2016

Assessment of a solid-state fermentation product in contemporary and lupin-containing diets for commercial finfish

Bowyer, Peter Herve A.

Assessment of a solid-state fermentation product in contemporary and lupin-containing diets for commercial finfish

by

Peter Hervé A. Bowyer

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

Doctor of Philosophy

(March 2016)

This work was supported by Alltech Inc., with contribution from Innovate UK under the Lupins in UK Agriculture and Aquaculture initiative.

Acknowledgements

I would like to thank the following people for their help and support, which has extended from the first sparks of fascination with fish, over two decades ago, to the completion of this PhD at present.

Firstly, Professor Simon Davies for fuelling my enthusiasm in this area, providing me with great opportunities, guiding me through many projects over the years and being not only a director of studies but a great friend.

Dr Daniel Merrifield, for his direction, management and expertise during this work.

John Sweetman, my industrial supervisor, for his instrumental guidance and trust in my own ideas.

Great thanks are given to Dr Pearse Lyons for his generous financial backing, which has ultimately allowed this thesis to exist. Along with Dr Karl Dawson, Dr Jorge Arias and Dr Keith Filer, for their support, encouragement and opportunities provided.

Much gratitude is extended to Voller, Benno and Dr Antonio Vizcaino, for their friendship, wine/beers/coffee and commitment to helping me during long days of handling live fish, bits of dried fish…and their faeces. We shall say the results of who still owes each other a coffee remain highly debateable thus inconclusive.

Thank you to the technical staff at Plymouth University and Alltech for their genius know-how and involvement with this work. Liz Preston, Glen Harper, Pete Bonds, Matt Emery, Dr Andrew Fisher, Mike Hocking, Dr Ana Rodiles and Patrick Becker, you have all been integral to this research.

Inexpressible thanks are given to Dave Fuller and Dan Young at Exmoor Fisheries, without whom, this work could not have been completed. Your generosity, patience, commitment and port have been integral to this work.

Cheers to all of my friends and fellow expert mega-wrasse fishers; you have kept me sane throughout. It doesn't look like I will be doing another season at Holywell.

To Maman, Dad and Anne-Fabienne, I am eternally grateful for your acceptance of my unusual love of fish and continual love, support and belief over the past 26 years. I couldn't have asked for a better upbringing and family foundations.

Merci Mémé et Pépé, pour mes premières expériences de la pisciculture - avec les gardons et carpes du Pratt, dans le lavoir! De m'avoir emmené à la pêche et tous les supports financières qui mon permis de suivre ma carrière.

Lastly, to my wife, Tess. Your love, friendship and endless support continually drive me towards the next chapter, both in writing and life.

Ethical review 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 30/10402.

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

ABSTRACT

This body of research explores the dietary application of a bioactive, solid-state fermentation (SSF) product in contemporary and lupin-containing diets of Nile tilapia (*Oreochromis niltoticus*) and rainbow trout (*Oncorhynchus mykiss*). Consequently, the work provides holistic assessment of the influences of SSF products on animal health and performance; depending upon rearing temperature, nutritional physiologies, feed formulations and extrusion conditions; alongside information on the performance of lupins in aquafeeds.

The SSF product (at 0.1 % inclusion) improved growth performance of Nile tilapia fed diets containing lupins. Phosphorous retention appeared higher when the SSF product was included in a yellow lupin (*Lupinus luteus*) diet whilst Mg retention was significantly higher in fish fed narrow-leaf lupin (*Lupinus angustifolius*). The experimental ingredients did not appear to show any clear effects upon midgut macrostructure. At an ultrastructural level, the fish fed yellow lupin alone, displayed poorest brush border characteristics but those fed yellow lupin and the SSF product showed signs of amelioration since they did not differ significantly from those fed narrow-leaf lupin.

Focus was then turned towards a yellow lupin-based diet in rainbow trout, with two inclusion levels of the SSF product. The SSF product significantly improved growth performance and feed efficiency at 0.5 %, with values closer to a fishmeal-based diet than the lupin control. The SSF product increased the digestibility of protein and energy and bioavailability of numerous elements. However, the digestibility and bioavailability of certain nutrients, e.g. fibre and Zn, were only increased with a 0.5 % inclusion. The SSF product influenced vertebral Ca:P ratio but no effect on vertebral morphology was identified. Fish fed yellow lupin kernel meal displayed high Mn concentrations throughout a number of tissues. The intestinal environment was explored in depth, revealing large differences dependent upon SSF product inclusion rate. Fish fed a 0.1 % inclusion exhibited deteriorated brush border characteristics and high diversity of microbes, including increased proportions of key salmonid pathogens. Those fed a 0.5 % inclusion displayed signs of increased surface area at an ultrastructural level, reduced goblet cell numbers and a low microbial diversity; with domination of one particular family, Enterobacteriaceae. Activities of alkaline phosphatase and leucine aminopeptidase within the anterior intestine also appeared to be influenced by SSF product inclusion. Variations in haemato-immunological parameters were also observed between the treatments.

In the final experiment, the SSF product was applied, pre-extrusion, to a contemporary rainbow trout formulation, at 0, 0.5, 1.0 and 1.5 %. No significant improvements to performance were identified following SSF product inclusion. However, crude protein digestibility from SSF-supplemented diets was significantly higher than the control and tendencies towards elevated retention of Ca, P, and Mg were apparent. *In vitro* analysis of free-phosphate release was conducted upon pre- and post-extruded diets, at varying temperatures. This indicated that neither extrusion conditions (105 °C) nor an ambient temperature of 10 °C were sufficient to cease P-liberating capabilities by the SSF product; suggesting that the two conditions combined limited the product's efficacy in practice.

This research evidenced that SSF products can be an effective means of improving the available nutrient profiles of compound diets for both omnivorous, warmwater and carnivorous, temperate finfish. Lupins are a promising alternative protein source but their nutritional value can be substantially improved by SSF product application. Exploration of the effects of SSF products on the intestinal environment revealed that both negative and positive effects on intestinal health can occur, which is highly dependent upon product inclusion rate. The holistic approaches adopted within this series of studies have seldom been performed on monogastric animals and thus provide valuable, transferable information for advancing knowledge in the application of SSF products, exogenous enzymes and lupins to farm animal feeds, in general.

LIST OF CONTENTS

Acknowledgements II
Copyright Statement III
Ethical Review Statement IV
ABSTRACT V
LIST OF CONTENTS VI
LIST OF TABLES XI
LIST OF FIGURES XII
LIST OF PLATES XIII
Author's Declaration XV
Work presented XVI
Publications XVII
LIST OF ABBREVIATIONS XVIII

CHAPTER 1. Aquaculture, aquafeeds and exogenous enzymes 1
1.1 Aquaculture and global protein security 1
1.2 Macronutrient provision and acquisition in finfish nutrition 2
1.3 Micronutrient provision and acquisition in finfish nutrition 5
1.4 Measuring nutrient digestibility and bioavailability in finfish 12
1.5 Aquafeed ingredients and sustainability 13
1.6 Anti-nutritional factors 17
1.7 Lupins 19
1.7.1 Lupins as an alternative protein 19
1.7.2 Lupin anti-nutritional factors 20
1.8 Exogenous enzymes in animal nutrition 22
1.8.1 Background 22
1.8.2 NSP-degrading enzymes 24
1.8.2.1 Background 24
1.8.2.2 Cellulase 25
1.8.2.3 Xylanase 28
1.8.2.4 Mannanase 30
1.8.2.5 Pectinase 31
1.8.3 Phytase 32
1.8.4 Synergistic enzymes and cocktails 41
1.9 Solid-state fermentation 47
1.9.1 Background 47

1.9.2 Synergen™ 48
1.10 Research rationale 47
1.11 Research aims 50

CHAPTER 2. General methods 52
2.1 Experimental animals and housing 52
2.2 Experimental ingredients and diets 53
2.3 Feeding regimes 53
2.4 Biomass sampling 53
2.5 Euthanasia 54
2.6 Anaesthesia 54
2.7 Proximate composition 54
2.7.1 Dry matter 54
2.7.2 Crude protein 55
2.7.3 Crude lipid 56
2.7.4 Crude fibre 57
2.7.5 Ash 58
2.7.6 Gross energy 58
2.8 Mineral and trace element analysis 59
2.8.1 Digestion 59
2.8.2 ICP OES 60
2.9 Performance calculations 61
2.10 Somatic index calculations 62
2.11 Nutrient digestibility and mineral bioavailability calculations 62
2.12 Nutrient retention 63
2.13 Protein efficiency and utilisation 63
2.14 Haematological and serological analysis 64
2.14.1 Haematocrit 64
2.14.2 Haemoglobin 65
2.14.3 Serum glucose 65
2.14.4 Serum lysozyme 66
2.15 Light microscopy (intestine) 67
2.16 Transmission electron microscopy 68
2.17 Statistical analysis 70

CHAPTER 3. Application of Synergen™ in Nile tilapia diets containing yellow and narrow-leaf lupin varieties 71
3.1 Introduction 71

3.2 Materials and methods 73
3.2.1 Experimental design 73
3.2.1 Experimental animals and housing 73
3.2.2 Experimental ingredients and diets 74
3.2.3 Feeding and biomass sampling 77
3.2.4 Performance calculations 77
3.2.5 Whole carcass sampling 77
3.2.6 Feed and carcass compositional analysis 77
3.2.7 Haematological and serological parameters 77
3.2.8 Intestinal histology 78
3.2.8.1 Sampling 78
3.2.8.1 Light microscopy 78
3.2.8.1 Transmission electron microscopy 79
3.2.9 Statistical analysis 80
3.3 Results 81
3.3.1 Growth performance and feed efficiency 81
3.3.2 Carcass composition and nutrient retention 82
3.3.3 Haematology and serology 84
3.3.4 Intestinal morphology 86
3.4 Discussion and conclusion 89

CHAPTER 4. Application of Synergen™ in the diets of rainbow trout containing high yellow lupin inclusion 102
4.1 Introduction 102
4.1.1 Salmonid culture 102
4.1.2 Trends in salmonid diet formulation 104
4.1.3 Sustainability in salmonid nutrition 105
4.2 Materials and methods 107
4.2.1 Experimental design 107
4.2.2 Experimental animals and housing 107
4.2.3 Experimental ingredients and diets 109
4.2.4 Compositional analyses 109
4.2.5 Feeding regimes 111
4.2.6 Biomass sampling and control (*N*-phase) 112
4.2.7 Performance calculations 112
4.2.8 Faecal sampling (*D*-phase) 112
4.2.9 Whole carcass sampling 113
4.2.10 Tissue mineral concentrations 113

4.2.11 Vertebral histomorphometry 114
4.2.12 Haematological and serological parameters 116
4.2.13 Intestinal histology 117
4.2.13.1 Sampling 117
4.2.13.2 Light microscopy 118
4.2.13.3 Transmission electron microscopy 119
4.2.13.4 Scanning electron microscopy 119
4.2.14 Endogenous protease activity 122
4.2.14.1 Sample collection 122
4.2.14.2 Enzyme extraction 122
4.2.14.3 Total alkaline protease 123
4.2.14.4 Trypsin 123
4.2.14.5 Chymotrypsin 124
4.2.14.6 Leucine aminopeptidase 125
4.2.14.7 Alkaline phosphatase 125
4.2.15 Statistical analysis 126
4.2.16 Intestinal microbiology 126
4.2.16.1 Sampling 126
4.2.16.2 DNA isolation and PCR 127
4.2.16.3 High-throughput sequence analyses 128
4.3 Results 129
4.3.1 Fish and feed performance 129
4.3.2 Nutrient digestibility and mineral bioavailability 130
4.3.3 Carcass composition and tissue mineral concentrations 132
4.3.4 Vertebral histomorphometry 135
4.3.5 Haematology and serology 136
4.3.6 Intestinal morphology 137
4.3.7 Intestinal proteolytic enzyme activity 142
4.3.8 Intestinal microbiota 143
4.4 Discussion 154
4.4.1 Fish performance and feed efficiency 154
4.4.2 Macronutrient availability and status 155
4.4.3 Micronutrient availability, status and osteology 158
4.4.4 Intestinal morphology, microbiota and proteolytic activity 164
4.4.5 Modulation of serum lysozyme 171
4.5 Conclusion 172

CHAPTER 5. Pilot-scale investigation of Synergen™ efficacy in salmonid culture under commercial conditions: from feed manufacture to fish rearing **174**
5.1 Introduction 174
5.1.1 Formulations and physical criteria for aquafeeds 174
5.1.2 Aquafeed manufacture 175
5.1.3 Retention of bioactive functionality under feed processing conditions 176
5.2 Materials and methods 179
5.2.1 Experimental design 179
5.2.2 Experimental system and animals 180
5.2.3 Diet formulation 180
5.2.4 Diet manufacture 182
5.2.5 Feeding regimes 183
5.2.6 Sampling 183
5.2.7 Proximate composition and element concentrations 184
5.2.8 Calculations 184
5.2.9 *In vitro* free phosphate release 184
5.2.10 Statistical analysis 186
5.3 Results 186
5.3.1 Fish and feed performance 186
5.3.2 Somatic indices 187
5.3.3 Nutrient and trace element availability and retention 187
5.3.4 Serum lysozyme 189
5.3.5 *In vitro* free phosphate release 190
5.4 Discussion and conclusion 191

CHAPTER 6. Solid-state fermentation application in aquafeeds: present knowledge and future direction **196**
6.1 Overview of programme of research 196
6.1.1 Improving nutrient profiles 197
6.1.2 SSF products and the intestinal environment 201
6.2 Future work 204
6.2.1 The gastrointestinal environment 205
6.2.2 Ingredients and diets 206
6.2.3 Life-stages 208
6.2.4 Overcoming thermal constraints 210
6.3 Overall conclusions 211

7 APPENDICES **213**

7.1 Experimental systems 213

7.2 Histological staining procedures.....214

8 REFERENCES..... **216**

LIST OF TABLES

Table 1.1: Reported concentrations and ranges of macro and trace elements found in commonly used and prospective protein sources for aquafeeds; with particular emphasis towards Lupins - *Lupinus albus* (white lupin*), Lupinus angustifolius* (narrow-leaf lupin) and *Lupinus luteus* (yellow lupin) 10

Table 2.1: Summaries of conducted experiments.....53

Table 3.1: Feed formulations, proximate compositions and mineral concentrations of the experimental Nile tilapia diets 76

Table 3.2: Fish and feed performance values of the dietary treatments 83

Table 3.3: Concentration of macronutrients and minerals in the whole carcasses of the experimental Nile tilapia..... 83

Table 3.4: Ultrastructural measurements of Nile tilapia midgut after 49 days of feeding diets containing lupins, with and without Synergen™ 88

Table 4.1: Feed formulations, proximate compositions and element concentrations of the experimental rainbow trout diets.....110

Table 4.2: Fish and feed performance values of the dietary treatments 130

Table 4.3: Apparent macronutrient digestibility coefficients (ADC) (%) and apparent mineral bioavailability coefficients (ABAC) (%) of the experimental lupin-based and fishmeal-based rainbow trout diets.....131

Table 4.4: Whole carcass composition of fish fed the experimental diets 132

Table 4.5: Tissue mineral concentrations of liver, muscle, vertebrae and caudal fin in experimental fish 134

Table 4.6: Bone histomorphometric indices of the 47^{th}-48^{th} vertebrae of rainbow trout fed the experimental diets.....135

Table 4.7: Haematological and serological parameters of rainbow trout fed the experimental diets.....136

Table 4.8: Morphological parameters of the posterior intestine of the lupin-fed fish, with and without Synergen™ inclusion 138

Table 4.9: Proteolytic enzyme activities in digesta and mucosa of fish fed the lupin based diet, with and without SYN inclusion 142

Table 4.10: Alpha diversity metrics of allochthonous microbial populations within the dietary treatments 143

Table 5.1: Formulations, proximate compositions and element concentrations of the experimental, extruded rainbow trout diets 181

Table 5.2: Fish and feed performance fingerling rainbow trout fed experimental extruded diets, with and without Synergen inclusion, over a 9 week period 187

Table 5.3: Apparent digestibility and bioavailability coefficients of nutrients obtained via faecal collection of fingerling rainbow trout fed extruded diets with and without Synergen™ inclusions 188

Table 7.1: Haematoxylin and eosin staining procedure 214

Table 7.2: Periodic acid Schiff's staining procedure 215

Table 7.2: Mallory's Trichrome with haematoxylin staining procedure 215

LIST OF FIGURES

Figure 1.1 Percentage contributions of ingredient types in commercial Norwegian salmon (*Salmo salar*) diets: 1990 to 2013 (Nofima 2014). 14

Figure 1.2: World lupin production, by country: 2012 to 2013 (FAOSTAT, 2016) 20

Figure 1.3 Phosphorous and ash digestibility coefficients from rainbow trout fed 2000 or 4000 FTU kg^{-1} microbial phytase under incremental water temperatures (Vandenberg *et al.,* 2012). 39

Figure 3.1: Phosphorous, magnesium and zinc retention within the carcass of the experimental Nile tilapia (mean + S.E. *n* = 2) 84

Figure 3.2: Haematological and serological parameters of the experimental Nile tilapia at day 49 (mean + S.E. *n* = 6). 85

Figure 3.3: Midgut macrostructural parameters of Nile tilapia fed diets containing lupins, with and without inclusion of Synergen™; displaying independent dietary treatments (*n* = 6) and grouped variables (*n* = 12) 87

Figure 4.1: Allochthonous microbiome relatedness of fish fed a lupin-based diet, with and without inclusions of SYN 143

Figure 4.2: Abundance and proportional contributions of phyla within the allochthonous microbiomes of the dietary groups. 145

Figure 4.3: Proportional contribution of selected bacterial phyla within the digesta of the dietary groups. A = Fusobacteria. B = Firmicutes. 146

Figure 4.4: Proportional contribution of selected bacterial phyla within the digesta of the dietary groups. A = Proteobacteria. B = Spirochaetes. 147

Figure 4.5: Venn diagram displaying the numbers of shared and exclusive genera within the allochthonous microbial population of the dietary groups. 148

Figure 4.6: Proportional contributions of identified genera within the microbiomes of sampled individuals. OTUs presented are those which express >0.1 % contribution towards the total number of sequences identified 151

Figure 4.7: Allochthonous genera belonging to the family Enterobacteriaceae, of which were found to differ significantly between dietary treatments 152

Figure 4.8: Miscellaneous allochthonous genera found to differ significantly between dietary treatments 153

Figure 5.1: Somatic indices (AU) of fingerling rainbow trout after 9 weeks feeding with experimental extruded diets, with and without Synergen™ inclusions 187

Figure 5.2: Element retentions (%) of fingerling rainbow trout fed experimental extruded diets, with and without Synergen™ inclusions, after 9 weeks 189

Figure 5.3: Serum lysozyme activity (U/ml) of fingerling rainbow trout fed experimental extruded diets, after 9 weeks 190

Figure 5.4: *In vitro* free phosphate release of pre- and post-extruded feed samples used within the feeding study, at varying ambient temperatures 191

LIST OF PLATES

Plate 1.1: Common digestive configurations of cultured finfish 6

Plate 3.1: Methodological demonstrations of Nile tilapia midgut macrostructural appraisal 79

Plate 3.2: Methodological demonstrations of Nile tilapia midgut ultrastructural appraisal, using TEM micrographs 79

Plate 3.3: TEM micrographs of Nile tilapia midgut brush border after 49 days of feeding diets containing lupins, with and without Synergen™ 88

Plate 4.1: Transverse section of 47/48th vertebrae of rainbow trout, stained with Mallory's trichrome and H&E, displaying gross anatomical structure and appraised features 116

Plate 4.2: Rainbow trout dissection displaying intestinal morphological portions and sample site selections (not proportionally to scale) 117

Plate 4.3: Light and electron micrographs of rainbow trout posterior intestine displaying appraisal methods 121

Plate 4.4: Histological sections of 47-48th vertebrae of fish fed the experimental diets 135

Plate 4.5: Intestinal ultrastructure of fish fed the basal lupin diet (LC) after 10 weeks 139

Plate 4.6: Intestinal ultrastructure of fish fed a 0.1 % Synergen™ inclusion (LS0.1) after 10 weeks 140

Plate 4.7: Intestinal ultrastructure of fish fed a 0.5 % Synergen™ inclusion (LS0.1) after 10 weeks 141

Plate 7.1: Warmwater RAS utilised for experimental work under Chapter 3, using Nile tilapia 213

Plate 7.2: Temperate RAS utilised for experimental work with rainbow trout under Chapter 4.....213

Plate 7.3: Temperate flow-through system utilised for experimental work conducted with rainbow trout under Chapter 5.....214

Author's declaration

At no time during the registration for this degree of Doctor of Philosophy has the author been registered for any other university award.

Word count: 58,762

Signed: P.H.Bowyer

Date: 01/09/2016

WORK PRESENTED

Unlocking the Nutritional Potential of Alternative Feed Ingredients: Lupins and Solid-State Fermentation
Bowyer, P. H.
Oral Presentation
Virginia Tech Department of Food Science and Technology Seminar Series. Blacksburg, VA, U.S.A.
May 2015

Effects of a Solid-State Fermentation Product on Growth Performance, Intestinal Health and Apparent Nutrient Digestibility in Rainbow Trout (*Oncorhynchus mykiss*) fed Diets with High Lupin Inclusion
Bowyer, P. H., Vizcaino, A. J., Merrifield, D. L., Sweetman, J., Davies, S. J.
Oral Presentation
World Aquaculture Society: Aquaculture America 2015. New Orleans, LA, U.S.A.
February 2015

Lupins in Aquaculture – dissemination of results
Davies, S. J., Adeoye, A. and Bowyer, P. H.
Oral Presentation
LUKAA: Final Meeting. Shropshire, U.K.
January 2015

Effects of a Solid-State Fermentation Product on Growth Performance, Intestinal Health and Apparent Nutrient Digestibility in Rainbow Trout (Oncorhynchus mykiss) fed Diets with High Lupin Inclusion
Bowyer, P. H., Vizcaino, A. J., Merrifield, D. L., Sweetman, J., Davies, S. J.
Oral Presentation
World Aquaculture Society: Aquaculture Europe 2014. San Sebastian-Donostia, Spain
October 2014

Growth Performance Evaluation of Rainbow Trout (Oncorhynchus mykiss) Fed Practical Diets Containing a Solid-State Fermentation Product
Poster Presentation
Bowyer, P. H., Merrifield, D. L., Sweetman, J., Davies, S. J.
World Aquaculture Society: Aquaculture America 2014. Seattle, WA, U.S.A.
February 2014

Development of an in vitro technique for assessing diets and feed ingredients for Tilapia
Adeoye, A., Bowyer, P. H., Merrifield, D. L. and Davies, S. J.
Oral presentation
10th International Symposium on Tilapia in Aquaculture. Jerusalem, Israel.
October 2013

PUBLICATIONS

Lupins and a by-product of solid-state fermentation influence performance and gut morphology of Nile tilapia (*Oreochromis niloticus*)
Bowyer, P. H., Harper, G. M., Merrifield, D. L., Sweetman, J. and Davies, S. J.
Submitted to: Czech Journal of Animal Science

By-product of solid-state fermentation improves nutrient digestibility and mineral bioavailability of yellow lupin-based rainbow trout diet
Bowyer, P. H., Merrifield, D. L., Sweetman, J., Davies, S. J.
In preparation

By-product of solid-state fermentation influences intestinal morphology and microbiome in rainbow trout fed a yellow lupin-based diet.
Bowyer, P. H., Rodiles, A., Vizcaino, A. J., Merrifield, D. L., Sweetman, J., Davies, S. J.
In preparation

LIST OF ABBREVIATIONS

ABAC	Apparent bioavailability coefficient
ADC	Apparent digestibility coefficient
AI	Anterior intestine
ALP	Alkaline phosphatase
ANF(s)	Anti-nutritional factor(s)
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
AU	Arbitrary unit
BAPNA	N-a-benzoyl-DL-arginine 4-nitroanilidehydrochloride
BL	Blue/Narrow-leaf lupin (*Lupinus angustifolius*)
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
BW	Body weight
CF	Crude fibre
CL	Crude lipid
CP	Crude protein
CTI	Centrum thickness index
DDGS	Distiller's dry grains with solubles
DGGE	Denaturing gradient gel electrophoresis
DM	Dry matter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP	Decimal place
DPX	Distyrene plasticiser xylene
EAA	Enterocyte apical area
EDTA	Ethylenediaminetetraacetic acid
EH	Enterocyte height
EM	Electron microscopy

ETAS	Estimated total absorption surface area
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed conversion ratio
FM	Fish meal
FTU	Phytase units
FW	Final fish weight
GC	Goblet cell
GE	Gross energy
GI	Gastro-intestinal
GIT	Gastro-intestinal tract
GM	Genetically modified
GMO	Genetically modified organisms
Hb	Haemaglobin
Hct	Hematocrit
H&E	Hematoxylin and eosin
HSD	Honest significant difference
HSI	Hepatosomatic index
ICP OES	Inductively Coupled Plasma Optical Emission Spectroscopy
IP	Intraperitoneal cavity
IU	International units
IW	Initial fish weight
K-F	K-factor index
LAP	L-Leucine Aminopeptidase
LPC	Lupin protein concentrate
LKM	Lupin kernel meal
LM	Light Microscopy
LOD	Limit of detection
LPW	Lamina propria width
LSD	Least significant difference

LYZ	Lysozyme
MG	Midgut
MOS	Mannan-oligosaccharide
MS222	Tricaine methanesulfonate
MTI	Muscularis thickness index
MVCT	Microvilli counts
MVCV	Microvilli coverage
MVD	Microvilli diameter
MVH	Microvilli height
MVL	Microvilli length
NGS	Next-Generation Sequencing
NR	Nutrient retention
NRC	National research council
NSP(s)	Non-starch polysaccharide(s)
NSPase(s)	Non-starch polysaccharide-degrading enzyme(s)
OTU	Operational taxonomic unit
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PER	Protein efficiency ratio
PI	Posterior intestine
PP	Plant-derived protein
PR	Perimeter ratio
QIIME	Quantitative Insights into Microbial Ecology
RAS	Recirculating aquaculture system
RNA	Ribonucleic acid
RSM	Rapeseed meal
SAPNA	N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide

SBM	Soyabean meal
SCM	Stratum compactum
SCR	Stratum circulae
SD	Standard deviation
SE	Standard error
SEM	Scanning Electron Microscopy
SFM	Sunflower meal
SGR	Specific growth rate
SLON	Stratum longitudinale
SPC	Soya protein concentrate
SSF	Solid-state fermentation
TAE	Tris-Acetate-EDTA
TAP	Total alkaline protease
TCA	Trichloroacetic acid
TEM	Transmission Electron Microscopy
Tris	Tris (hydroxymethyl) amino-methane
U	Enzyme unit
VPA	Vertebral perimeter to centrum area ratio
WG	Weight gain
WL	White lupin (*Lupinus albus*)
YL	Yellow lupin (*Lupinus luteus*)
YO	Yttrium oxide

CHAPTER 1. Aquaculture, aquafeeds and exogenous enzymes

1.1 Aquaculture and global protein security

With an ever-increasing global population, comes an inherent, indispensable requirement for a greater supply of food. Since 2003, the global population has risen by approximately one billion people (7.3 billion in mid-2015) and is expected to exceed 9.7 billion by 2050 (United Nations, 2015). Clearly, such a substantial rise will require an increase in food production, on an unprecedented scale. This is in the face of a global population where 800 million people already suffer from malnourishment (FAO, 2014a). Furthermore, the situation is not merely a question of feeding more mouths. The rise in population, particularly in developing countries, is forecasted to have an associated growth in wealth; this will inevitably lead to changes in dietary habits as more commodities become financial available to more people (FAO, 2014a). Already observed and set to continue, the most prominent dietary change is that of a greater consumption of animal protein per capita. Between 1960 and 2012, apparent fish consumption per capita is estimated to have almost doubled from 9.9 kg to 19.2 kg, respectively (FAO, 2014a). Therefore the question is how to feed more mouths, with more high-quality animal protein. Sustainable production systems, which can satisfy demand, must be implemented in order to achieve future success in food security, population well-being and sustainable development.

