribution data were dominated by the Firmicutes at phylum level, displayed in Figure 3.3b. The Firmicutes accounted for 76.17% of the total read sequences of all treatments. The Bacteroidetes were the next most dominant phylum (11.16%), followed by the Fusobacteria (4.32%), Proteobacteria (4.08%), Actinobacteria (1.50%), reads from the kingdom bacteria (phylum unknown) (1.50%), and the Chloroflexi (0.82%). Other phyla present in the sample-set, each with fewer than 0.2% of the total reads per phylum, combined accounted for 0.45%. Reads associated with the “kingdom: bacteria”, but of unknown phylum, accounted for an elevated percentage of the treatment reads of fish fed the 10% Sol diet ( $7.27\% \pm 6.61$ ) compared to all other diets. The proportion of all other phyla was unaffected by dietary treatment (Table 3.7b.).

The sequence distribution data at genus level is displayed in Figure 3.4b. The most abundant genus was *Enterococcus*, representing 46.52% of the total reads. *Bacteroides* represented the next most abundant genus (7.65%) followed by *Bacillus* (6.66%), order: Bacteroidales (genus unknown) (3.49%), *Weissella* (3.43%), *Cetobacterium* (3.36%), family: Ruminococcaceae (genus unknown) (2.21%), *Peptostreptococcus* (1.99%) and *Macrococcus* (1.60%). The remaining genera present represent <1.5% of total reads.

*Enterococcus*, the most abundant genus as a percentage of total reads was significantly ( $P < 0.05$ ) elevated in the 20% Vital and SPC fed fish ( $75.36\% \pm 20.17$  and  $54.54\% \pm 21.08$ , respectively) compared to the 10% Hydro and 10% Sol fed fish ( $16.65\% \pm 6.38$  and  $13.74\% \pm 5.12$ , respectively). Fish fed the 10% vital diets had significantly lower proportion of *Enterococcus* ( $38.79\% \pm 19.03$ ) than the 20% vital fed fish ( $P < 0.05$ ), but the abundance was not significantly different from the other treatments ( $P > 0.05$ ). Statistical difference ( $P <$

0.05) between proportions of phyla and genera contributing > 0.2% of total reads in each dietary treatment is displayed in Table 3.7b.



**Figure 3.3b.** Allochthonous bacterial communities in the posterior intestine of rainbow trout post 66 day feeding with experimental diets. Data are represented as bacterial phylum as a proportion of a total, expressed as a percentage. Data excludes phyla with less than 0.2% of the total reads

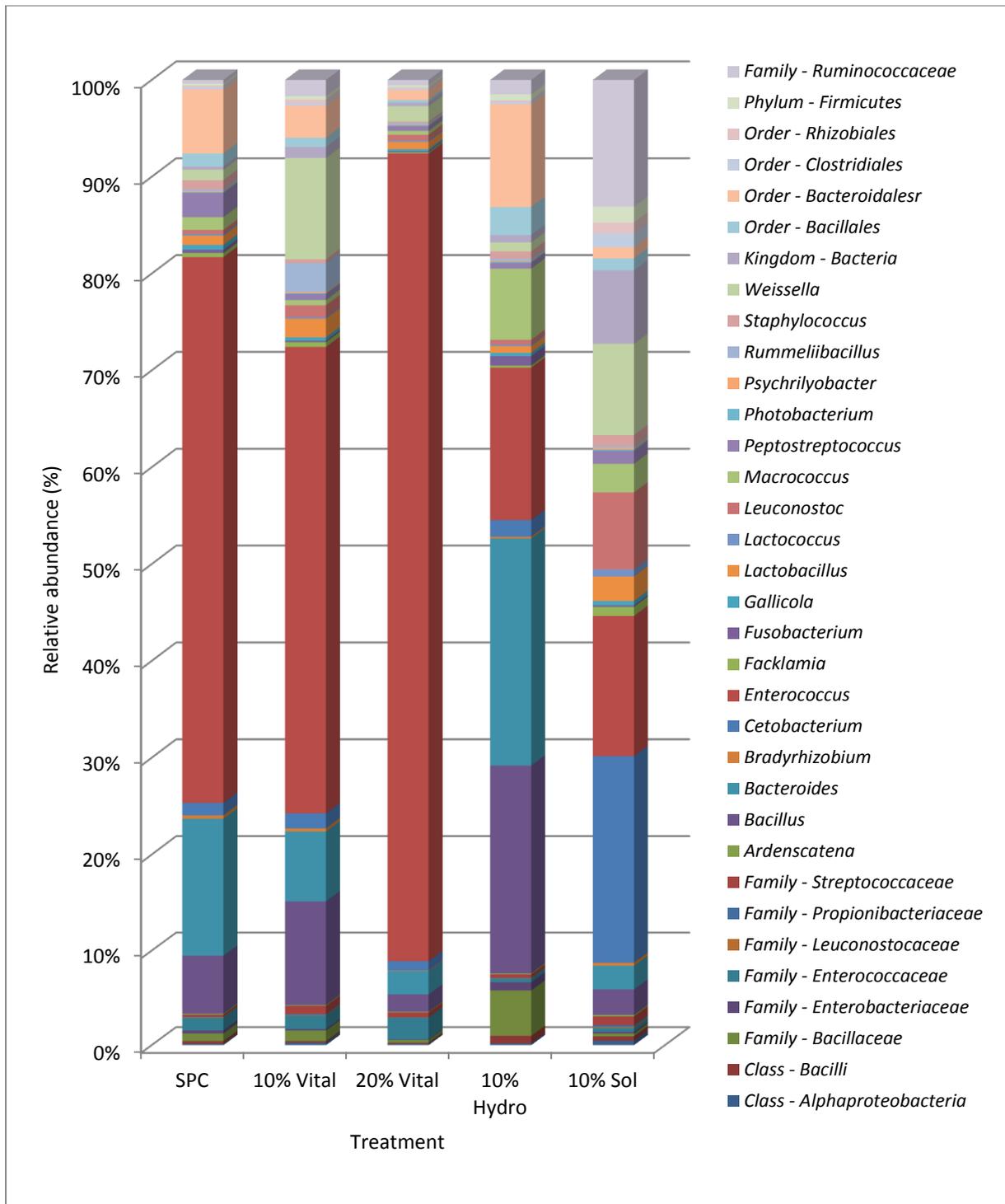


Figure 3.4b. Allochthonous bacterial communities in the posterior intestine of rainbow trout post 66 day feeding with experimental diets. Data are represented as bacterial Genus as a proportion of a total, expressed as a percentage. Data excludes genera with fewer than 0.2% of the total reads

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**Table 3.7b.** Allochthonous bacterial communities in the posterior intestine of rainbow trout post 66 day feeding with experimental diets. Data are represented as phyla and genus percentage means  $\pm$  SD. Data excludes phyla and genus with less than 0.2% of the total reads. Kruskal-Wallis with post hoc Tukey-Kramer. Superscripts denote significance, significance accepted at  $P < 0.05$ .

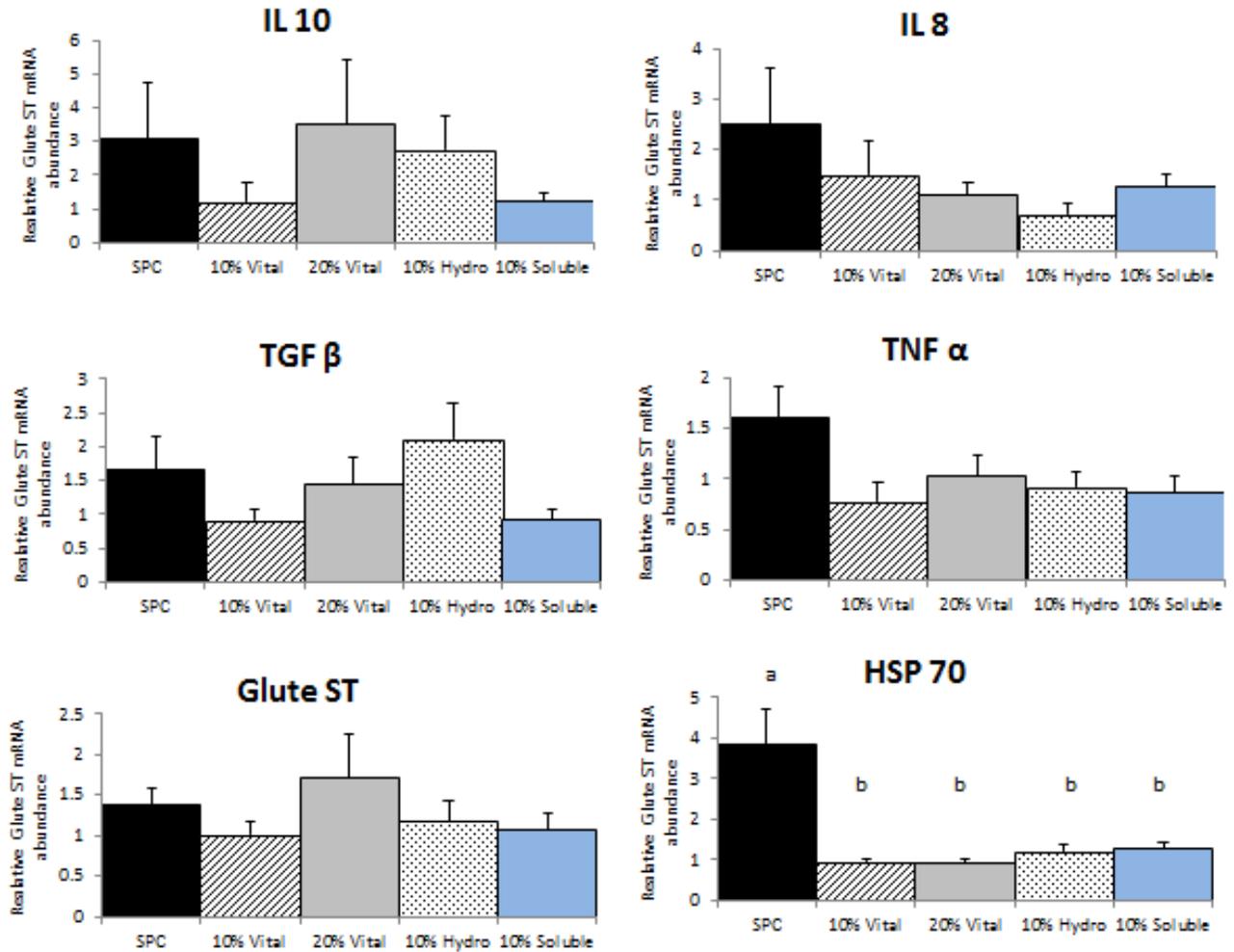
Taxon	SPC	10% Vital	20% Vital	10% Hydro	10% Sol
<b>Phylum</b>					
Actinobacteria	1.32 $\pm$ 0.65	1.78 $\pm$ 1.59	0.5 $\pm$ 0.22	0.59 $\pm$ 0.18	1.12 $\pm$ 0.73
Bacteroidetes	17.7 $\pm$ 14.92	10.59 $\pm$ 10.9	3.06 $\pm$ 1.7	23.56 $\pm$ 22.07	4.54 $\pm$ 6.2
Chloroflexi	0.11 $\pm$ 0.05	0.13 $\pm$ 0.06	0.08 $\pm$ 0.03	0.1 $\pm$ 0	0.12 $\pm$ 0.09
Firmicutes	76.62 $\pm$ 15.29	80.63 $\pm$ 10.89	92.47 $\pm$ 3.57	69.52 $\pm$ 24.25	66.27 $\pm$ 27.97
Fusobacteria	1.66 $\pm$ 0.52	1.77 $\pm$ 0.22	1.39 $\pm$ 0.67	2.49 $\pm$ 0.55	13.63 $\pm$ 25.13
kingdom - Bacteria	0.3 $\pm$ 0.12 <sup>b</sup>	1.59 $\pm$ 1.92 <sup>b</sup>	0.4 $\pm$ 0.23 <sup>b</sup>	0.77 $\pm$ 0.87 <sup>b</sup>	7.27 $\pm$ 6.61 <sup>a</sup>
Proteobacteria	2 $\pm$ 1.25	3.05 $\pm$ 2.1	1.44 $\pm$ 1.32	2.56 $\pm$ 1.87	6.05 $\pm$ 6.45
<b>Genus</b>					
<i>Ardenscatena</i>	0.08 $\pm$ 0.02	0.09 $\pm$ 0.01	0.08 $\pm$ 0.03	0.1 $\pm$ 0	0.11 $\pm$ 0.09
<i>Bacillus</i>	5.73 $\pm$ 3.97 <sup>b</sup>	11 $\pm$ 8.05 <sup>b</sup>	1.79 $\pm$ 0.21 <sup>b</sup>	25.02 $\pm$ 12.28 <sup>a</sup>	2.37 $\pm$ 1.16 <sup>b</sup>
<i>Bacteroides</i>	12.1 $\pm$ 10.17	7.23 $\pm$ 7.39	2.15 $\pm$ 1.19	16.24 $\pm$ 15.12	3.02 $\pm$ 4.2
<i>Bradyrhizobium</i>	0.41 $\pm$ 0.69	0.37 $\pm$ 0.34	0.1 $\pm$ 0.11	0.19 $\pm$ 0.11	0.25 $\pm$ 0.11
<i>Cetobacterium</i>	1.24 $\pm$ 0.39	1.48 $\pm$ 0.19	1.01 $\pm$ 0.18	1.74 $\pm$ 0.3	13.28 $\pm$ 25.14
Class - Alphaproteobacteria	0.01 $\pm$ 0	0 $\pm$ 0	0.11 $\pm$ 0.23	0.01 $\pm$ 0.01	0.01 $\pm$ 0.01
Class - Bacilli	0.29 $\pm$ 0.11 <sup>b</sup>	0.14 $\pm$ 0.03 <sup>b</sup>	0.13 $\pm$ 0.02 <sup>b</sup>	0.91 $\pm$ 0.65 <sup>a</sup>	0.4 $\pm$ 0.27 <sup>ab</sup>
<i>Enterococcus</i>	54.54 $\pm$ 21.08 <sup>ab</sup>	38.79 $\pm$ 19.03 <sup>bc</sup>	75.36 $\pm$ 20.17 <sup>a</sup>	16.65 $\pm$ 6.38 <sup>c</sup>	13.74 $\pm$ 5.12 <sup>c</sup>
<i>Facklamia</i>	0.45 $\pm$ 0.47	0.44 $\pm$ 0.35	0.22 $\pm$ 0.3	0.22 $\pm$ 0.08	0.88 $\pm$ 0.63
Family - Bacillaceae	0.73 $\pm$ 0.5 <sup>b</sup>	1.12 $\pm$ 0.74 <sup>b</sup>	0.29 $\pm$ 0.05 <sup>b</sup>	5.57 $\pm$ 2.68 <sup>a</sup>	0.28 $\pm$ 0.06 <sup>b</sup>
Family - Enterobacteriaceae	0.26 $\pm$ 0.23	0.14 $\pm$ 0.12	0.04 $\pm$ 0.01	0.52 $\pm$ 0.73	0.17 $\pm$ 0.22
Family - Enterococcaceae	1.39 $\pm$ 0.59 <sup>ab</sup>	1.08 $\pm$ 0.7 <sup>ab</sup>	1.92 $\pm$ 1.13 <sup>a</sup>	0.41 $\pm$ 0.16 <sup>b</sup>	0.39 $\pm$ 0.14 <sup>b</sup>
Family - Leuconostocaceae	0.05 $\pm$ 0.05	0.07 $\pm$ 0.03	0.02 $\pm$ 0.02	0.03 $\pm$ 0.01	0.11 $\pm$ 0.08
Family - Propionibacteriaceae	0.03 $\pm$ 0.01	0.11 $\pm$ 0.07	0.04 $\pm$ 0	0.06 $\pm$ 0.01	0.17 $\pm$ 0.18
Family - Ruminococcaceae	0.36 $\pm$ 0.09 <sup>b</sup>	2.38 $\pm$ 2.92 <sup>ab</sup>	0.5 $\pm$ 0.21 <sup>b</sup>	1.5 $\pm$ 2.09 <sup>b</sup>	12.42 $\pm$ 11.96 <sup>a</sup>

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Family - Streptococcaceae	0.21±0.15	0.98±0.72	1.55±3.15	0.28±0.16	0.79±0.67
<i>Fusobacterium</i>	0.29±0.24	0.16±0.09	0.26±0.43	0.59±0.82	0.16±0.1
<i>Gallicola</i>	0.53±0.63	0.34±0.12	0.23±0.15	0.33±0.08	0.42±0.23
Kingdom - Bacteria	0.3±0.12 <sup>b</sup>	1.59±1.92 <sup>ab</sup>	0.4±0.23 <sup>b</sup>	0.77±0.88 <sup>b</sup>	7.28±6.62 <sup>a</sup>
<i>Lactobacillus</i>	0.96±0.75	2.17±1.42	1.83±3.28	0.75±0.3	2.36±1.87
<i>Lactococcus</i>	0.15±0.1	0.15±0.1	0.41±0.79	0.13±0.04	0.61±0.66
<i>Leuconostoc</i>	0.42±0.2 <sup>b</sup>	1.36±0.78 <sup>b</sup>	1.3±2.19 <sup>b</sup>	0.5±0.13 <sup>b</sup>	7.68±6.68 <sup>a</sup>
<i>Macrococcus</i>	1.26±0.56 <sup>b</sup>	0.52±0.09 <sup>b</sup>	0.39±0.06 <sup>b</sup>	8.23±5.6 <sup>a</sup>	2.6±2.38 <sup>b</sup>
Order - Bacillales	1.32±0.87 <sup>b</sup>	1±0.49 <sup>b</sup>	0.36±0.34 <sup>b</sup>	3.31±1.44 <sup>a</sup>	1.06±0.87 <sup>b</sup>
Order - Clostridiales	0.02±0	0.05±0.03	0.02±0.01	0.03±0.01	0.15±0.15
Order - Rhizobiales	0.14±0.11	0.43±0.71	0.12±0.11	0.12±0.07	0.7±1.33
Peptostreptococcus	2.78±4.17	0.62±0.19	0.8±0.94	0.61±0.14	1.28±1.51
<i>Photobacterium</i>	0.08±0.03 <sup>ab</sup>	0.09±0.02 <sup>ab</sup>	0.06±0.02 <sup>b</sup>	0.1±0.02 <sup>ab</sup>	0.13±0.03 <sup>a</sup>
Phylum - Firmicutes	0.21±0.07	0.35±0.07	0.24±0.1	0.61±0.73	1.33±1.5
<i>Psychrilyobacter</i>	0.09±0.04 <sup>ab</sup>	0.1±0.02 <sup>ab</sup>	0.06±0.01 <sup>b</sup>	0.1±0.02 <sup>ab</sup>	0.14±0.04 <sup>a</sup>
<i>Rummeliibacillus</i>	0.19±0.06 <sup>b</sup>	3.03±2.3 <sup>a</sup>	0.17±0.04 <sup>b</sup>	0.26±0.1 <sup>b</sup>	0.19±0.05 <sup>b</sup>
<i>Staphylococcus</i>	0.92±0.81	0.41±0.17	0.17±0.11	0.77±0.37	0.92±0.91
<i>Weissella</i>	1.06±0.54 <sup>b</sup>	11.75±6.26 <sup>a</sup>	2.9±3.94 <sup>ab</sup>	0.99±0.27 <sup>b</sup>	9.4±8.55 <sup>ab</sup>

### 3.3.3b. Gene expression

Relative gene expression of IL-10, IL-8, TGF- $\beta$ , TNF- $\alpha$ , Glute ST and HSP70 to reference genes are presented in Figure 3.5b. The relative expressions of the pro-inflammatory cytokines TNF $\alpha$  and IL-8 were unaffected by dietary treatment. The anti-inflammatory cytokine IL-10, the immune-regulatory cytokine TGF-  $\beta$  and glutathione utilisation and antioxidant status gene Glute ST were all also unaffected by dietary treatment. However, HSP70 expression, which is up-regulated in response to a variety of stressful conditions, was significantly down-regulated in all wheat gluten treatments compared to the SPC diet ( $P < 0.05$ ).

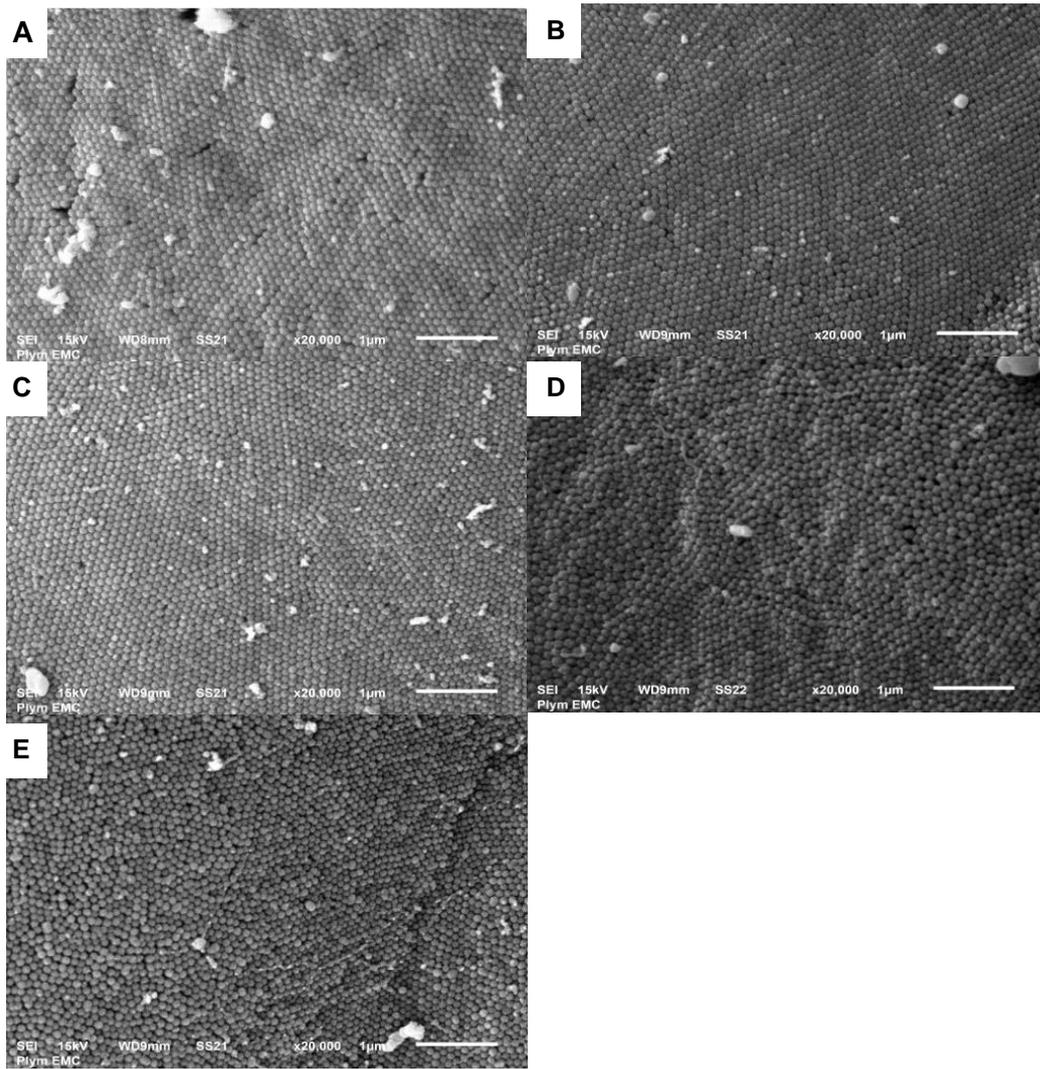


**Figure 3.5b.** Relative mRNA abundance of IL-10, IL-8, TGF  $\beta$ , TNF  $\alpha$ , Glute ST and HSP70 to reference genes in the posterior intestine of rainbow trout post 66 day feed trial. Superscript letters denote significant difference ( $P < 0.05$ ) between treatments.  $n = 6$  per treatment. Data are means  $\pm$  SE.

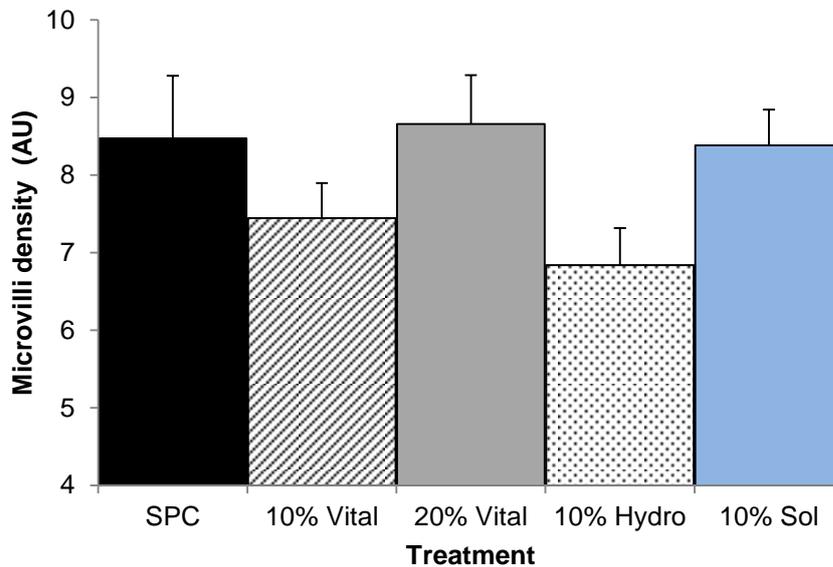
### *3.3.4b. Intestinal histology*

#### 3.3.4.1b. Scanning electron microscopy

Scanning electron micrographs of the posterior intestine of the rainbow trout revealed no qualitative effects induced by the inclusion of wheat gluten in the experimental diets compared to the SPC treatment. Gross structure qualitative analysis showed evenly shaped and distributed enterocytes with no signs of necrosis or gross damage. Quantitative analysis of microvilli density (arbitrary units) was also unaffected by the dietary regimes fed to experimental fish, despite numerical trends (Figure 3.7b.). Microvilli form and distribution looked uniform across treatments with densely packed microvilli, with no sign of patchy or damaged areas (Figure 3.6b.).