Aquaculture is recognised as one of the fastest growing sectors of the agri-business industry. Although growth has been slowing over the past two decades, aquacultural production nonetheless doubled from 32.4 million tonnes in 2000 to 66.6 million tonnes in 2012 (FAO, 2014a). With most of the world's major capture fisheries either reaching maximum harvestable levels or collapsing entirely, in turn making many populations vulnerable to extreme poverty and malnourishment, aquaculture has been able to provide an important compensatory strategy to seafood availability (FAO, 2014a). Latest figures indicate that in 2012, aquaculture contributed 41 percent of total global fishery

production; its input to global food fish supply was forecasted to have surpassed 50 percent in 2015, and reach 60 percent by 2030 (FAO, 2014a). In comparison to terrestrial meat, farmed fish (66 million tonnes) has long exceeded sheep and goat meat production (13.4 million tonnes in 2009) (FAO, 2009) and in 2011, a significant milestone was reached when beef production (63 million tonnes) was also eclipsed (Earth Policy Institute, 2013). Therefore, farmed fish are the third most produced livestock globally, after poultry and pig. Of great note, the expansion of aquaculture has consistently exceeded population growth in recent years (FAO, 2014a); therefore it is seen as a solution to supplying a vast proportion of the ever-increasing global demand for healthy, high-quality protein, in general. However, in order to raise animals for the supply of protein to the global market, they themselves must receive adequate provision of high-quality protein, among all other essential nutrients.

1.2 Macronutrient provision and acquisition in finfish nutrition

Although regularly grouped as a single unit in discussion of farm animal nutrition, aquaculture livestock represent a highly diverse group of animals. From a nutritional perspective, many of the major cultured aquaculture species are in fact no more similar to one another than poultry, pigs, or even ruminants; even though these terrestrial livestock classes are viewed as vastly different to one another by nutritionist. The following schematic diagram outlines the typical digestive configurations of various cultured finfish (Plate 1.1). The diversity in digestive morphologies of aquacultured species arises from the fact that these animals range from finfish to crustaceans, planktonivores to carnivores and agastrics to monogastrics. Consequently, these animals express large differences in terms of their nutrient requirements, digestive capabilities and nutritional sensitivities, and as such feeding strategies vary greatly.

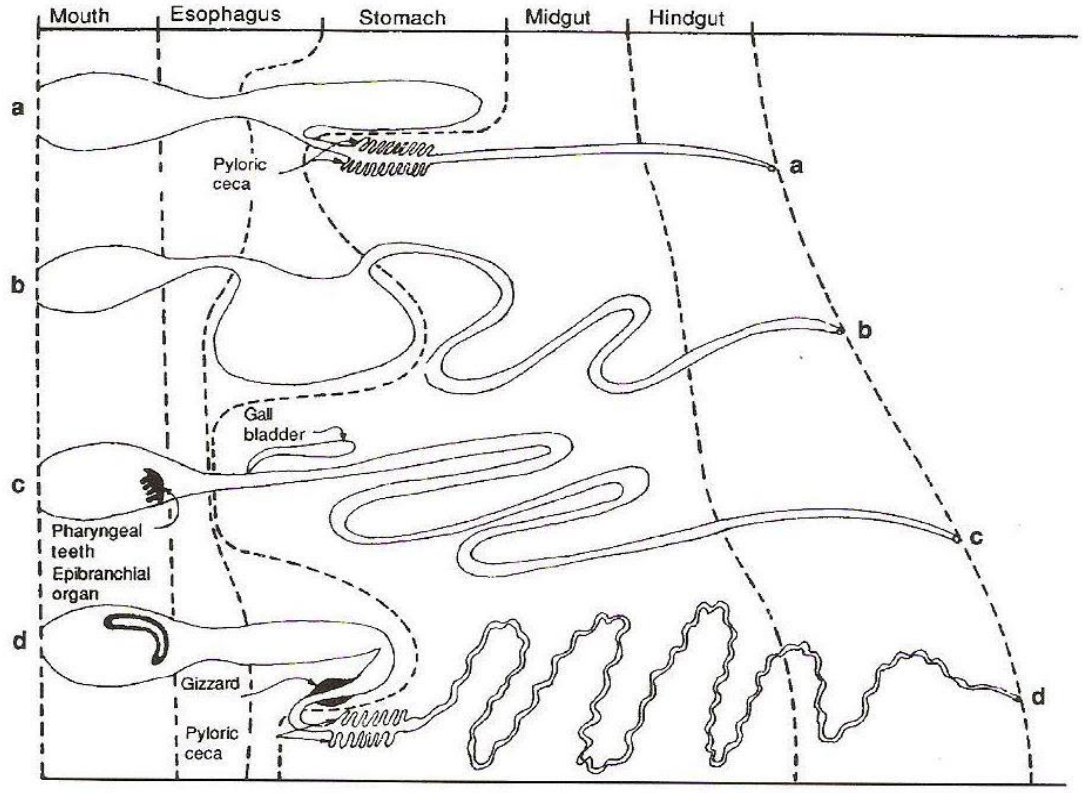

Plate 1.1 Common digestive configurations of cultured finfish. (a) Euryphagous carnivore, with Y-shaped stomach (e.g. salmon, trout, cod, halibut). (b) Euryphagous omnivore emphasising animal foodstuffs, with pouched stomach/intestinal sack (e.g. catfish and tilapia). (c) Euryphagous omnivore emphasising plant foodstuffs, without stomach (e.g. carp). (d) Stenophagous planktivore, with gizzard (e.g. milkfish). From Smith (1989).

In practice, the dietary crude protein level necessary for on-growing grass carp (*Ctenopharyngodon idella*), a phytophagous species, is ≤ 25 %, on a dry matter basis (DMB) (NRC, 2011). Crude protein levels required for optimal growth of on-growing Nile tilapia (Oreochromis niloticus), an omnivorous species, are 28 to 30 % on a DMB (NRC, 2011). In the diets of the carnivorous salmonids, Atlantic salmon (*Salmo salar*) and Rainbow trout (*Oncorhynchus mykiss*), at grow-out stages, the required dietary crude protein levels are between 40 and 45 %, DMB (NRC, 2011). However, there is no true requirement for crude protein since this depends on a well-balanced supply of amino acids and as such, this is dictated by the quality and source of the feed ingredients.

Ten amino acids are essential in the diets of finfish: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Wilson, 2002). However, requirements of these essential amino acids vary between species. For example, the methionine requirement of Atlantic salmon is 0.7 % of the diet, at 0.6 % cysteine; whilst in Nile tilapia, this value is 0.5 % of the diet, at 0.5 % cysteine (NRC, 2011). The lysine requirement of trout and salmon is 1.9 to 2.0 % of the diet, whilst in Nile tilapia it is approximately 1.4 % of the diet (NRC, 2011). For detailed collections of amino acid requirements in finfish and shrimp, readers are directed towards NRC (2011) and Wilson (2002).

Due to their trophic feeding levels and associated digestive configurations, utilisation capacity and tolerances of carbohydrates greatly differ between species. Carbohydrates are not the principal source of energy or carbon for most fish but they can still play an important role in the nutrition of certain species (Dabrowski and Guderley, 2002). Those which do feed at a lower trophic level, consuming plant or algal matter, generally possess morphological adaptations which initiate and facilitate the digestion of carbohydrates; such as pharyngeal mills and gizzards (see Plate 1.1). Although tilapia possess no morphological adaptations of these kinds, commercial diets for these species may contain up to 40 % carbohydrate, including 8 to 10 % crude fibre (NRC, 2011). On the other hand, salmonids thrive on diets devoid of carbohydrates. Modern salmonid diets do contain carbohydrates; however, their presence is due to functional properties that they bring to the feed manufacturing process (e.g. starch for pellet expansion) when deliberately included in formulations, or as an unavoidable consequence of the ingredients which are now used in commercial formulations (i.e. plant proteins) (Young and Forte, 2016b). These topics will be discussed further, throughout this body of work.

The nutrient requirement and limit figures, given so far, represent those of each species at grow-out stages; therefore, it is also important to note that these are subject to change throughout the animal's life-time. Younger life-stages, as well as broodstock, typically have higher requirements of macronutrients (NRC, 2011); due to heightened

requirements for growth and development. Many species which fulfil their nutritional needs via consumption of plant matter, as an adult, in fact begin their lives as predatory carnivores, following yolk sac absorption. At first-feeding, the digestive system of larval fish is rarely fully formed; existing as a simple tract (Rust, 2002), lacking the differentiation presented in Plate 1.1.

Across species and stage of ontological development, the digestive capabilities of fish, like all animals, are coordinated by their endogenous hydrolytic secretions (Rust, 2002) and as such, endogenous enzyme profiles vary between species (Hidalgo *et al*., 1999). However, digestive enzymes can be futile in ensuring optimal nutrition if overall digestive health is not upheld. The intestine is the common premier site for diet contact and nutrient acquisition, under all circumstances, be it life-stage or species. This organ comprises of a particularly complex network of biological units, which collectively allow its normal health and function. Intestinal health is governed not only by its plethora of enzymes but also its architecture and colonising microbiota. Without health of each of these components, optimal functioning of the organ cannot exist, meaning that acquisition of optimal or required macronutrients become restricted. In turn, optimal health and performance cannot be achieved from the animal. For these reasons, intestinal health is increasingly being viewed as an underpinning factor to optimising nutrition.

Optimal nutrition in finfish, like in all animals, depends also on the adequate provision of micronutrients. This is a topic of great complexity in fish and is relatively poorly understood at present.

1.3 Micronutrient provision and acquisition in finfish nutrition

Micronutrients can be defined as compounds or elements which are required in trace amounts for crucial roles in growth, development and metabolic function yet they cannot be synthesised by the organism. Namely, these comprise of vitamins and minerals.

These play an equally important role in supporting life as the macronutrients, yet they are somewhat overlooked on a regular basis. In context with the experimental work detailed later in this body of research, minerals will be the topic of focus herein.

In a review by Davis and Gatlin (1996), 10 elements were proposed as generally essential in the diets of fish; these being calcium, phosphorous, magnesium, potassium, iron, zinc, copper, iodine, selenium and manganese. This number seems to be lower than those identified in terrestrial animals (Davis and Gatlin, 2006) although it is likely that physiological requirement are similar, with a trace importance of elements such as cobalt, chromium and molybdenum (Watanabe *et al.*, 1997), to name but a few. Accurately defining dietary mineral requirements in fish is very challenging, most notably due to the water solubility of many elements. Indeed many of the essential elements can be derived, to varying extents, from surrounding water (Lall, 2002). However, much the same as dietary ingredients, water sources vary considerably in their nutrient profile. This has meant that specific requirements for elements in fish species remains poorly defined. Ultimately, feeds must supply adequate provision of the known element requirements so as to guarantee the normal health and function of the species under various rearing conditions.

A number of nutritional pathologies have been witnessed in commercial finfish farming, with attribution to improper provision of micronutrients. Furthermore, numerous scientific investigations have explored the biological consequences of restricted mineral provision. One of the predominant roles of minerals is contribution to ossification, thus the animal's structural integrity and form. Phosphorous (P) deficiency is undisputedly known to cause significant increases in skeletal abnormalities and reduced bone mineralisation (Sullivan *et al*., 2007a; Sullivan *et al*., 2007b; Fontagne *et al*., 2009; Fox and Davies, 2011; Le Luyer *et al*., 2014; Fjelldal *et al*., 2016), which may also impair growth (Albrektsen *et al*., 2009; Tang *et al*., 2012). In a study on Nile tilapia, adequate dietary P was shown to be critical since it was the only tested element, out of Mg, Na, K, Fe, Zn, I and Ca, which resulted in reduced animal performance when omitted from the

diet; despite the water being dosed with phosphate fertilizer (Dato-Cajegas and Yakupitiyage, 1996). Calcium (Ca) is nonetheless similarly critical in the growth and development of fish. However, it is now generally recognised that dietary dependence is somewhat lower than P considering much, if not all, of the animal's requirement can be satisfied via absorption from surrounding water (Lall, 2002). In freshwater, fish achieve this by uptake through the gills and skin, whilst in seawater, drinking becomes the route of acquisition (Lall, 2002). In Atlantic salmon, reduced bone mineralisation after dietary Ca deprivation has been reported to be reversed following the smoltification process (Berge *et al*., 2009); possibly to be expected considering the marine environment is Ca-rich. Perhaps more surprisingly, delayed ossification of fry due to low dietary Ca concentrations has similarly been observed to be compensated for, relatively rapidly, during freshwater stages (Fontagne *et al.*, 2009). This suggests that the diadromous, euryhaline nature of salmonids is not to a major issue in terms of waterborne Ca uptake. Nevertheless, dietary supply of Ca may be important in times of deprived water availability or when developmental requirement is augmented. Wild Atlantic salmon reabsorb minerals from their scales during their reproductive migration back into freshwater, indicating that requirement is increased during sexual maturation (Kacem *et al.*, 2013). This could be attributed to physiological processes, gonad maturation and/or morphological changes; for example, the natural development of kype in cock-fish (Witten and Hall, 2003). Natural periodic phenomena such as these must remain a consideration in domesticated finfish.

Together, Ca and P form hydroxyapatite, the major compound found in bone. However, a number of other trace elements are also important in bone formation and integrity, such as magnesium (Mg), zinc (Zn) and manganese (Mn) (Bigi *et al*., 1992; Seo *et al*., 2010; Strause and Saltman, 1987). Dietary concentrations of these trace elements can have significant effects upon bone mineralisation in fish; with deficiencies leading to deformity (Yamamoto *et al*., 1983; Satoh *et al*., 1983) and restriction of growth potential (Knox *et al.*, 1983). It is of great importance to recognise that numerous factors may

cause skeletal abnormalities in fish; genetics, pathogen infections, environmental parameters and human handling are all well-known causes of this (Deschamps *et al*., 2009). However other, more subtle, parameters may similarly influence skeletal characteristics. Owen *et al*. (2012) observed architectural variations in the vertebrae of juvenile rainbow trout under differing flow rates (0 and 2 body lengths per sec.). This is of great interest when we consider the different housing strategies used in finfish farming; for example ponds, recirculating tanks, cages and raceways, or likewise, fluctuating current speeds within these units. These different strategies are adopted or present within the production of individual species, as well as those which differ from one another.

It has been observed that temporary but plentiful supplementation of macrominerals, during times where environmental stressors are heightened, may mitigate the risk of poor ossification. This was demonstrated during seawater transfer of salmon, resulting in reduced incidences of skeletal abnormalities much later when the fish are slaughtered at market size (Fjeldall *et al*., 2009). The numbers of fish suffering from skeletal deformity are variable between operations due to the numerous factors involved. However, currently, under rearing conditions which could be considered as realistically optimal, skeletal deformities can still be relatively high. In the production of rainbow trout, individuals expressing skeletal deformity, up to fingerling size, may still represent 5 to 10 % of the original ova (personal observation). All things considered, correct micronutrition can be seen as a safeguard to the stressors which threaten bone characteristics in commercial farming. In achieving this, significant benefits may be seen throughout production by retaining the largest possible number of animals which display highly marketable morphometries.

Deficiency of trace elements may cause other morphological effects, aside from skeletal architecture and composition. Copper (Cu), Mn, Mg and Zn deficiencies have all been observed to induce clinical signs of cataracts in salmonids (Richardson, 1985; Yamamoto *et al*., 1983; Read *et al.*, 2014); whilst Zn deficiency has been reported to increase

incidences of fin erosion (Yamamoto *et al*., 1983). Both of these symptoms can seriously reduce performance and product value. In extreme cases, increased mortality has been attributed to trace element deficiency (Read *et al.*, 2014).

Due to their varied and integral function in metabolic function and immunological responses, macrominerals and trace elements play an indispensable role in maintaining countless physiological processes. These include maintenance of cellular pH, osmotic balances and active transport pathways as well as participation in the structure and function of hormones (e.g. iodine and chromium) and enzymes (Cu, Zn, Mg, Mn and Se) (Watanabe *et al*., 1997). For this reason, availability is essential for normal physiological functioning and health.

Animal feed formulations are regularly altered to suit ingredient prices and availability, as such there is risk of mineral deficiencies or the flexibility of formulations becomes limited. There are in fact two considerations relating to these risks. The first is, what are the total concentrations of each essential element, in a given ingredient?

Table 1.1 displays reported concentrations of elements in some commonly used and prospective aquafeed ingredients. Evident from the information provided, protein sources vary tremendously in their macro and trace element concentrations, under various categorising levels. For example, Ca and P concentrations in animal-derived by-products are far higher than those derived from autotrophs and microbes. Legumes (e.g. soya, lupins and fababean) contain more K than grains (e.g. barley, wheat and triticale). Thereafter, more specifically, we can observe examples such as very high Mn in lupins compared to all other ingredients presented. The values reported should not be treated as definitive; crops in particular are highly susceptible to variation due to genetic engineering and soil mineral concentrations. However they serve to demonstrate that substitutions between ingredients, based upon protein/amino acid supply, will seldom maintain a natural, constant, micronutrient provision.

Table 1.1 Reported concentrations and ranges of macro and trace elements found in commonly used and prospective protein sources for aquafeeds; with particular emphasis towards Lupins - *Lupinus albus* (white lupin*), Lupinus angustifolius* (narrow-leaf lupin) and *Lupinus luteus* (yellow lupin).

		Macro elements (g/kg)						Trace elements (mg/kg)				
	Form	Ca	P	K	Na	Mg	S	Fe	Zn	Mn	Cu	Source
Fishmeal	N. Atlantic	23.8	19.0	13.0	10.0	2.0	0.6	250	100	10	5	Premier Nutrition, 2005
Fishmeal	LT herring	16.9	14.7	8.6	10.9	2.1	9.6	166	64	4	2	Leeming, 2013
Fishmeal	Capelin	26.6	21.3	13.0	9.0	1.5	4.0	250	100	15	5	Premier Nutrition, 2005
Fishmeal	Whitefish	46.1	23.0	6.3	5.7	1.3	7.6	303	130	16	12	Leeming, 2013
Fishmeal	U.K. offal	65.0	36.0	9.0	8.5	1.5	4.4	280	90	12	3	Premier Nutrition, 2005
Poultrymeal	Meat	32.6	19.6	6.1	3.0	1.1	7.4	122	76	11	7	Leeming, 2013
Algae	Various, whole	1-30	7-15	7-24	8-27	3-7	4-14	>1400	28-64	45-454	8-102	Tibbetts *et al.*, 2014
Yeast	Brewer's	2.5	12.5	17.0	1.0	2.0	4.0	150	50	30	35	Premier Nutrition, 2005
Yeast	Extract	1.4	6.5	18.3	0.3	1.5	4.4	276	147	22	5	Leeming, 2013
Soyabean	Protein conc.	3.0	8.0	22.5	0.1	3.1	4.0	.	40	.	5	Premier Nutrition, 2005
Soyabean	Meal	3.0	6.0	21.0	0.3	2.8	4.3	150	50	43	15	Premier Nutrition, 2005
Soyabean	Meal	2.7	5.9	18.1	0.3	2.7	4.4	108	50	23	10	Leeming, 2013
Rapeseed	Meal	8.0	11.0	12.5	0.2	5.5	4.5	160	55	55	7	Premier Nutrition, 2005
Cottonseed	Meal	1.9	10.0	12.5	0.5	5.0	4.7	150	60	20	10	Premier Nutrition, 2005
Groundnut	Meal	1.6	6.0	11.5	0.2	3.0	3.0	300	55	40	15	Premier Nutrition, 2005
Wheat	Gluten meal	1.0	1.3	1.5	0.3	.	.	54	.	.	.	Premier Nutrition, 2005
Maize	Gluten meal	0.4	3.0	1.0	0.5	0.4	6.5	200	41	7	10	Premier Nutrition, 2005
Maize	Gluten meal	.	3.0	0.4	0.4	0.2	8.3	45	16	3	6	Leeming, 2013
Triticale	Seed	1.1	3.3	5.0	0.1	1.1	1.5	50	40	25	8	Premier Nutrition, 2005
Fababean	Seed	0.8	5.8	13.1	*n.p.*	1.2	2.3	62	49	8	18	Lizarazo *et al*., 2015
Barley	De-hulled	0.6	3.6	4.7	0.1	1.0	1.5	85	25	16	5	Premier Nutrition, 2005
L. albus	Meal	2.5	4.0	1.1	0.2	1.5	2.4	30	35	1500	7	Premier Nutrition, 2005
L. albus	Meal	1.3-1.4	3.3-4.7	10.7-14.3	*n.p.*	1.5-1.9	*n.p.*	38-62	43-52	350-901	7-8	Porres *et al*., 2007
L. albus	De-hulled	1.0	5.4	11.6	0.7	1.8	3.0	39	49	1370	8	Hung *et al*., 1988
L. albus	α-GAL-free	1.5-1.8	3.0-3.2	5.7-7.8	*n.p.*	0.7-1.0	*n.p.*	35-38	42-45	780-820	7-8	Porres *et al*., 2007
L. angustifolius	Meal	3.4	3.6	11.1	*n.p.*	1.8	4.0	38	35	20	6	Lizarazo *et al*., 2015
L. angustifolius	Meal	1.4-1.6	5.4-6.1	12.9-13.0	*n.p.*	1.9-2.2	*n.p.*	42-43	37-38	76-84	10	Porres *et al*., 2007
L. angustifolius	De-hulled	1.2	4.2	7.8	0.3	2.0	3.4	52	39	74	6	Hung *et al*., 1988
L. angustifolius	α-GAL-free	1.8-1.5	5.4-5.5	7.9-9.4	*n.p.*	1.6-1.7	*n.p.*	29-33	41-42	87-88	6-9	Porres *et al*., 2007
L. luteus	*n.p.*	3.0	5.1	12.6	0.1	3.1	*n.p.*	130	70	80	20	Rutkowski *et al*., 2015
L. luteus	Meal	1.1-1.3	7.2-7.6	12.1-14.2	*n.p.*	2.9-3.1	*n.p.*	58-71	59-64	56-68	11-13	Porres *et al*., 2007
L. luteus	α-GAL-free	1.5-1.6	7.8-8.2	9.0-10.4	*n.p.*	3.2-3.3	*n.p.*	72-76	77-79	76-85	11	Porres *et al*., 2007

Abbreviations and symbols: LT = low temperature; α-GAL-free = α-galactosides extracted from ingredient; n.p.= information not presented by author(s); . = not detected.
Please refer to source references for details of further ingredient specifications (e.g. trade names, manufacturers, species, cultivars, processing conditions).

Having determined element concentrations within an ingredient, it is then paramount to consider in what form they are present.

The complexity of plant nutrient matrices, in particular, often results in elements being in a bound form, most notably due to the presence of phytate. This compound severely limits mineral bioavailability and is discussed at further length throughout this chapter, as a key focus of this body of research as a whole (see Sec. 1.5 and 1.7.3).

In order to guarantee that element requirements are met, heavy supplementation of associated elements (notably P), in freely available forms, is implemented during aquafeed manufacture. Although this practice aims to uphold nutritional and metabolic health, nutrient loading of diets in such a manner can incur deleterious consequences in the wider context of fish farming. Leeming (2013, p.2-3) highlighted that excess mineral loading with neglect of endogenously present minerals in ingredients, equates to vast economic losses every year considering the financial cost of inorganic supplements. Moreover, this increases potential detriment to surrounding ecosystems as unabsorbed elements are excreted; thus environmental- and societal-implicated costs may be similarly vast. A number of studies have investigated downstream effects of inorganic pollutants from trout farming, in particular, due to the limited containment and dilution of effluent from these operations. These studies have largely concluded that P discharge in particular, correlates with reduced biodiversity and signs of eutrophication (Camargo *et al*., 2011; Mayor and Solan, 2011; Bartoli *et al*., 2007). We may also bring into question the manner in which minerals are harvested for animal feed supplements. Mining is a highly controversial topic, which draws upon issues such as environmental damage (Mudd, 2010) and human health and welfare (Viviers and Boudler, 2010). The long-term sustainability of accessible mineral reserves is also highly questionable, considering the resource is finite. Furthermore, we will require more inorganic fertiliser in the years to come for intensifying arable farming which will inevitably cause great competition for inorganic resources between agri-sectors. This is despite the fact that demand for crops will increasingly be for animal feeds, with aquaculture included.

1.4 Measuring nutrient digestibility and bioavailability in finfish

Measuring the digestibility and bioavailability of nutrients in finfish is a challenging topic. The principle behind apparent digestibility coefficient (ADC) methods centres upon defining the loss of nutrients between the ingested feed and the excreta from the animal. The values are described as 'apparent coefficients' since endogenous digestive excretions cannot be accounted for (Hardy and Barrows, 2002). 'True' coefficients are only possible with the use of a control diet completely lacking in the nutrient to be tested (Hardy and Barrows, 2002), which is neither practical nor ethical in many cases within finfish. Apparent digestibility coefficients are performed by the introduction of an inert digestibility marker into the feed (yttrium oxide or chromic oxide), the concentration of which can be analysed in the feed and faeces for determining relative concentrations of other nutrients before and after digestion (Hardy and Barrows, 2002). Broadly, there are two strategies for obtaining faecal samples from fish; collection prior to excretion from the anus and collection after excretion (Hardy and Barrows, 2002; Blyth *et al*., 2015).

Stripping faeces from the hind portion of the intestine can be achieved in species with a digestive configuration similar to the one displayed in Plate 1.1a (e.g. salmonids). Species with a convoluted intestinal configuration (e.g. tilapia) cannot be easily stripped of faeces as placing pressure upon the hindmost portion of the intestinal tract to extract faecal material cannot be performed quickly and efficiently. The stripping method is further limited to fish of a larger size; for example, fingerling salmonids cannot be ethically stripped of faeces due to a high risk of damaging internal organs. Provided conditions allow its use, stripping is nonetheless seen as a relatively highly reliable method for determination of ADCs (Blyth *et al*., 2015). Alternatively, fish may be sacrificed and faeces removed via dissection, which can theoretically achieve the same sample collection as stripping. However, this strategy is highly susceptible to ethical scrutiny due to the availability of less invasive techniques, where loss of life is not necessary (e.g. stripping and settlement). Common to both methods involving faecal collection prior to excretion, scrutiny also exists in samples potentially being collected

before the digestive capacity of the animal has undergone its full extent, resulting in conservative values (Blyth *et al*., 2015).

Collection of faeces after excretion into the surrounding water can be performed by syphoning tanks or collecting faeces from specially designed faecal traps; referred to as settlement methods (Hardy and Barrows, 2002). Syphoning faeces is highly labour intensive and results in a high level of disturbance to the animals; as such, this method is not preferable. Meanwhile, traps must ensure that faeces settles quickly and remains chilled to prevent further degradation by bacteria. In experimental nutrition studies, ensuring that faeces rapidly settles may be challenging since ingredients can influence faecal physical characteristics (Ogunkoya *et al*., 2006). As previously mentioned in Sec. 1.3, the solubility of certain nutrients in water can hinder accurate definition of those acquired or required by fish. This is true of both micronutrients and macronutrients on the topic of digestibility, as leaching of nutrients can quickly occur from excreted faeces (Blyth *et al*., 2015). Consequently, the major limitation of settlement methods is the possibility of over-reported digestibility values due to leaching and/or continued fermentation by bacteria.

Defining nutrient availabilities from ingredients and diets is integral to research and development within the aquaculture nutrition sector and should be attempted with the highest rigour and reliability possible, under the given conditions. Conducting such analyses is particularly important since the digestibility of specific ingredients is not the same between species, given their variations in digestive configurations and further, aquafeeds and their constituent ingredients are continuously being altered due to a variety of commercial pressures.

1.5 Aquafeed ingredients and sustainability

Historically, much of the aquaculture industry has depended upon fishmeal (FM) for the supply of protein and essential amino acids within finfish diets; whilst lipid was similarly

supplied from fish oil (FO). However, in correspondence with the before mentioned situation of declining capture fishery productivity, under Sec. 1.1, sourcing sufficient quantities of pelagic marine fishes for FM and FO production has become unsustainable from both a socioeconomic and environmental standpoint; which is further aggravated by the increasing volumetric demand for aquafeed. Simply, traditional inclusion rates are no longer viable. As a result, changes in the diets of commercially produced finfish have occurred in an opposite manner to that of the human population. Farmed fish diets are now increasingly being dominated by protein and lipids derived from plants, as appose to marine-derived sources (Fig. 1.1).

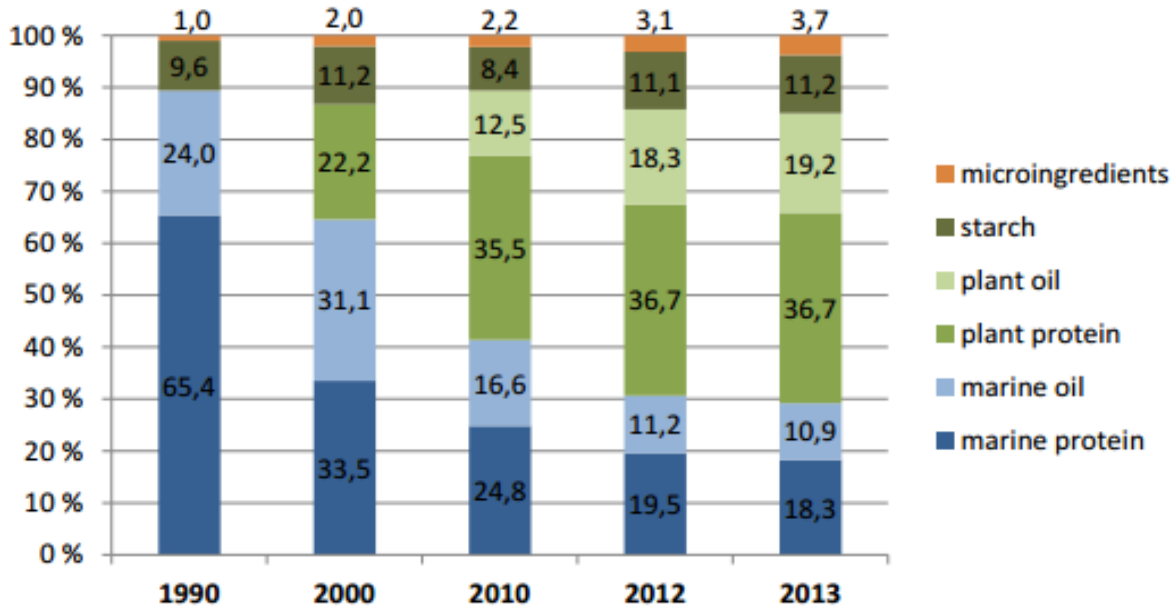

Figure 1.1 Percentage contributions of ingredient types in commercial Norwegian salmon (*Salmo salar*) diets: 1990 to 2013. Taken from Nofima (2014).

The substitution of FM with alternative plant proteins is a well-established and heavily researched topic. These ingredients are indeed attractive; although prices have fluctuated considerably in the past decade, their cost remains highly competitive (Hardy, 2010). Furthermore, after processing, they generally provide balanced essential amino acid profiles and their protein fractions are often relatively digestible by fish (Hardy, 2010). Lapses in these aspects can be relatively easily mitigated; for example, deficient amino acids can be supplemented into diets (Wilson, 2002). Although this does represent added cost, this process remains inexpensive when performed in bulk,

considering the financial savings on using plant proteins (Nunes *et al*., 2014). Some crops which have become well-established in formulations include soya, wheat, maize and rapeseed. These are generally regarded as far superior options for promoting sustainability within the aquaculture industry, compared with FM.

In developed countries, livestock producers are now regularly seeking the maximum sustainability credentials within their operations; this is in order to produce competitive products on a market where consumers are critical, food-conscious and are typically more prosperous. At retail, demonstration of credentials is clearly displayed by certifications and eco-labels, which present a visual stimulus for purchase by conscious consumers; subsequently, suppliers compete intensively for accreditation. Due to high competition, certifications are increasingly harder to obtain as standards are introduced and raised by awarding bodies. Therefore, there is great scope for any solution, throughout the production process, which can provide a producer with an edge over its rivals, whilst improving sustainability. Although the past and current major drive has been to reduce FM inclusions, attention is now also being turned towards the sustainability of plant-derived ingredients.

In Europe, the political and societal climate has been highly sceptical of genetically modified (GM) foods since their appearance on the continent almost two decades ago, such is that only one GM crop is licenced within the E.U., yet it is still banned for cultivation in most of its member states (Lucht, 2015). However, ironically, the E.U. imports vast quantities of GM crops every year, for sustenance of its livestock (Espinoza and Giraud-Heraud, 2012). Consumers are now beginning to question whether they can morally accept consumption of animal products fed GM crops. Due to their perception of genetic manipulation, this appears non-conducive to their ethos of investing in healthy, 'natural', ecologically-sound products, on a day-to-day basis (Lucht, 2015). Consequently, there may be a developing market for certified GM-free animal products. However, we must also focus, perhaps more importantly, upon the issue of importation. This issue is vastly more transparent than the argument of genetic modification.

Importation, to a level of absolute dependency, is risky from both socioeconomic and environmental sustainability perspectives. In terms of societal implications, large numbers of jobs involved in the feed supply-chain are outsourced. Economic implications include high costs and heavy investment in foreign economies. From an environmental perspective, the 'carbon-footprint' of transportation and little to no control over agronomic practices in the country of origin must be considered. Lastly, 'protein security' becomes unguaranteed, as dependence is placed on other nations for supply and competition for the product is on the global market.

The vast majority of these imports into the E.U. is soya (*Glycine max* L.), originating from the Americas (Soya UK, 2015). Demand for soyabean within the E.U. is great due its nutritional value but there is a distinctly large deficit in this commodity compared with other oil and protein crops (Espinoza and Giraud-Heraud, 2012). In perspective, the E.U. imports on average 30 million tonnes of soya per year; however, less than 15 percent is certified GM-free (Lucht, 2015). France, for example, is particularly opposed to the cultivation of GM crops but it supplements its modest 140,000 tonne per year production of soya with around 4 million tonnes of imports (Espinoza and Giraud-Heraud, 2012). Meanwhile, In the U.K., soyabean was the second most consumed ingredient in terrestrial livestock diets in 2015 (UK Government, 2015), whilst it was most likely the most consumed in aquafeeds; U.K. soya production is negligible (Soya UK, 2015). Following these concerns, calls have been made for diversification in available feed ingredients; preferentially those which are 'home-grown', thus also non-GM. All in all, alternative proteins should no longer be viewed, or categorised, as alternatives to fishmeal. Alternative proteins must be seen as any ingredient with potential to better the sustainability credentials of any commercially used counterpart, whilst upholding nutritional quality. The latter consideration of maintaining nutritional value is one which is particularly problematic in finfish nutrition with current trends towards increasing the inclusion of plant-derived ingredients in commercial diets.

1.6 Anti-nutritional factors

Despite large advancements, the inclusion of plant proteins in finfish diets, particularly with carnivorous species, has been limited due to the presence of nutritional boundaries. As such, the promotion of aquaculture feed and production sustainability has been hindered. Asides from nutrient deficiencies and imbalances, these boundaries exist as anti-nutritional factors (ANFs). ANFs can be defined as naturally occurring compounds which when ingested, can interfere with the consumer's optimal digestive process, resulting in limitations to the acquisition of nutrients and/or nutritional pathology. ANFs occur substantially in plant-derived ingredients, typically originating from predator defence mechanisms and nutrient stores (Francis *et al.*, 2001; Kumar *et al*., 2012). The classification of these compounds as ANFs depends upon whether the animal lacks digestive capabilities to effectively catalyse their breakdown and subsequently mitigate their potentially deleterious effects; stemming from the consumption of feedstuffs which the digestive system has not evolved to encounter. In the field of finfish nutrition, common ANFs include protease inhibitors, alkaloids, gossypol, saponins, tannins, phytate and non-starch polysaccharides (NSPs).

Until present, a variety of methods have been implemented to dismantle the nutritional boundaries that ANFs pose in animal feed rations. This has been in a bid to maximise the nutritional profile of raw materials, by-pass the animals digestive configurations and natural capabilities and subsequently, optimise the nutritional function, health status and performance of livestock.

Firstly, genetic improvements can be applied to crops by selectively breeding from desirable phenotypes, such as the identification and emergence of low-phytate mutant soya lines (Jervis *et al.*, 2015; Al-Amery *et al*., 2015). However, selection of mutant lines for beneficial traits, followed by breeding programmes of desirable cultivars, can be a tedious process; perhaps not favourable when we immediately require intensification of food and animal feed sources. Secondly, we may consider efforts in

producing genetically modified (GM) crops but as previously mentioned, the media, legislation and consumer opposition may limit the development of GM crops due to perception of environmental risks (Lucht, 2015). Beyond agronomic stages, ingredient refining is heavily practiced in the commercial sector. The first major step is that of dehulling (Young and Forte, 2016b). This simple process removes husks, which are grossly indigestible to monogastrics, mostly due to their high NSP content (Krogdahl *et al*., 2005). Because of their composition, husks contain little to no nutritional value and their composition restricts and/or impairs digestive accessibility to the valuable nutritious fractions of the ingredients (Krogdahl *et al*., 2005). Secondly to note, is the advent and extensive manufacture of glutens, protein concentrates and isolates, such as soya protein concentrate (SPC); these aim to increase the protein value of ingredients via solubilisation and removal of other compounds, particularly starch (Glencross *et al*., 2004a). Lastly, heat treatment of ingredients has become a routine procedure (Young and Forte, 2016b). Although multifunctional in sterilising and modifying ingredient composition or characteristics, heat treatment can target a number of ANFs such as lectins, protease inhibitors and cyanogens (Francis *et al*., 2001). However, numerous other ANFs are heat stable, such as tannins, gossypol, saponins and phytate; or excessive treatment risks detriment to nutritious fractions of the ingredient such as heat-sensitive amino acids (e.g. lysine), which may cross-bridge, leading to complexation of protein (Francis *et al*., 2001; Hefnawy, 2011). Therefore many ANFs remain problematic, requiring a different processing approach for deactivation.

Although undeniably successful to an extent, questions can be asked of the efficiency of current mechanical and chemical techniques involved in the removal of ANFs. These forms of processing firstly require substantial investment in the form of machinery, labour and financial expenditure. For example, the production of SPC incurs a significantly higher financial cost than that of standard defatted soya meal (FAO, 1992). Furthermore, the methods implemented in modern feed processing are rarely 100 percent efficient. The complex nature of the plant nutrient matrix means that compounds

considered ANFs are often bound to desirable, nutritious fractions, such as complexes formed between saponins and amino acids (Potter *et al*., 1993). ANFs may also be present in a relatively free state, in unremoved physical components of seeds and pulses, for example phytate and NSPs in protein-rich kernels.

1.7 Lupins

1.7.1 Lupins as an alternative protein

Lupins have been described as "[…] the only high protein, high energy, nitrogen-fixing, grain legume that can compete nutritionally with imported soya in livestock and fish diets [in the U.K.]" (Soya UK, 2015). Three species of lupin are available for commercial cultivation; these being, white lupin (*Lupinus albus*), yellow lupin (*Lupinus luteus*) and the narrow-leaf lupin (*Lupinus angustifolius*), sometimes referred to as blue lupin. White, yellow and narrow-leaf lupin kernel meals contain approximately, 41, 52 and 44 % crude protein, respectively (Sipsas, 2003); whereas de-hulled soyabean meal contains on average 47.5 % crude protein (NRC, 1998).

Each of these three species allows opportunities for cultivation in different climates and soil types, so they can be considered an option for many arable farmers. Consequently, these legumes have been identified as an attractive option for farmers in both developed countries, such as those in the E.U. and Oceania, as well as in developing countries, for example Ethiopia (Yeheyis *et al*., 2012). Therefore, efforts to study their nutritional potential will be of benefit throughout the global social stratification.

At present lupin production in the U.K. is small; approximately 80 percent of its volume is grown as a mixture with cereals (e.g. spring triticale) and cut as forage, with the remainder grown as pure stands for on-farm feeding as a high-protein diet (Soya UK, 2015). However, until present, confusion over true nutritional value, appropriate species selection and crop management strategies has resulted in a lack of uptake from the

British feed sector. As a result of low volumes, bin space in feed mills cannot be justified, so lupins are seldom considered in compound feed formulations. Nonetheless, other nations have been successful in adopting lupins as a valuable feed-source for their livestock. Australia is the world's largest producer of lupins (Fig. 1.2). The adoption of lupin crops was particularly productive in Western Australia, due to the vast swathes of sandy, acidic soil, which are unsuitable for the cultivation of many other legumes and grains. Production in 1999 reached a high of 1.5 million tonnes, but has since fallen to under 400 thousand tonnes in 2014 (Government of Western Australia, 2015). This decrease in productivity has been caused by dismissal from farmers due to a fall in lupin prices and weed control issues (Government of Western Australia, 2015) but encouragingly, not the crops nutritional value.

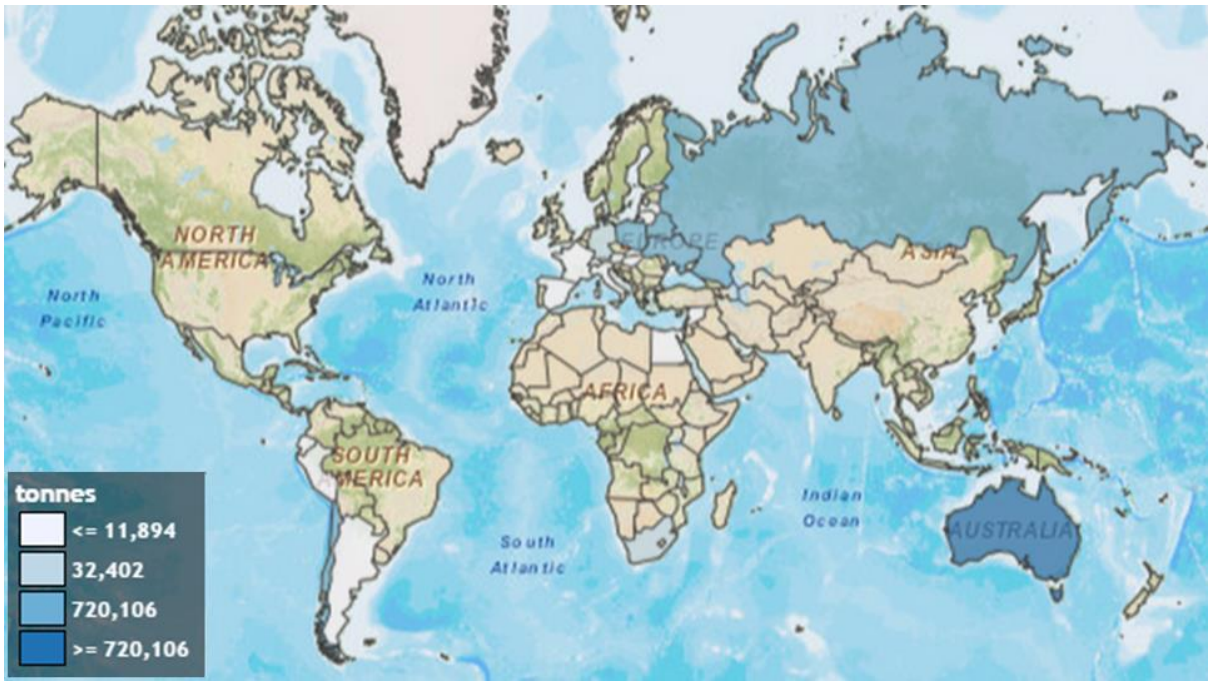

Figure 1.2 World lupin production, by country: 2012 to 2013 (FAOSTAT, 2016).

1.7.2 Lupin anti-nutritional factors

As previously discussed, lupins are seen to be strong competitors to the protein content of soya, but ANFs nonetheless limit the inclusion of lupins in animal diets. Initially, alkaloids were a major limiting factor but breeding strategies to reduce this ANF, to create 'sweet lupin' varieties, has since all but eliminated this deterrent.

In a study on Nile tilapia, increasing levels of soyabean meal substitution, with whole and dehulled narrow-leaf lupin were both observed to depreciate growth performance (Chien and Chiu, 2003), suggesting that inferiority exists between lupins and soya. The NSP content of lupins is particularly high; even after dehulling, contents remain significantly higher than other plant-protein competitors (Van Barneveld, 1999). Furthermore, lupins tend to contain significant quantities of oligosaccharides (Van Barneveld, 1999), notably α-galactosyl homologues of sucrose, which can similarly impede upon the digestive process (Glencross *et al.*, 2003). To promote the development and redevelopment of lupin inclusions in feed rations, it would be of great benefit to further improve their overall nutritional profile, so as to produce a more competitive product and reduce performance, health and welfare constraints surrounding these ingredients. Glencross *et al.* (2004a) suggested that plant breeders should be tasked with reducing the oligosaccharide content of high-NSP lupin cultivars, or further processing could be implemented with the manufacture of lupin-protein concentrates (LPCs) and isolates, thus allowing greater inclusion in finfish diets. The requirement for these approaches has more recently been brought to light by Lucas *et al*. (2015), in an exhaustive review on the development of lupins as a protein crop in Europe.

The debate upon how to reduce ANF content has already been addressed but to briefly reiterate, agronomy and processing will rarely be 100 percent efficient. Especially without the use of GM, one of the major driving forces behind lupin promotion, it is highly optimistic to assume we can alter the nutritional profile of a crop to a point where all of its possible ANFs are removed to a level where they pose no threat to the multitude of commercially farmed ruminant and monogastric livestock species. Heavy implementation of specialist ingredient processing steps will also contribute to a spike in the price of the ingredient, limiting its cost-effectiveness and not providing substantial return to the arable farmer, thus impeding uptake. Consequently, there is a niche for strategies which may improve the nutritional value of lupins.

The aquaculture industry is facing great challenges in providing and optimising adequate nutritional strategies for its growing volumetric production and diversity of species. However, the issue of ANFs in livestock diets is certainly not unique to aquaculture. The poultry and pig industries have and continue to experience their fair share of animal health, production performance, and land management issues caused by ANFs. To target specific ANFs, untreatable by mechanical or chemical means, the terrestrial monogastric livestock sector began to research biological treatment of ingredients. This was attempted using one of the most specific processes in nature, enzymatic degradation.

1.8 Exogenous enzymes in animal nutrition

1.8.1 Background

Initially, the application of exogenous enzymes in animal feeds was discounted due to belief that any supplementary enzymes applied to feed would be denatured in the digestive proteolytic activity of the stomach and anterior intestine (Bedford and Partridge, 2010). However in the mid 1940's, it was first reported that enzymes could improve growth and feed efficiency in high fibrous poultry diets, suggesting resistance to endogenous enzymatic action (Hastings, 1946). An extensive series of investigations into the application of feed enzymes followed suit. This movement was driven by the theory that specific enzymes from exogenous sources could compliment the plethora of endogenous enzymes already found in the digestive system of the exposed animal. These exogenous enzymes would break down previously indigestible ANFs within the animal's diet and mitigate their deleterious effects, whilst at the same time liberating nutrients and ultimately enhancing the nutritional value of feeds. Furthermore, exogenous enzymes were considered as a potential waste management tool.

In order to be an effective nutritional strategy, exogenous enzymes must ultimately be cost-effective, since the major driver for their inclusion in animal feed rations is reducing

cost (Bedford and Partridge, 2010). This depends upon degradation of specific, targeted nutritional components which reduces anti-nutritional effects and/or releases available nutrients at levels which equate to financial gain through improved growth, feed efficiency or animal survival via promotion of health. Alternatively, they may improve profit by allowing cheaper ingredients to be utilised, thus reducing the cost of feed altogether (Coweison *et al*., 2006). Consequently, the key attributes required for an exogenous enzyme to be cost-effective are the initial product price, specificity to contemporary formulations and retention of high activity under the conditions within the animal digestive system (Bedford and Partridge, 2010).

Over the past several decades, investigation into exogenous enzyme has revealed a wealth of positive findings which address contemporary issues faced in intensive animal farming. Subsequently, exogenous enzymes have been driven to a status of being key functional ingredients in terrestrial monogastric feeds (Campbell and Bedford, 1992). Exogenous enzymes have particularly revolutionised nutrition and production in the poultry industry (Acamovic, 2001), whilst they continue to gain momentum as a prolific nutritional tool in the porcine industry (Bedford and Partridge, 2010). Between 1998 and 2008, the animal feed enzyme market grew at an average of 13 % per year and in 2010, was worth in excess of US$ 650 million (Bedford and Partridge, 2010). This exponential growth is predicted to continue in both poultry and pig nutrition as research and new technologies increase, broadening the commercial application of enzymes as functional feed additives. Increasing demand for enzyme products in developing countries is also predicted (Bedford and Partridge, 2010); therefore, they will most certainly contribute greatly to the requirement for intensifying animal production in these regions. At present the market is dominated by NSP–degrading enzymes (NSPases) and phytases (Bedford and Partridge, 2010). Although the poultry meat and egg, and to an extent pork industries, readily accept exogenous enzymes as a standard dietary components (Bedford and Schulze, 1998; Bedford, 2003; Cowieson *et al.*, 2006; Bedford and Partridge, 2010), they are implemented much less extensively as digestive tools in the

other areas of livestock production. The effective use of enzymes in monogastric nutrition is undeniably least understood in finfish and shrimp. Therefore, if aquaculture is to supply the third greatest provision of animal protein to the global population in years to come, it is suggested that the industry considers following in the footsteps of its terrestrial counterparts by adopting approaches to mitigating ANFs and improving feed efficiency through the application of enzymology. To date, some research efforts have been made with the goal of increasing our understanding and validating applications in a range of aquafeeds.

1.8.2 NSP-degrading enzymes

1.8.2.1 Background

As previously mentioned, NSPs are the major components of dietary fibre which are found in abundance in plant-derived ingredients destined for use in animal feeds. These NSPs may be in the form of cellulose or non-cellulosic polysaccharides, typically present as linked monomers of hexoses and pentoses (Sinha et al. 2011). Much variation exists in the NSP profiles between different ingredients. Cereal grains, such as maize and wheat, are composed of xylans and ß-glucans, whilst soyabean and canola meals contain mainly galactans, galactomannans, mannans and pectic polysaccharides (Brufau *et al.*, 2006; Bedford and Partridge, 2010). The quantity of NSPs found within ingredients equally varies a considerable amount. In wheat, total NSP content is typically around 25 %, whilst lupins typically contain around 50 %, being the primary energy store in these particular seeds (King *et al.*, 2000; Sinha *et al.*, 2011).

With increasing dependence on the use of plant-derived ingredients in animal feeds, NSP presence in the diets of farmed fish is set to increase in both quantity and diversity. However, being non-ruminants, fish are unable to digest these complex carbohydrates due to scarcity or complete absence of NSPases in their gastrointestinal tract (Bedford and Schulze, 1998). NSPs exert a range of inhibitory or limiting effects on the digestive process and subsequent nutrient acquisition, categorising them as ANFs; but their

biochemical composition makes them potential energy sources for the consumer, if effectively degraded. Following successes in poultry and pig nutrition (Campbell and Bedford, 1992; Bedford and Schulze, 1998; Bedford and Partridge, 2010), application of NSPases in aquafeeds has been investigated in a bid to increase diet digestibility and ultimately promote fish health and performance.

1.8.2.2 Cellulase

Cellulose is the most abundant biopolymer on earth providing plants with the majority of their cell wall structure (Kleywegt *et al.*, 1997). Up to 15,000 D-glucose monomers, linked by ß (1-4) bonds make up this water-insoluble ß-glucan polymer (Brown, 2004). Due to its high degree of polymerisation, cellulose exhibits a strong resistance to enzymatic hydrolysis (Michael and Ely, 2011). Although cellulose may appear to be somewhat inert as a molecule in digestion, its hydrolysis holds potential for augmenting the provision of nutrients (Amirkolaie *et al.*, 2005). Firstly, because its strong structural function can result in protection of encapsulated substrates from digestive enzymes (Brufau *et al.*, 2006). This means that although the composition of an ingredient, or diet, may be seen to satisfy an animal's nutrient requirements, bioavailable nutrient content may be far reduced by elevated cellulosic content. Secondly, the monomers of cellulose could theoretically be metabolised by the consumer. In poultry nutrition, supplementary ß-glucanases provided some of the earliest successes in dietary enzyme technologies and have been particularly successful in association with commercial diets containing grains, such as barley (Campbell and Bedford, 1992; Bedford and Schulze, 1998) so aquaculture research has followed suit.

Before discussing exogenous cellulases in feeds, it is worthy to note that there have been a number of reports suggesting that cellulase activity is present in the digestive system of numerous species of fish. These include phytophagous grass carp (*Ctenopharyngodon idella*) and omnivorous tilapia (*Oreochromis mossambica*); but much,

if not all, of this appears attributable to the animal's gut microbiota (Das and Tripathi, 1991; Chakrabarti *et al.*, 1995; Stellwag *et al.*, 1995; Saha *et al.*, 2006) and not the fish's own endogenous secretions. As gut microflora is very susceptible to modulation through a number of parameters, it is unrealistic to assume a guaranteed activity of cellulase and further unlikely that sufficient levels will be present to degrade large proportions of cellulose.

As a forage type feedstuff, shredded duckweed and exogenous cellulase fed to the largely herbivorous grass carp improved growth performance in a 2 month feeding trial (Zhou *et al.*, 2013). In this study, cellulase, amylase and protease activity were observed to increase in the intestine following cellulase supplementation. It was suggested that results potentially originated from the proliferation of certain genera among the gut microbiome of cellulase-supplemented fish (Zhou *et al.*, 2013), an area warranting further investigation.