**Figure 3.6b.** Scanning electron micrographs of the posterior intestine of rainbow trout fed experimental diets; SPC (A), 10% Vital (B), 20% Vital (C), 10% Hydro (D) and 10% Sol (E) for 66 days. Scale bars = 1 µm.



**Figure 3.7b.** Threshold analysis of scanning electron micrographs of posterior intestine micro villi density of rainbow trout. Data are means  $\pm$  SE.

#### 3.3.4.2 Light microscopy

Figure 3.8b illustrates representative PAS with alcian blue and H & E stained sections of the rainbow trout posterior intestine at the end of the trial. Goblet cell counts conducted on the PAS stained sections revealed no significant difference ( $P > 0.05$ ) between treatments. Intraepithelial leukocytes, per  $100 \mu\text{m}^{-1}$ , were significantly increased in the 10% Vital and 10% Sol treatments ( $0.45 \pm 0.03$  and  $0.44 \pm 0.03$ , respectively) compared to the SPC treatment ( $0.34 \pm 0.02$ ) ( $P < 0.05$ ). Significant difference between treatments was also observed in regard to lamina propria width (Table 3.8b).

**Table 3.8b.** Histological parameters of the posterior intestine of rainbow trout fed experimental diet for 66 days. Data are means  $\pm$  SE. significance indicated by superscript letters accepted at  $P < 0.05$ .

	SPC	10% Vital	20% Vital	10% Hydro	10% Sol
Goblet cells ( $100 \mu\text{m}^{-1}$ )	1.41 $\pm$ 0.06	1.49 $\pm$ 0.05	1.5 $\pm$ 0.07	1.33 $\pm$ 0.05	1.57 $\pm$ 0.07
Intraepithelial leukocytes ( $100 \mu\text{m}^{-1}$ )	0.34 $\pm$ 0.02 <sup>b</sup>	0.45 $\pm$ 0.03 <sup>a</sup>	0.39 $\pm$ 0.02 <sup>ab</sup>	0.38 $\pm$ 0.03 <sup>ab</sup>	0.44 $\pm$ 0.03 <sup>a</sup>
Lamina propria width ( $\mu\text{m}$ )	9.48 $\pm$ 0.41 <sup>bc</sup>	11.83 $\pm$ 0.44 <sup>a</sup>	9.41 $\pm$ 0.37 <sup>c</sup>	10.29 $\pm$ 0.35 <sup>abc</sup>	11.03 $\pm$ 0.36 <sup>ab</sup>

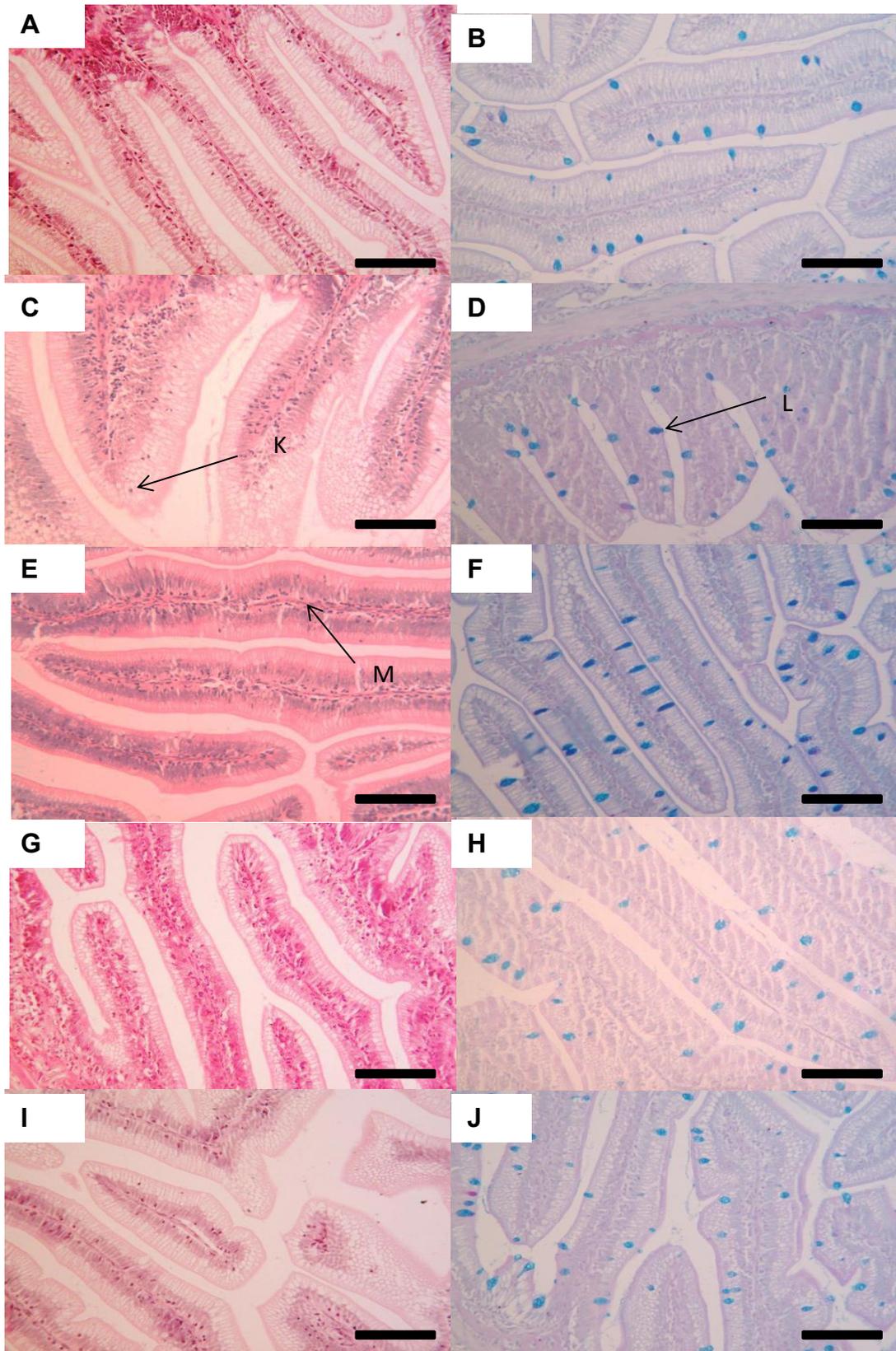


Figure 3.8b. Light micrographs of the posterior intestine of rainbow trout fed SPC (A & B), 10% Vital (C & D), 20% Vital (E & F), 10% Hydro (G & H) and 10% Sol (I & J) treatments for 66 days. H & E staining (A,C,E,G,I) and PAS staining (B,D,F,H,J). Arrows identify (K) Intraepithelial leukocytes, (L) goblet cells and (M) lamina propria. Scale bars = 100  $\mu$ m.

### 3.4b. Discussion

The objective of the current investigation was to evaluate the effect of vital, hydrolysed and soluble wheat gluten inclusions in soya based diets on rainbow trout growth performance, gut microbiology, histology and immunological status of the posterior intestine. Microbiological, molecular, and microscopy techniques were utilised to achieve these objectives at the end of the feed trial.

Throughout the trial fish performed well, with FCR's values lower than 1. Wheat gluten inclusions had no significant effect on FCR's compared to the soy protein control diet, yet the 20% vital treatment performed significantly better than the two hydrolysed wheat gluten treatments. The same trend was observed for protein efficiency ratios, yet no effect on SGR or mean end point fish weight was observed across all treatments (mean fish weight  $P = 0.052$ ). Comparable growth performance has been observed when utilising wheat gluten as a replacement of both fishmeal in Atlantic salmon (*Salmo salar*) (Storebakken et al., 2000) and plant based proteins in rainbow trout (Tusche et al., 2012). Storebakken *et al.* (2000) also observed improved protein apparent digestibility coefficients, as did Storebakken *et al.* (2015), in line with the numerical trend observed for protein efficiency ratios in vital wheat gluten fed fish. Improved growth performance (weight gain) has been reported in rainbow trout with wheat gluten replacement of fishmeal and soy protein with additional amino acid supplementation (Davies et al., 1997), and decreased FCR's have been observed in hybrid tilapia (*Acipenser schrenckii* ♀ × *Huso dauricus* ♂) (Qiyou et al., 2011). Despite no significant difference in mean fish weight at the end of the trial, significance was very nearly achieved between the SPC and 20% vital wheat gluten treatment ( $P = 0.052$ ), significance could well have been achieved if the trial had continued longer with the numerical trends in

FCR and SGR. Observations of the performance of different wheat gluten types, hydrolysed vs vital have never been analysed before, let alone in rainbow trout. Comparable 10% inclusions of vital and hydrolysed wheat gluten had no significant difference in growth performance. The positive performance of all treatments including the SPC treatment, with an FCR lower than 1 may have masked a possible beneficial effect of wheat gluten inclusions, unable to improve on very good growth performances observed in the basal treatment. Wheat gluten inclusions had no effect on haematological and serological parameters compared to the SPC control. Ten % vital wheat gluten however had significantly higher haemoglobin levels ( $\text{mg } 100\text{ml}^{-1}$ ) than 10% soluble wheat gluten treatment ( $41.186 \pm 5.52$  and  $32.174 \pm 5.58$ , respectively), a parameter never investigated for wheat gluten inclusions or types before, and to this authors knowledge no effect has been observed with soy protein inclusions in salmonids (Hemre et al., 2005; Olli et al., 1995). As packed cell volume was unaffected by dietary treatment, it would suggest the 10% vital wheat gluten inclusion increases the amount of haemoglobin present in each cell compared to the 10% soluble wheat gluten treatment fish, in turn increasing oxygen carrying capacity and possibly increasing resistance to anoxia.

Carcass composition analysis post 66 day feeding of experimental diets revealed no effect of wheat gluten inclusions on carcass moisture or protein content. Carcass lipid was significantly reduced in the two vital wheat gluten treatments compared to the soy protein control. Previous studies have observed lipid content to be unaffected by wheat gluten inclusions in rainbow trout compared to fishmeal based basal diets (Rodehutschord et al., 1994; Davies et al., 1997). The effect of increased plant protein inclusions has been reported to increased carcass lipid levels, observed in black-chinned tilapia (*Sarotherodon*

*melanotheron*) (Koumi et al., 2008) and European seabass (*Dicentrarchus labrax*) (Kaushik et al., 2004). Increased lipogenesis has also been observed in rainbow trout fed diets replacing total fish oil and protein with plant based products, resulting in the increase in expression of genes associated with lipid biosynthesis (Panserat et al., 2009). The reduction in carcass lipid observed with vital wheat gluten inclusion may be a result of a more advantageous amino acid profile and reduced carbohydrate in vital wheat gluten (as discussed in section 1.3.4) compared to the replaced soy protein concentrate, favouring utilisation throughout the body over lipid deposition. Hydrolysed wheat glutens with an added level of refinement had no effect on lipid content compared to the SPC treatment. Carcass ash (minerals) was not significantly affected by dietary inclusions of wheat glutens compared to the SPC treatment. However, the 10% hydrolysed was significantly elevated compared to the vital wheat gluten treatments. Carcass ash and skeletal mineralisation have previously been linked to phosphorous availability (Le Luyer et al., 2014), and may be elevated as a consequence of increased availability of phosphorous and other minerals with additional hydrolysis compared to the vital wheat gluten. Carcass energy was significantly increased in the 10% hydrolysed treatment compared to all others in line with the highest observed lipid content. Energy was significantly reduced in the 20% vital treatment in line with lower observed lipid levels.

The use of high throughput analyses of the allochthonous microbial community, along with the PCR-DGGE in the preliminary trial are among the first to analyse the effect of wheat glutens on the intestinal microflora of fish. Even more so, to the author's knowledge, it is the first investigation into the effect of wheat glutens on the allochthonous microbial populations of rainbow trout. The only other investigation observed the utilisation of

hydrolysed wheat gluten (6%) as a replacement for other plant proteins in processed animal protein based diets with Asian seabass (Apper et al., 2016). Alpha refraction analysis of Good's coverage reveals estimations of > 98.9%, indicative of a fully sampled microbiome. Bray Curtis analysis revealed two main clusters. The first cluster consisting of the hydrolysed treatments (10% Hydro and 10% soluble) and the second cluster of the vital (10% vital and 20% vital) and the SPC treatment. Two samples, 10% soluble replicate #6 and 10% hydro replicate #6, are distinct from both clusters, and may represent the intra-treatment variability identified by Mansfield *et al.* (2010). Observed species, Chao 1 (species richness) and Shannon-Wiener index (bacterial diversity) were unaffected by dietary treatment. Phylogenetic distance was significantly greater in the 10% vital treatment than the 10% hydro treatment.

The sequence distribution data reads of 16S rRNA was dominated by the Firmicutes at phylum level, accounting for 76.17% of the total read sequences of all treatments, in line with the observations of Mansfield *et al.* (2010) when increasing plant protein source inclusions. The Bacteroidetes were the second most dominant phylum (11.16%), followed by the Fusobacteria (4.32%), Proteobacteria (4.08%), Actinobacteria (1.50%) and Chloroflexi (0.82%). The proportion of identified phyla was unaffected by dietary treatment and have all previously been described as part of the commensal intestinal bacteria of fish (Wong et al., 2013; Wu et al., 2012; Mansfield et al., 2010; Navarrete et al., 2010b; Dehler et al., 2016; Gajardo et al., 2016).

The sequence distribution data at genus level, of those genera representing > 0.2% of the total reads, revealed the most abundant genus was *Enterococcus*, representing 46.52% of

the total reads and  $75.36 \pm 20.17\%$  of the reads in the 20% vital treatment. The reads for *Enterococcus* were significantly elevated in the 20% vital treatment compared to the 10% vital treatment and the two hydrolysed wheat gluten treatments (10% Hydro and 10% Sol). The hydrolysed treatments also showed significantly fewer reads than the SPC treatment. The genus *Enterococcus*, a member of the order Lactobacillales, has been reported in the intestine of both wild and cultured rainbow trout (Trust and Sparrow, 1974; Wong et al., 2013; Ringø and Gatesoupe, 1998), and contain potentially probiotic species. In the present trial, BLAST searches using the most abundant sequences from the *Enterococcus* OUT's consistently revealed *E. faecium* to be the dominant species, and was also observed in all treatment replicates in the short term trial in Chapter 3a. *E. faecium* is notably the most studied probiotic species of the genus *Enterococcus*, with reported applications in numerous fish species (Chang and Liu, 2002; Merrifield et al., 2010; Avella et al., 2011), and accepted by the European Union as an authorized probiotic feed additive for humans and terrestrial animals (70/524/EEC., 2004). Increased neutrophil activity and increased resistance to *Aeromonas hydrophila* have been reported in *Cyprinus carpio* fed diets containing *E. faecium* and elevated rainbow trout leukocyte levels observed when fed diets containing *E. faecium* and *Bacillus* probionts synergistically (Merrifield et al. 2010). However, the presence of pathogenic species relevant to rainbow trout in the genera *Enterococcus* must be noted, primarily *Enterococcus seriolicida*. Interestingly, the relative abundance of *Enterococcus* reads was significantly reduced in the intestine of fish fed the hydrolysed diets compared to the SPC and 20% vital fed fish, suggesting the inclusion of hydrolysed wheat gluten products reduces the competitiveness of *Enterococcus* species in the posterior intestine. This could be for a number of reasons, such as a change in the intestinal conditions being less optimal for *Enterococcus*, giving other species a competitive advantage, in turn increasing their relative

abundance, and reducing that of *Enterococcus*. *Bacillus*, class Bacilli, Family Bacillaceae and *Macrocooccus* were all significantly elevated in the 10% hydro treatment. Family Ruminococcaceae and *Leuconstoc* were significantly elevated in the 10% soluble treatment.

Of the *Bacillus* reads identified by high throughput sequencing, the majority belonged to *B. coagulans*. The highest percentage of reads associated with the genera *Bacillus* was observed in the 10% hydro treatments, in line with observations from Chapter 3a for *B. coagulans*. The probiotic potential of *B. coagulans* has been investigated in common, grass and koi carp (*Cyprinus carpio*, *Ctenopharyngodon idella* and *Cyprinus carpio koi*, respectively) (Xu et al., 2014; Lin et al., 2012; Wang, 2011). Abdhul *et al.* (2015) reported *B. coagulans* to produce bacteriocinogenic antimicrobials, and improved growth performance was observed with probiotic supplementation in common carp by Xu *et al.* (2014) and grass carp by Wang (2011). Lin *et al.* (2012) observed a synergistic effect with *B. coagulans* and prebiotic chitosan oligosaccharides (COS) which improved growth performance, as well as immunological parameters. Leukocyte counts, respiratory burst activity, phagocytic activity, lysozyme activity, superoxide dismutase activity and disease resistance to *Aeromonas veronii*, a known carp pathogen, were all improved with dietary supplementation of the pro and prebiotic. Similar results on lysozyme, myeloperoxidase, and respiratory burst activities were also observed by Xu *et al.* (2014) in common carp.

Other genera of interest observed in the allochthonous microbiota of sampled rainbow trout include *Weissella* (3.43% of total reads) and *Leuconstoc* (1.23% of total reads). *Weissella* have been routinely identified in the intestines of trout and Atlantic salmon (Wong et al., 2013; Reveco et al., 2014; Mansfield et al., 2010; Hovda et al., 2012; Apper et

al., 2016). In the current study, the relative abundance of *Weissella* was significantly elevated in the 10% vital treatment, representing  $11.75 \pm 6.26\%$  of the treatments allochthonous microbiota. Of the *Weissella* reads identified by high throughput sequencing, the majority belonged to *Weissella confusa*. *W. confusa* has been identified as a potential probiotic species for juvenile Asian sea bass (Rengpipat et al., 2008), with no information to this authors knowledge on its effects on salmonids. Rengpipat et al. (2008) reported that *W. confusa* inhibited *Aeromonas hydrophila*, a known seabass pathogen, *in vitro* using a well agar diffusion method, and subsequently provided improved survival against *A. hydrophila* in an *in vivo* challenge study. Improved growth performance was also observed with *W. confusa* fortified feed by Rengpipat et al. (2008). Apper et al. (2016) observed significantly increased *W. confusa* reads with 6% soluble hydrolysed wheat gluten inclusion compared to all other dietary treatments, numerically observed in the present investigation with Solpro inclusion, but not significantly so.

Species of the genus *Leuconostoc* have been identified in the GI tract of fish, including Atlantic salmon, rainbow trout and brown trout (Ringø and Gatesoupe, 1998; Balcazar et al., 2007a; Balcazar et al., 2007c; Mansfield et al., 2010), as well as in many dairy, fermented and meat products (Hemme and Foucaud-Scheunemann, 2004). Information on *Leuconostoc* presence in salmonids is increasing, being identified in Artic charr, rainbow trout and Atlantic salmon (Ringo et al., 1998; Balcazar et al., 2007c; Askarian et al., 2012). *Leuconostoc mesenteroides* was observed by Balcazar et al. (2007c) to increase *in vitro* resistance to numerous fish pathogens and enhance lysozyme and alternative complement activity in the serum of brown trout (Balcazar et al., 2007a). Balcazar et al. (2007b) also observed enhanced cellular and humoral immune functions in rainbow trout supplemented

*L. mesenteroides*, in turn elevating survival after exposure to *Aeromonas salmonicida*, the causative agent of furunculosis in salmonids. *Leuconostoc lactis* is another identified species with potential application as a probiotic in fish, although far less documented (Zhang et al., 2013). Apper-Bossard *et al.* (2013) identified the genus *Leuconostoc* in Asian seabass autochthonous microbiota and observed significant increase with the addition of hydrolysed wheat gluten (Solpro®), a trend also observed in the present investigation for rainbow trout. *Leuconostoc* was not identified in the allochthonous bacteria of Asian seabass, possibly as the result of low read abundances being below the threshold 0.5% abundance specified, and excluded from comparisons.

The relative transcriptional expression levels of a number of immunologically relevant genes was assessed in the current study as an indicator of the effect of dietary wheat gluten on the localised immunological status of the rainbow trout intestine. The pro-inflammatory cytokines TNF- $\alpha$  and IL-8, which play an important role in cell proliferation, differentiation, apoptosis, necrosis, and the induction of other cytokines (TNF- $\alpha$ ) and chemotaxis of neutrophils, T lymphocytes and basophils (IL-8) (Reyes-Cerpa et al., 2013; Abid et al., 2013) were unaffected by dietary inclusion. IL-10, produced by activated T cells and monocytes, is associated with forms of peripheral tolerance and is a major suppressor of inflammation and the immune response. Intestinal IL-10 transcriptional levels were unaffected by dietary wheat gluten inclusion in the present study, as was also the case for TGF- $\beta$ . TGF- $\beta$ , regulates cell differentiation, migration, proliferation and development as well as survival in leukocytic lineages (leukocytes, macrophages, granulocytes, NK (natural killer) cells and dendritic cells) (Reyes-Cerpa et al., 2013). Associated with suppression of autoimmunity and immune tolerance, TGF- $\beta$  has been reported to suppress TNF- $\alpha$  induced macrophage

activation in common carp and goldfish (Haddad et al., 2008; Kadowaki et al., 2009). The expression of the inflammatory cytokines in the present study showed high levels of inter treatment variation, reducing the opportunity for statistical significance to be observed between treatments, this has been previously identified by Mansfield *et al.* (2010) who noted high intra treatment sample variation.

Glutathione S-transferase, and the glutathione that it binds substances too, are two primary lines of defence against both acute and chronic toxicities of electrophiles and reactive oxygen/nitrogen species (Li, 2009). Glutathione S transferase is part of the Phase 2 detoxification enzymes superfamily, with the ability to detoxify both reactive oxygen species as well as toxic xenobiotics (Wu et al., 2004; Li, 2009). Detoxification is achieved through glutathione S transferase catalysing glutathione dependent conjugation and redox reactions (Li, 2009). No single biomarker of oxidative stress has been identified for utilisation in fish (Olsvik et al., 2011; van der Oost et al., 2003), attributed to the high degree of out breeding and genetic variation between and within fish species compared to laboratory reared mammals (Olsvik et al., 2011). Sitjà-Bobadilla *et al.* (2005) reported an increased glutathione redox status in blood and liver, and glutathione reductase when feeding the carnivorous sea bream (*Sparus aurata*) diets with increased plant protein content. The relative gene expression of Glute ST in the present study was unaffected by dietary inclusions of wheat gluten, however, the relative expressions of the HSP 70 gene was significantly down-regulated in the posterior intestine of wheat gluten fed fish.

HSP 70 is known to mediate the degradation and repair of denatured or altered proteins and is highly conserved at amino acid level (Basu et al., 2002; Kiang and Tsokos, 1998).

Levels of expression are known to be affected by various stressors, such as pathogen invasion and detrimental stocking densities (Sanden and Olsvik, 2009; Zhang et al., 2014; Gornati et al., 2004). A significant up-regulation of HSP 70 has been observed in the posterior intestine of Atlantic salmon (*Salmo salar*) fed soy bean meal based diets, compared to fishmeal controls, which is attributed to elevated cellular stress and subsequent increased cellular repair mechanisms and apoptosis (Bakke-McKellep et al., 2007b). Up-regulation has also been observed in responses to model toxicants (Sanden and Olsvik, 2009), genetically modified maize (Sagstad et al., 2007), and unfavourable rearing densities (Gornati et al., 2004). The observed down-regulation of the HSP 70 gene in the present trial would indicate a reduced level of stress in the posterior intestine of wheat gluten fed fish compared to those fed the soy protein based control diet. The effect of soy products on the posterior intestine of fishes is well documented, in many species causing enteritis. The up-regulated status of HSP 70 in both salmon (Bakke-McKellep et al., 2007a) and common carp (Urán et al., 2008) intestines when fed diets containing high levels of soya products reveals the increased stress applied to the intestinal tract. In the present study, a 10% substitution with hydrolysed or vital wheat gluten instigates a down-regulation of HSP 70 expression, potentially indicative of reduced stress. It can therefore be inferred that wheat gluten products have a reduced cellular stressor fraction than soya proteins, potentially due to their lower concentrations of ANFs. To the author's knowledge there is no previous literature on the effect of wheat gluten products on the localised intestinal immunity of fish.

Scanning electron microscopy revealed no dietary effect on the microvilli density of enterocytes in the posterior intestine at the end point of the feeding trial. Absorptive

surface area analysis of rainbow trout sampled in the present study was inhibited by the vast degree of intestinal folding present in the posterior region. Measurements of perimeter ratio (lumen to muscularis circumference) and fold height were unachievable due to intestinal folds leaving the two dimensional plane observable with 5µm microtome sectioning, producing incomplete folds for imaging and measurement. Microvilli density is a good indicator of enterocyte and intestinal health with deformities, damage and necrosis clearly apparent if present. Increased absorptive surface area has been observed in Asian seabass (Apper et al., 2016) as well as intestinal fold height/villous height in the intestine of hybrid sturgeon (*A. schrenckii* ♀ x *H. dauricus* ♂) and broilers with dietary hydrolysed wheat gluten inclusions (Qiyu et al., 2011; van Leeuwen et al., 2004). Increased absorptive area associated with wheat gluten has been attributed to the high levels of glutamine. Utilised as an energy source for rapidly proliferating cells, dietary glutamine supplementation has resulted in increased microvilli and enterocyte length of hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) and channel catfish (*Ictalurus punctatus*) (Cheng et al., 2012; Pohlenz et al., 2012).

In the present study goblet cell abundance in the posterior intestine was not affected by dietary treatment. Producing mucus utilised for lubrication, digestive function, barrier protection and pathogen translocation, goblet cells play a pivotal role in the intestine for both nutrition and health. Intraepithelial leukocyte (IEL) numbers in the posterior intestine were significantly elevated in the 10% vital and 10% soluble treatments compared to the SPC treatment. The lack of lymphoid structures in teleost intestinal folds enhances the importance of leukocytes in the epithelium and lamina propria as protection against pathogenic insult. Comprised of diffuse populations of phagocytes, natural cytotoxic cells

and lymphocytes, changes in IEL's populations can be interpreted in two manners. Firstly, as an increase in immune readiness for potential pathogen encounters, or secondly, an increase could be due to pathogen identification and an active immune response. The elevated IEL numbers observed in the present study combined with the survivability data and gene expression analysis of inflammatory cytokines IL-8 and TGF- $\beta$ , associated with leukocyte chemotaxis, would suggest there is no underlying pathogenic insult. Therefore, it could be concluded that the 10% inclusion of vital or soluble wheat gluten enhances the immune potential of the posterior intestine. Twenty % vital and 10% hydrolysed wheat gluten inclusions also numerically increased IEL counts, but not significantly so. Lamina propria width was significantly increased in the 10% vital treatment compared to the SPC and 20% vital treatments. Morphological changes in the posterior intestine have been well observed in salmonids in response to full-fat and solvent extracted soy bean meal, including the widening of the lamina propria (Rumsey et al., 1994; Bureau et al., 1998; Refstie et al., 2000; Ostaszewska et al., 2005) which has been associated with inflammation and chemotaxis of mixed leukocytes into the lamina propria and submucosa. The widening observed in the present study is again inconsistent with gene expression analysis regarding inflammation and level of leukocyte migration to the posterior intestine. There was no observed effect on lamina propria width with a higher inclusion of vital wheat gluten (20% vital wheat gluten treatment), or with 10% inclusion levels of hydrolysed type wheat gluten protein sources. In addition, Storebakken *et al.* (2000) observed little change to intestinal morphology with 50% of total protein in the diet supplied by wheat gluten (29.12% ingredient inclusion) for Atlantic salmon, with only one sampled fish exhibiting moderate, non-specific change. Apper *et al.* (2016) observed no effect on lamina propria width with 6% hydrolysed wheat gluten inclusion, consistent with the findings of the current study. The

reason for the increased lamina propria width as observed in the present study is therefore not clear.

### **3.5b. Conclusion**

Over the course of the current 66 day feed trial the incorporation of wheat gluten, regardless of processing level, as a substitute for soy protein concentrate in aquafeed for rainbow trout, had no effect on growth parameters compared to the soy protein control. The increased inclusion level of 20% vital wheat gluten outperformed 10% inclusion levels of hydrolysed products (10% hydrolysed and 10% Solpro) in terms of feed conversion ratio and protein efficiency ratio.

Wheat gluten inclusions and types had a modulatory effect on the allochthonous microbial population of rainbow trout. Overall population constituents at phylum level were unaffected by dietary inclusions of wheat gluten and dominated by Firmicutes, as observed by Desai *et al.* (2012) as a result of increasing plant protein inclusions. However, observations at genera level revealed significant differences between wheat gluten inclusion types and varying inclusion levels. Cluster analysis revealed that the bacterial communities from fish fed hydrolysed products had a higher level of similarity to one another than to the vital wheat gluten and SPC treatments, which clustered together. This would suggest the hydrolysed wheat gluten has a larger impact on the intestinal population than the vital wheat gluten, yet significant intra-treatment variation was observed in line with the observations of Mansfield *et al.* (2010) and Desai *et al.* (2012). Significant modulation of genera was observed across all dietary treatments. Vital wheat gluten enhanced the proportion of *Enterococcus* and *Weissella* in the 20% and 10% vital wheat gluten treatments,

respectively, compared to the SPC or other wheat gluten treatments. *Bacillus* and *Leuconostoc* relative abundance was significantly increased in the 10% hydrolysed wheat gluten and 10% soluble wheat gluten fed fish, respectively, compared to the SPC diet. These genera have all been shown to contain probiotic species, with the potential to aid the intestine in health and/or nutritional function when present in the microflora. This would indicate that the addition of wheat gluten products in aquafeed formulations has the ability to enhance probiotic genera within the allochthonous microbial population, without affecting the overall structure of the intestinal microbiota, as also noted by Wong *et al.* (2013).

The gene expression analysis revealed little effect on the localised immune response, with dietary inclusions of wheat gluten showing no effect on the pro-inflammatory cytokines IL 8 and TNF  $\alpha$  and the anti-inflammatory cytokines IL 10 and TGF  $\beta$ . Antioxidant status was also unaffected. The expression of HSP 70 however showed a significant down-regulation, indicating a reduced level of stress with wheat gluten inclusion compared to the SPC treatment, likely caused by a reduction in the abundance of dietary ANF's and the high levels of glutamine within wheat gluten providing the substrate and energy for highly proliferating intestinal cells. In addition, increased intestinal intraepithelial leukocyte numbers were observed in the wheat gluten fed fish, leading to a potentially enhanced non-specific immune response, highly important to teleosts.

In conclusion, wheat gluten products are a promising alternative plant protein source providing adequate growth performance with the added benefits of enhancing beneficial

bacterial genera in the posterior intestine, reducing intestinal stress and potentially enhancing the non-specific innate immune system of rainbow trout.

Further investigations into the utilisation of blended wheat gluten proteins in aquafeeds are needed, enabling increased levels of wheat gluten to be incorporated in aquafeeds, overcoming limitations of extruding processes. Additional evaluations of inclusion levels for optimal growth performance should also be investigated.

## **Chapter 4: The effect of commercially relevant blended wheat gluten on growth performance, condition and intestinal microbiota in juvenile rainbow trout (*Oncorhynchus mykiss*).**

### **4.1 Introduction**

As a result of previous feed trials and associated microbiological, molecular and microscopic analysis, wheat gluten products have been observed to be an appropriate alternative plant protein source for juvenile rainbow trout (chapter 3). Inclusions levels of up to 20% had no detrimental effect on growth performance, promoted beneficial bacterial species and reduced intestinal stress compared to a soy protein control. The structural characteristics of wheat glutes, which promote its pellet binding effects, raise commercial concerns over pelleting high vital wheat gluten inclusion diets, and can require blending hydrolysed and vital wheat glutes to enable extrusion and optimal pellet characteristics.

Starches have traditionally been utilised as pellet binders, despite limited digestibility in salmonids and the associated detrimental effect starches can have on blood glucose regulation with high carbohydrate levels (Krogdahl et al., 2004; Hemre et al., 1995; Storebakken et al., 2000; Bergot, 1979). Hydrocolloid binders, utilised mainly in moist feeds for ground dwelling grazing crustaceans and echinoderms (Tacon, 1987), produce water Table feeds, yet have been reported to reduce lipid and protein digestibility in salmonids (Storebakken, 1985; Storebakken and Austreng, 1987). The monomeric gliadins (30 to 100 kDa) which constitute half of the protein fractions of vital wheat glutes produce matrix viscosity and extensibility, whilst polymeric glutenin (100 kDa to >10,000 kDa),

comprising the other half of the protein fraction, generate cohesiveness and elasticity (Apper-Bossard et al., 2013). Through the feed preparation and extruding process, hydration, mixing, shearing and heating, wheat gluten form a variety of natural bonds (hydrogen, ionic, hydrophobic and covalent disulphide) binding and entrapping other ingredients in a cohesive network (Wieser, 2007; Apper-Bossard et al., 2013).

The addition of vital wheat gluten in extruded aquafeeds has been shown to increase pellet hardness and durability, with increasing vital wheat gluten inclusion from 10 to 20% (Apper-Bossard et al., 2013). Draganovic *et al.* (2011) also reported increased radial expansion of extruded wheat gluten pellets compared to a fishmeal based diet, and increased breaking strength. Increased pellet breakdown time was also observed in the wheat gluten inclusion pellets, attributed to vital wheat glutens water insolubility, a positive attribute for diets requiring high water stability. The addition of hydrolysed wheat gluten (HWG) has been reported to reduce die pressure, specific mechanical energy (SME) and torque required for extruding with increasing HWG inclusion from 0 to 13.46% of the diet (Storebakken et al., 2015). Radial expansion was also increased with up to 13.46% inclusion, however water stability decreased. The highest inclusion level of 26.94% was prevented from being extruded due to the visco-elastic nature of wheat gluten, and required significant reduction in water content (19-20% reduced to 11%) to enable the diet to be extruded, die pressure was reduced with increased SME. Water stability and durability were also reduced with the highest HWG inclusion level (Storebakken et al., 2015). A blend of the two wheat gluten products has enabled a patented extrusion process enabling high inclusion levels of wheat gluten to be incorporated into aquafeed.

Growth performance of rainbow trout fed varying levels of vital and hydrolysed wheat gluten in chapter 3 showed no dietary effect on growth performance. To this author's knowledge, there is currently no information on the application of blended vital and hydrolysed wheat gluten products in aquafeeds. Previous literature has observed comparable or significant improvements on growth parameters in various commercially relevant species when replacing fishmeal of soya proteins as discussed in section 1.3.2. Chapter 3 identified the 20% vital wheat gluten diet to be numerically advantageous over the other treatments, and was the basis of this investigations design.

The aim of the present investigation was to assess the effects of 20% vital, 20% hydrolysed (soluble) and three inclusion levels of a blend of the two wheat glutens (20%, 25% and 30%) on the growth performance, somatic indices and allochthonous microbiota of juvenile rainbow trout.

### **4.2 Materials and methods**

All experimental work involving fish was conducted under the UK Home Office project licence PPL 30/2644 and was in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Plymouth University Ethical Committee.

#### *4.2.1 Experimental design*

Five hundred and forty rainbow trout were obtained from Exmoor Fisheries (Somerset, UK). After a two week acclimation period, at the Aquatic Animal Nutrition and Health Research Facility at the university of Plymouth, the fish were graded and randomly distributed into 18, 120L fibreglass tanks (30 fish per tank; average weight =  $26.70 \pm 0.12\text{g}$ ) in a 7,000 litre closed

recirculation system. Over the course of an 56 day feeding trial, two inclusions of wheat gluten products at 20% (20% vital wheat gluten and 20% soluble wheat gluten) and three inclusions of a blend of soluble and vital wheat gluten (20%, 25% and 30% inclusion levels) were fed to rainbow trout at the expense of soy protein concentrate, along with a plant protein control. Dietary treatments were randomly attributed to triplicate tanks, and fed at a rate of 1.5 - 2.5 % of biomass per day in equal rations at 09:00, 13:00 and 17:00 daily. Feed was adjusted daily on a predicted FCR of 1, based on initial biomass weights. Rainbow trout were maintained at  $15 \pm 1^{\circ}\text{C}$  with a 12:12 light dark photoperiod. pH was maintained at  $7.0 \pm 0.5$  and  $>85\%$  dissolved oxygen. Temperature, pH and dissolved oxygen were monitored daily. Water ammonia, nitrite and nitrate were monitored weekly and maintained within the acceptable range for the species, and managed with water changes to negate any detrimental build-up of compounds.

### *2.2.2. Experimental diets*

Six experimental diets were formulated and manufactured at the University of Plymouth as described in section 2.3. Vital wheat gluten (Amytex<sup>®</sup>), and hydrolysed, soluble wheat gluten (Solpro<sup>®</sup>) were supplied by Tereos Syral (Marckolsheim, France). Each wheat gluten product was included in its own diet at an inclusion level of 20% (20% Vital and 20% Sol treatments), and a further three diets were manufactured with wheat gluten blended in a ratio of 18:2, vital to soluble wheat gluten at inclusion levels of 20%, 25% and 30% (20% Blend, 25% Blend and 30% Blend treatments), incorporated at the expense of soy protein concentrate, in the same formulation as a soya protein control diet (diet SPC). Additional L-Lysine was added to all diets to exceed the minimum nutritional requirements of rainbow trout. Proximate composition of all diets was carried out prior to the start of experimental

feeding as described in section 2.8. All diets were iso-nitrogenous and iso-lipidic. Diet formulation and proximate composition can be seen in Table 4.1.

**Table 4.1.** Dietary formulation and proximate composition (%).

	SPC	20% Vital	20% Sol	20% Blend	25% Blend	30% Blend
<b><i>Ingredient (%)</i></b>						
Herring meal <sup>5</sup>	10	10	10	10	10	10
Soya protein concentrate <sup>1</sup>	52.00	27.38	25.67	27.21	21.05	14.89
Soyabean meal <sup>7</sup>	10	10	10	10	10	10
Vital wheat gluten <sup>3</sup>	-	20	-	18	22.5	27
Soluble wheat gluten <sup>4</sup>	-	-	20	2	2.5	3
Corn starch <sup>6</sup>	7.69	13.56	14.05	13.61	15.06	16.52
Fish oil <sup>2</sup>	16.49	15.47	16.61	15.58	15.35	15.12
L-Lysine HCl <sup>6</sup>	1.85	1.59	1.67	1.6	1.53	1.47
Calcium carbonate <sup>8</sup>	1	1	1	1	1	1
Vitamin mineral premix <sup>9</sup>	0.5	0.5	0.5	0.5	0.5	0.5
CMC-binder <sup>6</sup>	0.5	0.5	0.5	0.5	0.5	0.5
Antioxidant mix <sup>10</sup>	0.03	0.03	0.03	0.03	0.03	0.03
<b><i>Proximate composition (%)</i></b>						
Moisture	5.27	6.00	5.37	5.95	5.89	5.91
Protein	45.73	45.45	45.41	45.38	46.07	46.00
Moisture	5.27	6.00	5.37	5.95	5.89	5.91
Lipid	17.08	16.81	17.26	16.81	17.28	17.23
ash	7.03	5.55	5.36	5.59	5.28	4.98

<sup>1</sup> SPC 60 (BioMar, DK); <sup>2</sup> Epanoil (Seven Seas, UK); <sup>3</sup> Amytex® (Tereos syral, FR); <sup>4</sup> Solpro®(Tereos syral, FR) <sup>5</sup> LT94 Herring meal (CC Moore, UK); <sup>6</sup> (sigma Aldrich, UK); <sup>7</sup> HP 100 (Hamlet, DK); <sup>8</sup> (Fisher Scientific, USA); <sup>9</sup> PNP Fish: Ash 78.7 %, Ca 12.1 %, Mg 1.56 %, P 0.52 %, Cu 0.25 g/kg, Vit. A 1.0 µg/kg, Vit D3 0.1 µg/kg, Vit. E 7 g/kg (Premier Nutrition, UK); <sup>10</sup> Ethoxyquin 0.075 gKg<sup>-1</sup>, BHT 0.05 gKg<sup>-1</sup>, Natural tocopherols 0.2 gKg<sup>-1</sup> (Premier Pet Nutrition, UK).

### *4.2.3. Sampling*

Throughout the course of the feeding trial, tank biomass was bulk weighed bi-weekly, and prior to end point sampling to allow the calculation of growth performance as described in section 2.5.

Two fish per tank were sampled at the end point of the 56 day trial for microbiology ( $n = 6$  per treatment). A further two fish per tank were euthanised and samples taken for microscopy ( $n = 6$  per treatment) and two fish per tank were also euthanised for analysis of carcass composition. Fish were euthanised via concussion followed by destruction of the brain, in accordance with the schedule one procedure of the Animals (Scientific Procedures) Act 1986. Fish for microbiological analysis were dissected and samples obtained under aseptic conditions. The intestine of the fish were excised post pyloric caeca to the anal vent, visceral fat removed and samples taken from identical areas of the posterior region as described in section 2.6. Digesta for microbiological analysis of allochthonous bacterial community was collected from the entire posterior intestine under aseptic conditions utilising sterile forceps and collected in PCR clean / sterile microcentrifuge tubes, before storage at  $-20^{\circ}\text{C}$ .

### *4.2.4. Proximate composition*

Proximate composition of diets and carcasses was carried out as described in section 2.8.

#### *4.2.5. Haematological and serological analysis*

Blood was taken from the caudal vein of four fish per tank at the conclusion of the feed trial, achieving an  $n = 12$  per treatment. Hematological and serological analysis was carried out as described in section 2.9.

#### *4.2.6. Somatic indices*

Post euthanasia, specific weights and lengths of experimental animals were taken for Fulton's K-factor, hepatosomatic and viscerosomatic indices as described in section 2.7.

#### *4.2.7. Microbiological analysis / PCR-DGGE and sequencing*

For PCR-DGGE analysis, digesta was sampled from two fish per tank, providing  $n = 6$  per treatment. DNA extraction and Denaturing gel gradient electrophoresis was carried out as described in sections 2.10.1. and 2.10.2., respectively, on five of the six samples taken per treatment. The sample omitted was selected at random providing a final  $n = 5$  per treatment, allowing all samples to be run on a single gel, allowing cross treatment comparison.

### **4.3 Results**

#### *4.3.1. Gross observations*

Over the course of the 56 day feed trial fish accepted the experimental diets well and grew consistently throughout the trial. Survivability was unaffected by dietary treatment.

#### 4.3.2. Growth performance and carcass composition

Growth performance was assessed at the end point of the 56 day feed trial, based on the final tank biomass weights. Assessment of growth performance was achieved through FCR, SGR, mean fish weight, PER, K factor and survivability (Table 3.3b). Rainbow trout in all treatments performed well throughout the trial with good appetites, achieving good growth performance with FCR's ranging from  $1.18 \pm 0.02$  in the SPC treatment to  $1.09 \pm 0.02$  in the 20% Sol diet. FCR was significantly improved ( $P < 0.05$ ) in all wheat gluten treatments compared to the SPC control. SGR, PER, and mean fish weight at the end point of the trial all showed the same significant improvements in all wheat gluten treatments compared to the SPC treatment ( $P < 0.05$ ). There were no significant differences observed between wheat gluten treatments for any growth performance parameters (Table 4.2)

Carcass protein post 56 day feed trial was unaffected by wheat gluten inclusions compared to the SPC treatment. The 20% Sol and 30% Blend treatments ( $14.94 \pm 1.74\%$  and  $14.64 \pm 0.29\%$  respectively) were however significantly elevated ( $P < 0.05$ ) compared to the 20% Vital treatment ( $12.75 \pm 1.51\%$ ). Carcass lipid was significantly increased ( $P < 0.05$ ) in the 20% Vital treatment ( $14.17 \pm 1.60\%$ ) compared to the SPC, 20% Blend and 30% Blend treatments ( $12.06 \pm 0.27\%$ ,  $11.62 \pm 1.36\%$  and  $12.09 \pm 0.81\%$  respectively), all other treatments showed no significant difference in lipid composition (table 4.3). Moisture was significantly decreased ( $P < 0.05$ ) in the 20% Sol and 30% Blend treatments ( $68.64 \pm 3.88\%$  and  $70.51 \pm 0.55\%$  respectively) compared to the SPC diet ( $72.17 \pm 0.94\%$ ), all other treatments showed no significant difference. Ash was significantly decreased in the 20% Vital ( $1.42 \pm 0.06\%$ ) and 20% Sol ( $1.41 \pm 0.15\%$ ) treatments compared to the SPC treatment ( $1.68 \pm 0.08$ ). Blended

wheat gluten inclusions were not significantly different from any other treatment ( $P>0.05$ ).

Data is presented in Table 4.3.

#### *4.3.3. Somatic indices and haematological parameters*

Condition factor (K-factor) and VSI were unaffected by dietary treatment post 56 day feed trial. Hepatosomatic index was significantly increased in the 30% blended wheat gluten treatment ( $1.17 \pm 0.09$ ) compared to the SPC, 20% Vital and 25% Blend treatments (Table 4.4).

Most haematological parameters, haemoglobin level and serum lysozyme activity were unaffected by dietary inclusion of wheat gluten at the conclusion of the feed trial. Packed cell volume (haematocrit) was significantly increased in the 20% Sol treatment ( $33.58 \pm 6.33$  %) compared to the 20% Vital treatment ( $26.33 \pm 6.19$  %).

**Table 4.2.** Growth performance of rainbow trout at the end of the feed trial.  $n = 3$ . Superscripts denote significance. Significance accepted at  $P < 0.05$ .

	SPC	20% Vital	20% Sol	20% Blend	25% Blend	30% Blend
FCR	1.18±0.02 <sup>b</sup>	1.11±0.03 <sup>a</sup>	1.09±0.02 <sup>a</sup>	1.10±0.02 <sup>a</sup>	1.10±0.03 <sup>a</sup>	1.10±0.02 <sup>a</sup>
SGR	1.89±0.03 <sup>b</sup>	1.99±0.04 <sup>a</sup>	2.02±0.05 <sup>a</sup>	2.02±0.05 <sup>a</sup>	2.01±0.06 <sup>a</sup>	2.02±0.03 <sup>a</sup>
Average fish weight end point (g)	78.38±1.48 <sup>b</sup>	83.91±1.24 <sup>a</sup>	85.46±2.06 <sup>a</sup>	84.62±1.84 <sup>a</sup>	85.00±1.28 <sup>a</sup>	84.16±1.77 <sup>a</sup>
Protein efficiency ratio	0.61±0.02 <sup>b</sup>	0.68±0.02 <sup>a</sup>	0.71±0.02 <sup>a</sup>	0.70±0.02 <sup>a</sup>	0.68±0.03 <sup>a</sup>	0.68±0.02 <sup>a</sup>
Survivability (%)	98.9±1.91	98.9±1.91	97.8±1.91	98.9±1.91	97.8±3.81	100±0.00

**Table 4.3.** Carcass composition of rainbow trout at the end of the feed trial.  $n = 3$ . Superscripts denote significance. Significance accepted at  $P < 0.05$ .

	SPC	20% Vital	20% Sol	20% Blend	25% Blend	30% Blend
<b><i>Carcass proximate composition (%)</i></b>						
Moisture	72.17±0.94 <sup>a</sup>	72.95±3.41 <sup>ab</sup>	68.64±3.88 <sup>b</sup>	71.72±1.96 <sup>ab</sup>	70.97±0.93 <sup>ab</sup>	70.51±0.55 <sup>b</sup>
Protein	13.69±0.38 <sup>ab</sup>	12.75±1.51 <sup>b</sup>	14.94±1.74 <sup>a</sup>	13.99±0.94 <sup>ab</sup>	13.8±1.08 <sup>ab</sup>	14.64±0.29 <sup>a</sup>
Lipid	12.06±0.27 <sup>b</sup>	14.17±1.6 <sup>a</sup>	12.24±0.34 <sup>ab</sup>	11.62±1.36 <sup>b</sup>	12.34±1.32 <sup>ab</sup>	12.09±0.81 <sup>b</sup>
Ash	1.68±0.08 <sup>a</sup>	1.42±0.06 <sup>b</sup>	1.41±0.15 <sup>b</sup>	1.59±0.1 <sup>ab</sup>	1.45±0.04 <sup>ab</sup>	1.45±0.15 <sup>ab</sup>
Energy (MJ kg <sup>-1</sup> dry weight)†	28.68±0.09	29.38±0.31	28.96±0.22	28.71±0.32	29.00±0.55	29.07±0.02

† calculated utilising the conversion factors of 23.6, 39.5, and 17.2 kJ g<sup>-1</sup> for protein, lipid, and nitrogen-free extract (NFE), respectively (Tytler and Calow, 1985).

**Table 4.4.** Somatic, Haematological and serological parameters of rainbow trout post 56 day feed trial.  $n = 12$ . Superscripts denote significance. Significance accepted at  $P < 0.05$ .