With regards to performance of omnivorous tilapia, supplementation of pure cellulase derived from *Aspergillus niger*, was described as ineffective in promoting growth and nutrient digestibility (including crude fibre) in canola meal diets (Yigit and Olmez, 2011). In an investigation into soaked and un-soaked soyabean meal with 0.2% cellulase in carnivorous rainbow trout diets, the enzyme supplementation was similarly observed to not affect performance characteristics (Xavier *et al.*, 2012). However, carcass crude protein was increased with cellulase inclusion in both soaked and un-soaked soya, correlating with an observed increase in endogenous protease activity (Xavier *et al.*, 2012) in correspondence with work by Zhou *et al*.'s (2013). Carcass ash was also increased in fish fed cellulase-treated, soaked soyabean (Xavier *et al.*, 2012). Greater efficacy of ß-glucanase has been documented in soyabean diets fed to rainbow trout with increasing digestibility of crude protein, lipid, ash, phosphorous and dry matter (DM), as well as substantial increases in energy retention (Dalsgaard *et al.*, 2012). However, no improvements to nutrient digestibilities were observed by Dalsgaard *et al*. (2012) in sunflower and rapeseed meal based diets, suggesting limited flexibility in formulation. As

previously mentioned, barley was the original target substrate for ß-glucanase inclusions in poultry. In correspondence with this, supplementation of endo ß-1, 3 (4) glucanase in barley-based common carp (*Cyprinus carpio*) diets has been demonstrated to provide significant benefits to a number of desirable haematological parameters (Mohammadbeygi *et al*., 2012), indicating that improvements to fish health and immune status may also be achieved from successful degradation of cellulose.

Overall, research on pure cellulase supplementations in fish is sparse within the scientific literature. From findings to date, it appears cellulases provide varied results when introduced to the gastrointestinal tract of fish. The liberation of sufficient nutrients to promote growth is realistically a great challenge, owing to the use of complex compound feeds, significant cellulose removal from ingredients during dehulling and the mild deleterious nature of dietary cellulose; all of which will easily mask benefits to be gained by cellulase applications.

It is of note that although exogenous cellulolytic supplementation aims in part to augment availability of energy to the fish, it is imperative that this kind of work is treated with care and enzymes are applied in moderation. Many fish species (particularly carnivores) have low tolerances to glucose; therefore, elevated dietary levels may lead to hyperglycaemia and diabetic-like symptoms (Stone, 2003a; Stone *et al.*, 2003b; Booth *et al.*, 2006; Booth *et al.*, 2013), for example glycogen deposition and liver damage. Even species considered very similar in their nutritional requirements may vary in their tolerance of glucose; for example, European seabass have been shown to be less tolerant than gilthead seabream (Enes *et al.*, 2011). In general, this topic is still not well understood thus heightening the risks involved in providing more available sugars to such species.

Further development of enzyme products targeting cellulose-degradation should scrutinise the complexity of cell walls, considering the array of other NSPs present. One should also carefully consider the sourcing species from which to harvest enzyme activity,

as their cellulolytic enzyme profiles can vary considerably (Knott *et al.*, 2014). Higher levels of non-specific enzymes may, for instance, be a more desirable option considering variations in substrates encountered between feeds; therefore allowing greater flexibility in dietary formulations. For example, the filamentous fungi, *Trichoderma reesei,* secretes two cellobiohydrolases, at least four endoglucanases, and a ß-glucosidase (Kleman-Leyer *et al.*, 1996; Kleywegt *et al.*, 1997). Its predominant endoglucanase (Endoglucanase I) is considered non-specific as it hydrolyses not only cellulose and barley ß-glucan but also ß (1-4) linkages in xylans (Kleywegt *et al.*, 1997; Knott *et al.*, 2014).

1.8.2.3 Xylanase

Xylans are the second most abundant plant-derived polymer on earth (Bedford and Partridge, 2010). These hemicellulose components, consist of ß (1-4) linked xylose units, which can be substituted with (2-3) linked arabinose residues (Sinha *et al.*, 2011). Being such a common, naturally occurring polysaccharide, xylans are found in high quantities in plant-derived feed ingredients, notably grains (Adeola and Bedford, 2004; Krogdahl *et al.*, 2005; Sinha *et al.*, 2011). Xylans are particularly soluble fractions of NSPs; therefore they can substantially increase intestinal viscosity (Bedford and Partridge, 2010). This leads to numerous digestive implications in both terrestrial and aquatic livestock, such as restriction of digestive enzyme-substrate interaction, hindered movement of digesta through the intestinal tract and finally inhibition of nutrient uptake at the intestinal mucosa (Refstie *et al.*, 1999; Adeola and Bedford, 2004; Leenhouwers *et al.*, 2006; Leenhouwers *et al.*, 2007a; Leenhouwers *et al.*, 2007b; Amerah *et al.*, 2011). Clearly, neglect of high dietary xylan levels can impede upon optimum nutrition and production.

Interest in xylanases has been substantial since the dawn of the feed enzyme industry, with great successes in commercial applications after rapid licencing of xylanase-related supplements (Mathlouthi *et al.*, 2002; Adeola and Bedford, 2004; Adeola *et al.*, 2007; Jozefiak *et al.*, 2010; Barekatain *et al.*, 2013). Whether xylanases can be as effective as a dietary tool in finfish nutrition continues to be a topic of research and debate.

In a rainbow trout study, xylanase was observed to have no effect upon the digestibility of a number of tested nutrients in a sunflower meal-based diet (Dalsgaard *et al.*, 2012). Conversely, in a soya-based diet, the product was observed to promote protein digestibility. Whilst in a rapeseed meal-based diet, the enzyme product improved lipid and DM digestibility. Despite indications of improved nutrient provision in soya and rapeseed-based diet, no detectable improvements to performance were found in this study (Dalsgaard *et al.*, 2012). Based upon, Dalsgaard *et al*.'s (2012) report, there is strong indication benefits of xylanase are highly dependent on substrate and resulting performance enhancement may be minimal. A commercial preparation of primarily xylanase was shown to improve fish performance, feed efficiency and nitrogen retention in practical diets fed to Japanese seabass (*Lateolabrax japonicas*) (Ai *et al.*, 2007). Carcass protein content was also influenced by xylanase supplementation (Ai *et al.*, 2007). Furthermore, phosphorous and nitrogen excretions were indicated to be suppressed (Ai *et al.*, 2007). Although promising overall, it must be considered that additional enzymes present in the product may have contributed to the observed results in Ai *et al*.'s (2007) study. Supplementation of another predominantly xylanase enzyme product was reported to result in depressed growth and feed conversion of common carp fed high wheat bran diets, suggested as potentially attributable to low fish tolerance of xylose (Kazerani and Shahsavani, 2011). Similarly to concerns about cellulase, it has been highlighted that exogenous xylanase treatment of aquafeeds should be treated with an air of caution as some fish, particularly carnivores, have a low tolerance of xylose (Stone, 2003a; Stone *et al.*, 2003b).

Overall, research into the effects of xylanase in aquafeeds is severely limited, making it difficult to sufficiently evaluate their potential efficacy. Results to date again appear variable, particularly in terms of providing sufficient hydrolytic action to reliably improve animal performance, correlating with many findings in terrestrial monogastrics (Widyaratne *et al.*, 2009). Xylanases are nevertheless relatively highly regarded in poultry nutrition and this is not without founding that they can benefit production.

Therefore, research should continue to identify and refine optimal circumstances for xylanase inclusions.

1.8.2.4 Mannanase

Mannans are a common NSP throughout many plant ingredients in animal feed formulations. These hemicellulose components consist of a ß (1-4) linked backbone either containing solely D-mannose residues (mannans) or mannose and D-glucose residues (glucomannans) (van Zyl *et al.*, 2010). They are most prevalent in palm-kernel meal and copra meal, making up not only the majority of the NSP fraction but in fact 35 and 30% of the total ingredient content, respectively (Krogdahl *et al.*, 2005; Sundu *et al.*, 2006). ß-mannan contents as high as these severely limit inclusion of such ingredients in diets; therefore they can be viewed as a major limiting factor in broadening our arsenal of potential alternative proteins. These NSPs also have a significant presence in numerous other commonly used plant ingredients including dehulled soyabean meal (Mehri *et al.*, 2010), so they are nonetheless present in contemporary formulations. Similarly to xylans, elevated mannan content in poultry diets is seen as a potential risk due to their solubility (Sundu *et al.*, 2006). There is evidence to suggest that this phenomenon can equally occur in finfish with subsequent suppression of feed utilization and animal performance (Hossain *et al.*, 2001). Inclusions of ß-mannanase in broiler diets incorporating a range of plant ingredients have been shown to improve performance and digestibility, reduce feed intake with maintenance or improvement in performance, stimulate immunity and positively influence gut morphology and microbiota (Chartchai *et al.*, 2006; Zou *et al.*, 2006b; Mehri *et al.*, 2010; van Zyl *et al.*, 2010; Chegeni *et al.*, 2011). To the author's knowledge, research to date remains limited to only two focused studies in fish.

Caspian salmon (*Salmo trutta caspius*) fed a commercial diet preparation with a spray coating of ß-mannanase displayed significant improvement of growth and condition factor, with tendencies towards improved feed efficiency and survival (Zamini *et al*. 2014). Zamini *et al.* (2014) also presented white blood cell counts to be positively

influenced by the supplement, suggesting that ß-mannanase could be a potential contributor to stimulation of the innate immune system, correlating with poultry findings (Zou *et al.*, 2006a; Mehri *et al.*, 2010). On the other hand, ß-mannanase was reported to have no effect upon performance or nutrient digestibility in soyabean-based diets for rainbow trout (Yigit *et al*., 2014).

It is of surprise that no more work has been conducted on mannanase supplementations in fish, so as it stands, this topic remains unclear with virtually all speculation of its potential in fish originating from investigations with poultry and pigs. Much further work is therefore required to determine the feasibility of ß-mannanase supplementation in aquafeeds and confirm its abilities of improving performance, health status and nutrient digestibility. In theory, due to the domination of soya products in many commercial diets, which to reiterate have a high mannan content, potential for mannanase in aquafeeds is promising. Investigation into whether ß-mannanase supplementation can benefit the intestinal microflora also deserves much further research when considering the numerous positive findings of mannan-oligosaccharide (MOS) applications as prebiotics in fish (Dimitroglou, 2010; Dimitroglou *et al.*, 2010; Merrifield *et al.*, 2010).

1.8.2.5 Pectinase

Pectic polysaccharides, or pectins, are large molecules mainly comprised of galacturonic acid residues joined by α (1-4) glycosidic linkages, who serve as a binder of plant cell walls and play a role in ripening of fruits (Bedford and Partridge, 2010; Sinha *et al.*, 2011). Although generally a less potent ANF than other NSPs, pectins may still increase intestinal viscosity and may contribute considerably in maintaining nutrient matrixes within plant ingredients, thus inhibiting hydrolysation of their individual components by other enzymes (Igbasan *et al.*, 1997; Harholt *et al.*, 2010). With only one study currently available which provides information on the potential activity of exogenous pectinase in finfish diets, this type of enzyme is the least explored in aquafeed applications.

A commercial carbohydrase complex targeted at degradation of cell walls, was shown to positively influence uronic acid levels in pretreated soyabean meal, rapeseed meal and sunflower cake (Denstadli *et al.*, 2011a). This indicates that a pectinase fraction of the complex was able to act upon a number of substrates matrixes commonly used in aquafeeds. Surprisingly, the pretreated ingredients from this study appeared to not contribute to increased performance or digestibility of main nutrients when subsequently fed to rainbow trout (Denstadli *et al.*, 2011a).

Because pectins principally bind more potent ANFs (Bedford and Partridge, 2010), benefits of supplementing pectinases alone are likely to be fairly limited. This has been widely recognised and as a consequence pectinases are available in commercial enzyme cocktails rather than pure isolations (Bedford and Partridge, 2010). However, despite pectinase presence being quite common in commercial products, a lack of investigation into uronic acid levels in the gastrointestinal tract makes it difficult to elucidate and quantify the efficacy of these pectinase inclusions. With regards to forwarding research in this enzyme it is important to note that dicotyledon cell walls have typically a much greater pectin content than monocotyledons (Jarvis *et al.*, 1988). Therefore the use and efficacy of pectinases may be more relevant in dicot-based diets, such as those with high rapeseed, sunflower, lupins and soyabean content as appose to those based on grains. Considering that dicotyledons comprise the vast majority of the plant-derived portion of modern aquafeeds, pectinase additions could be more relevant and applicable to the aquaculture sector than the production of pigs and poultry.

1.8.3 Phytase

Phosphorous (P) is an essential nutrient for growth and function in fish, being an integral component of nucleic acids, numerous coenzymes, cell membranes and particularly skeletal tissue (Cashman and Flynn, 1999; Kumar *et al.*, 2012). Unlike many other minerals, fish have a very limited capacity to absorb P from their aqueous surroundings; therefore dietary levels of this mineral must be provisioned in quantities

sufficient to fully satisfy the animal's requirements. With high FM inclusions, P provision was a minor concern due to its high content and bioavailability within the ingredient. However, up to 80 percent of P found in plant-derived meals is bound in its storage form of phytic acid [inositol hexakisphosphate (IP6)] also known as phytate (*myo*-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) when a salt (Ellestad *et al.*, 2003; Kumar *et al.*, 2012). Despite shifts in dietary formulations, there is still, typically, ample levels of total P naturally present in the ingredient matrix of plant-based aquafeeds. However, inorganic forms must now be supplemented into diets to satisfy requirements because the bioavailability of P from plant sources is severely restricted. This is due to the fact that the endogenous enzyme activity of monogastric and agastric animals is devoid of phytate-degrading properties, thus sufficient quantities of naturally present P cannot be liberated (Ellestad *et al.*, 2003; Debnath *et al.*, 2005a). Furthermore, phytate may chelate with a large number of mineral cations (K, Mg, Ca, Zn, Fe and Cu) and additional complexes with proteins, amino acids and lipids, which can affect digestibility and subsequent nutrient bioavailability (Cowieson *et al.*, 2006; Denstadli *et al.*, 2006; Morales and Moyano, 2010; Kumar *et al.*, 2012). Clearly, phytate can be seen as a potent, broad-scale threat to satisfying the animal's nutritional needs. Environmental concerns relating to excessive P excretion from livestock are also a major driving force in improving P availability (Debnath *et al.*, 2005a); with risks being potentially most severe in aquaculture. Whilst dietary inorganic P supplementations meet the nutrient requirements of fish, the remaining phytate-bound P remains untapped and can cause excessive nutrient loading in effluent water, which in turn can contribute to eutrophication of aquatic environments surrounding farming operations (Amirkolaie, 2011; Bian *et al.*, 2012; Hlavac *et al.*, 2014). Additionally, the high cost of supplementing inorganic P into diets contributes significantly to operational feeding costs. Lastly, the social and environmental sustainability issues surrounding mining of this resource must also be considered (Mullaney *et al.*, 2000). In summary, not only does phytate pose severe constraints on maximising feed efficiency, animal performance and health, it also negatively influences socioeconomic and environmental sustainability of

operations, both directly and indirectly.

Despite its significant absence in monogastric and agastric animals, phytase (*myo*-inositol hexakisphosphate phosphohydrolase) is a widely distributed digestive enzyme among life forms (Kumar *et al.*, 2012). Microbial sources are particularly prolific and numerous commercial phytase products derived from the likes of *Aspergillus niger* and *Escherichia coli* are now available (Campbell and Bedford, 1992; Kumar *et al.*, 2012). As an exogenous enzyme supplement, phytase is an extensively used dietary tool in swine and poultry nutrition, regularly featuring in modern terrestrial monogastric feeds (Mullaney *et al.*, 2000; Cowieson *et al.*, 2006; Bedford and Partridge, 2010). Over the past decade, the aquafeed industry has also seen some degree of inclusion in dietary formulations (Debnath *et al.*, 2005a; Kumar *et al.*, 2012), however its functionality as a practical and effective dietary addition remains under investigation.

Microbial phytase supplementation in diets for *Pangasius pangasius* fingerlings has been demonstrated to have promising effects upon fish performance and feed efficiency (Debnath *et al.*, 2005b). Subsequent digestibility analysis by the authors revealed dry matter and protein digestibility to be improved with phytase-treated diets. Debnath *et al.* (2005b) investigated phytase inclusion ranging between 150 and 2000 FTU kg^{-1} (phytase unit [FTU] is defined as the amount of enzyme activity which liberates 1 micromole of inorganic phosphorus per minute at pH 5.5 and 37 °C at a substrate [sodium phytate] concentration of 5.1 mmol L^{-1}). Although its benefits were observed throughout enzyme treatments, Debnath *et al.* (2005b) demonstrated an optimum inclusion of 500 FTU kg^{-1} under the culture conditions, which is not dissimilar to a typical pig and poultry inclusion rate. Similarly to the previous investigation, it was reported that faecal dry matter and crude protein could be significantly reduced by inclusion of phytase in soya-based rainbow trout diets; moreover, digestibility of all essential amino acids (bar tryptophan), along with alanine, aspartic acid, cysteine, glutamic acid, glycine, proline and serine could be improved (Cheng *et al.*, 2004). In a later study, inclusion of microbial phytase in high-plant diets for rainbow trout was again shown to significantly improve dry matter

and crude protein digestibility, along with ash (Vandenberg *et al.*, 2012). It has also been demonstrated that P excretion by fish can be significantly reduced by phytase and further reduced by tailoring substrate (soyabean meal) inclusion levels in rainbow trout diets (Castro *et al.*, 2011). However, this investigation showed nitrogen excretion to be unaffected by phytase supplementation (Castro *et al.*, 2011). Growth and feed efficiency were shown to be positively influenced by a 2000 FTU kg^{-1} supplementation in the diets of juvenile red sea bream (*Pagrus major*) (Laining *et al.*, 2012). Carcass ash and protein appeared to also be influenced by phytase inclusion (Laining *et al.*, 2012), corresponding with previously mentioned findings. Similar results were also observed in Nile tilapia although at much lower phytase activity (<300 FTU kg^{-1}) whereby growth, feed conversion, protein efficiency ratio, specific growth rate and nutrient deposition were all promoted by the enzyme supplementation into a fishmeal-free diet (Liebert and Portz, 2005). A subsequent study by Liebert and Portz (2007) reported increased crude protein digestibility coefficients following phytase supplementation in tilapia diets, supporting findings in previously described species. On the other hand, the supplementation of 2500 IU g^{-1} phytase in Japanese seabass (*Lateolabrax japonicas*) diets was displayed as having no significant effect on soluble P excretion, protein retention, fish performance and feed efficiency (Ai *et al.*, 2007).

Work on the agastric rohu (*Labeo rohita*) fed sub-optimal protein level diets with phytase and citric acid inclusion, reported improved growth, protein efficiency ratio and increased carcass ash following dietary inclusion of both ingredients (Baruah *et al.*, 2007a; Baruah *et al.*, 2009). Haematological analysis of the rohu also found indications of immunomodulatory effects, proposed as originating from the increased availability of minerals (Baruah *et al.*, 2009). Attempts to alter the pH of the gut by lowering it towards an optimum for phytase activity appeared successful in this series of investigations and will be discussed at length later in this review.

Specific focus on reducing dietary inorganic mineral inclusions through enzyme supplementation has also been a topic of research, considering potential for phytase to

degrade phytate-mineral chelates. Up to 50 percent replacement of monocalcium phosphate with neutral phytase in plant-based diets was reported to not affect performance and feed efficiency of gibel carp (*Carrasius auratus gibelio*) (Liu *et al.*, 2012). However, P and protein digestibility were significantly improved in this study when compared with the pure monocalcium phosphate inclusion diet (Liu *et al.*, 2012). These findings appeared to suggest that the use of phytase may not just be a solution to reducing the supplementation of inorganic P in diets, but may in fact be a means of more efficient provision of P to the fish. A study which investigated inclusion of microbial phytase in rainbow trout diets proposed that trace mineral supplementation could be neglected altogether, without affecting fish performance and body composition, giving strong indication of effective phytate-mineral chelate degradation in this instance (Cheng *et al.*, 2004). It has been suggested that ingredients with low P profiles, such as distiller's dry grain with solubles (DDGS) (Cheng and Hardy, 2004), are best suited to phytase inclusions as due to the enzyme's efficacy, high P diets may still lead to elevated dissolved and/or suspended waste output of the nutrient, much like the scenario of P supplemented diets (Dalsgaard *et al.*, 2009).

Overall, the majority of reports on fish and feed performance concurrently suggest that phytase could play an important role in maximising nutritional values of diets, such as mediating protein/amino acid acquisition, thus stimulating growth. Reports of carcass ash increases and abilities to reduce mineral supplements also point towards broad spectrum liberation of other minerals from chelates. Determination of exactly which minerals can be liberated has been a strong focus point of numerous studies to date.

The liberation and deposition of P and potentially other minerals was clearly highlighted through an observed increase in total ash and P content of both vertebrae and scale of Nile tilapia fed a phytase supplement (Liebert and Portz, 2005). At a later date, Liebert and Portz (2007) specified both Ca and P digestibilities could be improved with a range of inclusion rates of phytase derived from the yeast *Hansenula polymorpha* and Ronozyme® P. Faecal and hepatic loop chyme phytate-P was observed as overall lower

following phytase supplementation, further confirming its hydrolysation (Liebert and Portz, 2007). Laining *et al.*'s (2012) study on red sea bream observed plasma P and Mg levels to be increased, indicating an increase in bioavailability of these minerals. This was further supported by analysis of vertebral mineral content which indicated a substantial increase in the presence of P, Ca and Mg, whilst P in scales was also augmented (Laining *et al.*, 2012). Digestibility of Ca, Mg, phytate-P, total P, Mn and Zn but not Fe and Cu were reported to have been improved in three experiments on rainbow trout using soya protein concentrate, semi-purified soyabean meal and practical soyabean meal-based diets supplemented with phytase (Cheng *et al.*, 2004). Vandenberg *et al*.'s (2012) study similarly revealed phytase inclusion to significantly improve P, Ca, Mg, and Zn bioavailability, but conversely to Cheng *et al*. (2004), also Fe. More evidence of mineral liberation by phytase was demonstrated in *Pangasius pangasius*; apparent absorption of Ca, P, Mg, Mn, Zn, Fe, K, Cu and cobalt (Co), carcass Ca, P, Zn, Fe, Cu and Co as well as of bone Ca, P, K, Cu and Co were all greater in phytase-provisioned fish (Debnath *et al.*, 2005c). In further support, faecal ash was reduced and bone ash was increased (Debnath *et al.*, 2005c). Increases in bone mineralisation (Na, Ca, K, P and Fe) were also recorded in juvenile rohu following phytase and citric acid additions to the diet (Baruah *et al.*, 2005). In a later study on rohu, by Baruah *et al.* (2007b), increased absorption of Zn, Na, P, K, Mn, Mg, Fe and N was specified.

Although an array of different phytase sources have been analysed in research to date, giving generally positive results, their origin and characteristics should be a strong point of scrutiny prior to inclusion. Investigation of bacterial phytase from *Escherichia coli*, versus a fungal counterpart from *Peniophora lycii*, showed the fungi-derived phytase to have a single, higher optimal pH; meanwhile, bacterial phytase peaked at two separate lower pH (Morales *et al.*, 2011). Furthermore, simulated gastric conditions revealed proteolytic degradation to vary with 90% retention of activity in bacterial phytase whilst 90% loss was observed in fungal phytase (Morales *et al.*, 2011). A consideration of this

kind is imperative to aquacultural applications due to the range of digestive physiologies encountered between species.

Unavoidably, pH conditions encountered within the digestive system can be considered grossly sub-optimal for phytase activity. Research in poultry has provided strong evidence that phytate hydrolysis occurs predominantly in the fore-stomach, under low pH, causing phytate to become more water soluble (Selle and Ravindran, 2007). The absence of gastric digestion in many extensively cultured finfish, notably carp, may mean that phytase effectiveness becomes compromised. Supplementation of acid in diets (notably citric acid) may well hold the key to tackling this potential issue as demonstrated by previously discussed studies (Baruah *et al.*, 2005; Baruah *et al.*, 2007a; Baruah *et al.*, 2007b; Baruah *et al.*, 2009).

On the topic of conditions within the digestive system, we also face the issue of species rearing temperature. The poultry and pig industries can guarantee that doses of dietary phytase will become exposed to temperatures of around 40 °C, on a daily basis. This is simply not possible in aquacultural production; considering that fish are poikilothermic, they express an extensive range of optimal rearing temperatures between species and these ambient temperatures are often subject to strong seasonal fluctuations. Temperate species will undoubtedly be the greatest challenge, as their own optimum metabolic temperatures will most certainly not reflect that of exogenous phytases. In a previously mentioned rainbow trout study different inclusion rates provided dramatic non-linear responses to ambient temperature, observable through P and ash digestibility measurements (Vandenberg *et al.*, 2012). With a dosage of 2000 FTU kg^{-1}, a steep decrease in P and ash digestibility was observed between 15 and 10 $^{\circ}C$, however they were comparable between these temperatures when dosage rate was doubled (Fig. 1.3). Whilst typically experimental inclusions of phytase have been between 500 and 2000 FTU kg^{-1} in warmer aquaculture species, Vandenberg *et al.* (2012) gave a clear indication that far greater phytase inclusion levels may be required in temperate to cold water species. However, it is still unclear whether doses higher than previously investigated

have the potential to effectively degrade phytate in fish reared at temperatures sub 10 °C. This is despite the fact that salmonids are routinely reared under these conditions for a large proportion of the calendar year in many regions.

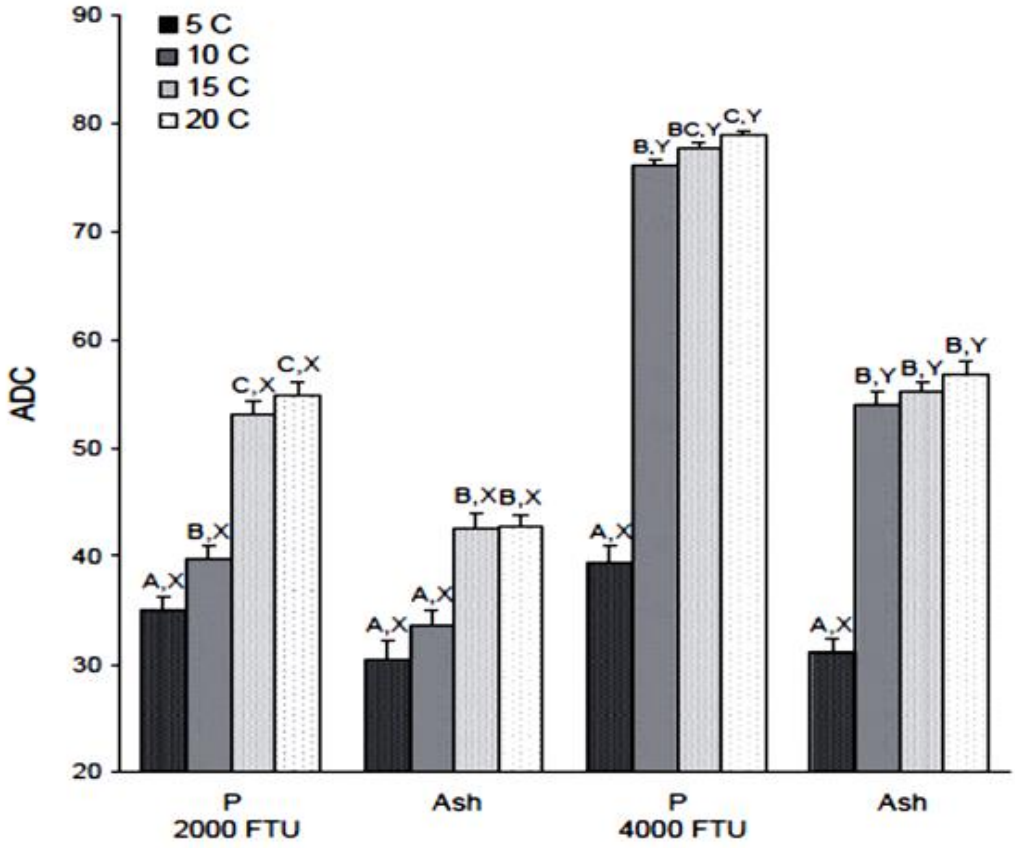

Figure 1.3 Phosphorous and ash digestibility coefficients from rainbow trout fed 2000 or 4000 FTU kg^{-1} microbial phytase under incremental water temperatures. $^{A-D}$ within individual variable denotes significant differences ($P < 0.05$). ^{XY} within individual temperature denotes significant differences ($P < 0.05$). Taken from Vandenberg *et al.* (2012).