	SPC	20% Vital	20% Sol	20% Blend	25% Blend	30% Blend
<b><i>Somatic indices</i></b>						
K-factor	1.31±0.10	1.31±0.06	1.21±0.25	1.37±0.10	1.36±0.08	1.37±0.09
HSI	1.02±0.14 <sup>b</sup>	1.13±0.12 <sup>b</sup>	1.25±0.58 <sup>ab</sup>	1.19±0.15 <sup>ab</sup>	1.10±0.13 <sup>b</sup>	1.17±0.15 <sup>a</sup>
VSI	14.1±0.99	13.55±1.08	15.99±7.9	13.89±1.1	14.46±2.9	13.67±0.99
<b><i>Haematological parameters</i></b>						
Haemoglobin (mg/100ml)	0.64±0.1	0.59±0.13	0.72±0.15	0.64±0.19	0.62±0.11	0.68±0.12
Haematocrit (%)	30.23±4.14 <sup>ab</sup>	26.33±6.19 <sup>b</sup>	33.58±6.33 <sup>a</sup>	27.11±7.44 <sup>ab</sup>	27.84±4.74 <sup>ab</sup>	29.23±4.98 <sup>ab</sup>
Lysosyme activity (AU)	899.4±294.2	948.2±416.2	794.4±378.8	826.6±421.3	847.5±501.3	908.7±411.8

HSI = hepatosomatic index, VSI = Viscerosomatic index

#### 4.3.4. Intestinal microbiology

##### 4.3.4.1. PCR-DGGE

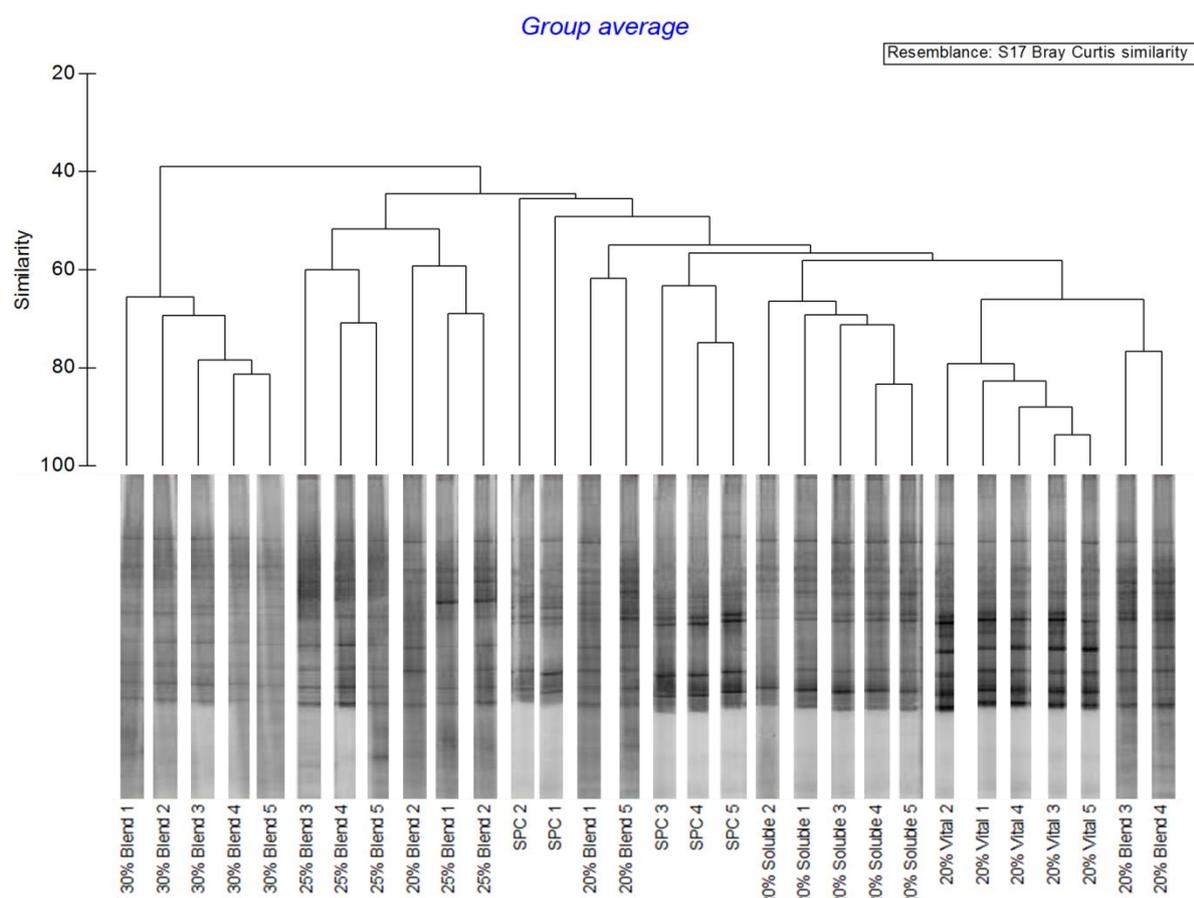
V3 16S rRNA PCR-DGGE fingerprints and associated dendrogram, showing a degree of clustering, from the allochthonous microbiota extracted from the posterior intestine at the end of the feeding are presented in Figure 4.1. Ecological parameters and pairwise comparisons are presented in Table 4.4.

The denaturing gradient gel revealed a total of 71 distinct OTU's within the digesta of sampled fish. Comparison of the experimental diets with the plant protein control revealed a relatively low level of similarity in bacterial communities (28.92 – 53.51 %) (Permanova), decreasing with increasing blended wheat gluten inclusion. 45.13% similarity was observed between the SPC and 20% Blend treatments, reducing to 28.92% similarity between the SPC and 30% Blend treatments (Table 4.4). All treatments were significantly different from one another ( $P < 0.05$ ). Intra-treatment similarity (SIMPER) significant differences ( $P < 0.05$ ) are presented in Table 4.4. OTU's per treatment and ecological parameters, diversity and richness, were unaffected by dietary treatment.

##### 4.3.4.2. DGGE sequence analysis

Twelve prominent OTU's were excised from the PCR-DGGE gel for sequence analysis. Only three of the excised bands yielded sequences of sufficient quality for taxonomic identification, despite numerous PCR enzymes and protocols tested.

The three bands returned sequences for *Weissella confusa* (96% alignment similarity), *Aerococcus* sp. (95% alignment similarity) and *Macrococcus caseolyticus* (99% alignment similarity). Species presence in replicates per treatment is presented in Table 4.7.



**Figure 4.1** PCR–DGGE fingerprint profiles with cluster analysis dendrograms of the posterior intestinal microbiota of rainbow trout at the end of the feeding trial.

**Table 4.6.** Allochthonous microbial community analysis from the PCR-DGGE of the bacterial communities in the posterior intestine of Rainbow trout fed experimental diets for 56 days. (ANOVA + *post hoc* Tukey's) Significance accepted at  $P < 0.05$ . Values expressed as means  $\pm$  standard deviation. Superscripts denote significance. Significance accepted at  $P < 0.05$ .

	Ecological parameters				PERMANOVA			
	OTU's	Richness	Diversity	SIMPER (similarity %)	df	f	<i>P-value</i>	Similarity (%)
SPC	18.6 $\pm$ 2.61	1.68 $\pm$ 0.24	2.88 $\pm$ 0.14	56.34 $\pm$ 9.33 <sup>d</sup>				
20% Vital	20.6 $\pm$ 1.52	1.83 $\pm$ 0.12	2.97 $\pm$ 0.08	83.43 $\pm$ 5.70 <sup>a</sup>				
20% Sol	16.8 $\pm$ 2.77	1.53 $\pm$ 0.24	2.8 $\pm$ 0.17	69.89 $\pm$ 6.26 <sup>bc</sup>				
20% Blend	17.6 $\pm$ 4.1	1.59 $\pm$ 0.36	2.83 $\pm$ 0.26	59.87 $\pm$ 8.07 <sup>cd</sup>				
25% Blend	15 $\pm$ 2.12	1.37 $\pm$ 0.19	2.69 $\pm$ 0.14	57.43 $\pm$ 8.80 <sup>d</sup>				
30% Blend	17 $\pm$ 1.73	1.58 $\pm$ 0.16	2.82 $\pm$ 0.1	70.78 $\pm$ 7.21 <sup>b</sup>				
Treatments					5	7.65	0.001	
Residuals					24			
Total					29			
Pairwise comparison						t		
SPC Vs 20% Vital						2.4528	0.01	53
SPC Vs 20% Sol						1.8857	0.006	53.51
SPC Vs 20% Blend						2.0892	0.006	45.13
SPC Vs 25% Blend						2.53	0.01	36.99
SPC Vs 30% Blend						3.6895	0.01	28.92
20% Vital Vs 20% Sol						3.2482	0.006	57.69
20% Vital Vs 20% Blend						2.3071	0.012	57.97
20% Vital Vs 25% Blend						3.0815	0.013	46.35
20% Vital Vs 30% Blend						5.2427	0.006	38.6
20% Sol Vs 20% Blend						1.841	0.007	56.34
20% Sol Vs 25% Blend						2.6789	0.008	44.09
20% Sol Vs 30% Blend						3.8522	0.012	41.52
20% Blend Vs 25% Blend						1.7407	0.007	50.3
20% Blend Vs 30% Blend						2.827	0.01	44.78
25% Blend Vs 30% Blend						2.9626	0.004	40.65

**Table 4.7.** Closest bacterial relatives (% similarity) of excised and sequenced bands from the PCR-DGGE of rainbow trout digesta samples from the posterior intestine, post 8 week feeding of experimental diets. Presence absence of bands within treatment replicates is indicated in column 2-7. Numbers represent bands present in number of replicates. 0 = not present in any replicate, 5 = present in all five treatment replicates.

Band ID	Band presence						Phyla	Nearest neighbour	Alignment similarity (%)	Accession number
	Replicates									
	SPC	20% Vital	20% Sol	20% Blend	25% Blend	30% Blend				
4	1	5	5	5	5	5	Firmicutes	<i>Weissella confusa</i>	96	AM117158.1
7	5	0	0	0	0	0	Firmicutes	<i>Aerococcus</i> sp.	95	KP152586.1
8	5	4	5	4	5	0	Firmicutes	<i>Macrococcus caseolyticus</i>	99	LN997935.1

#### 4.4. Discussion

The objective of the current investigation was to evaluate the effect of vital and hydrolysed (soluble) wheat gluten inclusions, and a blend of both, in soya based diets on the growth performance, organism condition and microbiology of the posterior intestine of rainbow trout. A 56 day feed trial combined with microbiological, condition indices and haematological analysis were utilised to achieve these objectives.

Throughout the trial fish performed well, with FCR's ranging from  $1.09 \pm 0.02$  (20% Sol) to  $1.18 \pm 0.02$  (SPC), with pellets readily accepted. FCR, SGR and PER was significantly improved ( $P < 0.05$ ) in the wheat gluten treatments compared to the SPC treatment. These improvements in growth parameters led to significantly larger final mean fish weights at the end point of the 56 day trial ( $P < 0.05$ ). Improved growth performance (weight gain) has been reported in rainbow trout with a 28.65% vital wheat gluten inclusion, at the expense of both fishmeal and soy protein with additional amino acid supplementation (Davies et al., 1997), and improved FCR's have been observed in hybrid sturgeon (*Acipenser schrenckii* ♀ × *Huso dauricus* ♂) supplemented 5% Solpro as a glutamine enhancer (Qiyu et al., 2011). Improved FCR has also been reported with a 6% inclusion of hydrolysed wheat gluten (Solpro®) in Asian seabass fed an animal by-product based diet (Apper et al., 2016). Investigations observing comparable growth performance results whilst utilising wheat glutes as alternative protein sources have previously been discussed in section 3.4. Storebakken *et al.* (2015) reported maintained growth performance in diets replacing 50% of fishmeal with hydrolysed wheat gluten, whilst increasing amino acid availability. Growth performance observed in the present investigation may be as a result of such amino acid availability in wheat gluten diets surpassing that of those in the SPC treatment. Significant

reductions in amino acid digestibility coefficients have been observed in Atlantic salmon when soya was incorporated into feeds (Anderson et al., 1992). Davies *et al.* (1997) identified elevated individual essential amino acid digestibility in wheat gluten inclusion treatments with additional lysine supplementation compared to a soya-fish-meal-based reference diet. ANF content may also play a role in the improved growth performance observed compared to the soya protein control. As a processed by-product, having undergone carbohydrate extraction, and hydrolysis in the case of Solpro<sup>®</sup>, wheat glens are considered a much 'cleaner' protein source and a partially purified ingredient (Davies et al., 1997). The higher degree of processing in wheat gluten results in lower ANF content, which have the potential to reduce nutrient digestibilities (as discussed in section 1.2.1), especially when compared to 'full fat' soya bean meal and other crude plant protein sources. Further to this effect is the greater alcohol soluble carbohydrate fraction in full fat soya products, responsible for decreasing nutrient digestibilities in Atlantic salmon (Olli and Krogdahl, 1995). The replacement of sub optimal protein sources in aquafeeds with high quality wheat gluten products could therefore be reasonably assumed to increase nutrient and amino acid digestibilities, improving growth performance in juvenile rainbow trout.

The effect of wheat gluten inclusion on the carcass composition at the end point of the investigation showed no significant difference in the protein content of sampled rainbow trout compared to the SPC treatment (table 4.3) as was observed in chapter 3b. The 20% Sol and 30% Blend treatments however were significantly elevated compared to the 20% Vital treatment. Carcass moisture content was significantly reduced in the 20% Sol and 30% Blend treatments, potentially an inverse effect of numerically higher observed protein levels in these treatments. Carcass lipid levels were significantly increased in the 20% vital wheat

gluten treatment compared to the SPC, 20% Blend and 30% Blend treatments. The increase in lipid content has not previously been associated with the inclusion of dietary wheat glutes, with reduced lipid levels observed with vital wheat gluten inclusion in chapter 3b. Investigations by both Rodehutsord *et al.* (1997) and Davies *et al.* (1997) observed no effect on carcass composition with wheat gluten inclusions with free crystalline amino acid supplementation. The effect of large plant protein inclusions has previously been reported to increased carcass lipid levels, observed in black-chinned tilapia (*Sarotherodon melanotheron*) (Koumi et al., 2008) and European seabass (Kaushik et al., 2004). Increased lipogenesis has also been observed in rainbow trout fed diets replacing total fish oil and protein with plant based products, resulting in the increase in expression of genes associated with lipid biosynthesis (Panserat et al., 2009). However, with the present investigation, experimental diets all contained equal 10% inclusions of fishmeal, and only soya protein concentrate removed for increased wheat gluten inclusions. The higher lipid content of vital wheat gluten (~6%, Tereos Syral product description) compared to soy protein concentrate(3%) (NRC, 1998), could play a role in the increased lipid carcass content, yet ensuring iso-lipid formulations should negate this factor and similar results would be expected with higher wheat gluten inclusion levels. Carcass ash was reduced in the 20% Vital and 20% Sol treatments compared to the SPC treatment. Significant differences observed in the present study are contradictory to those observed in chapter 3b, where hydrolysed wheat gluten products resulted in elevated carcass ash levels. Despite significant differences observed between treatments, carcass energy was unaffected by dietary treatment.

In the present study condition factor (K-factor), was unaffected by dietary inclusion. Blended wheat gluten treatments were numerically superior to all other treatments, yet not significantly so. Fulton's K-factor enables inferences on physiological and biological circumstance and has been shown to fluctuate through numerous stressors, including feeding condition, probiotic supplementation, feed formulation, parasitic infection and physiological factors (Le Cren, 1951; de Francesco et al., 2004; Merrifield et al., 2011). K-factors have been previously observed by Barnes *et al.* (2012) to be unaffected in rainbow trout after 70 days feeding with up to 50% inclusions of fermented soybean meal product, with 0% fishmeal. However de Francesco *et al.* (2004) observed increased K-factors with mixed plant protein inclusions in the same species after a 24 week feed trial. This increase in condition factor was attributed to increased mesenteric fat content, as could be assumed likely in the increased lipid content observed with enhanced plant protein inclusions (Koumi et al., 2008; Kaushik et al., 2004; Panserat et al., 2009).

In the present study carcass lipid was significantly increased in the 20% vital wheat gluten treatment. As discussed, increased plant protein inclusion has been shown to increase carcass lipid content, likely to be in the form of increased visceral fat. In the present study the viscerosomatic index (VSI) was unaffected by wheat gluten inclusions (Table 4.4). Chaiyapechara *et al.* (2003) observed increased carcass, fillet and visceral lipid in rainbow trout with high lipid diets, compared to a commercial control. Associated with the increased visceral fat, VSI was also significantly increased (Chaiyapechara et al., 2003). The present studies observations of unaffected VSI would suggest any additional carcass lipid was present in the fillet of experimental animals, and not in the visceral material, discarded during animal processing.

The hepatosomatic index of fish in the present study ranged from  $1.02 \pm 0.14$  in the SPC diet to  $1.25 \pm 0.58$  in the 20% soluble treatment, similar to investigations into plant protein replacement in rainbow trout diets (Barnes et al., 2012; Panserat et al., 2009), yet much higher values have been observed with soybean meal and other soya product inclusions (Gaylord et al., 2006; Kaushik et al., 1995). Hepatosomatic index (HSI) has been identified to be positively related to carbohydrate content in the diet (Daniels and Robinson, 1986; Kim and Kaushik, 1992). In the present study, HSI was significantly increased in the 30% Blend treatment compared to the SPC, 20% Vital and 25% Blend. This is unlikely to be as a result of carbohydrate content increase as the vital wheat gluten comprising 90% of the blended wheat gluten has a lower carbohydrate content than the soya products it is replacing (Apper-Bossard et al., 2013). HSI has also been observed to be inversely related to dietary phosphorous (Sakamoto, 1978). However, low levels of phytic acid in wheat gluten should increase phosphorous bio-availability as discussed in section 1.3. Wheat gluten inclusions have previously reported no impacts on HSI in rainbow trout (Tusche et al., 2012) and pacific white shrimp (Molina-Poveda and Morales, 2004), nor with increased plant protein inclusion in Atlantic salmon (Tacchi et al., 2012).

The substitution of soy protein concentrate with wheat gluten inclusions had no effect on serum lysosyme activity at the end of the feed trial as was observed in chapter 3b. Haemoglobin levels were observed to be elevated with 10% vital wheat gluten in chapter 3b, compared to a 10% soluble wheat gluten inclusion. In the present study no effect of wheat gluten inclusion level or type was observed on haemoglobin level. Haematocrit levels observed in the present study, around 30%, are indicative of healthy fish in good nutritional status (Tusche et al., 2012; Wells and Weber, 1991; Congleton and Wagner, 2006).

Haematocrit was significantly increased in the 20% Sol treatment compared to the 20% Vital treatment in the present study. Increase in haematocrit is associated with increased oxygen carrying capacity, but also an exponential rise in blood viscosity and increased stress on the circulatory system (Wells and Weber, 1991). The effect of wheat gluten inclusion on the packed cell volume of salmonids is poorly reported in the literature, however, Tusche *et al.* (2012) previously report no effect of up to 50% replacement of fishmeal with wheat gluten and potato protein.

The importance of the GI tract and the modulatory effects of feed ingredients on the intestinal microbiota have been well established, as discussed in chapter 1 and section 3.4a. The preliminary investigation and the long term feed trial in chapter 3b documented the effects of individual wheat gluten products on the allochthonous bacterial populations in the posterior intestine of rainbow trout. Clustering of the communities from the fish fed hydrolysed wheat protein and clustering of the communities from the fish fed vital and SPC treatments together were observed in both instances. Despite modulation of bacterial genera in sampled digesta, overall bacterial population at phylum level was unaffected by the dietary inclusions of wheat glutes. The effect of blending two wheat gluten meals, vital (Amytex®) and soluble hydrolysed (Solpro®) wheat gluten are currently unreported.

The PCR-DGGE analysis of the allochthonous bacterial community of the posterior intestine reveal that the inclusion of vital, soluble and blended wheat gluten products at 20% inclusion levels had little effect on the clustering of treatments as can be seen in Figure 4.1. Sample replicates generally clustered more closely with each other than with other treatments. The 20% Vital, 20% Sol, 2 of 5 20% Blend replicates and 3 of 5 SPC replicates

showed similarities of approx. 58%. Increased blended wheat gluten inclusion (25% and 30%) appeared to decrease intra-treatment similarity with the 20% inclusion replicates (approx. 45% similarity for the 25% Blend replicates, approx. 40% similarity for the 30% Blend replicates). The decreasing similarity to the 20% wheat gluten treatments and the SPC treatment indicates a dose response of intestinal microbiota change to blended wheat gluten inclusion.

Replicate similarity within treatments (SIMPER) was significantly elevated in the 20% vital wheat gluten treatment compared to all other treatments. Replicate similarity in the 20% Sol and 30% Blend treatments were also significantly elevated compared to the SPC treatment. These elevations in replicate similarity would indicate a stabilising effect of wheat gluten on the allochthonous microbial population compared to soya based proteins, as was observed with 15% vital wheat gluten inclusion in chapter 3a. Ecological parameters, OUT's, species richness and diversity were unaffected by dietary inclusions of wheat gluten, even at the highest inclusion levels compared to the SPC treatment. This is contradictory to the reduced species diversity observed with 15% hydrolysed (Merripro®) wheat gluten inclusion in chapter 3a. This could be as a result of different ingredient characteristics in the less heavily processed Solpro® hydrolysed wheat gluten, aligning it more closely to the vital wheat gluten. The pairwise comparisons (PERMANOVA) of the banding profiles and intensities of treatments reveal all treatments to be significantly different from one another ( $P < 0.05$ ) (Table 3.2a). This can be correlated with the higher degree of clustering within treatment replicates than with other treatments observed in Figure 4.1. This is again consistent with the observations of chapter 3a, for the higher inclusion levels of wheat gluten aquafeeds at the expense of soya products, and the clustering observed in chapter 3b.

PCR-DGGE band Sanger sequencing returned 3 sequences from 12 with sufficient quality for taxonomic identification (Table 4.7). Low sequence quality is likely assumed to be a result of two or more PCR products of very similar base pair length occurring in the band location excised for sequencing. This would result in a mixture of species sequences and make taxonomic identification unachievable. The three species which returned sequences high enough for taxonomic identification were all of the phyla Firmicutes. Firmicutes have been reported to be a major contributor to the intestinal microbiota, as well as Proteobacteria (Mansfield et al., 2010; Heikkinen et al., 2006; Huber et al., 2004; Navarrete et al., 2010; Pond et al., 2006), as discussed in section 1.4.1 and is consistent with the results observed and discussed in chapter 3.

*Weissella confusa* was identified (alignment similarity 96%) in all wheat gluten treatment replicates, but only in one of the SPC replicates. *Weissella* have been routinely identified in the intestines of trout and Atlantic salmon (Wong et al., 2013; Revoco et al., 2014; Mansfield et al., 2010; Hovda et al., 2012) and have been identified as a potential probiotic species for juvenile sea bass (*Lates calcarifer*) (Rengpipat et al., 2008) as discussed in section 3.4.. Increased *Weissella confusa* reads from high throughput sequencing have previously been observed with 10% vital wheat gluten inclusions in rainbow trout (chapter 3b), and with 6% soluble hydrolysed wheat gluten (Solpro®) by Apper et al. (2016) in Asian seabass (*Lates calcarifer*). Identification in all wheat gluten treatments and only in one replicate of the SPC treatment would support these previously observed results.

*Aerococcus* sp. was identified (95% alignment similarity) in all five SPC treatment replicates, and none of the wheat gluten treatment replicates. This would suggest the digesta and

intestinal conditions produced with the addition of 20% wheat gluten products in diets is sub optimal for this specific *Aerococcus* sp. and in turn prevents *Aerococcus* sp. proliferation or it is out competed by other commensal bacteria. *Aerococcus* spp. have not been noted as part of the normal intestinal microflora in extensive investigations on characterising the intestinal microbiota of rainbow trout by Wong *et al.* (2013), Mansfield *et al.* (2010), Ringo and Birkbeck (1999) or Ringø and Gatesoupe (1998), nor have they been noted with increased plant protein inclusions (Desai *et al.*, 2012). *Aerococcus* spp. have however previously been shown to be associated with farmed fish and the farmed environment, isolated by Michel *et al.* (2007), yet from unspecified samples. *Aerococcus viridans* is the most commonly associated *Aerococcus* species documented in the aquaculture and seafood environment, notably the causative agent of fatal gaffkaemia infection in lobsters (Genus *Homarus*). *A. viridans* has also been associated with streptococcosis in rainbow trout in a region of turkey (Özer *et al.*, 2008) as well as 30-40% mortality in tilapia (*Oreochromis niloticus*) (Ke *et al.*, 2012), but conversely isolated from farmed European seabass and identified as a potential probiotic, showing resistance to pathogenic and spoilage bacterial species (Bourouni *et al.*, 2012). *Aerococcus* spp. were not identified in chapter 3, and the absence of *Aerococcus* sp. in the allochthonous microbiota of wheat gluten treatments in the present investigation could be seen as beneficial to the health of the animal.

*Macrocooccus caseolyticus* (alignment similarity 99%) was identified in the SPC, 20% Sol and 25% Blend treatment replicates, and 4 out of 5 treatment replicates in the 20% Vital and 20% Blend treatments, and in no treatment replicates of the 30% Blend treatment (Table 4.7). *Macrocooccus* spp. and *Maccrocooccus*-like bacteria have been isolated and observed as normal constituents of the intestinal microflora of salmonids as described in section 3.5a,

and significantly larger number of reads associated with the genus *Macrocooccus* was observed with 10% hydrolysed wheat gluten inclusion in chapter 3b. To this authors knowledge there are no pathogenic reports of *Macrocooccus caseolyticus* in the aquaculture setting, with only one investigation identifying a *Macrocooccus* sp. associated with odontoma in one sampled marine pickhandle barracuda (*Sphyraena jello*), yet not associated as the causative agent of tumour development (Singaravel and Gopalakrishnan, 2015). *Macrocooccus caseolyticus* identified in the posterior intestine allochthonous microbiota could therefore be presumed an unassuming member of the microbial population, unable to freely proliferate in the conditions associated with the highest inclusion levels reported in the present study.

### 4.5. Conclusions

In conclusion, the application of wheat gluten inclusions, blended or single products had no obvious detrimental effects for juvenile rainbow trout. Increased growth performance with inclusion levels up to 30% support the positive results observed in this investigation as well as chapter 3. Blended wheat glutes, that allow higher percentage of wheat gluten inclusion in commercially extruded aquafeeds, achieved better growth performance than the control diet whilst maintaining healthy somatic indices, with no large scale impact on the intestinal microflora. These results suggest blended wheat gluten inclusions are a promising alternative protein sources for juvenile rainbow trout, allowing increased wheat gluten inclusions in extruded aquafeeds. Further investigation into sites of lipid deposition could help reveal the identified increase in carcass lipid with no effect on VSI. The application of wheat gluten with feed additives would also be an interesting area of research as pro-and prebiotics are being increasingly utilised in the aim to enhance organism health.

## **Chapter 5: The effect of blended dietary wheat gluten and scFOS on gut health, allochthonous intestinal microbial populations and growth performance of juvenile rainbow trout (*Oncorhynchus mykiss*).**

### **5.1. Introduction**

With the increased demand for the production of more sustainably sourced fish, the ever expanding aquaculture industry has realised the combined importance of animal nutrition and health. Throughout chapters 3 and 4 it has been observed that wheat gluten inclusions, an example of alternative plant protein sources, in commercially relevant low fishmeal aquafeeds have the ability to promote the aforementioned factors. Dietary feed additives are a further step, and an area of much interest, that have been examined with the potential for beneficial attributes in intensified production. Prebiotics, non-digestible feed ingredients fermented by intestinal microbiota, aimed at promoting beneficial intestinal microbiota whilst suppressing potentially pathogenic species, are one such additive that have received high research effort.

Since the first investigations in the late 1990's, a range of prebiotics have been investigated for the potential to improve growth performance, feed conversion, digestibility, survival, intestinal morphology, gastrointestinal enzyme activities, immune functions, the suppression of potentially pathogenic bacteria and enrichment of potentially beneficial gut bacteria (Ringø et al., 2014). Amongst the most commonly utilised prebiotics in aquafeed are inulin, mannanoligosaccharides (MOS), galactooligosaccharides, fructooligosaccharides (FOS) and short-chain fructooligosaccharides (scFOS). The application of prebiotics in

salmonids is well reviewed by Merrifield *et al.* (2010) Ringø *et al.* (2010) and Ringø *et al.* (2014). scFOS, characterised by a lesser degree of polymerisation than FOS, has not received a great deal of attention for use in salmonid aquaculture, yet has had promising results when supplemented in the diets of hybrid tilapia, white shrimp and European seabass as discussed in section 1.6..

Growth performance improvements and modulation of the intestinal microbiota have been demonstrated with scFOS supplementation in hybrid tilapia (*Oreochromis aureus*♂ × *O. niloticus*♀) (Lv *et al.*, 2007) and pacific white shrimp (*Litopenaeus vannamei*). There is however, little information on the effect of scFOS on immune parameters, intestinal health and intestinal stress is available in the literature. Guerreiro *et al.* (2014) observed no effect on innate immunology or haematology in turbot, whilst Abid *et al.* (2013) observed upregulation of immune-regulated genes with synergistic supplementation of scFOS with a probiotic. The present study therefore sought to investigate the effects of 0.3% supplementation of scFOS in high soy protein and wheat gluten aquafeeds for rainbow trout. The aim of the present study was to assess the effect of scFOS supplementation on the allochthonous microbial population of the posterior intestine, assess the effects on the intestinal immune and stress parameters and the effects on SCFAs concentrations in the posterior intestine.

SCFAs are scarcely studied in teleosts, and the literature is predominantly associated with herbivorous species (Kihara and Sakata, 1997; Clements *et al.*, 1994; Mountfort *et al.*, 2002; Smith *et al.*, 1996; Burr *et al.*, 2005). Produced via the fermentation of dietary fibre, such as prebiotics, SCFAs are an important energy source for rapidly proliferating enterocyte cells of

the intestinal tract (Guerreiro et al., 2015b). SCFAs have also been associated with the proliferation of lactic acid bacteria (Mountfort et al., 2002; Merrifield et al., 2010) and affecting lipid metabolism (Delzenne et al., 2002). Kihara (2008) observed increased concentrations of SCFAs in the hind gut of red sea bream (*Pagrus major*) fed with a fermentable oligosaccharide supplementation. The concentrations of lactic, acetic, butyric, formic and Valeric acids in the intestine of rainbow trout are currently unreported. This investigation aimed to ascertain the levels present in the intestine of trout fed soya protein and wheat gluten inclusion aquafeeds, and the affect the supplementation with fermentable scFOS has on their concentrations.

The analysis of immune-relevant inflammatory cytokines, stress biomarkers and cell proliferation associated genes at a molecular level will enable an insight into the effect of scFOS supplementation on juvenile rainbow trout intestinal health. The characterisation of teleost cytokine genes in recent years has aided the analysis of immune response activities, through the expression analysis of these immune-related genes (Abid et al., 2013; Alejo and Tafalla, 2011). The pro-inflammatory cytokines TNF- $\alpha$  and IL-8 are involved in cell proliferation, differentiation, apoptosis, necrosis, and the induction of other cytokines (TNF- $\alpha$ ) and chemotaxis of neutrophils, T lymphocytes and basophils (IL-8), whilst IL-1 $\beta$  is a key mediator in response to microbial tissue injury and invasion, activating lymphocytes or causing a cascade resulting in the release of other cytokines activating lymphocytes, macrophages and NK cells (Reyes-Cerpa et al., 2013; Abid et al., 2013). TGF- $\beta$ , regulates cell differentiation, migration, proliferation and development as well as survival in leukocytic lineages (leukocytes, macrophages, granulocytes, NK cells and dendritic cells) and can play an anti-inflammatory role (Reyes-Cerpa et al., 2013). Glute ST is involved in the protection from

reactive oxygen species, including intracellular metabolites. HSP 70, associated with cellular stress, and Casp 3, associated with apoptosis, will also be analysed to achieve the investigation aims.

In addition to these genes, microbiological and SCFAs analysis, growth performance and carcass composition analysis will be undertaken.

## **5.2. Materials and methods**

All experimental work involving fish was conducted under the approval of the Plymouth University Ethical Committee.

### *5.2.1 Experimental design*

Eight hundred and sixty four rainbow trout (XXX triploid genotype and wild phenotype) were acquired from the commercial production raceways at Exmoor fisheries (Somerset, UK) and stocked into 12, 120L tanks in a flow-through research system. Fish were stocked 72 fish per tank; average weight =  $14.65 \pm 0.07\text{g}$ , at an initial stocking density of  $8.79 \pm 0.04 \text{ Kg/m}^3$ . Flow through tanks were supplied with virgin spring water at approx. 6 L/min.

Over the course of a 70 days nutritional feed trial, four dietary treatments with and without 20% blended (vital and hydrolysed) wheat gluten and scFOS inclusions were fed to rainbow trout at the expense of soya protein concentrate (wheat gluten inclusion) and corn starch (scFOS inclusion). Dietary treatments were randomly attributed to triplicate tanks, and fed at a rate of 1.5 - 2% of biomass per day utilising commercially available clockwork belt feeders. Feed was adjusted daily on a predicted FCR of 1, based on initial biomass weights

and subsequent bi-weekly tank biomass weighing data. Rainbow trout were maintained at  $10.5 \pm 0.5^{\circ}\text{C}$  with an 18:6 light dark photoperiod utilising florescent lights and timers. Dissolved oxygen was maintained above 85%.

At the trial end point, additional samples were taken for carcass composition, molecular gene expression, microbiological and SCFA analysis.

### *5.2.2 Experimental diets*

Four experimental diets were formulated and manufactured at the University of Plymouth as described in section 2.3. Experimental wheat gluten products, vital wheat gluten (Amytex<sup>®</sup>) and hydrolysed, soluble wheat gluten (Solpro<sup>®</sup>), were supplied by Tereos Syral (Marckolsheim, France). A 20% inclusion level of blended vital and soluble-hydrolysed wheat gluten was formulated with wheat gluten incorporated at the expense of soy protein concentrate, in the same formulation as a soya protein control diet (diet SPC). A further two dietary treatments were formulated consisting of the SPC and 20% blend treatments each with the addition of 0.3% scFOS, at the expense of corn starch. scFOS (Profeed<sup>®</sup>) was provided by Tereos Syral (Marckolsheim, France). Proximate composition of all diets was carried out prior to the start of experimental feeding as described in section 2.8. All diets were iso-nitrogenous and iso-lipidic. Diet formulation and proximate composition are presented in Table 5.1.

**Table 5.1.** Dietary formulation and proximate composition (%).

	SPC	SPC+ FOS	20% Blend	20% Blend + FOS
<b><i>Ingredient (%)</i></b>				
Herring meal <sup>5</sup>	10	10	10	10
Soya protein concentrate <sup>1</sup>	52.00	52.00	27.21	27.21
Soyabean meal <sup>7</sup>	10	10	10	10
Vital wheat gluten <sup>3</sup>	-	-	18	18
Soluble wheat gluten <sup>4</sup>	-	-	2	2
Corn starch <sup>6</sup>	7.36	7.06	13.31	13.01
Fish oil <sup>2</sup>	16.49	16.49	15.58	15.58
L-Lysine HCl <sup>6</sup>	1.85	1.85	1.60	1.60
Calcium carbonate <sup>8</sup>	1	1	1	1
Vitamin mineral premix <sup>9</sup>	0.5	0.5	0.5	0.5
CMC-Binder <sup>6</sup>	0.5	0.5	0.5	0.5
scFOS <sup>11</sup>	-	0.30	-	0.30
Antioxidant mix <sup>10</sup>	0.03	0.03	0.03	0.03
<b><i>Proximate composition (%)</i></b>				
Moisture	5.08	5.39	5.35	5.66
Protein	46.94	46.6	47.39	47.48
Lipid	18.09	17.99	17.35	17.7
Ash	6.57	6.61	5.13	5.11

<sup>1</sup>SPC 60 (BioMar, DK); <sup>2</sup>Epanoil (Seven Seas, UK); <sup>3</sup>Amytex® (Tereos syral, FR); <sup>4</sup>Solpro®(Tereos syral, FR) <sup>5</sup>LT94 Herring meal (CC Moore, UK); <sup>6</sup>(sigma Aldrich, UK); <sup>7</sup>HP 100 (Hamlet, DK); <sup>8</sup>(Fisher Scientific, USA); <sup>9</sup>PNP Fish: Ash 78.7%, Ca 12.1%, Mg 1.56%, P 0.52%, Cu 0.25 g/kg, Vit. A 1.0 µg/kg, Vit D3 0.1 µg/kg, Vit. E 7 g/kg (Premier Nutrition, UK); <sup>10</sup>Ethoxyquin 0.075 gKg<sup>-1</sup>, BHT 0.05 gKg<sup>-1</sup>, Natural tocopherols 0.2 gKg<sup>-1</sup> (Premier Pet Nutrition, UK). <sup>11</sup>Profeed™ (Tereos syral, FR)

### *5.2.2. Sampling*

Throughout the course of the 70 day feeding trial, tank biomass was weighed bi-weekly, and prior to end point sampling to allow the calculation of growth performance as described in section 2.5.

At the end point of the 70 day feed trial, three fish per tank were euthanised and samples taken for intestinal microbiota analysis ( $n = 9$  per treatment). A further three fish per tank were euthanised and samples taken for gene expression and SCFA analysis ( $n = 9$  per treatment, per analyses). Three fish per tank were also euthanised for analysis of carcass composition. Fish were euthanised with an overdose of MS-222 ( $200 \text{ mgL}^{-1}$ , Pharmaq, UK) for 5 minutes, followed by destruction of the brain. Fish were dissected and intestine excised post pyloric caeca to the anal vent, visceral fat removed and samples taken from identical areas of the posterior region as described in section 2.6. Fish sampled for microbiological analysis of the allochthonous bacterial community of the posterior intestine were dissected and samples were taken under aseptic conditions utilising sterile forceps and collected in PCR clean / sterile microcentrifuge tubes, before storage at  $-20^{\circ}\text{C}$

### *5.2.3. Proximate composition*

Proximate composition of diets and carcasses was carried out as described in section 2.8.

#### *5.2.4. High –throughput intestinal microbiology*

Samples of posterior intestine digesta for high throughput sequence analysis were taken aseptically from two fish per tank, providing  $n = 6$  per treatment. DNA was extracted from 100 mg of sample as described in section 2.10.1. High throughput sequencing was carried out as described in section 2.10.4.

#### *5.2.5. Gene expression*

##### *5.2.5.1. RNA extraction, cDNA synthesis and real-time PCR*

Two fish per tank were sampled for gene expression analysis. RNA extraction, cDNA synthesis and real-time PCR were carried out as described in sections 2.10.5 and 2.10.6.

##### *5.2.5.2. Reference genes, genes of interest and analysis.*

Beta-actin ( $\beta$ -actin) and elongation factor 1-alpha (EF1- $\alpha$ ) were utilised as reference genes, as described in section 2.10.6. The absence of primer-dimers, amplification products in negative controls and acceptable primer efficiencies were insured prior to and whilst running samples.

Genes of interest analysed were the pro-inflammatory cytokines TNF- $\alpha$ , IL-8 and interleukin IL 1 $\beta$ . The anti-inflammatory cytokine: TGF-  $\beta$ . The cellular stress biomarker: HSP70, antioxidant status indicator: Glute-ST and apoptosis biomarker: Casp 3. Primers and their sequences can be found in Table 5.2.. Expression analysis was carried out as described in section 2.10.6.

**Table 5.2.** Primer information used for real-time PCR analysis.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Amplicon size (bp)	Acc. No	E-value	Annealing temp
$\beta$ actin	ACTGGGACGACATGGAGAAG	CCACCCTCAGCTCGTTGTAG	57	AJ438158.1	2.0	60
EF1- $\alpha$	AGGCTCCATCTTGGCTTCTC	GGGACCAGACTCGTCGTA	76	AF498320.1	2.1	60
IL-1 $\beta$	CTGCCTCAGGGTCTGGATCT	GCGATGATGAGGTTGGCAATG	74	AJ223954.1	2.0	58
IL-8	CGGAGAGCAGACGTATTGGTAA	GAGCTGGGAGGGAAACATCTC	58	HG917307.1	1.9	60
TNF- $\alpha$	TGGGTGTGAGTGACATCGTTAT	AGACCCTCAGCATCTGGTACT	87	HE717002.1	2.1	60
Glute-ST	CCTTCTCATTGGCTGACGTTAT	GCCGTAGACAGCCCAAAG	70	NM_001160559.1	2.0	58
CASP 3	AACAGATGTGGATGCTGGCTAT	CCGTCTGGTCATTGGCAACTT	80	AJ586436.1	2.1	60
TGF- $\beta$	TGCCTTGTGATTGTGGGAAAC	CCTCAGCTTGTTTCATCCCTGAT	68	AJ007836.1	2.1	60
HSP 70	TTGGCCGCAGTTTTGATGAT	CTCAAAGGGCCAATGCTTCAT	60	K02550.1	2.1	60

### 5.2.3. SCFA analysis

Digesta samples taken from the posterior intestine were snap-frozen in liquid nitrogen prior to storage at  $-20^{\circ}\text{C}$ . Samples were analysed for SCFA and lactic acid concentration utilising High Performance Liquid Chromatography (HPLC) according to the method of Niven et al. (2004) with some modifications in sample preparation as described by Akoy (2015). Briefly,  $0.1 \pm 0.01$  g of digesta was weighed into a clean 1.5ml micro centrifuge tube, and diluted 1:10 with MilliQ water. Samples were mixed for 10 minutes on a shaker plate at  $4^{\circ}\text{C}$  before centrifugation at  $17,000 \text{ xg}$  for 15 min at  $4^{\circ}\text{C}$ .  $500 \mu\text{l}$  of supernatant was retained in a new 1.5 ml micro centrifuge tube and  $18.5 \mu\text{l}$  7% (v/v) sulphuric acid was added to denature dissolved proteins and shift the acid dissociation equilibrium towards complete protonation of fatty acids. Samples were then vortexed for 30 seconds before centrifugation at  $13,000 \text{ xg}$  for 5 minutes. The supernatant was extracted utilising a 1ml polypropylene disposable syringe (Fisher Scientific, BD A-Line, UK) and filtered through  $0.2\mu\text{m}$  syringe filters (SMI-LabHut Ltd, Gloucester, UK), removing any particulate material still present. Samples were filtered into glass vials and sealed with crimp caps (11mm, Ruber/PTFE, Fisher Scientific, Loughborough, UK) before immediate running through the instrument.

Samples were run on a Dionex Ultimate 3000 with UV detector (220 nm) and agilent PL Hi-Plex H,  $300 \text{ mm} \times 7.7 \text{ mm}$  was utilized for the separation with a PL Hi-Plex H Guard Column  $50 \times 7.8 \text{ mm}$  of the same phase. 5mmol sulphuric acid was used as the eluent, pumped at a flow rate of  $0.5 \text{ mL/min}$  through the column (Agilent Technology, USA) ,maintained at  $25^{\circ}\text{C}$ .

Data analysis was carried out utilising Chromeleon® 7.1 Chromatography Data System Software (Dionex Softron GmbH, Germering, Germany). A calibration curve for each target

acid (lactic acid, butyric acid, acetic acid, formic acid and Valeric acid) was obtained from eight different concentrations (0.5 mM, 1 mM, 2.5 mM, 5.0 mM, 10.0 mM, 25.0 mM, 50.0mM and 1000 mM) of molecular grade standards.

### **5.3. Results**

#### *5.3.1. Gross observations*

Over the course of the 70 day feed trial fish accepted the experimental diets well and grew consistently throughout the trial. Survivability was unaffected by dietary treatment with all treatments achieving >99% survival.

#### *5.3.2. Growth performance and. Carcass composition*

Growth performance was assessed at the end point of the 70 day feed trial, based on the final tank biomass weights. Assessment of growth performance was achieved through FCR, SGR, mean fish weight, PER, K factor and survivability (Table 5.3). Rainbow trout in all treatments performed very well throughout the trial with good appetites, achieving positive growth performance, achieving FCR's from  $0.94 \pm 0.03$  (20% Blend + FOS treatment) to  $0.95 \pm 0.05$  (SPC + FOS treatment).

Growth performance was unaffected by dietary treatment. FCR, SGR, mean fish weigh at the end point, protein efficiency ratio and K factor were all not significantly different between treatments ( $P > 0.05$ ) (Table 5.3).

There was no significant differences between treatments for carcass moisture, crude protein, crude lipid or Ash levels ( $P > 0.05$ ) (Table 5.4.).

**Table 5.3** Growth performance of rainbow trout at the end of the feed trial. Data are presented means  $\pm$  standard deviation.  $n = 3$ .

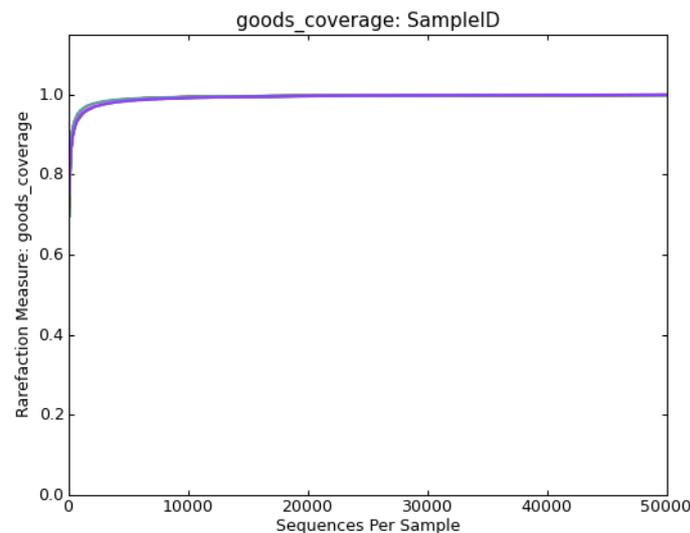
	SPC	SPC+ FOS	20% Blend	20% Blend + FOS
FCR	0.94 $\pm$ 0.01	0.95 $\pm$ 0.05	0.95 $\pm$ 0.03	0.94 $\pm$ 0.03
SGR	2.05 $\pm$ 0.01	2.05 $\pm$ 0.05	2.07 $\pm$ 0.03	2.07 $\pm$ 0.05
Mean weight end point (g)	60.15 $\pm$ 0.49	61.23 $\pm$ 1.02	61.66 $\pm$ 1.00	60.934 $\pm$ 1.85
Protein efficiency ratio	2.26 $\pm$ 0.028	2.27 $\pm$ 0.11	2.26 $\pm$ 0.07	2.24 $\pm$ 0.08
K-Factor	1.54 $\pm$ 0.10	1.47 $\pm$ 0.07	1.54 $\pm$ 0.06	1.54 $\pm$ 0.10
Survivability (%)	100 $\pm$ 0.00	99.08 $\pm$ 1.59	99.54 $\pm$ 0.78	100 $\pm$ 0.00

**Table 5.4** Carcass composition of rainbow trout at the end of the feed trial. Data are presented means  $\pm$  standard deviation.  $n = 3$ .

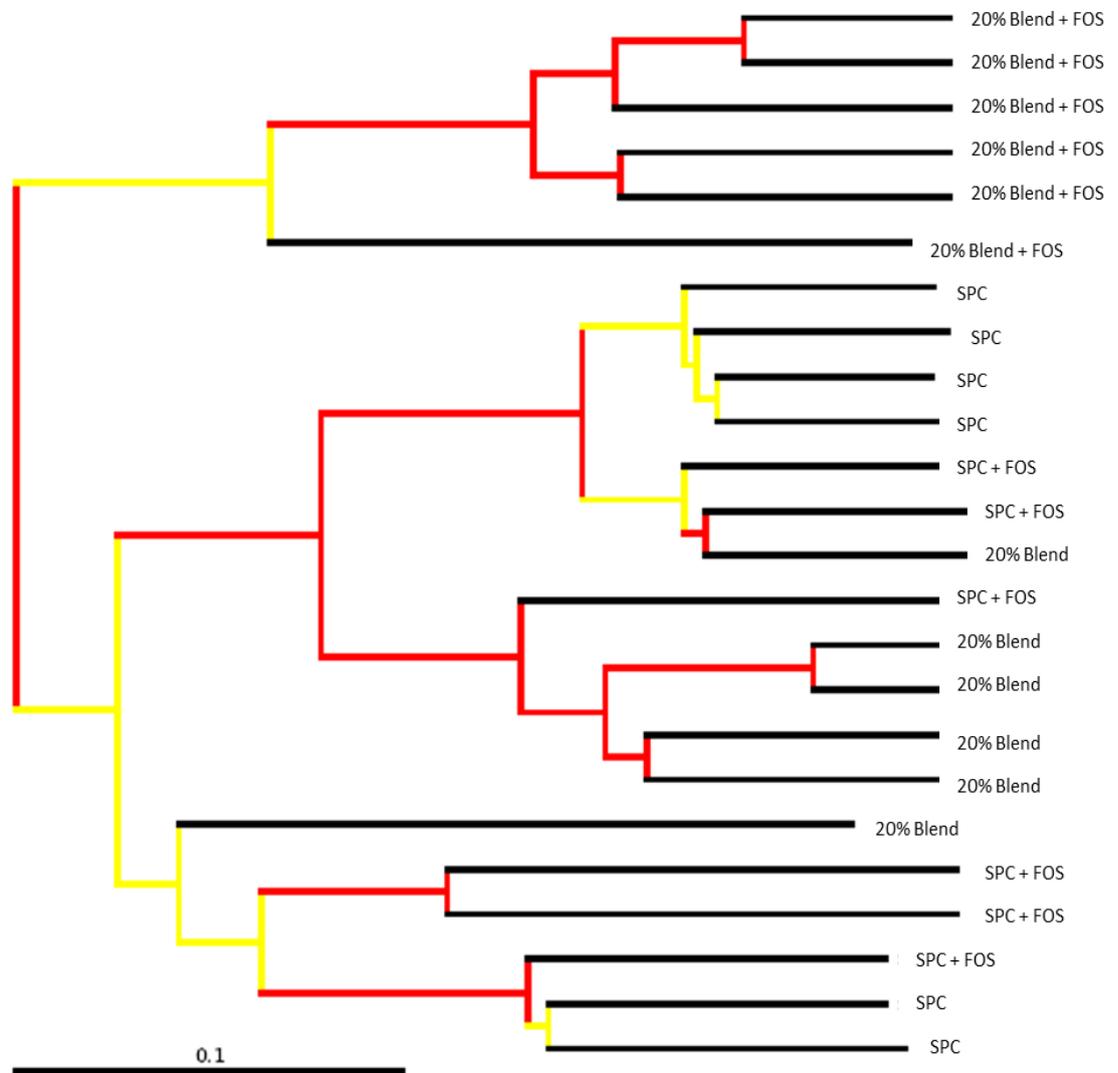
	SPC	SPC+FOS	20% Blend	20%Blend + FOS
<b><i>Carcass composition (%)</i></b>				
Moisture	72.2 $\pm$ 1.05	71.6 $\pm$ 2.09	71.8 $\pm$ 1.44	72.11 $\pm$ 1.00
Protein	12.57 $\pm$ 0.59	12.56 $\pm$ 0.80	12.4 $\pm$ 1.00	12.76 $\pm$ 0.53
Lipid	13.02 $\pm$ 0.5	13.01 $\pm$ 0.90	12.91 $\pm$ 0.75	12.56 $\pm$ 0.39
Ash	1.28 $\pm$ 0.07	1.24 $\pm$ 0.15	1.22 $\pm$ 0.02	1.2 $\pm$ 0.04

### 5.3.4 High-throughput microbiological analysis

Post trimming and quality control, 1,612,069 sequence reads were retained for downstream analysis, identifying 520 distinct OTU's. Alpha rarefaction analysis of Good's coverage reveals estimations of  $> 0.998$  for the total species present per sample. Refraction of Good's coverage plateaued after approx. 5,000 reads per sample (Figure 5.1.), suggesting that the bacterial communities were fully sampled and data are representative of the population. No significant difference was observed in alpha diversity parameters between treatments ( $P > 0.05$ ) (table 5.5.).