Further merit in increasing phytase inclusions from traditional recommended doses can be taken from the current movement in the poultry and pig industry whereby a method termed as 'superdosing' is being promoted. Superdosing centres upon administering very high doses of phytase, which aim to liberate P not only from IP6 but sequentially IP5, IP4, IP3 and IP2 also (Yu *et al*., 2012). Thereafter, the P from IP1 can be released by endogenous alkaline phosphatase, to produce inositol. The benefits of this strategy are emerging; these include evidence that IP6 to IP2 all inhibit the efficacy of endogenous

protease (Yu *et al*., 2012) and decrease Zn solubility in the digestive tract (Price *et al.*, 1992). As previously mentioned, 500 FTU kg^{-1} has been a standard dosage rate in monogastrics, however, superdoses between 1000 and 2000 FTU kg^{-1} are now being proposed by a number of industry leaders (AB Vista, 2013). Such doses have been demonstrated to destroy IP6 almost entirely and significantly reduce the quantities of IP5, IP4 and IP3, to very low levels; something which does not appear possible with traditional inclusion rates (Pontoppidan *et al*., 2012; Walk *et al*., 2014). We may therefore ask the question of whether superdosing, likely in excess of 2000 FTU kg^{-1}, could be worthwhile considering for achieving desirable outcomes in finfish.

Lastly, aquafeed processing conditions are of great concern when it comes to producing a feed containing phytate-degrading properties. Water is quick to degrade physical cohesiveness and cause nutrient leaching from feed. As aquafeeds must withstand these pressures of being introduced to an aqueous environment, their manufacturing process is harsh in comparison to pig and poultry feeds. In order to reduce nutritional and thus economical loss, they are subjected to high temperatures and pressures during the extrusion process. Typically, these parameters normally exceed 100 °C and 3000 k/pa respectively. Phytase is notoriously sensitive to denaturing by temperature and pressure so its application does not lend itself easily to this area of animal nutrition. Effective solutions to safeguard against denaturation are therefore fundamental to progression in using exogenous phytase sources in modern aquaculture feeds. Post-extrusion applications, such as liquid spray-coating, may be considered as a means of incorporating phytase in diets although this process will likely incur further labour and financial costs, counter-intuitive to previously highlighted cost implications in ingredient processing. Vast differences in thermostability of phytase from different sources also exist, for example bacterial phytase from *E. coli* has been suggested to be more stable than fungal derived counterparts (Morales *et al.*, 2011). Upon this topic, sparsity in investigations leaves numerous avenues to be explored.

In conclusion, phytase can undeniably produce exceptional results under the correct conditions. However one cannot simply ignore the other ANFs and potential nutrient sources previously discussed. Indeed, there may be merit in approaching the nutrient matrix in a holistic manner by tackling multiple problematic compounds simultaneously.

1.8.4 Synergistic enzymes and cocktails

Although successes have been achieved, individual enzyme inclusions in finfish diets have been proposed by many to be too specific (Bedford and Partridge, 2010). One could indeed consider them to be an overly reductionist approach to the issues faced in the shift towards alternative ingredients. Aquafeeds tend to be substantially more complex in their formulations compared to those of terrestrial livestock as they contain a wider mix of ingredients. Their diverse composition is driven by the before mentioned pressures to reduce FM, with no economically viable alternative that can be considered equal in its nutritional composition. As a direct result, substrate complexity is heightened, simultaneously introducing a plethora of ANFs, in variable proportions. Alternatively, from the more optimistic viewpoint, nutrient potential is heightened. Consequently, benefits of single enzyme inclusions become somewhat more negligible. This has been recognised to an extent by those investigating exogenous enzymes in aquafeeds. For example, an absence of improved performance in tilapia fry fed pure cellulase was attributed to inefficient or incomplete hydrolysis of fibre content by removal of possible synergistic interactions between cellulase, hemicellulase and pectinase which work together to degrade a larger compliment of cell wall components (Yigit and Olmez, 2011).

When one observes enzymatic hydrolysis by organisms in nature, it is most evident that the process is a concerted affair, whereby multiple enzymes are secreted by the organism in order to effectively degrade the complexity of substrates encountered in their natural diet and subsequently satisfy nutrient requirements. For example, carnivorous fish obviously possess an arsenal of proteolytic and lipolytic enzymes due to nutritional composition of their diet, but they have also been found to possess

endogenous activities of chitinase, particularly in gastric tissue (Fines and Holt, 2010; Lu and Ku, 2013; Abro *et al.*, 2014). Endogenous chitinases allow the rapid degradation of chitin, which encases invertebrates, providing digestive accessibility to the energy-rich protein and lipid fractions of prey sources. This supports the proposal that natural synergistic interactions between complements of enzymes are a more efficient means of maximal substrate degradation in enzyme-treated feeds.

Additionally, increased liberation of nutrients, such as minerals from phytate, may not necessarily equate to optimal bioavailability if ANFs which limit nutrient uptake, such as soluble NSPs, are not simultaneously tackled. It is also worthy to consider the specificity and sensitivity of enzymes. Since formulations are regularly adjusted to suit ingredient availability and costs, ANF profiles are likely to fluctuate, concurrently opening the possibility of fluctuating efficacy, if a single-component enzyme product is used.

These considerations have sparked investigation into multi-enzyme applications and the development of numerous commercial products which aim towards a more efficient degradation of complex structures. For example the plant cell wall matrix, as a mode of targeting multiple substrates; namely NSPs, proteins and phytate for reduction of ANFs and release of energy-rich, bioavailable nutrients.

It must also be highlighted that the digestive system of finfish is highly influenced by external thermal conditions, due to their poikilothermic nature. Thus, over time, conditions required for specific enzymes to work optimally, or at least efficiently, will fluctuate. Livestock producers demand efficient and predictable efficacy of any additional investments they make in the form of functional feed ingredient additions. Multi-component ingredients would seem to allow a greater chance of this being guaranteed in the culture of aquatic organisms.

Both growth performance and feed efficiency were observed as promoted in juvenile hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) when fed a commercial neutral protease, ß-glucanase and xylanase preparation (Lin *et al*. 2007). Similarly, a triticale-

based diet, supplemented with an enzymatic complex of xylanase and ß-glucanase (Natugrain Blend ®), was observed to have promoted dry matter and energy digestibility in Nile tilapia (Tachibana *et al.*, 2010). Conversely, Natugrain Blend ® supplementation in silver perch (*Bidyanus bidyanus*) fed diets containing lupin or wheat, displayed no signs of affecting dry matter, energy or protein digestibility (Stone *et al.*, 2003b). Variation in temperature between the two studies was negligible. As Natugrain Blend ® is designed for wheat-based poultry diets (Odetallah *et al.*, 2002); it is also interesting that no differences were observed in wheat diets for silver perch, thus, specificity could also be discounted. However, different enzyme application methods were utilized with aqueous mixing throughout the feed in the tilapia study and a spray-on technique being employed for silver perch. From this, it may be speculated that enzyme-substrate contact may have been comparatively reduced in the study by Stone *et al.* (2003b) causing a lack of improvement in enzyme-treated diets.

In an 8 week trial on juvenile Japanese seabass, a xylanase-based supplement and a glucanase, pentosanase, cellulase compound were both demonstrated to be effective promoters of growth, feed efficiency and nitrogen retention along with tendencies towards improve phosphorous retention (Ai *et al.*, 2007). Results of both of these supplements were found to not be significantly different from one another. However, an amalgamation of the two enzyme supplements resulted in further improvements to growth, feed efficiency and nutrient retention. Conversely, the inclusion of various enzyme complexes (Energex™, Bio-Feed™ Pro, Alpha galactosidase™) did not reveal any benefit to growth in rainbow trout fed dehulled lupin-based diets, nor did a mix of all complexes (Farhangi and Carter, 2007). However, inclusion of Energex™, a high hemicellulase complex, did significantly improve protein efficiency ratio, along with dry matter, crude protein and gross energy digestibility (Farhangi and Carter, 2007). Rainbow trout fed a broad spectrum enzyme cocktail (Superzyme™ CS) in soyabean-orientated diets also showed no signs of improved growth performance and negligible effects on nutrient digestibility over 16 weeks (Ogunkoya *et al.*, 2006). In this instance,

highly digestible diets were proposed as a limiting factor in the efficacy of the enzyme supplementation, supporting a paradigm that enzymes should be used as a means of augmenting the nutritional value of diets with sub-optimal characteristics, such as being NSP-rich. Although a lack of significant effects in these trials was pinned to issues surrounding formulations, much further work is required to confirm whether enzyme complexes can be effective in salmonid culture.

Ai *et al*.'s (2007) previously mentioned study on Japanese seabass gave interesting insight into potential benefits of NSP enzymes on nutrient excretions; interestingly, the cocktail of xylanase, glucanase, pentosanase and cellulase was superior to phytase at reducing total phosphorous excretions. Furthermore, ammonia nitrogen excretion was reduced by the multi-enzyme supplementation. Unfortunately, this work did not include attempts to determine NSPase-phytase interaction, thus this area remained unclear. NSPase-phytase synergies have been highlighted in poultry as an area warranting more research (Bedford and Partridge, 2010). Phytate and NSP can chelate in feedstuff and digesta, thus NSPases and phytase in combination could theoretically eliminate this nutritionally limiting occurrence (Slominski, 2011). In a study on rohu fingerlings, a phytase and cellulase cocktail was observed to consistently produce better fish performance characteristics than the individual enzyme inclusions alone (Xavier *et al.*, 2012). Xavier *et al.* (2012) also observed the enzyme combination to show significantly pronounced tissue crude protein, lipid and ash, which affirmed theories of beneficial interactions between these two enzymes.

An enzyme complex (Allzyme Vegpro®) containing a plethora of NSP enzymes, amylase, phytase and protease*,* was observed as highly effective in enhancing the apparent digestibility of dry matter, crude protein, gross energy, starch, calcium and phosphorous in a fishmeal-free Nile tilapia diet (de Oliveira *et al.*, 2007). Augmentation of dry matter, crude protein, crude lipid and gross energy digestibility, correlating with improved growth and feed efficiency was also observed following Allzyme Vegpro® treatment of 40% palm kernel meal diets, again in Nile tilapia (Ng *et al.*, 2002). At a later date, a

similar diet with inclusion of Allzyme SSF®, a complex derived from *Aspergillus niger*, showed enhanced performance of tilapia over a 60 day period (Moura *et al.*, 2012), which also correlates with performance enhancing successes seen in broiler chickens (Hooge *et al.*, 2010). Analysis of sugar levels in intestinal digesta revealed fructose, sucrose and glucose to be significantly higher in enzyme-fed fish (Moura *et al.*, 2012). Moura *et al.* (2012) attributed observed improvements to performance as being derived from increased bioavailable carbohydrates as energy sources, sparing protein for structural growth. However, as previously mentioned in this review, NSPases also appear to have an ability to augment protein digestibility so this explanation cannot be neglected.

As with all enzyme applications, substrate specificity may still be a critical factor in supplementation of multi-enzyme inclusions. Pre-treatment of fibrous ingredients used in aquafeeds with Ronozyme VP®, a complex of NSPases revealed the NSP content of soyabean meal and sunflower cake to be significantly reduced but this was not observed in rapeseed meal or field peas (Denstadli *et al.*, 2011b). However, inclusion of enzyme pre-treated soyabean meal and sunflower cake in rainbow trout diets did not promote nutrient digestibilities and furthermore, deterioration in feed efficiency was observed (Denstadli *et al.*, 2011b). The authors proposed that enzymatic action upon the ingredients achieved releases of oligosaccharides as appose to monomers, thus carbohydrate uptake was not possible. Although oligosaccharides can be potent prebiotics, they have been demonstrated to have negative effects on the nutritional value of feeds in high concentrations, by restricting the uptake of other nutrients (Glencross *et al.*, 2003). Nevertheless, copper absorption was significantly increased with enzyme pre-treatment in the feeding trial by Denstadli *et al*. (2011).

The application of exogenous digestive enzymes has typically been directed at young fish whose digestive systems are still under development, or enhancing performance of livestock for market-sale. However, benefits may also be possible in other livestock categories such as broodstock. AmecoZyme 2X®, a commercial preparation of NSPases,

protease, lipase, phytase and alphagalactosidase has been shown to provide benefits to reproductive performance of Nile tilapia, explained by an increase in available energy from non-protein sources (Tahoun *et al.*, 2011).

As discussed enzyme applications may have pronounced effects on nutrient excretions but it must also been considered how they may affect the physical properties of faeces. This has been a large consideration in terrestrial animal farming, particularly with regards to poultry and faecal 'stickiness', a result of high NSP content (Bedford and Schulze, 1998; Acamovic, 2001; Cowieson *et al.*, 2006). Changes to physical faecal characteristics with dietary exogenous enzyme inclusions were observed in rainbow trout by Ogunkoya *et al.* (2006), whereby faecal material cohesiveness and sinking speed were reduced with enzyme supplementation. Results of this kind may have profound effects in commercial aquaculture operations. The reported characteristics of the exogenous enzyme-associated faeces may be undesirable for land-based operations due to inhibition of waste recovery, whilst cage culture may benefit in increased dispersion of waste thus minimising localised pollution issues (Ogunkoya *et al.*, 2006). Despite the huge body of research efforts and applications of enzymes as a waste-management tool in poultry production, parallel efforts in aquaculture appear to be inconsequential in comparison. This neglected area of research deserves much more attention, as one must consider the downstream waste management benefits or implications of enzyme-treated feed.

Synergies between digestive enzymes are not limited to those artificially administered to the digestive system via feed. As previously mentioned, exogenous enzymes can affect endogenous counterparts, a phenomenon very much recognised in poultry nutrition (Cowieson *et al.*, 2006). The findings by Li *et al.* (2009) appeared to suggest that the degradation of phytate by exogenous enzymes could promote natural hydrolysis of starch by the fish. The same trend was observed with addition of a Roche preparation of NSPases, further indicating that the natural digestion of starch may be facilitated by a variety of non-native enzymes (Li *et al.*, 2009). This occurrence could accentuate the

provision of energy to the fish, in turn leading to improved production.

To date, research into synergistic interactions between exogenous enzymes has generated varied results. However, the theoretical potential for these inclusions to work effectively cannot be ignored. It is to be scrutinised that amalgamating enzymes from a variety of production sources may not necessarily be the most effective approach to the situation of degrading a natural nutrient matrix, considering the before mentioned argument of natural enzymatic complements. Therefore, it is suggested that more natural solutions, capable of delivering a multifaceted approach to the issue of dietary substrate complexity, must be found.

1.9 Solid-state fermentation

1.9.1 Background

Solid-state fermentation (SSF) can be defined as the fermentation of a substrate in the absence of free-flowing water. For millennia, this technology has been implemented around the globe, in a multitude of different ways, to great effect. By doing so, humans have learnt to harness its natural capabilities to benefit, and in some ways shape, society. The oldest known references to SSF come from the ancient civilisations of Egypt (Jacob, 1944). Having happened upon this phenomenon through curiosity, as early as 2600 BC, this civilisation began extensively implementing SSF of inedible flours to create bread (Jacob, 1944). The power of fermentation in the baking process extended thereafter through civilisations across the globe, with bread regularly featuring in key religious and socio-political events as a fundamental preserver of human well-being (Jacob, 1944). Other historical developments and applications include the manufacture of cheese, yoghurt, cured meats and condiments, such as soy sauce, to name but a few. As scientific understanding and advents in technology have progressed, developments in the practice of SSF have followed suit. At the present day, SSF implementation has grown from a poorly understood phenomena, involved in the production of artisanal

foods, to the underpinning technology behind the industrialised manufacture of high-value commodities and bioprocesses. All in all, this can be attributed to the discovery and growing scientific understanding of enzymology.

The use of SSF in improving animal feeds is not a novel concept but its use has been grossly restricted to the production of silage for ruminants. Firstly, the process allows preservation of the fodder for times when feed becomes unavailable or nutritionally poor. Secondly, it enhances its nutritional properties in a variety of ways, such as production of folic acid and vitamin B12 by the microorganisms involved (Santos *et al*., 2008). Ultimately, the product becomes more digestible, allowing the animal greater potential for assimilation of nutrients.

Applications of SSF in monogastric nutrition have been somewhat more limited. Indeed, the harvesting of enzymes has been prolific and contributed tremendously to pig and poultry production but SSF products in their crude, relatively unrefined form are yet to make a significant appearance in feed rations. This is despite their apparent simplicity and the previously discussed potential benefits of implementing natural complements of enzymes.

1.9.2 Synergen™

Synergen™ (Alltech Inc.; KY, USA), is a novel product of SSF intended for application within monogastric animal feed rations. The production process utilises wheat bran as its substrate, which is derived as a by-product from industrial food flour milling. This wheat crop originates from cultivation in Canada and northern Mexico, before later being processed in Mexico (La Espiga, Mexico City).

Prior to fermentation, the substrate is autoclaved at 110 °C for 15 min. to eliminate microbial contamination. After cooling to 37 °C the substrate is inoculated with a non-GMO strain of the filamentous fungi *Aspergillus niger*, which is cultured via submerged liquid fermentation. Following seeding, moisture content of the wheat bran is raised to 48 percent to begin the fermentation process. The moist, inoculated wheat bran (termed

koji) is incubated at 37 °C for approximately 100 hrs under a strictly controlled temperature, humidity and oxygen system. Following fermentation the koji is dried to cease the fermentation process and for preservation. This remaining product contains residual enzymatic activity which could theoretically reinstate its catalytic potential upon substrates in the gastro-intestinal tract of monogastrics. Due to the substrates nutritional composition, its key contributing components are those which are not present in the digestive enzyme arsenal of monogastrics.

1.10 Research rationale

It is clear that the aquaculture industry is facing growing pressures to improve its socioeconomic and environmental sustainability credentials. Although an attempt to improve sustainability, a shift in dietary formulations towards high plant protein inclusions has presented an extensive range of issues regarding animal performance, health and welfare; issues which stem from a lack of endogenous digestive capabilities. Conversely to studies in swine and poultry, exogenous enzyme applications in fish have been highly unpredictable and moderate in their overall success. Realistically, the environmental and substrate parameters encountered by exogenous enzymes in finfish diets may be overwhelmingly unfavourable to eliciting a response capable of equating to significant gain, be it in terms of animal performance, health, profitability or reducing potential environmental impact. Specifically, the challenge of dietary substrate complexity in this area is great and suggests adoption of a more complex approach than those previously observed. One must therefore consider multi-faceted products, with the grand aim of simultaneously degrading a plethora of ANFs, maximising nutrient release and ultimately allowing flexibility in feed formulations. It is suggested that SSF may be an avenue which fits this paradigm, thus it should be explored in more scientific detail. Additionally, we have a profound lack of knowledge in how exogenous enzyme sources may specifically influence the health of livestock. Gaining insight into these downstream

effects is paramount to advancing our understanding and progress in this field.

To date investigations into the efficacy of Synergen™ in aquatic species is limited to a few reports with no peer-reviewed articles. Furthermore, these reports have focused purely upon performance and feed efficiency so understanding downstream effects upon animal health status and digestive characteristics remains unexplored. Therefore, application in fish diets is yet to be validated.

1.11 Research aims

This body of work aims to evaluate the function and efficacy of SSF products for improving production and health of finfish by improving the available nutrient profiles of contemporary and novel feed formulations. Growth performance and nutrient availabilities will be assessed, in line with routine experimental designs in the assessment of exogenous feed enzymes. However, with increasing interest in promoting finfish intestinal health, this characteristic will also be a focus of exploration; using techniques which are routinely implemented in the evaluation of other feed ingredients, such as alternative protein sources and pro- and prebiotics. This is on the basis that changes in the nutrient profile of digesta within the intestine can have profound effects upon its morphology, endogenous secretions and microbiota (Merrifield *et al*., 2010).

With a call for diversification in the alternative ingredients available to the industry, but an inherent risk of poor digestibility and ANFs, the first study (Chapter 3) investigates the application of Synergen™, as well as two types of lupins, in a warmwater, omnivorous species, Nile tilapia. A comparison is conducted between yellow and blue lupin species, each with and without Synergen™ inclusion, to determine not only efficacy of the SSF product but also the viability of the different lupin species. The study firstly assesses performance and feed efficiency from a production standpoint. Thereafter, more in depth analyses aim to identify whether the three experimental ingredients affect intestinal morphology at both macro and ultrastructural levels.

The second investigation (Chapter 4) remains on the topic of Synergen™ inclusion in a lupin-based diet but in this instance focusing on yellow lupin. Furthermore, attention is turned to the feeding of rainbow trout, a temperate, carnivorous species. This species can be seen as much more sensitive to a shift towards a plant-based diet and its rearing temperature less conducive of continued fermentation by the experimental product within the gastro-intestinal tract. Beyond performance and feed efficiency, the study first aims to give in depth quantification of both macronutrient and mineral bioavailabilities. Subsequently, potential downstream receptors of increased nutrient influx are analysed, such as tissue mineral concentrations and vertebral morphology. A wider-scale assessment of intestinal health is implemented than previously to give a more holistic insight into effects. Again, macro and ultrastructure of the intestine is studied but with the inclusion of three-dimensional appraisal techniques. How substrate alteration in the intestine of fish may modulate the gut microbiome is also examined, using high-throughput sequencing. Further, the activities of endogenous proteolytic enzymes are examined to determine if an endo-exogenous interaction may be a contributing factor.

Lastly, attention is turned to the efficacy of Synergen™ in a pilot-scale on-farm trial in Chapter 5. Synergen™ is exposed to commercial extrusion in a low-cost rainbow trout formulation. It is then subsequently fed to a younger, more sensitive life-stage of rainbow trout, which is reared at a lower temperature than previously. Consequently, it is explored whether potential for *in vivo* fermentation is maintained, by tracking a number of desirable parameters relating to fish performance, feed efficiency, macronutrient digestibility and mineral bioavailabilities and retention. Following this, *in vitro* analysis is implemented with an aim of quantifying and explaining the effects of both feed manufacturing conditions and typical salmonid rearing temperatures on the efficacy potential of Synergen™.

Beyond immediate aims described, this work looks to provide a body of in-depth, quantitative information, which is transferable across monogastric livestock species, in the study of SSF product and exogenous enzyme inclusions in commercial feeds.

CHAPTER 2. General methods

2.1 Experimental animals and housing

All of the experimental animals utilised within these studies were obtained from dedicated commercial finfish farms. Nile tilapia (*Oreochromis niloticus*) and Rainbow trout (*Oncorhynchus mykiss*) were primarily selected as the experimental species due to their global importance on the aquaculture market. The occupied research facilities were all licenced to house the species in question and perform the nature of the work involved. These locations implemented the highest possible degree of biosecurity and consistency in environmental and chemical parameters throughout the duration of this work, so as to avoid all possible external influences upon the data provided. Due to the differences in experimental species and housing conditions, specific details of the animals, husbandry procedures and rearing conditions are detailed within their respective chapters. Images of the systems utilised can be found with the appendices (plate 7.1, 7.2 and 7.3). Summaries of the experiments are displayed in the table below.

Table 2.1 Summaries of conducted experiments

	Chapter 3	**Chapter 4**	**Chapter 5**
Species	Nile tilapia	Rainbow trout	Rainbow trout
Initial fish weight	36 g	44 g	6 g
Rearing temperature	26 °C	12.5 °C	10.5 °C
Bulk ingredient focus	Yellow lupin vs. narrow-leaf lupin	Yellow lupin, FM reference diet	Commercially-extruded pellets
Bulk diet formulation	15 % lupin inclusions	30 % yellow lupin	Least-cost, wheat inclusions
Synergen™ inclusions	0 and 0.1 %	0, 0.1 and 0.5 %	0, 0.5, 1.0 and 1.5 %
Nutrition trial duration	7 weeks	10 weeks	9 weeks
Analyses	- Performance - Element retention - Carcass composition - Gut histology - Haematology	- Performance - Nutrient digestibility - Element bioavailability - Carcass composition - Tissue element concentrations - Haematology - Gut histology - Gut microbiome - Gut protease activities - Vertebral histology	- Performance - Somatic indices - Nutrient digestibility - Element bioavailability - Element retention - Serum lysozyme - *In vitro* free phosphate release

2.2 Experimental ingredients and diets

All ingredients utilised within these investigations have been approved for use in animal diets and are available on the commercial market, albeit they may not be available from the suppliers specified herein. The experimental dietary formulations aimed to assess and optimise inclusion levels for the ingredients of interests, so as to provide novel data regarding their influences upon animal health and performance. All dietary formulations were performed so as to satisfy the animal's requirements of each nutrient, where information is available (NRC, 2011). Details pertaining to ingredient specifications and experimental diet manufacturing are presented within their respective chapters.

2.3 Feeding regimes

All animals were fed ration sizes deemed to be representative of commercial production. So as to partially limit nutrient provision and avoid masking the effects of the experimental ingredients, only fixed rations were utilised in the investigations. Details of the feeding methods and regimes used within the investigations are given in their respective chapters.

2.4 Biomass sampling

Biomass sampling was performed on a weekly or biweekly basis, by catching all individuals within a tank and weighing in bulk, in every instance. This was performed under methods which minimised the possible effect of oxygen deprivation, either by aeration or by constant water supply to bins prior to weighing. All weighing was performed to the accuracy of 1 gram, in tared bins of system water. Detailed methods and equipment used for weighing of the experimental animals are described within their respective chapters.

2.5 Euthanasia

All euthanasia was undertaken by initial immersion in 300 mg/L tricaine methanesulfonate (MS222) (Pharmaq ltd., Hants, UK) using system water buffered to its original pH with sodium bicarbonate ($NaHCO_3$). Aeration was continuously supplied via an air stone. Immersion was performed until deep anaesthetisation. The fish were subsequently removed from the anaesthetic and a sharp blow to the head, followed by destruction of the brain, was administered.

2.6 Anaesthesia

All methods requiring anaesthesia were performed by immersion in 200 mg/L MS222 (Pharmaq ltd., Hants, UK) using system water buffered to its original pH with sodium bicarbonate ($NaHCO_3$). Aeration was continuously supplied via an air stone.

2.7 Proximate composition

All chemical analyses were conducted according to AOAC (2016) guideline methods, as detailed in the following subsections.

2.7.1 Dry matter

Oven-drying of samples was performed by the following method (AOAC Method 934.01):

Before drying, diets were milled and the peritoneal cavities of whole carcasses were opened. The samples were dried at 105 °C in a fan-assisted oven (Genlab Ltd; Ches, UK) until constant weight was achieved.

Freeze-drying of samples was performed by the following method:

Before drying, the peritoneal cavities of whole carcasses were opened. The samples were subsequently dried in a Super Modulyo® freeze drier (Thermo Electron Corp; MA, USA), with ~09 x 10^{-3} mbar chamber evacuation and -50 °C operating temperature.

For both methods and all samples, dry matter (DM) was run in triplicate and calculated as follows:

$$Dry\ matter\ (\%) = 100 - (((W_w - W_d) / (W_w)) \times 100)$$

Whereby; W_w = wet weight (g) and W_d = dry weight (g).

2.7.2 Crude protein

Crude protein contents of diets, carcasses and faeces were determined via the Kjeldahl method (AOAC Method 2001.11), which quantifies nitrogen content and later crude protein, via a conversion factor. Prior to analysis, all diets and samples from Chapter 3 and 4 were dried according to the oven method and carcass and faecal material from Chapter 5 was dried according to the freeze-drying method, both of which are given in Sec. 2.7.1. Between 100 and 250 mg of dried sample (to 0.1 mg) was weighed into micro Kjeldahl tubes. Sample weight was determined by expected protein content; e.g. 100 – 150 mg was employed for carcass (high N content) and 200 – 250 mg for faecal matter (low N content). Into every tube, one catalyst tablet, containing 3 g K_2SO_4, 105 mg $CuSO_4$ and 105 mg TiO_2 (BDH Chemicals Ltd; Dor, UK) was added. The samples were then immersed in 10 ml of concentrated (≥95 %), low nitrogen, sulphuric acid (H_2SO_4) (Fisher Scientific; Leics, UK). Digestion was conducted using a Gerhardt Kjeldatherm 40 tube digestion block (Gerhardt Laboratory Instruments; DE) with the following process; 30 min at 100 °C, 1 hrs at 225 °C and 1 hrs at 380 °C. Following digestion, the samples were allowed to cool overnight. Next, the samples were distilled with an automated Vapodest 40 unit (Gerhardt Laboratory Instruments; DE) into boric acid to form ammonium borate complex. Resulting distillate was neutralised through addition of concentrated H_2SO_4 and titrated. The efficiency of nitrogen recovery was quantified through the inclusion of triplicate acetanilide standards; as well as visual checks against

duplicate purified casein standards, of known N content. Further, duplicate blanks were analysed.

Nitrogen content of the samples was calculated as follows:

$$Nitrogen\ (\%) = ((((TV_s - TV_b) \times AN \times MW_N) / SW)) / NE) \times 100$$

Whereby; TV_s = titration volume (ml) of the sample, TV_b = titration volume (ml) of the blank, AN = acid normality of H_2SO_4 (0.20), MW_N = the molecular weight of nitrogen (1.40067 g), SW = sample weight (g) and NE = efficiency of nitrogen recovery (%).

Thus, crude protein content was calculated as follows:

$$Crude\ Protein\ (\%) = N\ (\%) \times cf$$

Whereby; N = nitrogen content (%) and cf = conversion factor. A standardised conversion factor of 6.25 was implemented for carcass, diets and faecal matter (AOAC, 2012); whilst a conversion factor of 5.5 was utilised for analysis of lupin meals (Mosse, 1990).

2.7.3 Crude lipid

Crude lipid (CL) content of samples was determined via hot-solvent extraction (AOAC Method 920.39). Prior to analysis, samples were dried according to details given in Sec. 2.7.1. Approximately 3 g (to 1 mg) of sample was added to a cellulose thimble and plugged with cotton wool. The thimbles were then inserted into holders and suspended within extraction beakers containing anti-bumping granules (Fisher Scientific; Leics, UK) (of known weight to 1 mg); followed by the addition of 40 ml of petroleum ether. Lipid extraction was performed from using a 6 place Soxtherm unit (C. Gerhardt; DE) run at 200 °C, with an automated control system (Multistat, C. Gerhardt; DE). Following total petroleum ether evaporation and cooling in a fume cupboard, beakers and extracted lipid were weighed (to 1 mg). All samples were analysed in triplicate and lipid content was calculated as follows:

$$Crude\ lipid\ (\%) = (LE^* / SW) \times 100$$

$$^* determined\ by: FBW - IBW$$

Whereby; LE = lipid extract (g), SW = sample weight (g), FBW = final beaker weight (g), IBW = initial beaker weight (g).

2.7.4 Crude fibre

Crude fibre content of diets and faecal matter was determined via a digestion-incineration method, which solubilises and removes starch, sugars, protein and lipid, followed by incineration of residual carbohydrates allowing collective quantification of cellulose, hemicellulose and lignin (AOAC Method 950.02). Prior to analysis, samples were dried according to the oven method given in Sec. 2.7.1.

For faecal matter, residual de-fatted sample was taken from thimbles and approximately 1 g (to 0.1 mg) was weighed directly into dried, pre-weighed (to 0.1 mg) Fibretherm bags (Gerhardt Laboratory Instruments; DE), taking care not to gain contact with the thimble surface.

For diets, approximately 1 g (to 0.1mg) of dried sample was weighed directly into dried, pre-weighed (to 0.1 mg) Fibretherm bags (Gerhardt Laboratory Instruments; DE). Dried glass spacers were then inserted into bags. The samples were subsequently immersed in 40/60 petroleum ether solvent and agitated to extract lipid. This process was undertaken, with fresh petroleum ether, until the solvent appeared clear. The bags were then left to dry in a fume cupboard for 1 hrs. After drying, the samples were introduced to an automated 12 space Fibretherm extraction unit (Gerhardt Laboratory Instruments; DE). The solubilisation process utilised sulphuric acid (0.13 M) (Fisher Scientific; Leics, UK), sodium hydroxide (0.313 M) (Fisher Scientific; Leics, UK) and distilled water washes.

At the end of the extraction procedure, the bags, containing samples with extracted solubles, were removed and inserted into ashing crucibles. The samples, bags and crucibles were then dried at 105 °C for 14 hrs. The samples were subsequently cooled in a desiccator and weighed (to 0.1mg).

The crucibles and their contents were then incinerated for 6 hrs at 600 °C. Following incineration, the crucibles and contents were again moved to a desiccator to cool and subsequently weighed once more (to 0.1 mg).

The samples were analysed in association with blank runs utilising the same batches of bags and reagents used throughout the procedure.

CF content was calculated as follows:

$$Crude\ fibre\ (\%) = (((S_3 - S_1 - S_4) - (B_3 - B_1 - B_4)) \times 100) / S_2$$

Whereby; S_1 = initial bag weight (g), S_2 = initial sample weight (g), S_3 = sample after digestion and drying, S_4 = sample after incineration, B_1 = blank initial bag weight, B_3 = blank bag after digestion and drying and B_4 = blank bag after incineration.

Samples obtained from the end of the lipid determination method were corrected against corresponding lipid content.

2.7.5 Ash

Ash content of diets and carcasses was determined by incineration (AOAC Method 942.05). Prior to analysis, samples were dried according to the oven method given in Sec. 2.7.1. Into ceramic crucibles of known weights (to 0.1 mg), 400 to 600 mg (to 0.1 mg) of dry sample was added. The samples were subsequently incinerated in a muffle furnace (Carbolite ELF; Derbs, UK) at 550 °C for 12 hrs. Samples were cooled in a desiccator before weighing. All samples were analysed in triplicate. Percentage ash content was calculated as follows:

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

Whereby; FW = incinerated sample weight (g) and IW = initial sample weight (g).

2.7.6 Gross energy

Quantification of gross energy in diets and faecal matter was performed via oxygen bomb calorimetry, which measures heat of combustion as a means of obtaining calorific

values. Prior to analysis, the samples were dried according to the oven method given in Sec. 2.7.1. The analysis was conducted using a Parr 1356 Bomb Calorimeter (Parr Instrument Company; IL, USA). Approximately 1 g (to 0.1 mg) of sample was weighed into nickel crucibles and a fuse wire was shaped so as to become submerged, at its bend, within the sample. The crucible and fuse wire were then inserted into the bomb chamber. The chamber was filled with pure oxygen and lowered into the bucket containing 2 kg of municipal water. Following entry of the sample weight (to 1mg) into the computer system, the automated system combusted the sample, and recorded the internal temperature change within the jacket to generate a MJ gross energy per kg value. All analyses were performed in duplicate.

2.8 Mineral and trace element analysis

2.8.1 Digestion

Before digestion, samples were dried according to details given in Sec. 2.7.1. All dietary samples and tilapia carcasses were dried by the oven method. Individual tissues, faecal matter and trout carcasses were dried by the freeze-drying method. Diets and carcasses were homogenised using a household blender whilst faecal material and individual tissues were homogenised using a synthetic-coated pestle and mortar. Between 100 and 250 mg of sample (to 0.1 mg) was weighed into boiling tubes. The samples were then immersed in 10 ml of 70 % nitric acid (Fisher Scientific; Leics, UK). Digestion was conducted using a Gerhardt Kjeldatherm 40 tube digestion block (Gerhardt Laboratory Instruments; DE) with the following process; 1 hrs at 60 °C, 1 hrs at 90 °C, 30 min at 110 °C and 3.5 – 4 hrs at 135 °C. Digests were subsequently left to cool for a minimum of 2 hrs whilst covered. Once cooled, the samples were transferred to propylene vials and diluted to 50 ml with distilled water. Duplicate blanks of diluted nitric acid in distilled water were analysed in all instances. Tilapia diet samples were analysed in triplicate and carcasses were analysed in quadruplicate. Diets, faecal material and individual tissue

from rainbow trout study 1 were analysed in triplicate. Diets, faeces and carcasses from rainbow trout study 2 were analysed in quadruplicate. Replication was determined by sample availability in the case of faecal matter. Carcasses were analysed in quadruplicate so as to allow negation of erroneous reads (± 2σ) caused by the presence of un-homogenised, mineralised tissues (e.g. scales, fin rays, bone); whilst still maintaining n = 3 replication.

2.8.2 ICP OES

All mineral determination analyses were performed by Inductively Coupled Plasma Optical Emissions Spectroscopy (ICP OES). A Thermo Scientific iCAP 7400 series (Thermo Scientific Inc; MA, USA) with a cyclone spray chamber and a Burgener Peek Mira Mist® nebulizer (Burgener Research inc; ON, CAN) was utilised throughout.

Operating conditions were as follows:

Rf[1] power (kw)	1.15
Coolant gas flow (L/min)	12.0
Nebulizer gas flow (L/min)	0.50
Auxillary gas flow (L/min)	0.50
Additional flow (L/min)	0.00
Viewing height (mm ALC[2])	12.0

[1] Rf = radio frequency
[2] ALC = above load coil

For each element, the following wavelengths (nm) were implemented; Ca 317.93 and 315.89, Co 238.89, Cr 283.56, Cu 324.75, Fe 259.94, K 766.49, Mg 285.21, Mn 257.61, Mo 202.03, Na 589.59, P 177.50 and 178.28, S 180.73, Y 371.01, Zn 213.86.

Trace element concentrations, in parts per million (ppm), were calibrated against 4 external standard concentration curves, containing each element. Drift (± 1.0 %) was routinely checked after every 5 samples by reanalysing standards and correcting values where necessary.

Element concentrations of the samples were determined by the following calculation:

$$Element\ concentration\ (ppm) = V_d / IW \times C_d$$

Whereby ; V_d = digest volume (ml), IW = initial sample weight (g) and C_d = element concentration (ppm).

2.9 Performance calculations

Growth performance of fish and feed utilisation was assessed through calculation of weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR). All mortalities were accounted for in performance calculations.

WG was calculated as follows:

$$Weight\ gain\ (g) = FW_f - FW_i$$

Whereby; FW_f = final fish weight (g) and FW_i = initial fish weight (g).

SGR was calculated as follows:

$$Specific\ growth\ rate\ (AU) = 100 \times ((Ln\ W_f - Ln\ W_i) / DF)$$

Whereby; Ln = natural log, W_f = final tank biomass (g), W_i = initial tank biomass (g) and DF = days fed.

Days where fish were fed a ration of ≤ 0.75 % BW (e.g. before sampling) were not counted in SGR calculations, due to classification as a maintenance ration.

FCR was calculated as follows:

$$Feed\ conversion\ ratio\ (AU) = (W_f - W_i) / FI$$

Whereby; W_f = final tank biomass (g), W_i = initial tank biomass (g) and FI = feed intake (g).

2.10 Somatic index calculations

Somatic indices were calculated as indicators of fish condition and health, in accordance with methods described by Rawling *et al.* (2012). Fulton's K-factor (K-F) was used as an indicator of fish condition. To summarise, euthanised fish were measured from the tip of the snout to the fork of the tail, and weighed (to 1 mg).

K-F was calculated as follows:

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

Whereby; FW = fish weight (g) and FL = fork length (cm).

Hepatosomatic index (HSI) was used as an indicator of health status. To summarise, euthanised fish were weighed (to 1 mg) and were subsequently dissected, the corresponding whole livers were thus weighed (to 0.1 mg).

HSI was calculated as follows:

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

Whereby; LW = whole liver weight (g) and FW = fish weight (inc. liver) (g).

2.11 Nutrient digestibility and mineral bioavailability calculations

Apparent digestibility coefficients (ADC) and apparent bioavailability coefficients (ABAC) were conducted where experimental housing and species allowed the ethical collection of faecal matter, namely in rainbow trout. The methods utilised were stripping and settlement in juvenile and fingerling fish, respectively; in accordance with discussion on ethics and limitations given in Sec. 1.4. Full details of the collection materials and methods are given in their respective chapters.

ADC of nutrients were determined via proximate composition results of nutrients and ICP OES quantification of the inert marker, yttrium oxide (YO) in the diets and collected faeces.

ADC of nutrients was calculated as follows, according to methods described by Lupatsch *et al*. (1997):

$$\textit{Apparent digestibility coefficient (\%)} = 100 - (100 \times (Y_d / N_f) / (Y_f / N_d))$$

Whereby; Y_d = YO concentration in the diet, Y_f = YO concentration in the faeces, N_d = nutrient concentration in the diet, N_f = nutrient concentration in the faeces.

ABAC of dietary elements were determined via ICP OES quantification of element concentrations in the diets and collected faeces.

ABAC of elements was calculated as follows:

$$\textit{Apparent bioavailability coefficient (\%)} = 100 - (100 \times (Y_d / E_f) / (Y_f / E_d))$$

Whereby; Y_d = YO concentration in the diet, Y_f = YO concentration in the faeces, E_d = element concentration in the diet, E_f = element concentration in the faeces.

2.12 Nutrient retention

Apparent nutrient retention (NR) of dietary elements was determined using ICP OES quantification of their concentrations in the diets, initial fish carcasses and final fish carcasses, alongside biomass and feed intake data. Element concentrations were corrected back to a wet basis and all mortality weights were added to final tank weight before calculations were performed.

NR was calculated as follows:

$$\textit{Apparent nutrient retention (\%)} = 100 \times (((W_f \times E_f) - (W_i \times E_i)) / (FI \times E_d))$$

Whereby; W_f = final tank biomass (kg), W_i = initial tank biomass (kg), E_f = final element concentration of whole body (g/kg), E_i = initial element concentration of whole body (g/kg), E_d = element concentration in the diet (g/kg), FI = feed intake (kg).

2.13 Protein efficiency and utilisation

Quantification of dietary protein utilisation was achieved through calculation of protein efficiency ratio (PER) and apparent net protein utilization (ANPU).

Protein efficiency ratio was calculated as follows:

$$\textit{Protein efficiency ratio (AU)} = (W_f - W_i) / PI$$

Whereby; W_f = final biomass (g), W_i = initial biomass (g) and PI = total protein intake (g).

ANPU was calculated as follows:

$$\textit{Apparent net protein utilisation (\%)} = 100 \times (CP_f - CP_i) / ((PF / 100) \times ADC_{CP})$$

Whereby; CP_f = final crude protein content of carcass (g), CP_i = initial crude protein content of carcass (g), PF = total crude protein fed (g), ADC_{CP} = apparent digestibility coefficient of crude protein (%).

2.14 Haematological and serological analysis

Blood was sampled from the fish in order to obtain indicators of health and immune status, as well as a potential indication of nutrient uptake. The fish were deeply anaesthetised (200 mg/L MS222) and blood was extracted from the caudal vein, in line between the anal fin and caudal fin, with a 25 gauge needle and 1 ml syringe. The blood was subsequently transferred to microcentrifuge tubes (Protein LoBind, Eppendorf®; DE). For collection of serum, whole blood was left to clot on ice (~ 4 hrs) and then stored at

4 °C for 12 hrs; these samples were subsequently centrifuged at 2,500 x g for 5 min and serum was removed. The serum samples were stored at -80 °C until analysed.

2.14.1 Haematocrit

Haematocrit was quantified as a measure of the erythrocyte fractions in the blood, as described by Brown (1988). Fresh blood was drawn into heparinised capillary tubes (75 µl) and sealed with plasticine (Cristaseal, Hawksley; West Sussex, UK). The capillary tubes were subsequently centrifuged at 10,500 x g for 5 min. A Microhaematocrit Tube Reader (Hawksley; W Sussex, UK) was used to measure packed cell volume proportion (%) of the samples.

2.14.2 Haemoglobin

Haemoglobin was quantified, as an indicator of health and immune status, via a turbidimetric method as described by Rawling *et al*. (2012). From freshly drawn blood, 4 µl of sample was added to 1 ml of Drabkin's alkaline ferricyanide-cyanide solution (D5941, Sigma-Aldrich Co.; Dorset, UK) and incubated at room temperature (~20 °C) for ~4 hrs. Following incubation, absorbance was measured at 540 nm in a spectrophotometer (Helios Epsilon, Thermo Scientific; MA, USA). Haemoglobin was calculated as follows:

$$Haemoglobin\ (g/dl) = (Abs_{sa} / Abs_{st}) \times DF$$

Whereby; Abs_{sa} = absorbance of the sample, Abs_{st} = absorbance of the standard and DF = dilution facor (200).

2.14.3 Serum glucose

Glucose was measured in the blood as an indicator of health, immune and energy status as well as being a potential indicator of sugar uptake derived from fermentation of carbohydrates in the GIT. The method implemented was the Trinder glucose activity test, which is a turbidimetric coupled-enzyme assay. A glucose standard was prepared by

dissolving 0.1 % (w/v) of D-Glucose (G8270, Sigma-Aldrich; Dorset, UK) in distilled water saturated with benzoic acid ($C_7H_6O_2$). Phosphate buffer (100 mM, pH 7.0) containing 0.016 % (w/v) 4-Aminoantipyrine (G-7016, Sigma-Aldrich Co.; Dorset, UK), ~0.0002 % (w/v) peroxidase (P-8250, Sigma-Aldrich; Dorset, UK), 0.105 % (w/v) phenol (P-8250, Sigma-Aldrich Co.; Dorset, UK) and 0.1 % (v/v) Tween-20 was created as a colour reagent. Into microcentrifuge tubes, 3 ml of colour reagent and 50 µl of serum were dispensed. The blank consisted of 3 ml colour reagent and 50 µl of pure phosphate buffer and the standard consisted of 3 ml of colour reagent and 50 µl of D-Glucose standard solution. The solutions were mixed with a vortex and incubated in a water bath for 15 min at 28 °C. Following incubation, the sealed tubes were rapidly cooled in ice water (~2 °C) to cease glycolytic activity. Immediately after cooling, samples were transferred to vials and absorbance was measured at 550 nm. Each sample was run in duplicate. Glucose concentration within the serum was calculated as follows:

$$Glucose\ (mg/dl) = 100\ x\ (Abs_{sa} / Abs_{st})$$

Whereby; Abs_{sa} = absorbance of the sample and Abs_{st} = absorbance of the standard.

2.14.4 Serum lysozyme

Serum lysozyme activity was quantified, as an indicator of innate immune status, via a kinetic turbidimetric assay using methods adapted from Demers and Bayne (1997). *Micrococus lysodeikticus* (4698, Sigma-Aldrich Co.; Dorset, UK) was suspended in 0.05 M Na_2HPO_4 (pH 6.2) at a concentration of 200 mg/ml, as a substrate. Into microplate wells, 25 µl of serum was added, 175 µl of substrate solution was subsequently added using a multichannel pipette. The plate was immediately mechanically agitated and absorbance at 530 nm was read every 30 sec then on for 5 min (OPTImax microplate reader, Molecular Devices LLC; CA, USA). Each sample was run in quadruplicate. One unit (U) of lysozyme was defined as follows:

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

2.15 Light microscopy (intestine)

Histology of the intestine, via light microscopy, was undertaken as a means of quantifying intestinal health and potential surface area for digestion and absorption of nutrients at a macrostructural level. Specimen processing was conducted using standard histological methods. The analytical methods utilised thereafter were in accordance with those described by Standen *et al*. (2015), in tilapia and Dimitroglou *et al*. (2009, 2010) in rainbow trout and are described at length in their respective chapters.

Selected tissue samples were fixed in 10 % formalin and kept at 4 °C for 48 hrs in the case of tilapia mid gut and 72 hrs for rainbow trout gut. Following fixation, samples were transferred to 70 % ethanol at 4 °C for storage. The samples were removed from the alcohol and where possible, digesta was rinsed away with PBS and/or removed via forceps, taking care to avoid contact with tissue structure. The specimens were further dehydrated in graded ethanol concentrations in an automated tissue processor (Leica TP1020; Bucks, UK). Embedding was performed in paraffin wax (Leica EG1150 H; Bucks, UK).

All sectioning of specimens was conducted at 5 µm thickness using a Leica RM2235 microtome (Leica; Bucks, UK), with blocks being chilled on a cooling plate (Leica EG1150 H; Bucks, UK) prior to sectioning. Sections were mounted on glass slides and dried at 30 °C for 48 hrs.

All intestinal specimens were stained using a Leica Autostainer XL (Leica; Bucks, UK). This process was initiated by rehydration in graded ethanol concentrations; twice at 100 % followed by 90, 70, 50 % ethanol and a rinse with distilled water. Stains implemented were haematoxylin and eosin (H&E), and periodic acid-Schiff with Alcian blue (PAS) (Tables 7.1 and 7.2). Cover slips were mounted with DPX and left to dry at 30 °C.

Micrographs were captured with a Leica DMIRB microscope and Olympus E410 digital SLR camera, at varying magnifications.

Methodological approaches employed for the quantification of intestinal macrostructure are provided in their respective chapters.

2.16 Transmission electron microscopy

The intestine was further appraised with the use of electron microscopy techniques for quantification of intestinal health and functional topographical surface area, at an ultrastructural level. The ultrastructure of finfish is particularly sensitive to nutritional pathologies (Merrifield *et al*., 2010) but little is known on the effects of exogenous enzymes upon this parameter, thus the area was explored.

Transmission electron microscopy (TEM) dissection and tissue selection methods are detailed within their respective chapters. The processing methods utilised for TEM were in accordance with methods described by Dimitroglou *et al*. (2009, 2010) and are described below. Thereafter, analytical methods utilised were derived from a variety of sources and are described at length in respective chapters.

Intestinal samples were directly immersed in in 2.5 % glutaraldehyde fixative agent containing 1 part 0.1 M sodium cacodylate buffer (pH 7.2). Storage of samples was conducted at 4 °C. Samples were rinsed twice, for 20 min, in 0.1 M sodium cacodylate buffer to remove fixative. Approximately 2 mm of fixed tissue was selected from the middle portion of samples, discarding ends to omit possibilities of tissue damaged by handling. Postfixation was then conducted by immersion of the samples in 1 % osmium tetroxide (OsO_4) solution in 0.1 M sodium cacodylate buffer (pH 7.2), for 2 hrs. This fixation step was consecutively performed a total of 3 times, at 2 hrs intervals, using fresh fixative and buffer on each occasion. Following final fixation, residual fixative was cleared with a brief rinse in pure 0.1 M sodium cacodylate buffer. Samples were then dehydrated by immersion in graded levels of ethanol (30, 50, 70, 90 and 100 %), at 20 min intervals. Next, samples entered a fresh 100 % ethanol solution for 20 min and were then drained. Low viscosity resin premix (Agar Scientific; Essex,UK; no. AGR1078) and

absolute ethanol were used throughout resin infiltration stages. Samples were immersed in 30:70 (resin:ethanol) mix for ~14 hrs. Following this, samples entered a 50:50 mix for ~9 hrs. This was then replaced with a 70:30 mix for ~14 hrs. Finally, samples were immersed in 100 % resin for 24 hrs. Following resin infiltration, samples were inserted into polyethylene embedding capsules (BEEM®; PA, USA) and immersed in resin for curing. Polymerisation was conducted at 70 °C for 14 hrs.

All sectioning was conducted on a Reichert-Jung Ultracut E ultratome (Leica group; Bucks, UK). Blocks were trimmed and semi-thin (~0.5 µm) sections were cut using a glass knife. These sections were stained with methylene blue for pre-examination under LM. Once appropriate sample locations were indentified, ultra-thin (~80 nm) sectioning was performed with a diamond knife (Microstar Tech.; TX, USA). Sections were subsequently collected onto copper grids.

Saturated uranyl acetate – ethanol (90 %) solution was created, mixed by hand and subsequently centrifuged at 5,000 x g for 10 min, as a stain. Copper grids were placed, section side down, upon drops of the uranyl acetate supernatant and left to stain for 15 min, under dark conditions. The grids were subsequently rinsed in distilled water. Secondary contrasting was performed with lead citrate solution, again for 15 min; this was performed under CO_2 limiting conditions, in covered vessels containing sodium hydroxide (NaOH) granules.

Samples were screened with a 120 kV JEOL JEM-1400 transmission electron microscope (Tokyo, Japan) with a Gatan Orius 830 imaging system (CA, USA).

Methodological approaches employed for the quantification of intestinal ultrastructure are provided in their respective chapters but all centre upon either quantifying microvilli dimensions or organisation.

2.17 Statistical analysis

Details of statistical methods, models and software utilised are described in their respective chapters.

CHAPTER 3. Application of Synergen™ in Nile tilapia diets containing yellow and narrow-leaf lupin varieties

3.1 Introduction

The Nile tilapia (*Oreochromis niloticus*) is historically one of the oldest domesticated livestock species, with evidence of its culture dating back to the ancient Egyptian civilisation, some 4000 years ago (Chimits, 1957). Today, the species remains a staple, subsistence animal in many Asian and African populations; meanwhile, it continues to gain momentum in its commercial production, across the continents (FAO, 2014b). According to FAO (2014b), between 1990 and 2013, global production of Nile tilapia increased from approximately 1.5 million tonnes to 3.4 million tonnes. However figures relating to total global production are likely to be underestimations as much of the farming operations occur in rural areas of developing nations, thus non-reported productions are inevitable. A number of other tilapia species are also produced around the world, such as Mozambique tilapia (*O. mossambicus*) and blue tilapia (*O. aureus*), as well as hybrids (e.g. *O. mossambicus X O. niloticus*); each with regional importance. As it stands, tilapias are the second most farmed fishes in the world (FAO, 2014a) and this seems set to remain.

It is the tilapia's exceptional farming characteristics which have led this ease of culture and intensification. One of the most crucial factors in this success has been that of its dietary acceptance and nutritional requirements. Nile tilapia is an omnivorous species which has been observed to display plasticity of its feeding habits, but overall its natural diet consists of phytoplankton, macrophytes, periphyton, and invertebrates (Nijiru *et al.*, 2004). Considering tilapia naturally feed at a low trophic-level, compound feeds theoretically do not require fishmeal or fish oil inclusion, from an animal health perspective (FAO, 2014a). Although tilapia feeds have included fishmeal in the past, the global push towards reducing fishmeal inclusions has been met with relative ease within the tilapia industry. Typically, commercial tilapia diets now contain negligible, if any, quantities of fishmeal. However, it is not to be assumed that tilapia diets can be vastly

flexible or of poor quality if we aim for sustainable production systems. First and foremost, its importance in the agrimarkets and diets of certain communities means production cannot be compromised. Secondly, tilapia production is often a highly intensive process; with routine stocking densities reported in excess of 100 Kg / M^3 in cage culture (Garcia *et al*., 2013). Under such conditions we heighten risks of stress, disease and crowding-related injuries, all of which are aggravated through inadequate nutrition. In closed systems (e.g. ponds and RAS), we must also consider nutrient loading, which is concomitant with poor feed efficiency. However, feed formulations must still be undertaken at a highly cost-effective level considering that tilapia are a relatively low-value species (FAO, 2014b), thus the consumer expects an affordable end product. There is currently a wealth of plant-proteins available on the market which could theoretically be utilised in tilapia feeds. In fact, we can consider the arsenal of potential ingredients to be far greater than those suitable for many other commercially produced fish as tilapia feed manufacturing and culture is so widespread, meaning locally available ingredients are at the industry's disposal. Formulations heavily based upon local or regional agronomic assets could undoubtedly contribute significantly to the sustainable development of the tilapia industry. The species is highly popular in North America and is gaining popularity within Europe. Therefore, lupins present an alternative protein option for tilapia producers in the western world.