**Figure 5.1.** Alpha rarefaction curves of Good's coverage representing % of total species present within a sample as a function of the sequencing effort.



**Figure 5.2.** Bray-Curtis UPGMA UniFrac clustering of reads from treatment replicates of the allochthonous bacterial communities from the posterior intestine of rainbow trout, post 70 day feeding trial. Jackknife support is: Red (75-100%) and yellow (50-75%). Scale bar indicates 10% divergence.

**Table 5.5** . High throughput sequencing alpha diversity parameters, goods coverage estimations by treatment of the allochthonous bacterial communities in the posterior intestine of rainbow trout post 70 day feeding trial

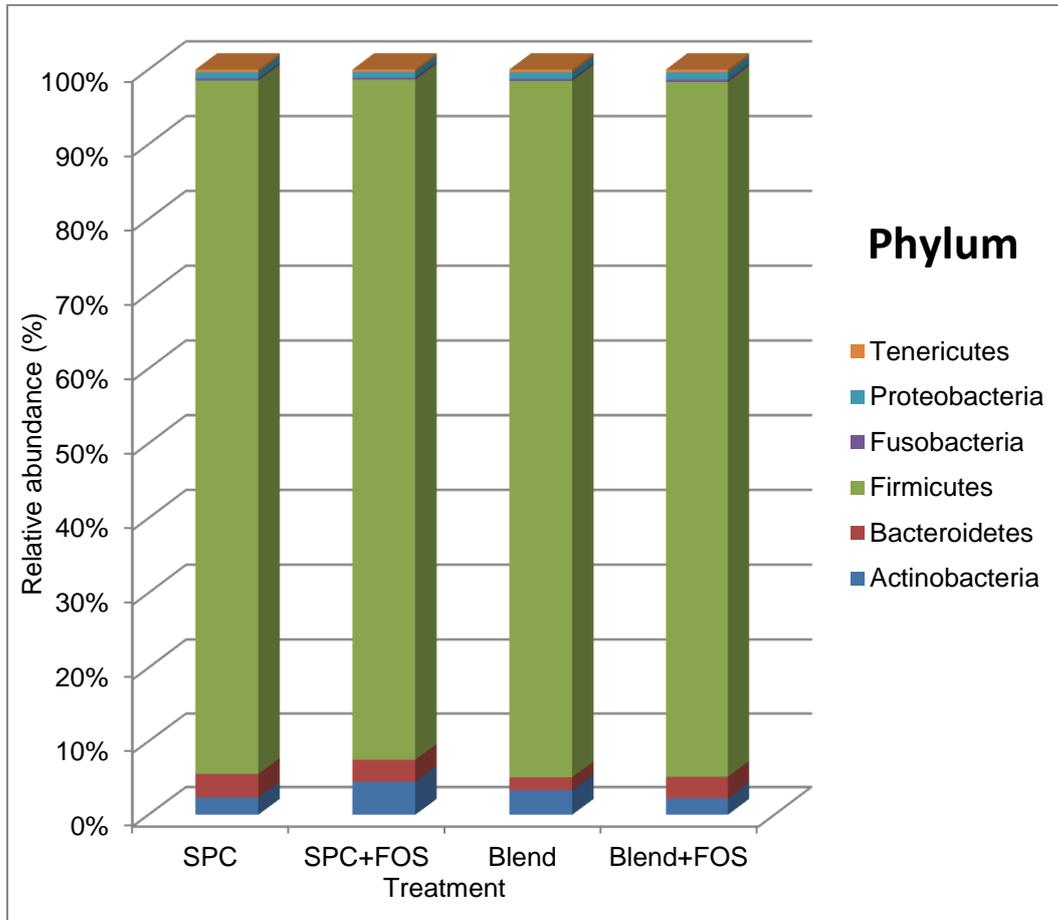
	Goods coverage	Shannon	Chao1	Observed species
SPC	0.999±0.000	4.44±0.20	461.24±18.96	411.5±30.85
SPC + FOS	0.998±0.001	4.6±0.22	458.2±9.37	393.33±15.15
20% Blend	0.999±0.000	4.84±0.20	455.42±30.67	399.17±20.22
20% Blend + FOS	0.999±0.000	4.63±0.17	469.22±14.04	420.83±27.53

The sequence distribution data were dominated by the Firmicutes at phylum level, as displayed in Figure 5.3. The Firmicutes account for 92.61% of the total read sequences of all treatments. The Actinobacteria were the next most dominant phylum (3.02%) followed by the Bacteroidetes (2.73%) and Proteobacteria (0.88%). Other phyla present in the sample-set, each with fewer than 0.2% of the total reads per phylum, combined accounted for 0.07%. Phylum composition of the allochthonous microbial community was unaffected by dietary treatment or scFOS inclusion.

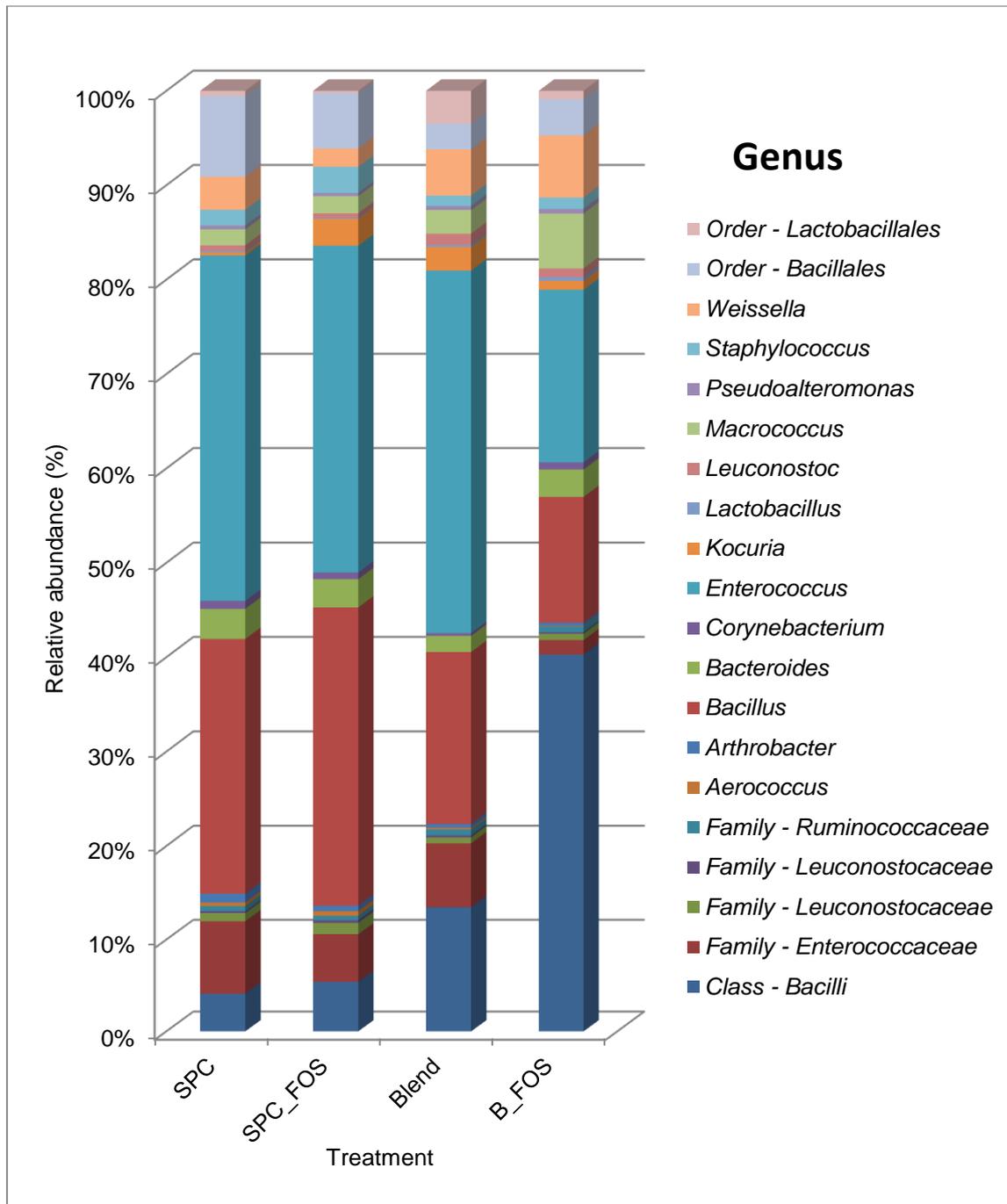
The sequence distribution data a genus level is displayed in Figure 5.4. The most abundant genus was *Enterococcus*, representing 31.17% of the total reads. *Bacillus* represented the next most abundant genus (22.14%) followed by class Bacilli (15.37%), order: Bacillales (genus unknown) (5.35%), family Enterococcaceae (genus unknown) (5.30%), *Weissella* (4.15%), *Macrococcus* (2.89%), *Bacteroides* (2.70%), *Staphylococcus* (1.63%) and *Kocuria* (1.51%). The remaining genera present represent <1.5% of total reads.

*Enterococcus*, the most abundant genus in terms of sequence distribution and as a percentage of total reads per treatment was significantly ( $P < 0.05$ ) reduced in the 20% blend + FOS treatment ( $18.16 \pm 6.94\%$ ) compared to the 20% blend treatment ( $37.22 \pm 10.38$ ). The proportion of reads for the genus *Enterococcus* was unaffected by dietary inclusion of wheat gluten or scFOS supplementation compared to the basal SPC diet, yet significantly reduced in the 20% blend + FOS treatment compared to that of the 20% blend. The percentage of reads associated with Family Enterococcaceae were unaffected in the SPC + FOS and 20% blend treatments compared to the SPC treatment, however, 20% blend + FOS had significantly fewer reads associated to family Enterococcaceae than the SPC treatment.

Reads associated with the class Bacilli were significantly elevated in the 20% blend + FOS treatment ( $39.49 \pm 4.68\%$ ) compared to the 20% Blend treatment ( $13.34 \pm 5.62\%$ ). Class Bacilli reads were also significantly elevated in both wheat gluten treatment compared to the SPC treatments ( $P < 0.05$ ). Reads associated with the genus *Kocuria* was statistically elevated in the SPC + FOS treatment ( $3.45 \pm 3.18\%$ ) compared to the SPC treatment ( $0.37 \pm 0.11\%$ ), however, neither SPC treatment was significantly different from the wheat gluten treatments. Statistical difference ( $P < 0.05$ ) between proportions of genera contributing > 0.2% of total reads in each dietary treatment is displayed in Table 5.6.



**Figure 5.3.** Allochthonous bacterial communities in the posterior intestine of rainbow trout fed the experimental diets. Data are represented as bacterial phylum percentage. Data excludes phyla with less than 0.2% of the total reads



**Figure 5.4.** Allochthonous bacterial communities in the posterior intestine of rainbow trout after feeding with the experimental diets. Data are represented as bacterial Genus percentage. Data excludes genera with less than 0.2% of the total reads.

**Table 5.6.** Allochthonous bacterial communities in the posterior intestine of rainbow trout at the end of the trial. Data are represented as means  $\pm$  SD. Kruskal-Wallis with post hoc Tukey-Kramer. Superscript letters denote significance, significance accepted at  $P < 0.05$ .

Taxon	SPC	SPC + FOS	20% Blend	20% Blend + FOS
<b>Phylum</b>				
Actinobacteria	2.44 $\pm$ 0.75	5.21 $\pm$ 3.52	3.29 $\pm$ 1.08	2.2 $\pm$ 0.42
Bacteroidetes	3.03 $\pm$ 0.96	2.86 $\pm$ 0.93	1.8 $\pm$ 0.71	2.96 $\pm$ 1.45
Firmicutes	92.88 $\pm$ 1.59	90.42 $\pm$ 3.32	93.23 $\pm$ 1.25	92.99 $\pm$ 2.22
Fusobacteria	0.29 $\pm$ 0.1	0.28 $\pm$ 0.06	0.3 $\pm$ 0.09	0.33 $\pm$ 0.14
Proteobacteria	0.89 $\pm$ 0.4	0.82 $\pm$ 0.15	0.89 $\pm$ 0.24	1.03 $\pm$ 0.4
Tenericutes	0.35 $\pm$ 0.18	0.31 $\pm$ 0.11	0.38 $\pm$ 0.12	0.38 $\pm$ 0.16
<b>Genus</b>				
<i>Aerococcus</i>	0.41 $\pm$ 0.39	0.56 $\pm$ 0.44	0.16 $\pm$ 0.15	0.05 $\pm$ 0.05
<i>Arthrobacter</i>	0.96 $\pm$ 0.48 <sup>a</sup>	0.57 $\pm$ 0.17 <sup>a</sup>	0.4 $\pm$ 0.23 <sup>b</sup>	0.35 $\pm$ 0.11 <sup>b</sup>
<i>Bacillus</i>	29.35 $\pm$ 15.24	28.92 $\pm$ 15.93	17.64 $\pm$ 7.14	12.21 $\pm$ 9.77
<i>Bacteroides</i>	2.96 $\pm$ 0.93	2.78 $\pm$ 0.95	1.72 $\pm$ 0.71	2.87 $\pm$ 1.43
Class- Bacilli	4.13 $\pm$ 2.39 <sup>c</sup>	4.78 $\pm$ 3.24 <sup>c</sup>	13.34 $\pm$ 5.62 <sup>b</sup>	39.49 $\pm$ 4.68 <sup>a</sup>
<i>Corynebacterium</i>	0.81 $\pm$ 0.37 <sup>a</sup>	0.75 $\pm$ 0.3 <sup>ab</sup>	0.29 $\pm$ 0.07 <sup>b</sup>	0.68 $\pm$ 0.28 <sup>ab</sup>
<i>Enterococcus</i>	33.94 $\pm$ 11.17 <sup>ab</sup>	35.26 $\pm$ 11.98 <sup>ab</sup>	37.22 $\pm$ 10.38 <sup>a</sup>	18.16 $\pm$ 6.94 <sup>b</sup>
Family- Enterococcaceae	7.08 $\pm$ 2.89 <sup>a</sup>	5.66 $\pm$ 3.93 <sup>ab</sup>	6.58 $\pm$ 2.59 <sup>a</sup>	1.54 $\pm$ 0.53 <sup>b</sup>
Family- Leuconostocaceae	0.92 $\pm$ 0.27	1.18 $\pm$ 0.5	0.6 $\pm$ 0.3	0.69 $\pm$ 0.28
Family- Leuconostocaceae	0.2 $\pm$ 0.08	0.3 $\pm$ 0.21	0.21 $\pm$ 0.1	0.17 $\pm$ 0.07
Family- Ruminococcaceae	0.56 $\pm$ 0.32	0.5 $\pm$ 0.16	0.61 $\pm$ 0.19	0.59 $\pm$ 0.2
<i>Kocuria</i>	0.37 $\pm$ 0.11 <sup>b</sup>	3.45 $\pm$ 3.18 <sup>a</sup>	2.43 $\pm$ 0.9 <sup>ab</sup>	0.97 $\pm$ 0.32 <sup>ab</sup>
<i>Lactobacillus</i>	0.12 $\pm$ 0.09 <sup>b</sup>	0.08 $\pm$ 0.04 <sup>b</sup>	0.21 $\pm$ 0.07 <sup>b</sup>	0.39 $\pm$ 0.12 <sup>a</sup>
<i>Leuconostoc</i>	0.61 $\pm$ 0.37	0.53 $\pm$ 0.32	1.19 $\pm$ 0.42	0.8 $\pm$ 0.91
<i>Macrococcus</i>	1.86 $\pm$ 1.31	1.69 $\pm$ 0.61	2.51 $\pm$ 1.37	5.97 $\pm$ 3.19
Order- Bacillales	8 $\pm$ 2.75 <sup>a</sup>	5.56 $\pm$ 1.3 <sup>ab</sup>	2.69 $\pm$ 0.53 <sup>c</sup>	3.82 $\pm$ 0.46 <sup>bc</sup>
Order- Lactobacillales	0.44 $\pm$ 0.24	0.25 $\pm$ 0.13	3.59 $\pm$ 3.98	0.78 $\pm$ 0.52
<i>Pseudoalteromonas</i>	0.42 $\pm$ 0.2	0.36 $\pm$ 0.11	0.44 $\pm$ 0.11	0.5 $\pm$ 0.2
<i>Staphylococcus</i>	1.67 $\pm$ 0.4 <sup>ab</sup>	2.5 $\pm$ 1.29 <sup>a</sup>	1.07 $\pm$ 0.64 <sup>b</sup>	1.2 $\pm$ 0.32 <sup>ab</sup>
<i>Weissella</i>	2.94 $\pm$ 2.39	1.96 $\pm$ 1.83	5.02 $\pm$ 4.92	6.39 $\pm$ 1.44

### 5.3.5. SCFA analysis of luminal contents

High performance liquid chromatography analysis of the posterior intestinal digesta revealed no dietary effect on SCFA concentrations ( $P > 0.05$ ). Formic acid ( $\text{CH}_2\text{O}_2$ ) was observed in the highest concentrations followed by acetic acid ( $\text{C}_2\text{H}_4\text{O}_2$ ), lactic acid ( $\text{C}_3\text{H}_6\text{O}_3$ ) and trace levels of Valeric acid ( $\text{C}_5\text{H}_{10}\text{O}_2$ ) (Table 5.7). Butyric acid nor Propionic acid were detected.

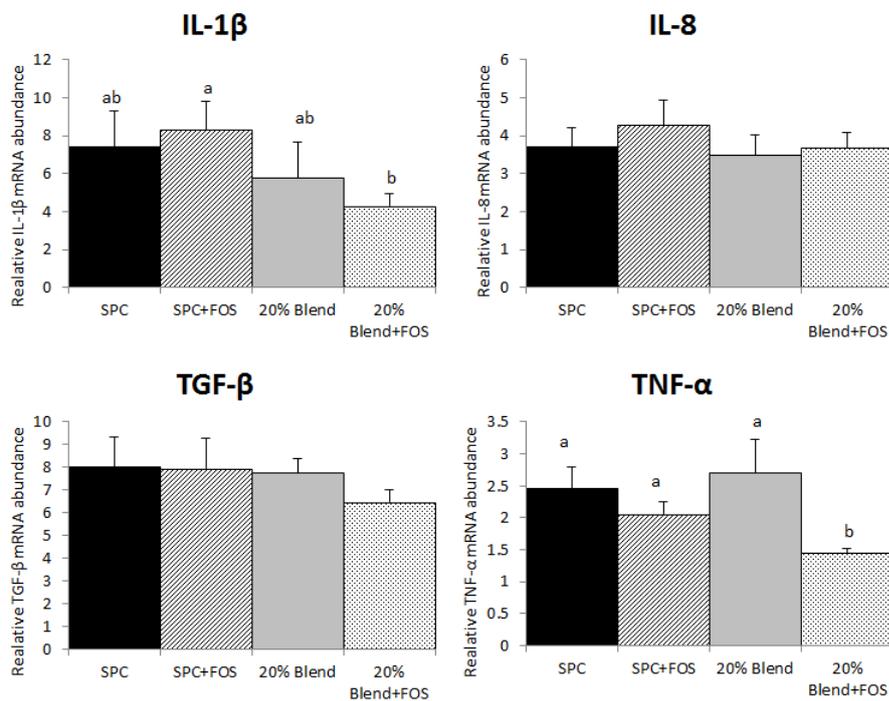
**Table 5.7.** SCFA concentrations ( $\text{mM g}^{-1}$ ) in the posterior intestine digesta of rainbow trout at the end of the feeding trial. Data are means  $\pm$  SD.

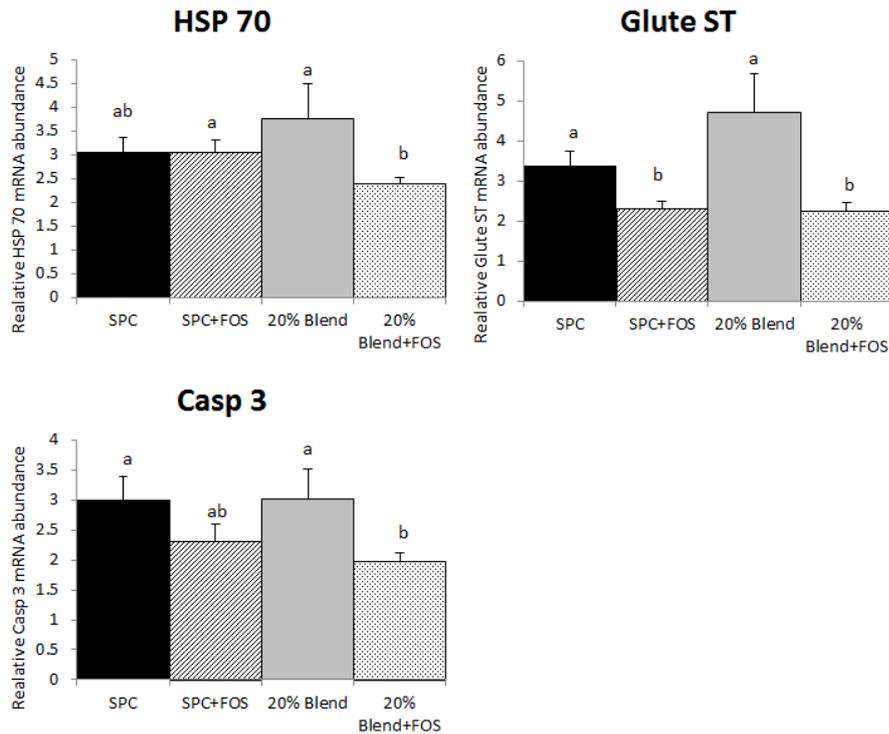
	SPC	SPC + FOS	20% Blend	20% Blend + FOS
Lactic	2.02 $\pm$ 0.48	1.95 $\pm$ 0.23	2.07 $\pm$ 0.18	1.99 $\pm$ 0.48
Acetic	2.71 $\pm$ 0.65	2.70 $\pm$ 0.44	2.53 $\pm$ 0.32	2.46 $\pm$ 0.78
Propionic	Not detected	Not detected	Not detected	Not detected
Butyric	Not detected	Not detected	Not detected	Not detected
Formic	4.34 $\pm$ 1.31	4.76 $\pm$ 1.18	4.45 $\pm$ 0.48	4.87 $\pm$ 1.99
Valeric	-0.41 $\pm$ 0.56	-0.32 $\pm$ 0.59	-0.45 $\pm$ 0.49	-0.89 $\pm$ 0.38

### 5.3.6. Gene expression.

Relative transcriptional level gene expression of IL-1 $\beta$ , IL-8, TGF- $\beta$ , TNF- $\alpha$ , Glute ST, HSP70 and Casp 3 are presented in Figure 5.5. The relative expressions of the pro-inflammatory cytokine TNF $\alpha$  was significantly reduced ( $P < 0.05$ ) with the addition of scFOS to the wheat gluten basal diet, and numerically reduced with scFOS addition to the SPC basal diet, but not significantly so. The pro-inflammatory cytokine IL-8 was unaffected by dietary treatment, whilst IL-1 $\beta$  was numerically lower with scFOS inclusion in the wheat gluten basal treatment,

and significantly down regulated in the 20% blend + FOS treatment compared to the SPC + FOS treatment. The anti-inflammatory cytokine TGF-  $\beta$  followed the same trend with numerically ( $P > 0.05$ ) reduced expression in the 20% blend + FOS treatment compared to the 20% blend treatment, however, no significant treatment effect was observed. Glutathione S-transferase was significantly down regulated with the inclusion of scFOS in both SPC and 20% blend basal diets ( $P < 0.05$ ). HSP70 expression was significantly reduced in the 20% blend + FOS treatment compared to the 20% blend and SPC + FOS treatments ( $P < 0.05$ ). Casp 3 gene expression was significantly down regulated in the 20% blend + FOS compared to the 20% blend treatment with the same, yet not significant, trend with addition of FOS to the SPC basal diet.





**Figure 5.5.** Relative mRNA abundance of IL-1  $\beta$ , IL-8, TGF  $\beta$ , TNF  $\alpha$ , Glute ST, HSP70 and Casp 3 in the posterior intestine of rainbow trout at the end of the feed trial. Superscript letters denote significant difference ( $P < 0.05$ ) between treatments.  $n = 6$  per treatment. Data are means  $\pm$  SE.

## 5.4 Discussion

The objective of the current investigation was to evaluate the effect of wheat gluten and scFOS inclusions in soya based diets on the growth performance, gut microbiology, immunological status and SCFA production in the posterior intestine of rainbow trout. Microbiological, molecular and column chromatography techniques were utilised to achieve these objectives at the end of the 70 day feed trial, as well as growth performance and carcass composition analysis.