This study aims to determine whether application of the SSF product (Synergen™) can improve the nutritional value of diets containing yellow or narrow-leaf lupins for Nile tilapia. Furthermore, considering these lupins will not be of absolute identical nutritional value, the study aims to identify which lupin species is more favourable for application within tilapia diets, both with and without supplementary addition of the SSF product. If the SSF product does effectively improve the nutritional value of the diets, then improved growth performance and feed efficiency are to be expected, along with increased deposition of liberated nutrients. Further, if exogenous degradation of ANFs which impinge upon intestinal health occurs, it is expected that amelioration of gut

macro or ultrastructure will be identifiable. The lupin with the most favourable nutritional profile, whether it is via greater nutrient quality or reduced ANFs, is expected to result in better animal performance and potentially a healthier gut surface.

3.2 Materials and methods

3.2.1 Experimental design

The feeding trial was designed to determine production performance of the tilapia fed the experimental diets, over a 7 week time period. To provide supportive information to the growth performance and feed efficiency data, midgut samples were temporally collected as the major indicator of nutritional health. A number of supplementary health indices, outside of the intestine, were also evaluated.

3.2.2 Experimental animals and housing

The nutritional trial was conducted at Plymouth University's West Aquarium research facility. Fingerling black Nile tilapia (*Oreochromis niloticus*) (~25 g) were obtained from North Moore Tilapia (Lincolnshire, UK). The animals utilised were derived from YY super-male stock. Upon entering the research facility, the fish were acclimatised for one hour. After acclimatisation, the fish were stocked into rectangular 80 L tanks on a ~2200 L recirculating system (RAS) (99 % water recycle). The system was powered by a 1.00 hp pump (Certikin, HPS100M; Oxfordshire, UK), supplying a flow rate of ~600 L/hr. Throughout conditioning and the experimental trial, adequate water quality was maintained by biological and mechanical filtration. Water chemistry (nitrate, nitrite, and ammonium) was tested bi-weekly. Temperature was maintained at 26.0 °C ± 1 via an inline heater (Elecro Titanium; Hertfordshire, UK). Dissolved oxygen was maintained above 76.0 % with air supplied via a low pressure side channel blower (Rietschle Ltd.; Hampshire, UK) to perforated piping below biological media and air stones within tanks. A 12 hr light: 12 hr dark photoperiod was implemented with fluorescent lights and timers. During the conditioning period, fish were exposed to a 7 day protozoa and fungicide treatment (Protozin, Waterlife; Middx, UK) along with a 7 day oral administration of

florfenicol (Florocol, MSD Animal Health; Bucks, UK), at 10 mg/kg BW per day; these were conducted as routine prophylactic measures. Following the conditioning period, the fish were graded by size and visual condition. Selected individuals were restocked into groups of 50 individuals in four sets of duplicate tanks (n = 2), with resulting average initial fish weight of 36.22 g ± 0.16; corresponding to a stocking density of 20.12 kg/m^3 ± 0.13. At day 26, biomass control was undertaken on the basis of system carrying capacity for maintenance of adequate water quality. During this procedure, six fish were randomly selected and removed from each tank, three of these were utilised for histological analysis, as described later in Sec. 3.2.7.

3.2.2 Experimental ingredients and diets

During the conditioning period, the fish were fed BioMar Efico Enviro (BioMar; DK) at approximately 2 % BW per day. This high-grade, commercial, salmonid feed was fed so as to not compromise digestive health prior to trial commencement, as it is deemed to have a low ANF content in the diets of tilapia.

Dehulled kernel meal from narrow-leaf lupin (*Lupinus angustifolius* cv. Sanabor) and yellow lupin (*Lupinus luteus* cv. Pootalong) were supplied by Soya UK (Hampshire, UK), after dehulling and milling by Alvan Blanch (Wiltshire, UK). These crops were cultivated in the U.K. as part of the Lupins in UK Agriculture and Aquaculture (LUKAA) initiative (Innovate UK). A 15 % inclusion of lupins was implemented in a manner deemed to partially replace a niche which would usually be held by soya products. A low ANF soyabean protein concentrate (Hamlet HP100, DK), specifically developed to contain a low content of oligosaccharides, was utilised as the marginally predominant protein source.

Synergen™ was supplied by Alltech Inc. (KY, USA) from industrial batches manufactured under the conditions detailed in Sec. 1.4.2.

Four experimental diets were formulated, using FeedSoft Pro™ (TX, USA), so as to satisfy all known nutrient requirements of Nile tilapia (NRC, 2011) and be isonitrogenous,

isolipidic and isocaloric (Table 3.1). These were two basal diets, with 15 % yellow or narrow-leaf lupin inclusion. Synergen™-supplemented diets were manufactured by including the SSF product at 0.1 % at the expense of corn starch.

The dietary bulk ingredients (bar corn starch) were mixed thoroughly in a Hobart Legacy mixer (Hobart Food Equipment, AU; model: HL1400-10STDA). Meanwhile, corn starch, vitamin/mineral premix, antioxidants and SYN (where included) were thoroughly combined. The starch carrier and additives were slowly added to the bulk ingredients and mixed for a further 30 min. Oil was subsequently added to the ingredient mix and left to combine homogenously for 30 min, followed by the addition of tepid water to achieve an appropriate consistency for pellet manufacture. Once an appropriate consistency was achieved, the dietary mix was immediately cold-press extruded (PTM Extruder System, Devon, UK; model: P6) and manually cut, using a knife, to form 2 mm diameter pellets. The moist pellets were oven-dried at 35 °C for 48 hrs (Genlab, Cheshire, UK; model: MINO 200 F). The diets were packaged in sterilised containers and kept at room temperature (~15 °C) until used.

Table 3.1
Feed formulations, proximate compositions and mineral concentrations of the experimental Nile tilapia diets.

	Diets			
	YLC	YLS	BLC	BLS
Ingredient (g / kg)				
Soyabean protein concentrate[1]	303.87	303.87	334.13	334.13
Corn Starch [2]	301.24	300.24	275.93	274.93
Yellow Lupin [3]	150.00	150.00	_	_
Narrow-leaf Lupin [4]	_	_	150.00	150.00
Fishmeal [5]	120.00	120.00	120.00	120.00
Maize Gluten Meal [6]	50.00	50.00	50.00	50.00
Fish Oil [7]	30.00	30.00	30.00	30.00
Corn Oil [8]	23.89	23.89	18.94	18.94
Vitamin/Mineral Premix [9]	20.00	20.00	20.00	20.00
Antioxidant [10]	1.00	1.00	1.00	1.00
Synergen™ [11]	_	1.00	_	1.00
Proximate composition				
Moisture (%)	2.39	2.47	2.22	2.08
Crude protein (%)	37.24	36.91	37.72	37.96
Crude lipid (%)	7.63	7.61	7.71	7.64
Ash (%)	6.15	6.19	5.35	5.84
NFE [12]	46.59	46.82	47.00	46.48
Mineral concentration				
Ca (g/kg)	5.22	4.63	6.40	5.92
P (g/kg)	6.85	6.64	6.97	6.93
Mg (g/kg)	1.97	1.99	1.81	1.86
Zn (mg/kg)	105.91	100.95	91.75	93.87

[1] HP100 (Hamlet, DK), [2] (Sigma Aldrich, UK), [3] dehulled (kernel meal) *Lupinus luteus* cv. Pootalong (Soya UK), [4] dehulled (kernel meal) *Lupinus angustifolius* cv. Sanabor (Soya UK), [5] LT94 herring meal (CC Moore, UK), [6] Glutalys (Roquette, FR), [7] Epanoil (Seven Seas, UK), [8] (Sainsburys ltd., UK), [9] 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), [10] Tocopherol, ethoxyquin, butylated hydroxytoluene (BHT) (Premier Nutrition, UK), [11] (Alltech Inc., KY, USA), [12] nitrogen free extract = dry matter – (crude protein + crude lipid + ash).

3.2.3 Feeding and biomass sampling

Throughout the trial, the animals were fed fixed rations between 2.0 and 3.5 % BW per day, which were incrementally reduced as the fish grew. Rations were immediately adjusted following mortalities. The fish were starved for a 24 hrs period prior to biomass sampling. The fish were weighed in bulk, by tank, on a weekly basis. This was performed to an accuracy of 1 gram. Feeding rations were subsequently calculated for the following 6 days.

3.2.4 Performance calculations

All calculations were performed as detailed in Sec. 2.9. Final fish weight was calculated at day 56 after adhering to the routine feeding methods; this was conducted by weighing all the remaining fish individually to 0.5 gram accuracy. All mortalities were accounted for in performance calculations.

3.2.5 Whole carcass sampling

Sampling for whole carcass was performed by pooling ≥200 g of wet fish per replicate tank; this was performed at day 0 for an initial composition of the stock fish and again at day 49 (n = 2). The whole fish were oven-dried according to procedures detailed in Sec. 2.7.1 and ground using a household blender.

3.2.6 Feed and carcass compositional analysis

Proximate composition was undertaken following methods described in Sec. 2.7. Mineral concentration was conducted in correspondence with methods described in Sec. 2.8. Nutrient retention (NR) calculations were performed in accordance with Sec. 2.12.

3.2.7 Haematological and serological parameters

At day 49, 3 fish per tank (n = 6) were heavily sedated and blood was collected from the caudal vein as described in Sec. 2.14. Haemoglobin (Hb), haematocrit (Hct), serum

glucose and serum lysozyme were analysed according to procedures detailed in Sec 2.14.1, 2.14.2, 2.14.3 and 2.14.4, respectively.

3.2.8 Intestinal histology

3.2.8.1 Sampling

Sampling of intestinal tissue was scheduled at days 0 (light microscopy only), 26 (light microscopy only) and 49 of the feeding trial. Six fish were sampled from the initial stock population, and 3 fish per tank were sampled at days 26 and 49 of the trial (n = 6). Following euthanasia procedures described in Sec. 2.5; the IP cavity of fish was opened and the entire GIT was removed from the animal at the oesophagus and anus. Intraperitoneal fat deposits were carefully separated from the gut tissue, whilst the GIT was simultaneously disentangled. The intestinal tract was detached below the stomach sack and at the anus. The midmost portion of the intestinal tract was identified and ~7.5 mm of tissue was removed from the location. The first ~5 mm of this tissue (from the anterior end) was separated for LM, whilst the last ~2.5 mm was taken for transmission electron microscopy (day 49 only).

3.2.8.2 Light microscopy

Midgut samples were processed and sectioned in accordance with the methods detailed in Sec. 2.15, followed by staining with H&E as described in Sec. 2.15 (Table 7.1). Micrographs were collected using methods and equipment detailed in Sec. 2.15. All appraisals were conducted using ImageJ 1.45 (National Institutes of Health, USA).

The micrographs were appraised for enterocyte height through measurement of the cells at 100 intermittent locations per fish (Plate 3.1A).

For calculation of surface area potential (perimeter ratio), micrographs were converted to binary tone form and the outer mucosal perimeter (OP) and inner functional border (IP) were measured (Plate 3.1B). Perimeter ratio (PR) was calculated as follows, in accordance with methods described by Standen *et al*. (2015):

PR = OP / IP

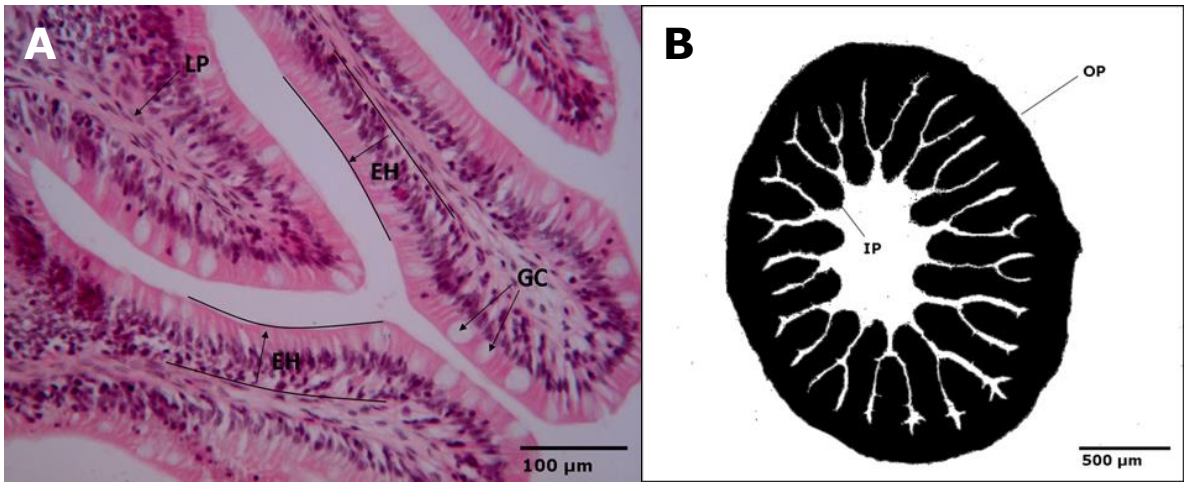

Plate 3.1 Methodological demonstrations of Nile tilapia midgut macrostructural appraisal. ***A*** = Tilapia midgut folds stained with H&E, displaying the height of enterocytes (EH), lamina propria (LP) and goblet cells (GC). ***B*** = Tilapia midgut silhouette, displaying outer mucosal perimeter (OP) and intestinal functional border (IP).

3.2.8.3 Transmission electron microscopy

The collected midgut samples were processed, sectioned and screened as described in Sec. 2.16. Microvilli height (MVH) and microvilli diameter (MVD) measurements were performed in accordance with methods described in Sec. 2.16. Microvilli counts (MVCT) (no. / 1 μm^2) were performed using locations displaying an aerial view of the brush border, with 10 quadrant counts per fish (Plate 3.2A); measured over 5 micrographs from differing locations. Two sets of coordinates were pre-determined as standardised locations for quadrant placement on each micrograph. The top-left corner of quadrants was placed upon said coordinates. The methods utilised followed quadrant methods largely adopted in ecological surveys for random samples to be collected.

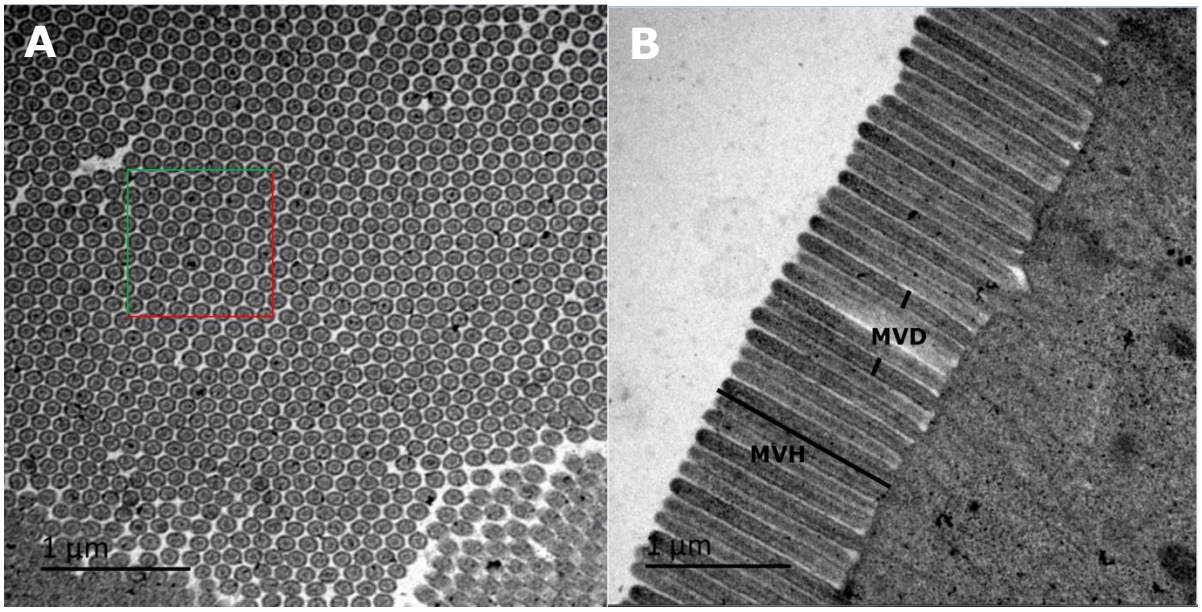

Plate 3.2 Methodological demonstrations of Nile tilapia midgut ultrastructural appraisal, using TEM micrographs.
A = Aerial view of brush border, displaying 1 μm^2 quadrant; microvilli which lay upon *green* edges are counted, whilst those on *red* edges are discounted.
B = lateral view of brush border, displaying microvilli height (MVH) and microvilli diameter (MVD).

Estimated total absorption surface area (TAS) (μm^2 per 1 μm^2 of epithelial base plan [μm^2 / 1 μm^2]) was calculated to provide a three-dimensional estimate of functional topography, utilising two-dimensional measurements (MVD, MVL and MVCT) obtained from the specimens and Pi. This method was adapted from Vizcaino *et al*. (2014) and was performed for each fish as follows:

$$TAS\ (\mu m^2 / 1\mu m^2) = ((2\pi \times \tfrac{1}{2}MVD \times MVL) + (2\pi \times \tfrac{1}{2}MVD^2)) \times MVCT$$

Whereby; π = Pi, MVD = mean microvilli diameter (µm), MVL = mean microvilli length (µm) and MVCT = mean microvilli counts (no. / 1 μm^2).

3.2.9 Statistical analysis

All statistical analyses were performed using SigmaPlot 13.0. All values expressed herein as percentages were arcsine-transformed prior to statistical analysis. Fish and feed performance, carcass compositional parameters and nutrient retention results were

analysed via Two Way Analysis of Variance (ANOVA) in partnership with Fisher's LSD Method for pairwise multiple comparisons. Variables tested were lupin type (yellow and narrow-leaf) and SYN presence (control and 0.1 % SYN), with a test of interaction between these two variables. Enterocyte height (EH) and absorptive surface area (PR) among treatments were analysed via Three Way ANOVA in partnership with Fisher's LSD Method for pairwise multiple comparisons. Variables tested were lupin type, SYN presence and time point (day 0, 26 and 49), with interactions assessed between the three variables. Two Way ANOVA was utilised for statistical analysis of midgut ultrastructural measurements (TEM) in partnership with Fisher's LSD Method for pairwise multiple comparisons. Analyses were performed upon ranks, where data expressed non-normal distribution and are referred to as such within.

3.3 Results

3.3.1 Growth performance and feed efficiency

The final average fish and feed performance results are displayed in Table 3.2. By day 49, the YLC, YLS, BLC and BLS fed fish had increased in biomass by approximately 177, 213, 170 and 207 %, respectively. The mean final weights (FW) of the experimental animals did not significantly differ as a consequence of lupin type ($P > 0.05$) but a significant increase was observed in Synergen™ (SYN) treatments compared with the controls ($P = 0.02$); this was independent of lupin type. Likewise, overall weight gain (WG) was unaffected by lupin type ($P > 0.05$) but SYN-fed fish gained significantly more weight than those fed the control diets ($P = 0.02$), although again this was independent of lupin type. No significant differences were observed in FCR based on lupin type ($P > 0.05$), SYN presence ($P > 0.05$) or as a consequence of interaction between the two variables ($P > 0.05$). However, numerical tendencies appear to display a decrease in FCR following SYN inclusion. Lupin type was not observed to display a significant effect on specific growth rate (SGR) ($P > 0.05$). However, SYN inclusion was observed to

significantly increase SGR (P = 0.03), with no dependence on lupin type ($P > 0.05$). No significant effect of lupin type was observed upon protein efficiency ratio (PER) ($P > 0.05$). SYN inclusion was observed to impart a near significant increase upon PER ($F = 5.87$, P = 0.07), with no observed dependence upon lupin type ($P > 0.05$). No significant effects on K-factor (K-F) were observed as a consequence of lupin type, SYN presence or interaction between the experimental ingredients ($P > 0.05$). Following prolonged observation of animal behaviour, mortalities were deemed to stem from hierarchal, territorial aggression, confirmed thorough post-mortems revealing no signs of pathology. Mortality rate was accelerated following the loss of individuals causing a reduction in stocking density and consequently this increased aggressive behavioural interaction between individuals. The trial was preemptively terminated at day 49 on these grounds, given environmental enrichment increases aggression in this species (Barreto *et al.*, 2011) and supplementing stocking density is non-conducive of experimental growth trials. Thus, survival is discounted as a statistically meaningful analysis of nutritional status within the experimental animals. The survival rates serve to explain discrepancies between growth indices (FCR and SGR) and weights (FW and WG). Including fish euthanised at day 26, a total of 1553, 2094, 2112 and 1638 g of fish were removed from the YLC, YLS, BLC and BLS treatments respectively, between days 0 and 49.

3.3.2 Carcass composition and nutrient retention

No significant effect of lupin type, SYN inclusion or interaction between the two dietary variables was identified in any of the analysed carcass nutrient and mineral concentrations (all $P > 0.05$), the results of which are displayed in Table 3.3.

Table 3.2
Fish and feed performance values of the dietary treatments

	Diet					
	YLC	YLS	BLC	BLS	Sig.	Interaction
IW (g)	36.24 ± 0.28	36.40 ± 0.11	36.20 ± 0.45	36.02 ± 0.03	NS	-
FW (g)	100.67 ± 5.03	114.12 ± 1.09	98.01 ± 3.10	110.61 ± 2.11	*P* = 0.02	C vs S
WG (g)	64.43 ± 5.32	77.72 ± 1.20	61.66 ± 2.43	74.59 ± 2.09	*P* = 0.02	C vs S
SGR	2.13 ± 0.13	2.36 ± 0.01	2.22 ± 0.01	2.37 ± 0.11	*P* = 0.03	C vs S
FCR	1.39 ± 0.13	1.23 ± 0.04	1.28 ± 0.00	1.21 ± 0.10	NS	-
PER	1.98 ± 0.14	2.32 ± 0.00	2.18 ± 0.13	2.24 ± 0.13	NS	-
K-F	2.04 ± 0.13	2.06 ± 0.03	2.01 ± 0.07	1.96 ± 0.02	NS	-
Survival (%)	88.64 ± 0.00	81.82 ± 0.03	81.82 ± 0.03	89.77 ± 0.02	-	

Abbreviations: YLC = yellow lupin basal diet; YLS = yellow lupin basal + Synergen™ (0.1%); BLC = narrow-leaf lupin basal diet; BLS = narrow-leaf lupin basal + Synergen™ (0.1%); IW = initial fish weight; FW = final fish weight; WG = weight gain (g); FCR = feed conversion ratio; SGR = specific growth rate; K-F = k-factor condition index; Sig. = statistical significance; NS = not significant; C = grouped control diets; S = grouped SYN 0.1% diets.
Values expressed as mean ± S.D (n=2). Statistical test: Two-Way ANOVA + Fisher's LSD (lupin type x SYN presence).
[a, b] Diets possessing the same superscript in the same row are not significantly different (P≤0.05), no superscripts indicate no significant difference between any diets

Table 3.3
Concentration of macronutrients and minerals in the whole carcasses of the experimental Nile tilapia

	Diet					
	YLC	YLS	BLC	BLS	Sig.	Interaction
Macro composition (%)						
Moisture	71.68 ± 0.00	71.37 ± 0.00	71.67 ± 0.00	71.63 ± 0.00	NS	-
Crude protein	14.50 ± 1.78	14.83 ± 0.06	14.72 ± 0.34	14.75 ± 0.78	NS	-
Crude lipid	10.08 ± 0.57	8.79 ± 0.17	8.56 ± 1.05	8.70 ± 0.95		
Ash	2.88 ± 0.19	3.51 ± 0.38	3.15 ± 0.03	3.01 ± 0.48	NS	-
Nitrogen-free extract	1.29 ± 0.34	1.63 ± 0.42	1.84 ± 0.87	1.63 ± 0.42	NS	-
Mineral conc.						
Ca (g/kg)	7.37 ± 0.33	8.88 ± 1.19	9.01 ± 2.04	8.25 ± 1.25	NS	-
P (g/kg)	4.59 ± 0.27	5.43 ± 0.69	5.47 ± 0.99	5.09 ± 0.62	NS	-
Mg (g/kg)	0.23 ± 0.02	0.27 ± 0.02	0.29 ± 0.04	0.26 ± 0.02	NS	-
Zn (mg/kg)	14.03 ± 0.70	15.54 ± 0.95	16.33 ±3.90	16.19 ± 0.51	NS	-

Abbreviations: YLC = yellow lupin basal diet; YLS = yellow lupin basal + Synergen™ (0.1%); BLC = narrow-leaf lupin basal diet; BLS = narrow-leaf lupin basal + Synergen™ (0.1%); Sig. = statistical significance; NS = not significant.
All values expressed as mean concentration of whole carcass (on wet basis) ± S.D. (n=2). Statistical test: Two-Way ANOVA (lupin type x SYN presence).

As displayed in Fig. 3.1, no significant effect of lupin type, SYN inclusion or interaction between the two dietary variables was identified in P retention ($P > 0.05$). A significant effect of lupin type was observed upon Mg retention ($P = 0.01$) with the yellow lupin treatments displaying lower retention of this mineral; no significant effect of SYN or SYN-lupin interaction was identified ($P > 0.05$). No significant effect of lupin type, SYN inclusion or interaction between the two dietary variables was identified in Zn retention ($P > 0.05$).