Throughout the trial fish performed well, with FCR's lower than 1 observed. Wheat gluten inclusion or scFOS supplementation had no significant effect on FCR's compared between the two basal treatments and scFOS supplementation in each. The same non-significant differences were observed for SGR, protein efficiency ratio and mean fish weight at the conclusion of the 70 day feed trial. ( $P > 0.05$ ). Comparable growth performance has been observed when utilising wheat gluten as a replacement of both fish meal in Atlantic salmon (Storebakken et al., 2000), plant based proteins in rainbow trout (Tusche et al., 2012) and was observed and discussed chapter 3b. The supplementation of scFOS has been previously observed to result in comparable growth performance in European sea bass (Guerreiro et al., 2015c), turbot (*Scophthalmus maximus*) (Guerreiro et al., 2014) and pacific white shrimp (Li et al., 2007), yet contradictory results observing improved growth performance have been reported in Pacific white shrimp, gilthead sea bream (*Sparus aurata*) and hybrid tilapia (Lv et al., 2007; Zhou et al., 2009; Zhou et al., 2007; Guerreiro et al., 2015b). The only investigations utilising prebiotics in salmonids observed increased weight gain in rainbow trout, and comparable growth performance to the basal control diet in Atlantic salmon with FOS supplementation (Ortiz et al., 2013; Grisdale-Helland et al., 2008). As discussed in

section 3.4b, the excellent growth performance observed by the soy protein basal control may have not left enough scope for nutritional improvement, with fish performing optimally on the cold extruded/pressed diets in a flow-through, virgin water experimental system. Survivability and condition factor (K-factor) were unaffected by the dietary inclusion of scFOS to basal diets or wheat gluten inclusion. Carcass lipid, protein ash and moisture content was unaffected by dietary treatments, as was observed in chapter 3b with 20% vital wheat gluten inclusions and chapter 4 with 20% blended wheat gluten inclusion compared to the same basal soy protein control. No effect on carcass composition was observed with scFOS supplementation in the mainly carnivorous gilthead sea bream and turbot (Guerreiro et al., 2015b; Guerreiro et al., 2015a). Decreased crude protein in rainbow trout fillets have been observed with FOS supplementation, however, with a non-significant trend for increased lipid (Ortiz et al., 2013).

The effect of scFOS on the intestinal microbiota has been previously evaluated in Pacific white shrimp and hybrid tilapia utilising selective agar or PCR-DGGE (Lv et al., 2007; Zhou et al., 2009; Zhou et al., 2007). To the author's knowledge, as yet, there is no previous literature on the effect of scFOS supplementation on the allochthonous microbial populations of rainbow trout, or analysis conducted utilising high throughput techniques. Chapter 3 and 4 investigated and discussed the effects of wheat gluten products and inclusion levels on the allochthonous microbial community.

Alpha refraction analysis of Good's coverage reveals estimations of > 99.8%, indicative of a fully sampled microbiome. Bray Curtis reveals two main clusters. The first cluster consisting of the 20% blend + FOS treatment replicates and the second cluster of the SPC, SPC + FOS

and the 20% blend treatment replicates, indicating greater similarity within these treatments than with sample replicates from other clusters. Ecological parameters of the posterior allochthonous bacterial community, observed species, Chao 1 (species richness) and Shannon-Wiener index were unaffected by dietary treatment. Quantitative analysis of microbiome ecological parameters has yet to be investigated with scFOS or FOS supplementation as a result of the lack of culture independent analysis. Dietary supplementation of MOS, however, has been reported to increase intestinal bacterial species richness and diversity in gilthead sea bream fed fishmeal based diets, whilst having no effect in fish fed soybean meal based diets (Dimitroglou et al., 2010). Moriñigo (2011) observed reduced bacterial species richness in the intestine of gilthead sea bream with inulin supplementation and identified this as an area of research in its infancy with the need for further research by Ringø et al. (2014).

The sequence distribution data of 16S rRNA reads is dominated by the Firmicutes at phylum level, accounting for 92.61% of the total read sequences of all treatments, in line with the observations of Mansfield et al. (2010) when increasing plant protein source inclusions. Actinobacteria were the next most dominant phylum (3.02%) followed by the Bacteroidetes (2.73%) and Proteobacteria (0.88%). The proportion of identified phyla was unaffected by dietary treatment or the supplementation of 0.3% scFOS, and have all previously been described as part of the commensal intestinal bacteria of fish (Wong et al., 2013; Wu et al., 2012; Mansfield et al., 2010; Navarrete et al., 2010b; Gajardo et al., 2016).

The sequence distribution data at a genus level, of those genera representing > 0.2% of the total reads, revealed the most abundant genus was *Enterococcus* sp., representing 31.17%

of the total reads and approx. 35% of the reads of the SPC, SPC + FOS and 20% blend treatments. The reads for *Enterococcus* sp. were significantly reduced with the supplementation of scFOS to the 20% blend diet, and unaffected between all other treatments. The genus *Enterococcus*, a member of the order Lactobacillales, has been reported in the intestine of both wild and cultured rainbow trout (Trust and Sparrow, 1974; Wong et al., 2013; Ringø and Gatesoupe, 1998; Bakke-McKellep et al., 2007), and contain potentially probiotic species as described in section 3.4. Dimitroglou *et al.* (2009) observed an increase in culturable *Enterococcus* spp. with MOS supplementation of a commercial aquafeed for rainbow trout, whilst Bakke-McKellep *et al.* (2007) reported reduced numbers with inulin supplementation compared to the soy bean meal control. Interestingly, as observed in chapter 3b, *Enterococcus* levels were not significantly different from the SPC treatment. The effect of scFOS supplementation on *Enterococcus* spp. was not identified or reported by Zhou *et al.* (2009; Zhou et al., 2007), Lv et al. (2007) or Li et al. (2007). The same significant reduction of family Enterococcaceae was observed with 0.3% supplementation of scFOS.

A bacterial OTU identified as belonging to the class Bacilli accounted for a significantly larger population of the allochthonous microbiota of the posterior intestine in the 20% blend + FOS treatment than all other treatments. The 20% blend treatment alone had a significantly larger proportion of OTU class Bacilli than the SPC treatments, which were not significantly different with or without scFOS supplementation. The class Bacilli consists of two orders: Bacillales, importantly containing the family Bacillaceae and Staphylococcaceae and the *Bacillus* and *Staphylococcus* genera. The other order, Lactobacillales, importantly contains the families Enterococcaceae, Lactobacillaceae and Leuconostocaceae containing the

genera *Lactobacillus*, *Enterococcus*, *Leuconostoc* and *Weissella* amongst others. BLAST identification of reads of the class Bacilli OTU identified sequences to be most closely aligned to *Staphylococcus pasteurii*. With increased research effort into the microbiota of fish, the genus *Staphylococcus* has been identified in the intestinal microbiota of many fish, including salmonids, eels and Atlantic cod (Bakke-McKellep et al., 2007; Esteve and Garay, 1991; Ringø et al., 2006b; Ringø et al., 2006a; Askarian et al., 2012), dominating the hind gut of Arctic charr (*Salvelinus alpinus* L.) and the adherent bacteria in the distal and posterior intestine of Atlantic salmon (Askarian et al., 2012; Ringø et al., 2006a) utilising 16S rRNA gene sequencing and culture dependent practices, respectively. Dehler *et al.* (2016) identified *Staphylococcus* spp. in the core microbiome of Atlantic salmon parr in differing fresh water environments, at a low abundance, utilising deep sequencing yet did not quantify a value. *Staphylococcus* spp. was not identified in the 12 most abundant species of Atlantic salmon housed in sea water utilising high-throughput 16s rRNA sequencing, yet was present in the anterior and posterior distal digesta at lower levels (Gajardo et al., 2016). *Staphylococcus pasteurii* has been isolated from the autochthonous bacteria of the anterior and posterior intestine of Atlantic salmon (Askarian et al., 2012) and has not been associated with any fish disease. It cannot be ignored, however, that some *Staphylococcus pasteurii* strains display antibiotic resistance, and are a possibly linked to human infection (Savini et al., 2009). A bacterial OTU identified to order level Bacillales, identified through BLAST searching the most common reads as another *Staphylococcus* spp., was significantly more abundant in the SPC treatment compared to the 20% blend treatments.

Reads associated to the genus *Lactobacillus*, although accounting for less than 1% of the microbiota of any treatment, were significantly increased in the 20% blend + FOS treatment

compared to all other treatments. *Lactobacillus* spp. have been identified and associated with the core microbiome of salmonids, and are among species targeted for enrichment in the intestine (Gajardo et al., 2016; Dehler et al., 2016; Ringø et al., 2010; Heikkinen et al., 2006; Balcázar et al., 2008). *Lactobacillus* spp. have been shown to be enriched in grain based diets (Wong et al., 2013) and numerically with increasing scFOS supplementation in hybrid tilapia (Lv et al., 2007). Zhou et al. (2007) observed significantly increased *Lactobacillus* counts with 0.16% supplementation of scFOS, as was observed at much higher resolution in the present study, utilising high-throughput techniques. *Lactobacillus* levels were unaffected by dietary treatment in chapter 3b, consistent with the observations in the present study between the SPC and 20% blend treatments. The supplementation of 0.3% scFOS's affect in wheat gluten inclusion diets could suggest a beneficial synergistic effect of scFOS and blended wheat gluten inclusions, with many *Lactobacillus* spp. being investigated as probiotics in a range of species (Suzer et al., 2008; Aly et al., 2008; Nayak, 2010; Merrifield, 2014).

Supplementation of scFOS in the SPC diet revealed an increase in the proportion of genus *Kocuria* reads in the treatment. *Kocuria* have been observed utilising deep sequencing across two variable regions of the 16S rRNA gene in the posterior intestine allochthonous microbiota of Atlantic salmon (Dehler et al., 2016) and culture dependent techniques in rainbow trout (Kim et al., 2007; Bakke-McKellep et al., 2007). *Kocuria* was observed in the allochthonous microbiota of fish fed basal and wheat gluten diets in chapter 3b, yet constituted <0.2% of the total reads. Sharifuzzaman and Austin (2010) observed increased resistance of rainbow trout supplemented with *Kocuria* as a probiotic against *Vibrio anguillarum* and *Vibrio ordalii*, significantly reducing mortalities. The innate immune

response was reported to be enhanced with probiotic supplementation. However, poor probiotic persistence in the intestine was observed with *Kocuria* sp. supplementation (Sharifuzzaman et al., 2014). The ability of scFOS supplementation at 0.3% to enhance the genus *Kocuria* in the allochthonous microbiota of rainbow trout in soy protein based diets is potentially beneficial, aiding protection from vibriosis.

Reads associated with the genus *Arthrobacter* were significantly reduced in the wheat gluten inclusion treatments compared to the SPC treatments. scFOS supplementation had no effect in either diet. The genus *Arthrobacter* was identified in chapter 3b, yet below the threshold of 0.2% of the total reads, as well as at low levels in the gut microbiota of Atlantic salmon (Dehler et al., 2016; Gajardo et al., 2016) and rainbow trout (Wong et al., 2013; Ringø et al., 2016). *Artherobacter* species have been identified as potential probiotics for Atlantic cod larvae (Lauzon et al., 2010b; Lauzon et al., 2010a) and the genus was only observed in soy bean meal fed Atlantic salmon by Bakke-McKellep *et al.* (2007), and not in Atlantic salmon fed fishmeal based diets. The present studies observations are in line of that of Bakke-McKellep *et al.* (2007), observing significantly elevated reads in the higher soy protein treatments.

The relative expression levels of a number of immunologically relevant genes were asses as an indicator of the effect of wheat gluten inclusions and scFOS supplementation on the localised immunological status of the posterior intestine. Posterior intestinal samples were analysed for changes in gene expression associated with epithelial damage, inflammation, oxidative and cellular stress, as the posterior region has been associated with the greatest inflammatory impact (Mansfield et al., 2010; Bakke-McKellep et al., 2007; Heikkinen et al.,

2006). To the author's knowledge this is the first investigation to assess the effect of scFOS on immunologically relevant genes in rainbow trout. The Pro-inflammatory cytokines IL-8 and TNF- $\alpha$  together contribute in host defence against bacterial invasion or colonisation (Cerezuela et al., 2013). IL-8, associated with chemotaxis of neutrophils, T lymphocytes and basophils was unaffected by dietary treatment in the present study, as also observed in chapter 3b and in the entire intestinal length of Atlantic cod supplemented with MOS and  $\beta$ -glucan (Lokesh et al., 2012). TNF- $\alpha$ , which plays an important role in cell proliferation, migration, differentiation, apoptosis, necrosis, and the induction of other cytokines was significantly down regulated in the 20% blend + FOS treatment compared to all other treatments. Dietary protein source or scFOS supplementation of soy protein based diets did not affect TNF- $\alpha$  expression, as was observed in chapter 3b. with wheat gluten inclusions to the basal SPC diet. Cerezuela *et al.* (2013) observed an upregulation of IL-8 in inulin supplemented sea bream anterior intestine. Down regulation of TNF- $\alpha$  has been observed previously by Qin *et al.* (2014) with chito-oligosaccharides supplementation in gut of hybrid tilapia, whilst up regulation has been observed in the head kidney of rainbow trout with fermentable fibre and Immunogen supplementation (Yar Ahmadi et al., 2014a; Yar Ahmadi et al., 2014b). The contradictory results of prior prebiotic investigations reduces the ability to make inferences on the effect of prebiotics on the intestinal immunomodulation.

Down regulation of the pro-inflammatory cytokine IL-1 $\beta$  was observed in the 20% blend + FOS treatment compared to the SPC + FOS treatment. Expression between all other treatments was unaffected. IL-1 $\beta$ , a key mediator in response to microbial tissue injury and invasion, activating lymphocytes or causing a cascade resulting in the release of other cytokines activating lymphocytes, macrophages and NK cells (Reyes-Cerpa et al., 2013; Abid

et al., 2013). The effects of scFOS on IL-1 $\beta$  have yet to be reported, however, Lokesh *et al.* (2012) observed unaffected expression of IL -1  $\beta$  in the anterior and posterior intestine and rectum of Atlantic cod supplemented with MOS, and anterior and rectum with  $\beta$  glucan supplementation. The down regulation of IL-1 $\beta$  would suggest the addition of scFOS to diets containing 20% wheat gluten modulated the intestinal microbiota in such a way microbial tissue injury and invasion was reduced compared to scFOS supplementation of the SPC + FOS treatment, suggesting a synergistic effect of wheat gluten and scFOS. Cytokine expression in rainbow trout has been far more extensively studied within probiotic applications (Standen et al., 2013; Kim and Austin, 2006; Panigrahi et al., 2007; Panigrahi et al., 2011; Abid et al., 2013) and have been observed to increase IL-1 $\beta$  and TNF- $\alpha$  expression, as a reported effect of an immunologically elevated state, as no observations or pathogenic invasion or intestinal stress were observed in other investigated parameters. The level of pro-inflammatory cytokine expression indicative of pathogenic invasion or detrimental effects of macronutrients has not been quantified and inferences must be made using the health status of entire organisms to assess the function of observed changes in cytokine expression. Prebiotic studies in mammals have observed immunomodulation in the same manner as the present study. Zenhom *et al.* (2011) report reduced intestinal inflammation and the reduction of TNF- $\alpha$  and IL-8 expression in human intestine supplemented with oligosaccharides. Probiotic studies in humans have also observed similar results, with prebiotic inhibition of pro-inflammatory cytokines and increased TGF- $\beta$  and IL-10 expression (anti-inflammatory) (Haller et al., 2002; Smits et al., 2005; Jeon et al., 2012), increased anti-inflammatory TGF- $\beta$  has also been observed in hybrid tilapia supplemented with chito-oligosaccharides (Qin et al., 2014). Synergistic administration of *Bifidobacterium longum*, inulin and oligofructose in a human trial also resulted in reduced TNF- $\alpha$  and IL-1 $\beta$  levels

(Furrie et al., 2005). The anti-inflammatory cytokine TGF- $\beta$ , which plays a regulatory role in differentiation, migration, proliferation and development (Reyes-Cerpa et al., 2013), was unaffected by dietary treatment, as observed in chapter 3b, or scFOS supplementation in the present trial. The observations in the present study would suggest a synergistic effect of wheat gluten and scFOS to modulate the pro-inflammatory cytokine expression in the anterior intestine. TNF- $\alpha$  expression was significantly reduced in the 20% blend + FOS treatment compared to all others, and IL-1 $\beta$  was significantly reduced in the 20% blend + FOS compared to the SPC + FOS treatment. The application of scFOS in wheat gluten inclusion diets could likely be assumed to affect the microbial population as to reduce the number of antigens recognised by the gut associated lymphoid tissue (GALT) of the fish, and in turn a lower expression of down-stream immune-relevant inflammatory cytokines would be observed.

Heat shock protein 70 has been utilised as an indicator of cellular stress in numerous studies. HSP 70 is known to mediate the degradation and repair of denatured or altered proteins and is highly conserved at amino acid level (Basu et al., 2002; Kiang and Tsokos, 1998). Levels of expression have been shown to be affected by many factors such as pathogen invasion and detrimental stocking densities (Sanden and Olsvik, 2009; Zhang et al., 2014; Gornati et al., 2004). The significant up regulation of HSP 70 has been observed in the posterior intestine of Atlantic salmon fed soy bean meal based diets compared to fishmeal controls, attributed to increased cellular repair and apoptosis (Bakke-McKellep et al., 2007b). Upregulation has also been observed in responses to model toxicants (Sanden and Olsvik, 2009), genetically modified maize (Sagstad et al., 2007) and unfavourable diet compositions (Martin et al., 2003; Hemre et al., 2004). Wheat gluten inclusion in the SPC diet had no

effect on HSP 70 expression in the present investigation, contradictory to results observed in chapter 3b., where down regulation was observed across all wheat gluten treatments. Down regulation however was observed in the 20% Blend + FOS treatment compared to the 20% blend and SPC + FOS treatments. Numerical reduction in relative expression was observed between the 20% blend + FOS and SPC treatment, but not statistical. Down regulation of HSP 70 with scFOS supplementation in a wheat gluten inclusion diet may be another synergistic effect of scFOS and wheat gluten. Reduced HSP70 is generally associated with reduced stress, indicating less detrimental effects occurring in the posterior intestine as HSP 70 codes for proteins in stressful conditions (Iwama et al., 2004). Reduced relative HSP 70 expression has previously been associated with prebiotic supplementation of chito-oligosaccharides in the intestine of hybrid tilapia (Qin et al., 2014) and head kidney of rainbow trout supplemented fermentable fibre and Immunogen (Yar Ahmadi et al., 2014a; Yar Ahmadi et al., 2014b). The observed alterations in HSP 70 gene expression may again be as a result of the modulation of intestinal microbiota, as reduced HSP 70 expression has been previously observed with probiotic administration to a number of species (Rollo et al., 2006; Avella et al., 2010; Mohapatra et al., 2014; Ran C, 2015).

Relative expression of caspase 3 saw similar trends to HSP 70 expression. Caspase 3, a cell death effector caspase, is the most important terminal caspase, without which apoptosis cannot occur (Rojas et al., 2012) and is often activated after failed DNA damage responses (Krumschnabel et al., 2010). The present investigation observed significant down regulation in the 20% blend + FOS treatment compared to the SPC and 20% Blend treatments. No significant difference was observed between the two scFOS supplemented diets, nor was there any significance between the SPC + FOS treatment and the SPC and 20% blend

treatments. The effect of prebiotic supplementation on expression of caspase 3 in the intestine of fish is poorly reported. Torrecillas *et al.* (2015) however observed up regulation of caspase 3, potentially as a cell regulatory response to down regulated TGF- $\beta$ . Elevated caspase 3 has been observed in rat cell culture as a response to increased oxidative stress (Carvour *et al.*, 2008). The mirroring of caspase 3 expression to oxidative stress is also observed in the present study.

Glutathione S transferase expression was significantly reduced in both scFOS treatments compared to the basal formulations, which were not significantly different from one another, as was observed in chapter 3b. Glutathione S-transferase, and the glutathione that it binds substances too, are two primary lines of defence against both acute and chronic toxicities of electrophiles and reactive oxygen/nitrogen species (Li, 2009). Glutathione S transferase is part of the Phase 2 detoxification enzymes superfamily, with the ability to detoxify both reactive oxygen species as well as toxic xenobiotics (Wu *et al.*, 2004; Li, 2009). Detoxification is achieved through glutathione S transferase catalysing glutathione dependent conjugation and redox reactions (Li, 2009). Prebiotic supplementation on oxidative stress parameters is another poorly studied field. Nugroho and Fotedar (2014) observed increased glutathione S transferase activity in cultured crayfish (*Cherax cainii*) with MOS supplementation, whilst Sitjà-Bobadilla *et al.* (2005) reported an increased glutathione redox status in blood and liver, and glutathione reductase with increased plant protein content for carnivorous gilthead sea bream as discussed in chapter 3b. The limited available information on the effect of prebiotics on oxidative stress increases the difficulty of cross comparison, yet reduced glutathione S transferase combined with reduced apoptosis (casp 3 expression) and intestinal stress (HSP 70 expression) could feasibly be considered a

beneficial effect of scFOS supplementation in wheat gluten inclusion diets, potentially reducing the energy requirements of the intestine.

SCFAs are produced by the fermentation of indigestible dietary fibre by specific bacteria in the posterior intestine. Substrates for fermentation escape enzymatic digestion and include many oligosaccharides that are utilised as prebiotics. Fermentation of oligosaccharide by bacterial species in the posterior intestine in fish, colon in mammals, produces numerous metabolite groups including organic acids (e.g. SCFA, lactic acid and succinic), of which SCFAs are a major constituent (Kihara and Sakata, 2002; Kihara, 2008; Roy et al., 2006; Nicholson et al., 2012). Individual SCFAs are utilised by the host in different ways at different sites, from the intestine, to muscular tissue and the liver (Montagne et al., 2003). Utilised by the enterocytes of the GI tract as an energy source, SCFAs play a vital role in the maintenance of the epithelium as well as promoting bacterial species, modulating lipid metabolism and stimulation of the immune system (Maslowski and Mackay, 2011; Marcil et al., 2002; Roy et al., 2006).

No effect of scFOS supplementation was observed on juvenile rainbow trout posterior intestine concentrations in the present study. The effect of diet formulation and feed additives on SCFAs production focus around lactate, acetate, propionate and butyrate (Mountfort et al., 2002; Geraylou et al., 2012; Kihara, 2008; Kihara and Sakata, 2002; Mahious, 2006). In the present study formic acid was observed at the highest concentrations in the posterior intestine of juvenile rainbow trout. Dietary formic acid supplementation of aquafeed for rainbow trout has previously been observed to increase mineral digestibilities, specifically phosphorous, calcium and magnesium (Vielma and Lall,

1997). Increased apparent digestibility was attributed to the increased pH of the distal intestinal contents with formic acid supplementation, through regulation of the intestinal pH by the host organism (Vielma and Lall, 1997). Kihara (2008) observed no effect on formic acid concentrations in the water from red sea bream posterior intestinal contents supplemented with dietary lactosucrose.

Lactic and acetic acid concentrations were also unaffected in the present study. Unaffected lactic acid concentrations have been observed with inulin and oligofructose supplementation in the spiral intestine of Siberian sturgeon (*Acipenser baerii*) (Mahious, 2006). Concentrations remained around  $0.46\text{mM g}^{-1}$ , considerably lower than the observed approx.  $2\text{mM g}^{-1}$  in the present study. Acetic acid was also unaffected in the spiral intestine and accounted for approx. 80% of total SCFA production. Geraylou *et al.* (2012) observed similar dominance of acetate in total SCFA concentrations in Siberian sturgeon, however reported acetate and consequently total SCFA concentrations were increased in the posterior intestine with supplementation of a specific arabinoxylan-oligosaccharide.

Observations of SCFA in the intestine of three marine herbivores (*Kyphosus sydneyanus*, *Odax pullus* and *Aplodactylus arctidens*) observed increased concentration from proximal to the distal intestine, as would be expected, with increased fermentation occurring in the posterior intestine (Mountfort *et al.*, 2002). Acetate was observed in the highest concentrations, followed by propionate and butyrate. In the present study propionate and butyrate were undetected. Although detected in Siberian sturgeon, butyrate and Propionic acid constituted a much smaller proportion of total SCFAs (Geraylou *et al.*, 2012; Mahious, 2006). Butyrate and propionic are both rapidly absorbed and utilised in the organism which

could lead to reduced observations in sampled digesta, as a consequence SCFA levels observed in *in vitro* studies are considerably higher than those observed in *in vivo* studies (Mahious, 2006; Kihara and Sakata, 2002). Additional noise in biological samples also limits the detection of low concentrations. Kihara (2008) observed increased propionic acid with lactosucrose supplementation, yet failed consistently detect butyric acid. The increase in SCFAs is attributed to the promotion of specific bacteria by the prebiotic, enabling greater fermentation of the otherwise indigestible carbohydrate. Geraylou *et al.* (2012) observed the stimulation of lactic acid bacteria and *Clostridium* sp. and increased acetate, butyrate and total SCFA production.

## 5.5 Conclusions

The results in the present investigation demonstrate that wheat gluten inclusion and the supplementation with the prebiotic scFOS in both soy protein concentrate based diets and diets containing 20% blended vital and hydrolysed wheat gluten, had no detrimental effect on growth performance, condition factor, survival or carcass composition. Chapter 4 identified the ability of wheat gluten products, in inclusion levels from 20 to 30%, to increase growth performance. However, the present study, as also observed in chapter 3b, report very good FCR's and SGR's in the basal soy protein diet treatments, reducing the scope for improved growth performance with feed additives or alternative protein sources.

As observed with microbiological analysis in chapters 3 and 4, wheat gluten inclusion has again been observed to modulate the intestinal microbiota of juvenile rainbow trout. With scFOS supplementation to the basal diets or with wheat gluten inclusion alone, the structure of the allochthonous microbiota remained unchanged at phylum level, as also observed in

chapter 3. Modulation of the intestinal microbiota occurred at genus level. *Enterococcus* was the most observed genus in both chapter 3b and the present study. Twenty % blended wheat gluten inclusion enhanced the proportion of OTUs of *Arthrobacter*, *Staphylococcus pasteurii* and reduced order Bacillales. scFOS supplementation of soy the protein basal treatments resulted in enhanced *Kocuria* reads, whilst scFOS supplementation of wheat gluten treatments increased *Staphylococcus pasteurii* and *Lactobacillus* populations compared to all other treatments, an apparent synergistic effect.

An apparent synergistic effect was also observed in immuno-relevant and stress related gene expression in the posterior intestine. scFOS supplementation in 20% wheat gluten diets resulted in significant downregulation of the pro-inflammatory cytokine TNF- $\alpha$ , and numerical, yet not significant, down regulation of IL-1 $\beta$ . The combined scFOS and wheat gluten also resulted in down regulation of HSP 70, Casp 3 and Glute ST. Glute ST saw significant down regulation with scFOS supplementation to both basal diets.

Despite the observations of the modulation of intestinal microbiota, SCFA production/concentrations in the posterior intestine was unaffected by dietary treatment, with formic acid in the highest concentrations, followed by acetic and lactic. Butyric and propionic acid was not detected.

In conclusion, the wheat gluten and scFOS supplementation alone show promising results in the present study. However, the addition of 20% wheat gluten combined with scFOS supplementation showed a beneficial synergistic effect in both gut microbiota modulation and gene expression of immune-relevant and stress related genes, with promising applications in aquafeeds. Further investigations into the effect of prebiotics on the

intestinal microbiota utilising “next generation” culture independent techniques would provide greater detail as to mechanisms of action, and prebiotic specific effects. Immune-relevant Gene expression analysis of prebiotic supplemented fish post challenge would also constitute an interesting area of further research, enabling insights into possible enhanced immune states.

### **Chapter 6. General discussion.**

This body of research, consisting of four *in vivo* feeding trials, was conducted to assess the effects of wheat gluten products and scFOS (Profeed®) supplementation on the health and production of juvenile rainbow trout. These investigations provide novel insights into the effect of three distinct commercial wheat gluten products (vital Amytex®, hydrolysed Merripro® and soluble hydrolysed Solpro®) of differing levels of refinement. These were included as a substitute for soy protein concentrate to test their potential in complete diets for juvenile rainbow trout. The potential of a prebiotic feed additive to modulate the health of rainbow trout when fed soya based feeds as well as with wheat gluten inclusions was also evaluated. Emphasis has been placed on the effects in the intestine, which plays a key role in not only nutrition but defence against a host of water borne pathogens. Observations of growth performance, physiological and haematological parameters, intestinal allochthonous microflora, intestinal morphology and biomarkers for immune status and stress level of the intestine were conducted in order to achieve a holistic view of the experimental products.

The short-term trial (chapter 3a) investigated the, at the time, unreported, effects of wheat gluten products (Amytex® and Merripro®) on the allochthonous microbial population and also the general intestinal condition after a short exposure to the experimental diets. The aim of the preliminary trial was to observe the initial impacts of wheat gluten inclusions, ensuring the absence of any large scale deleterious effects on the GI tract after commencing two weeks of experimental feeding. The PCR-DGGE data from this chapter revealed the allochthonous intestinal microbial community OTU numbers and richness were unaffected

by dietary inclusions of wheat gluten. Microbial diversity, however, was significantly decreased in the 15% hydrolysed treatment compared to the plant protein basal diet. Observations of species differences between experimental treatments, and the clustering of the hydrolysed treatments apart from the basal plant protein and vital wheat gluten treatments suggested a degree of dietary induced modulation. All treatments were approximately 35% similarity to one another, and with the relative low resolution of the intestinal microbiota analysis achievable with PCR-DGGE, compared with high throughput sequencing, it cannot be disregarded that a core allochthonous community are resistant to diet based variation as observed by Wong *et al.* (2013). Indeed, all isolated DGGE bands that were sequenced yielded sequences belonging to the Firmicutes, a phylum previously observed at high abundances in the intestinal microbiota of rainbow trout (Mansfield *et al.*, 2010; Desai *et al.*, 2012; Heikkinen *et al.*, 2006; Huber *et al.*, 2004; Navarrete *et al.*, 2010; Pond *et al.*, 2006). The Sanger sequencing of DGGE bands revealed sequences aligned at varying degrees to the genera *Enterococcus*, *Macrococcus*, *Lactobacillus* and family Clostridiales were present across all treatments. An OTU aligned at 89% to *Bacillus coagulans* was observed solely in the hydrolysed wheat gluten treatments, whilst an OTU aligned at 86% to *Enterococcus faecium* was found only in the PPC and vital wheat gluten treatments. The observations of normal allochthonous phyla, species and genera with the clustering observed in DGGE band fingerprints would suggest a degree of wheat gluten inclusion based modulation, and even gluten type modulation, but overall a normal allochthonous microbial population with no obvious deleterious modulations. This, at the time of conducting the trials, was unreported. Low power scanning electron microscopy confirmed no large scale detrimental effects on the intestinal epithelial cells, and provided

confidence for a longer duration feed trial with a more holistic, higher resolution approach to analyse the effects of wheat gluten products.

The short term experiment, therefore, led onto a longer, 66 day, nutritional feed trial (chapter 3b) investigating growth performance, intestinal microbiota and health impacts of wheat gluten products, vital Amytex<sup>®</sup>, hydrolysed Merripro<sup>®</sup> and soluble hydrolysed Solpro<sup>®</sup>, as a substitute for soy protein concentrate in aquafeed for rainbow trout. The data from this chapter revealed no impact of wheat gluten inclusions on growth performance compared to the basal soy protein concentrate treatment. Numerical improvements in the vital wheat gluten treatments (Amytex<sup>®</sup>) SGR's and FCR's were observed, resulting in a numerically heavier mean fish weight at the conclusion of the trial. These may have yielded significance if the trial had been extended beyond 66 days, typically considered sufficient for trout growth trials, achieving threefold biomass increase. The FCR and PER of the 20% vital wheat gluten treatments were significantly improved compared to the hydrolysed treatments (Merripro<sup>®</sup> and Solpro<sup>®</sup>), presumed to be due to increased inclusion level and reduced processing level, though differences between diets make cross comparisons difficult. The formulation of the SPC diet was designed to be sub-optimal in the experimental conditions expected, allowing improvement with dietary inclusions of alternative protein sources to be observed if they were to occur. However, the growth performance observed in the SPC treatment with an FCR <1 reduced the scope for large scale improvements in a closed recirculating aquarium facility with cold pressed diets. Carcass composition analysis revealed no effect of wheat gluten inclusion on carcass protein levels; however, lipid content was reduced in the vital wheat gluten treatments, contradictory to the previously

observed unaffected carcass lipid contents with wheat gluten inclusions in rainbow trout (Rodehutscord et al., 1994; Davies et al., 1997).

The utilisation of high throughput sequencing in chapter 3b allowed expansion of what had been observed with PRC-DGGE in chapter 3a. Wheat gluten inclusion, and wheat gluten types, were again observed to have a modulatory effect on the allochthonous intestinal microbial population of juvenile rainbow trout. The high throughput sequencing allowed a far greater resolution of microbial analysis of the posterior digesta, observing overall population constituents at phylum level to be unaffected by dietary inclusions of wheat gluten. As with chapter 3a, dominance by Firmicutes was observed, as has been previously observed by Desai *et al.* (2012) as a result of increasing plant protein inclusions. Observations at genera level again revealed significant differences between wheat gluten inclusion types and varying inclusion levels. Cluster analysis revealed hydrolysed products sample replicates to have a higher level of similarity to one another than to the vital wheat gluten and SPC treatments, which themselves clustered together. This would suggest the hydrolysed wheat gluten has a larger impact on the intestinal population than the vital wheat gluten, yet significant intra-treatment variation was observed in line with the observations of Mansfield *et al.* (2010) and Desai *et al.* (2012). Significant modulation of genera was observed across all dietary treatments. The relative abundance of *Enterococcus* was highest, representing 46.52% of the total reads. Vital wheat gluten enhanced the proportion of the lactic acid bacteria *Enterococcus* and *Weissella* in the 20% and 10% vital wheat gluten treatments, respectively, compared to the SPC or other wheat gluten treatments. The relative abundance of *Bacillus* and *Leuconostoc* was significantly increased

in the 10% hydrolysed wheat gluten (Merripro®) and 10% soluble (Solpro®) wheat gluten treatments, respectively, compared to the SPC diet. These genera have been shown to contain probiotic species, with the potential to aid intestinal health and/or nutrition of fish when present in the microflora. This would indicate that the addition of wheat gluten products in aquafeed formulations has the ability to enhance probiotic genera within the allochthonous microbial population, without affecting the overall structure of the intestinal microbiota, as also noted by Wong *et al.* (2013) in rainbow trout and Reveco *et al.* (2014) in Atlantic salmon with the addition of plant proteins.

The gene expression analysis focused on the immune relevant genes: IL-10, TNF- $\alpha$ , TGF- $\beta$  and IL-8, as well as intestinal and oxidative stress biomarkers: HSP 70 and Glute ST. Results revealed little effect on the localised immune response, with dietary inclusions of wheat gluten showing no effect on the pro-inflammatory cytokines IL-8 and TNF- $\alpha$  and the anti-inflammatory cytokines IL-10 and TGF- $\beta$ . Antioxidant status was also unaffected. The expression of HSP 70 however showed a significant down regulation, indicating a reduced level of stress with wheat gluten inclusion compared to the SPC treatment, likely caused by the high levels of glutamine within wheat gluten providing the substrate and energy for highly proliferating intestinal cells, and reduced ANF content in wheat gluten diets. Histological assessment revealed an increase in the intraepithelial leukocyte numbers, significantly so in the 10% vital and 10% soluble treatments compared to the SPC treatment, leading to a potentially enhanced non-specific immune response, highly important to teleosts exposed to a range of water borne pathogens, with the GI tract being a first line of defence against pathogenic assault. No effect on epithelial cell condition, areas of damage

or necrosis, were observed with scanning electron microscopy, nor was microvilli density affected by wheat gluten inclusion.

The results from chapter 3 demonstrate beneficial effects of wheat gluten inclusions across all inclusion types compared to the SPC treatment. The 20% vital wheat gluten inclusion revealed the most promising growth performance, with no observed detriment with an increased inclusion level compared to the other wheat gluten treatments. Including 20% vital wheat gluten in commercial aquafeeds however presents further complication due to the cohesive and visco-elastic nature of the protein source. These attributes negate the need for pellet binders, an additional source of indigestible carbohydrate in salmonids, yet prevent extrusion at high inclusion levels with commercial extruders. To achieve higher inclusion levels wheat gluten must be blended, vital (Amytex®) with soluble (Solpro®) wheat gluten which, as a result of the hydrolysis process it undergoes, has different pellet binding characteristics compared to vital wheat gluten. As such, chapter 4 focused on the growth performance, condition and effect on allochthonous microbiota of 20% inclusions of vital (Amytex®) and hydrolysed-soluble (Solpro®) wheat gluten alone, as well as 20, 25 and 30% inclusions of the two blended.

The application of  $\geq 20\%$  wheat gluten products in the feed for juvenile rainbow trout resulted in significantly improved growth performance and significantly heavier fish at the conclusion of the 56 day feed trial. Improved growth performance in all wheat gluten inclusion treatments is assumed to be as a result of improved amino acid digestibility as identified by Davies *et al.* (1997) and Storebakken *et al.* (2015). Growth performance overall was generally worse than observed in chapter 3b between comparable diets, however, the

sub-optimal performance of the basal SPC treatment allowed improvements in growth performance in wheat gluten treatments to be observed. The blend treatments of Amytex<sup>®</sup> (vital) and Solpro<sup>®</sup> (soluble-hydrolysed) wheat gluten had no effect on carcass lipid or protein content compared to the basal diet, although increased lipid was observed in the 20% vital wheat gluten treatment compared to the SPC, 20% and 30% Blend and treatments, contradictory to the results from chapter 3b. Protein was also significantly decreased in the 20% vital treatment compared to the 20% soluble and 30% blend treatments.

Somatic indices indicated that all fish were in good condition with Fulton's K-factor ranging from  $1.21 \pm 0.25$  to  $1.37 \pm 0.10$ . K-factor was numerically superior in the blended wheat gluten treatments, however, not significantly so. Viscerosomatic indices were also unaffected by dietary treatment, indicating observations of increased carcass lipid was probably not as a result of visceral fat deposition. Hepatosomatic indices were significantly elevated in the 30% blended treatment compared to the SPC, 20% Vital and 25% Blend treatments. Associated with dietary carbohydrate levels and phosphorous availability, inclusions of wheat glutens would be assumed to have no impact on liver size, as observed in previous studies utilising wheat glutens in rainbow trout (Tusche et al., 2012) and pacific white shrimp (Molina-Poveda and Morales, 2004). Haematological parameter analysis showed no detrimental effect in the wheat gluten inclusions, haemoglobin levels and lysozyme activity was unaffected. Increased packed cell volume (haematocrit) was observed in the 20% Soluble treatment compared to 20% Vital treatment, enhancing oxygen carrying capacity of blood, yet with an associated increase in viscosity and the associated stress on the circulatory system.

Microbiological analysis was consistent with previous trials, observing no obvious overall change to the allochthonous microflora composition. Genus level modulation was observed with increased *Weissella confusa* levels, a potential probiotic in other fish species (Rengpipat et al., 2008), in the wheat gluten treatments compared to the SPC treatment, and reduction of *Aerococcus* sp., a potential fish pathogen. *Macrocooccus caseolyticus* was observed in all but the highest inclusion level of blended wheat gluten. Microbiological analysis was hampered by poor returns of PCR products sent for Sanger sequencing, most likely as a result of more than one OTU being present in a single PCR-DGGE band excised for sequencing.

Chapter 4 revealed the application of wheat gluten inclusions; as either single products or blended together, had no obvious detrimental effects for juvenile rainbow trout. Increased growth performance with inclusion levels up to 30% support the other positive results observed in chapter 4 as well as chapter 3. Blended wheat glutes, that allow higher percentage of wheat gluten inclusion in commercially extruded aquafeeds, provided improved rainbow trout growth performance compared to the control diet whilst maintaining healthy somatic indices, with no large scale impact on the intestinal microflora. These results led on to an investigation into the effects of scFOS, a prebiotic feed additive, with and without wheat gluten inclusions in soya based diets.

Feed additives in recent years have become a major area of research for potential health promoting effects in aquatic species (Ringø et al., 2010; Merrifield, 2014; Ringø et al., 2014). The uses of prebiotics, focusing on modulating the intestinal microbiota through fermentation of indigestible dietary fibre, often oligosaccharides, are one such feed additive

receiving great attention. FOS and scFOS are among a plethora of prebiotics investigated as discussed in section 1.5. Chapter 5 was designed to assess the potential of scFOS individually and in combination with 20% blended wheat gluten inclusion on the health and performance of juvenile rainbow trout. A 20% blend inclusion of wheat gluten was utilised in the investigation on the basis of results of chapters 3 and 4. Twenty % inclusions are achievable with blended formulation, improved growth performance with no observed detrimental health effects to the intestine or allochthonous microflora were observed, and the economic cost of higher inclusions would likely be unachievable in the commercial sector.

Chapter 5 investigated the allochthonous microbial population and the expression of the genes: IL-1  $\beta$ , IL-8, TNF- $\alpha$ , TGF-  $\beta$  associated with inflammatory responses and the genes: Casp3, HSP 70 and Glute ST associated with intestinal stress and oxidative stress in the posterior intestine of juvenile rainbow trout post 10 week feed trial. SCFA analysis of the posterior digesta was also undertaken to observe potential effects of scFOS supplementation on intestinal microbial SCFA production.

The results revealed supplementation with the prebiotic scFOS in soy protein, or in diets containing 20% blended vital and hydrolysed wheat gluten, to have no effect on growth performance, condition factor, survival or carcass composition compared to the basal diets (SPC or 20% Blend). No significant difference in growth performance or other performance parameters was observed with 20% blended wheat gluten inclusion alone. Chapter 4 identified the ability of wheat gluten products at inclusion levels from 20 to 30%, to increase growth performance. However, in chapter 5, as was also observed in chapter 3b, trout

displayed very good FCR's and SGR's in the basal soy protein diet treatment, reducing the scope for improved growth performance with feed additives or alternative protein sources.

As observed with microbiological analysis in chapters 3 and 4, experimental diets again were observed to modulate the intestinal microbiota of juvenile rainbow trout. With scFOS supplementation to the basal diets or with wheat gluten inclusion alone, the structure of the allochthonous microbiota remained unchanged at phylum level, as also observed in chapter 3, with dominance maintained by Firmicutes. Modulation of the intestinal microbiota was observed at genus level. *Enterococcus* was again the most abundant genus as was the case in chapter 3b, representing 31.17% of the sequence total reads. Twenty % blended wheat gluten inclusion enhanced the proportion of OTUs of *Arthrobacter*, *Staphylococcus pasteurii* and reduced order Bacillales. scFOS supplementation of the soy protein basal treatments resulted in enhanced *Kocuria* reads, whilst scFOS supplementation of wheat gluten treatments increased *Staphylococcus pasteurii* and genus *Lactobacillus* populations compared to all other treatments, an apparent symbiotic effect.

An apparent symbiotic effect was also observed in immuno-relevant and stress related gene expression in the posterior intestine. scFOS supplementation in 20% wheat gluten diets resulted in significant downregulation of the pro-inflammatory cytokine TNF- $\alpha$ , and numerical, yet not significant, down regulation of IL-1 $\beta$ . The combined scFOS and wheat gluten also resulted in significant down regulation of HSP 70, Casp 3 and Glute ST. Glute ST expression saw significant down regulation with scFOS supplementation to both basal diets. HSP 70, observed in chapter 3b to be down regulated with wheat gluten inclusions, was unaffected with 20% blended wheat gluten inclusion in chapter 5.

Despite the observations of the modulation of intestinal microflora, SCFA production/concentrations in the posterior intestine were unaffected by dietary treatment, with formic acid in the highest concentrations, followed by acetic and lactic acid. Butyric and propionic acids were not detected. Observations of SCFAs in the digesta could however be under reporting the true levels produced, due to the high rate of absorption in the posterior intestine. This has been observed with higher concentrations reported in *in vitro* compared to *in vivo* studies on the same species (Mahious, 2006; Kihara and Sakata, 2002).

This body of research adds a wealth of information in regards to the health, allochthonous microflora, and growth performance of juvenile rainbow trout in response to wheat gluten inclusions and scFOS supplementation. Throughout the three experimental chapters limitations in methodologies and practices must be noted, as they have the potential to affect the results observed. Firstly, molecular techniques rely on the efficient and complete DNA/RNA extraction and amplification through polymerase chain reactions (PCR). Variation in extractions and un-intentional PCR bias may be introduced prior to further down-stream analysis and sequencing. A PCR-DGGE notable limitation, with possible relevance to chapters 3a and 5, is the ability of more than one sequence migrating to the same point on the gradient gel, forming a band identified as a single OTU. This phenomenon can result in lower observed OTU's per sample and can prevent successful identification with Sanger sequencing. High-throughput analysis also has limitations associated with the analysis of short reads, and often multiple 16S rRNA copy numbers per bacterium. The 16S rRNA gene has been heavily relied upon for bacterial sequencing due to its universal phylogenetic distribution, but copy numbers are inconsistent between species (Wintzingerode et al.,

1997). These variations can lead to over or under estimates of taxa abundance. Further information, for readers with interests in molecular technique strengths and weaknesses, can be found in Jackson et al. (2000), Kuczynski et al. (2012) and Větrovský and Baldrian (2013). Limitations were also observed with light microscopy. The high extent of intestinal folding prevented consistent measurements of intestinal fold lengths, and perimeter ratio calculations that have high relevance to observing changes in absorptive surface area of the intestine.

The utilisation of microbial and molecular techniques, high throughput sequencing and gene expression analysis, utilised through this body of research have improved the understanding of rainbow trout intestinal health and microbiota. High throughput sequencing enables an overview of the taxonomic profile of the microbiota, highlighting changes at varying taxonomic levels between samples. These observable changes, however, cannot fully reveal the extent to which modulation affects the entire intestinal system. The utilisation of metagenomic analysis enables investigators to assess effects on the genetic potential of the microflora (Ghanbari et al., 2015). Further research in the intestinal microbiota field, especially in fish, should focus on the functional roles played by the microbiota by applying metabolomics, metaproteomics and metatranscriptomics (Ghanbari et al., 2015). Metatranscriptomics identifies active species through their gene expression in complex communities, whilst metabolomics and metaproteomics have the ability to identify roles microbes play in the intestine through the analysis of proteins and metabolites (Franzosa et al., 2015). It should be noted, however, that extraction of bacterial RNA from fish digesta samples is extremely challenging and likely to be an impediment to progress in this field.

Furthermore, the analysis of cytokines and immune relevant genes provide only a snapshot of large, complex molecular pathways associated with inflammatory responses in the intestine. The use of proteomic approaches, potentially on intestinal mucus as a first step, would improve our understanding of these pathways and their end functions (Rodrigues et al., 2012; Almeida et al., 2015).

The utilisation of wheat glutens in aquafeeds was evaluated throughout this body of research. Encouraging results have been observed in growth performance, microbiota modulation and gene expression, with no signs of detrimental impacts to the intestine. The promising amino acid profile, high in glutamine, and its low ANF content play into strengths of wheat glutens as an alternative protein source for the replacement of fishmeal and soya products for juvenile rainbow trout. The limitations of the extrapolation of results from short feeding trials utilising juvenile fish (fingerling to sub-adult) must also be acknowledged. The present research focused solely on juvenile fish, with the maximum average weight achieved in the investigations reaching  $85.46 \pm 2.06$ g. Throughout the production cycle varying nutritional requirements are apparent. Further investigation into the potential role of wheat glutens for aquafeeds for first feeders and fry, where there are more stringent nutritional requirements for amino acids and energy, would be of great interest. Likewise, more attention could be applied to fish at later stages such as harvest size fish (300-400g) where economic cost of feed is of more significance. There would also be merit in applying these studies to brood-stock fish, quantifying the effects on ova production, fecundity and quality as well as milt production in male fish.

It must also be noted the requirement for free crystalline lysine supplementation throughout this programme of investigations. The low lysine content of wheat gluten makes this essential amino acid the limiting factor, especially in higher wheat gluten inclusions in trout, as it is with cereal grains in pigs (Adeola et al., 2001). The addition of free amino acids incurs an additional cost to wheat gluten inclusion; although with increased production, prices are lower than historically noted. Wheat gluten alone is itself more expensive than other alternative plant protein sources such as soya products, potentially resulting in high inclusions being economically unviable. This could ultimately lead to lower inclusion levels and wheat gluten products being utilised as functional ingredients for its physical characteristics as well as biological impacts. Indeed, 6% inclusion levels of Solpro® have been observed to modulate the intestinal microbiota of Asian seabass (Apper-Bossard et al., 2013), as did 7.5% inclusions of Amytex® and Merripro® for rainbow trout in chapter 3a.

Abiotic factors such as temperature variation may play an important role when one considers the potential use of wheat gluten and prebiotics in salmonid aquaculture. Observed alterations, especially in the microbiota, may be significantly affected by seasonal variation. Winter water temperatures, which can regularly fall below 10°C, will have consequences on the gut microbiota and the general intestinal activity. The physical characteristics of the digesta may also be affected, and warrant further investigation. Viscosity, hydration rates and intestinal transit/evacuation rates may all be affected by wheat gluten inclusions and a range of abiotic factors. These variances may also lead to alterations in digestibility that would form basis of another interesting area for investigation, along with palatability and satiation response. The effect of wheat gluten inclusions on

enzyme excretion in the mid intestine and pyloric caeca would also be of interest for further investigation. Direct enzyme assays for the activity of pepsin, trypsin, chymotrypsin, amylase, lipase and various specific carbohydrase enzymes, such as maltase, may result in interesting findings as a result of wheat gluten inclusions at the expense of soya products. Soybean meal especially is high in trypsin inhibitors as well as many other ANFs.

Another area of noted is that the present studies are confined to rainbow trout as a model salmonid species. However, production of Atlantic salmon is a major industry in Scotland, Norway, Chile, Tasmania and Canada, combined producing 2,188,391 Tonnes in 2014 (FAO, 2016). Given the fish in – fish out ratio concerns in the relation to fishmeal use in intensive salmon production, there is considerable effort in seeking reliable and effective alternative plant proteins for formulations of diets for salmon. The findings in these studies for rainbow trout have indicated that it would be feasible and of benefit to undertake similar research for various stages of Atlantic salmon production.

In conclusion, through intensive research in the aquaculture sector over the past two decades, it has become established that to achieve a more sustainable and productive industry, the combination of nutrition, health status and genetics must be optimised. The genetic selection of brood stock fish is conducted by farms themselves if hatcheries are on site, or by multinational company's producing millions of eggs for intensive rearing. This in its self is a whole other area of research, and the fastest growing, largest fish may not be the genetically fittest and best animal for production systems. The new approval of genetically modified fish in the USA and Canada has also opened up a vast area for discussion for the future of genetics in aquaculture. Indeed, Dupont-Nivet et al. (2009) observed the

occurrence of genotype × diet interactions when feeding juvenile rainbow trout an all plant-protein diet. The findings from the present investigations, however, have the ability to improve knowledge for best practices and formulations to improve the nutrition and the health status of intensively reared salmonids. Indeed, results observed in aspects of this body of work have identified the ability of wheat gluten products, solely or combined, and scFOS to modulate the intestinal microbiota, improve growth performance and reduce intestinal stress and inflammation biomarkers when included at the expense of soy protein concentrate in aquafeeds for juvenile rainbow trout.

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