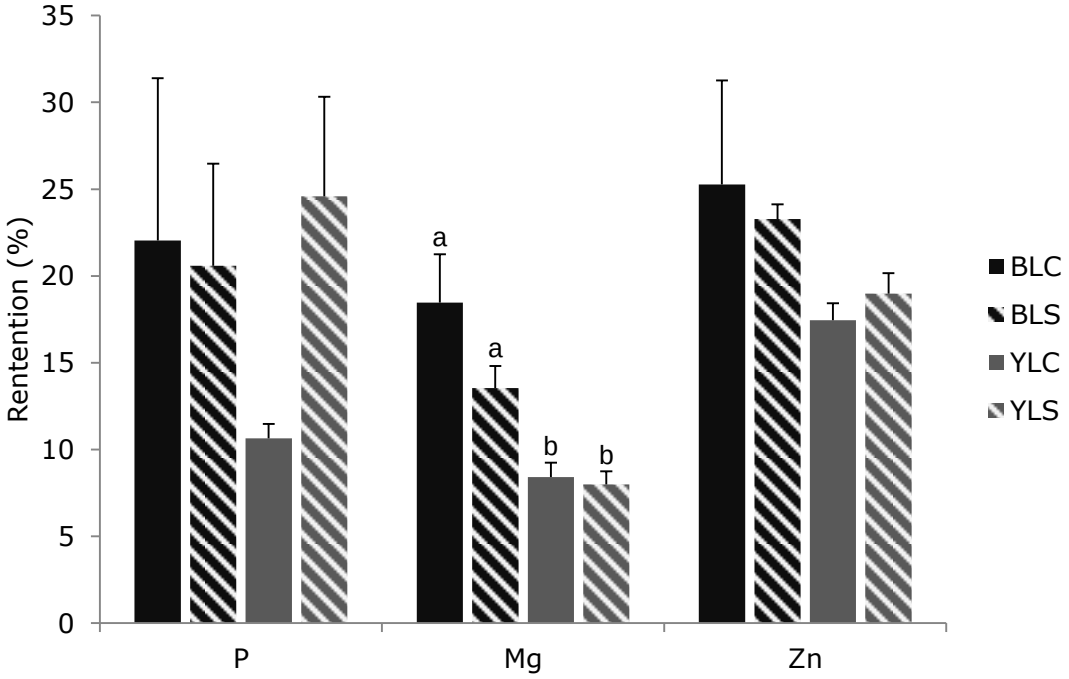

Figure 3.1
Phosphorous, magnesium and zinc retention within the carcass of the experimental Nile tilapia (mean + S.E. *n*=2). Solid black bar = BLC, dashed black bar = BLS, solid grey bar = YLC, dashed grey bar = YLS. Bars with the same suffix are not significantly different (based upon the lupin type variable), no suffix denotes no significant difference.

3.3.3 Haematology and serology

At day 49 of the trial, no significant differences were observed in haematocrit (% PCV) ($P > 0.05$), haemoglobin (g/dl) ($P > 0.05$), blood glucose (mg/dl) ($P > 0.05$) or serum lysozyme (U/ml) ($P > 0.05$). Results of haematological and serological analyses are displayed in Fig 3.2.

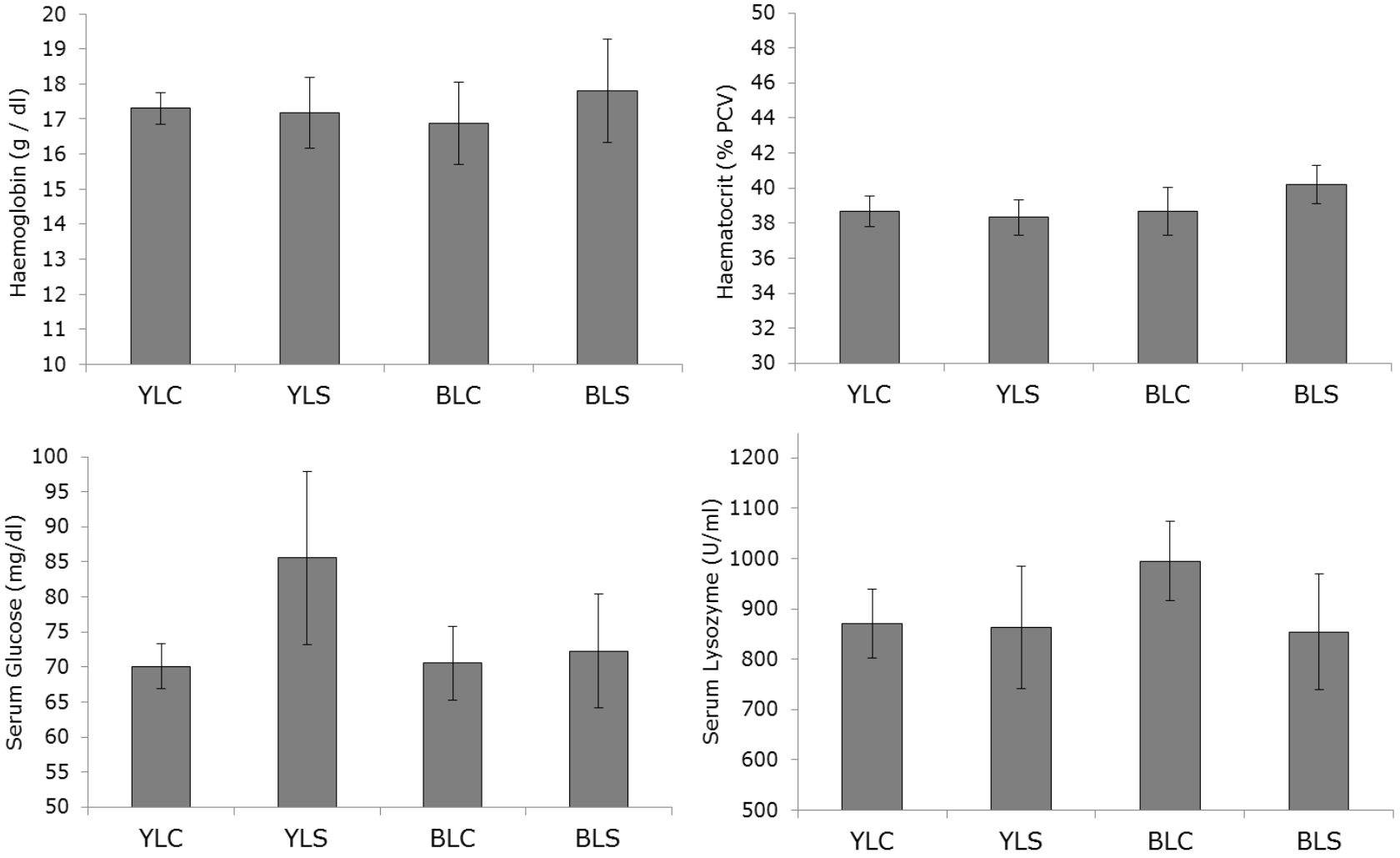

Figure 3.3. Haematological and serological parameters of the experimental Nile tilapia at day 49 (mean + S.E. *n* = 6).

3.3.4 Intestinal morphology

Graphical representation of average enterocyte heights (EH) over the trial period are displayed in Fig 3.3A. No significant effect of lupin type or SYN inclusion alone was identified upon ranked EH over all of the time points. However, time was observed to significantly affect ranked EH ($P = 0.02$), with the overall ranks at day 26 significantly differing from day 0 ($P = 0.01$) and likewise, ranks at day 49 differing from day 0 ($P = 0.01$) (not graphically presented). Within the controls, EH ranks were observed to not be significantly different between days 0 and 26 ($P > 0.05$), however, the values were significantly different between days 26 and 49 ($P = 0.03$) and days 0 and 49 ($P = 0.01$). With the SYN diets, a significant difference in EH ranks were observed between days 0 and 26 ($P < 0.01$) and days 26 and 49 ($P = 0.02$), however, no difference was observed between days 0 and 49 ($P > 0.05$). Furthermore, a significant interaction between SYN inclusion and time was observed upon EH ranks ($P = 0.01$). The controls displayed significantly lower EH ranks than the SYN treatments at day 26 ($P = 0.02$), whilst the trend was significantly the inverse at day 49 ($P = 0.04$).

Graphical representation of intestinal perimeter ratios (PR), over the trial period, is displayed in Fig 3.3B. No significant effects of dietary ingredients alone, or as interactions, were observed upon PR ($P > 0.05$). Time was observed to significantly affect this grouped parameter alone ($P < 0.01$), between days 0 and 26 ($P < 0.01$) and consequently days 0 and 49 ($P < 0.01$). No difference was detected between the overall values recorded between days 26 and 49. Significant effects of time upon PR appeared independent of lupin type and SYN presence. However of note, a near significant interaction between lupin type and SYN presence was detected ($F = 2.63$, $P = 0.08$), as a consequence of values observed within the yellow lupin diets.

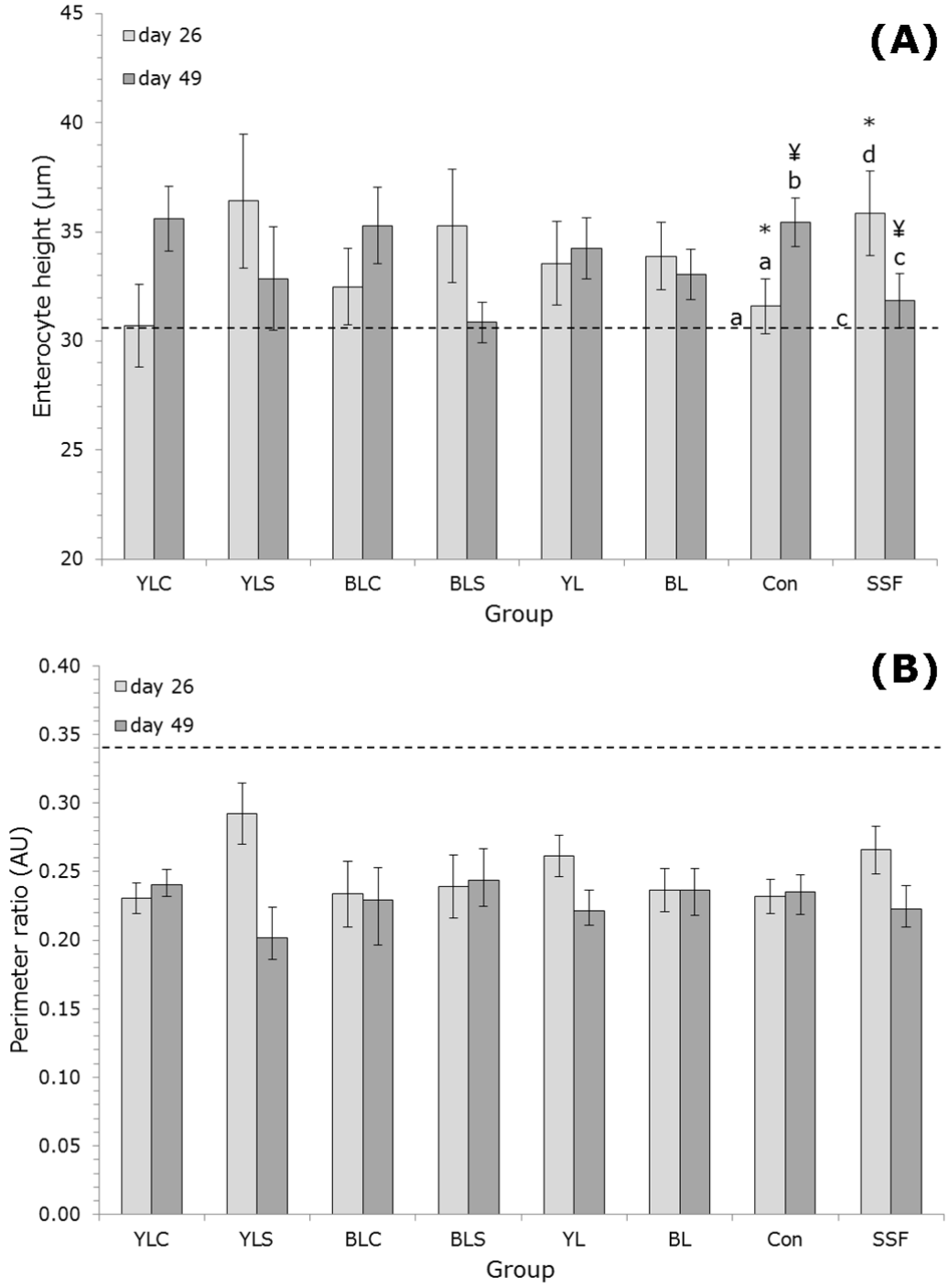

Figure 3.3
Midgut macrostructural parameters of Nile tilapia fed diets containing lupins, with and without inclusion of Synergen™; displaying independent dietary treatments (*n* = 6) and grouped variables (*n* = 12).
(A) Enterocyte height (µm). **(B)** Perimeter ratio (PR). Values are displayed as mean ± S.E. *Dashed line* = day 0 (*n* = 6).
YLC, YLS, BLC, BLS = independent dietary treatments; YL = grouped yellow lupin diets, BL = grouped narrow-leaf lupin diets; Con = grouped control diets, SSF = grouped SSF-containing diets. Same *letter* superscripts (a, b, c, d) in the same group are not significantly different ($P > 0.05$). Bars of the same time point displaying * or ¥ are significantly different from one another ($P \leq 0.05$).

Table 3.4
Ultrastructural measurements of Nile tilapia midgut after 49 days of feeding diets containing lupins, with and without Synergen™.

	Diet					
	YLC	YLS	BLC	BLS	Sig.	Interaction
MVCT (/μm^2)	50.13 ± 5.00	49.78 ± 10.56	52.28 ± 9.74	54.75 ± 7.66	NS	-
MVD (μm)	0.12 ± 0.00	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.00	*P* = 0.03	Y vs NL
MVH (μm)	1.12 ± 0.12	1.25 ± 0.08	1.30 ± 0.11	1.21 ± 0.12	*P* = 0.03	C vs C
TAS (μm^2 / 1μm^2)	21.60 ± 1.78	24.77 ± 3.80	24.91 ± 3.82	24.56 ± 2.19	NS	-

Abbrevations: Y = grouped yellow lupin treatments; NL = grouped narrow-leaf treatments; C = control diet. All values expressed as mean ± S.D. (n=6). Statistical test: Two-Way ANOVA (lupin type x SYN presence).

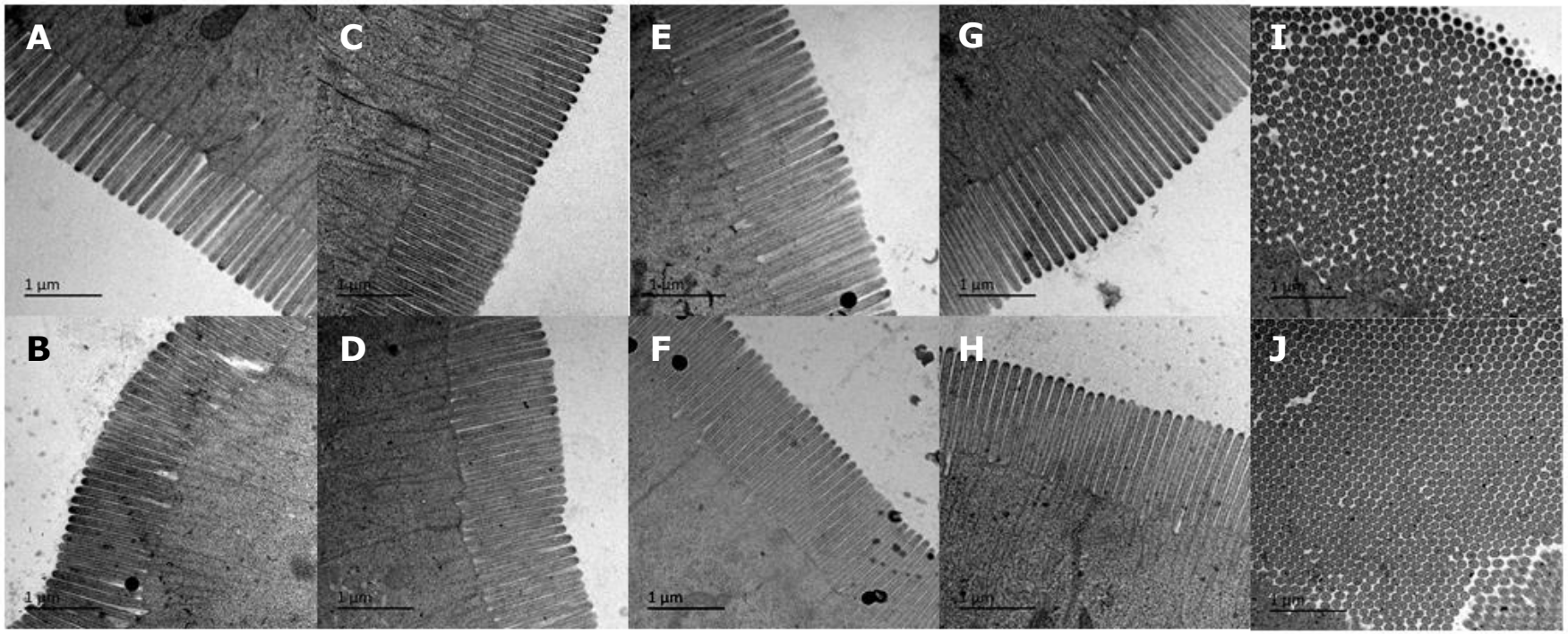

Plate 3.3
TEM micrographs of Nile tilapia midgut brush border after 49 days of feeding diets containing lupins, with and without Synergen™. A & B = YLC; C & D = YLS; E & F = BLC; G & H = BLS; I = YLC; J = YLS. Scale bars all display 1 μm.

Results of the ultrastructural appraisal of midguts taken from the experimental animals are displayed in Table 3.4, with micrographs exhibited in Plate 3.3. No significant effects of lupin type, SYN presence or an interaction between the two variables were observed upon microvilli counts (MVCT) ($P > 0.05$). Microvilli diameter (MVD) was observed to be significantly affected by lupin type alone ($P = 0.03$), with significantly highest MVD in the yellow lupin diets ($P = 0.03$). No effect of SYN inclusion alone or through an interaction between the ingredients was detected ($P > 0.05$). No significant effect of grouped lupin type or SYN presence alone was observed upon Microvilli height (MVH) ($P > 0.05$). However, a significant interaction between lupin type and presence of SYN was detected ($P = 0.03$), the difference was detected to lie within the control diets, in the absence of SYN, with significantly lower MVH in YLC than BLC ($P = 0.01$). There was no significant difference between the diets containing SYN ($P = 0.53$). To note, a near significant difference was detected between the YLC and YLS treatments ($P = 0.06$). No significant effect of lupin type, SYN presence or an interaction between the two dietary variables was observed upon estimated total absorption surface area (TAS), at the ultrastructural level ($P > 0.05$).

3.4 Discussion and conclusion

In the present study, comparison between the lupin basal diets seems to suggest that there was no significant effect of lupin kernel meal (LKM) type on performance. This appears to be in correspondence with findings by Glencross *et al*. (2006), whom did not observe any difference in performance between rainbow trout fed a yellow (cv. Wodjil) lupin protein concentrate (LPC) or a narrow-leaf (cv. Gungarru) LPC. A later study by Glencross *et al*. (2011), again in rainbow trout, similarly did not detect any significant difference between yellow (cv. Wodjil) and narrow-leaf (cv. Myallie) LKM. However, it must be noted that effective cross-cultivar comparisons can be particularly problematic as agronomic activities can alter nutritional traits either at the breeding or cultivation

stage. Demonstrating this, inclusions of different narrow-leaf lupin cultivars in the diets of tiger prawn (*Penaeus monodon*) resulted in differences of FCR between prawns fed the different cultivars (Smith *et al.*, 2007). Salini and Adams (2014) similarly reported significant differences in FCR between white lupin (*L. albus*) cultivars and tendencies towards differences in narrow-leaf cultivars in Atlantic salmon. In an Ethiopian cultivation study, Yeheyis et al. (2012) identified a significant interaction between growing location and cultivar in a number of nutritional parameters. The present study utilised lupins grown in the U.K., conversely to the vast body of information which exists upon the utilisation of lupins grown in Oceania and to a fair extent central Europe. Nonetheless, in the study by Salini and Adams (2014) which investigated a number of white, narrow-leaf and yellow lupin cultivars, in the diets of Atlantic salmon, it was reported that the FCR of fish fed the only yellow LKM cultivar, Pootalong, was higher than all other dietary treatments and significantly greater than the narrow-leaf LKM cultivar, Jindalee. This appeared to show that Pootalong was of the most inferior nutritional quality. The YLC diet in the present study, also of the cultivar Pootalong, expressed numerically inferior SGR, FCR and PER compared to the BLC diet, therefore there may be a tendency towards correspondence with the findings of Salini and Adams (2014).

Following inclusion of Synergen™ (SYN), the fish displayed significant improvements to their overall weight gain, final weight and SGR, in both lupin varieties. These findings were supported by strong tendencies towards reduced FCRs which were reflected in a near significant difference. The lack of corresponding significance in FCR parameters is suggested as attributable to low replication of the study paired with the dampening of numerical differentiation in this parameter. The greatest improvements to performance were observed in the SYN-supplemented yellow lupin diet. Contrary to these findings, a previous study investigating the supplementation of a similar product (Allzyme®-SSF) in narrow-leaf lupin diets for swine, revealed no effects on growth performance and was attributed to a lack of enzyme-NSP specificity by the authors (Kim *et al.*, 2011). However, multi-enzyme supplementations in yellow lupin meal diets for poultry

(Olkowski *et al*., 2010; Olkowski, 2011) and swine (Kim *et al*., 2008) have been observed to promote performance in a manner which corresponds with results in the present trial. No statistically significant differences in PER were found among the dietary parameters. However, the effect of SYN on this parameter was observed to be close to significance. Observation of the numerical trends seems to display a marked increase in PER of SYN fed fish, again particularly with regards to the yellow lupin diet. In correspondence with these results, Allzyme®-SSF was observed to improve protein efficiency in a practical broiler formulation (Yadava *et al*., 2009), indicating that the solid-state fermentation process can impart residual bioactivity capable of improving the animal's efficacy in converting dietary protein to body mass. From the results of this investigation, there is strong indication that SYN altered the nutritional characteristics of the diets in some way, once ingested. Due to differences in the overall nutritional profiles of yellow and narrow-leaf lupins and the multi-functional potential of SYN, a number of nutrient categories can be explored in an attempt to identify probable causes of the observed results.

In nutritional studies, focus is typically turned to crude protein content and amino acid profile but it is of worth to assess protein families when they are particularly distinct from those encountered in the norm or when diets are formulated to be isonitrogenous and amino acid profiles are unavailable, as is the case in this study.

The composition of lupin protein is characterised by its main seed storage proteins; these are classified as α, β, γ, and δ-conglutins. A recent study by Foley *et al.* (2015) determined that the vast majority of conglutins in yellow lupin (cv. Pootalong) belong to the δ family; whilst the dominant conglutins in narrow-leaf lupins appear to be those belonging to the β family, followed by δ-conglutin. Expression of δ-conglutin in cv. Pootalong appears to be over double that of narrow-leaf cultivars (Foley *et al*., 2015). A recent *in vitro* assessment of yellow lupin (cv. Wodjil) seed meal identified the presence of a δ-conglutin with particularly low-digestibility when faced with a digestive enzyme extract from Atlantic salmon (Ogura *et al.*, 2013). Specifics of why this protein is of low-

digestibility remain a question at this time as optimal conditions required for effective degradation of δ-conglutin, as a whole, are yet to be rigorously investigated. However, it is possible that a greater concentration of δ-conglutin could impair protein availability in diets containing yellow lupin. Therefore, this could be a contributing factor to the poorer performance and PER of fish fed the YLC diet.

The most studied lupin conglutins are those belonging to the γ family, this is due to potential anti-hyperglycaemic properties for humans (Schiarea *et al.*, 2013). Glycosylated γ-conglutin possesses a saccharide chain which promotes return to a folded, trypsin-resistant conformation following encounter of acidic surroundings (Duranti *et al*., 1995). In addition to this, it was later proposed that γ-conglutin is not liable to any proteolytic action above pH 4.0 and exhibits "all or none" degradation (Capraro *et al.*, 2009). Further evidence now suggests that γ-conglutin is resistant to both trypsin and pepsin; whilst proteolytic degradations of conglutins are collectively dominated by chymotrypsin (Czubinski *et al*., 2014). Nile tilapia have a limited stomachal capacity and residence time, predominantly relying upon protein digestion at the proximal intestine, under alkaline pH conditions (Uscanga *et al.*, 2010; Hlophe *et al*., 2014). As such, capacity to effectively degrade lupin conglutins may be slightly limited. Deglycosylation, to cleave the problematic saccharide side-chain, of γ-conglutin can be achieved through the action of *N*-glycosidase (Duranti *et al*., 1995) but at this time, it is not possible to determine whether this enzyme was present at the critical stage when catalysis could occur. Concentrations of γ-conglutin appear to be slightly higher in yellow lupin but it must also be noted that the γ family appears to be the smallest conglutin fraction in both species investigated (Foley *et al*., 2015).

Explanation of the results observed on the basis of protein degradation is possible; indeed, similar SSF processes have reported proteases as present in the final functional additive (Passos *et al*., 2015). However, the wheat bran substrate is low in protein so the operational fungus species, *Aspergillus niger*, is likely to produce a limited capacity for continued fermentation of this nutrient class. Due to the predominantly fibrous

nature of wheat bran, a large proportion of residual bioactivity is likely to be specific to carbohydrate fractions. Therefore the influence of residual carbohydrases can be considered as an explanation of the observed results. Observations have indeed indicated that all lupin conglutins are glycosylated, containing mannose, galactose and glucosamine residues (Eaton-Mordas and Moore, 1978; Ferreira *et al*., 1995). Thus, even though lupin-derived protein is generally considered to be of relatively high digestibility to fish (Glencross *et al*., 2003, 2005, 2006, 2008) there is theoretical potential for a significant facilitation of degradation via carbohydrase action within the digestive system. This could be considered for both glycoprotein and carbohydrate fractions alike.

Generally, carbohydrate utilisation by fish is poor and this is also true of tilapia, in a commercial context, despite its omnivorous nature (Shiau, 1997; Lin *et al.*, 1997). Instead, carbohydrates may impart undesirable characteristics upon the GIT and its contents, classifying them as ANFs. These regularly arise from increased viscosity of the digesta as discussed throughout Sec. 1.3.2. Extensive reviews of carbohydrate utilisation have been published by a number of authors (Krogdahl *et al*., 2005; Hemre *et al*., 2002; Stone, 2003a), the general consensus affirming that complex carbohydrates are grossly unavailable outside of strictly herbivorous fish but lower molecular weight carbohydrates (i.e. mono- and disaccharides) tend to be readily available. If low molecular weight carbohydrates become available to the animal then they may be utilised as metabolisable energy, reducing the cost of gluconeogenesis (Cowey *et al.*, 1977). Ultimately, the non-carbohydrate carbon substrates previously utilised for glucose synthesis, notably proteins, are spared for growth. Carbohydrate induced protein-sparing has been widely acknowledged as effective in a vast number of finfish species, throughout the trophic feeding levels; these include turbot (*Scopthalmus maximus*) (Zeng *et al*., 2015), *Solea senegalensis* (Guerreiro *et al*., 2014), Brook trout (*Salvelinus fontinalis*) (Amin *et al*., 2014), *Dentex dentex* (Perez-Jimenez *et al*., 2015) and Nile tilapia (Azaza *et al*., 2015), to name but a few. Furthermore, glucose absorption can be impaired by dietary fibre (Shiau *et al*., 1989), so vigorous degradation of fibre could

accentuate the absorption of monosaccharides by the fish. Blood glucose levels were examined as a possible indicator of carbohydrate degradation and monosaccharide uptake; however, no significant differences were detected between lupin types, nor were any effects observed following SYN application. However, the methods implemented must be scrutinised and questioned whether definitive. Blood samples were collected from fish following a fasting period of approximately 24 hours, in accordance with facility husbandry and welfare guidelines. Although glucose turnover in fish is low in comparison with mammals and birds (Polakof *et al.*, 2012), the post-feeding delay may have presented sufficient time for restoring baseline blood glucose levels. It has been reported that omnivorous fish achieve this much more rapidly than obligate carnivores (Legate *et al*., 2001). It may also be of consideration that the blood glucose lowering peptide, γ-conglutin, could play a role in maintaining blood glucose levels in lupin-fed fish. Nonetheless, previous work on yellow lupin applications in turkey feeds similarly did not detect any alteration to serological parameters (Krawczyk *et al*., 2015)

It is also important to consider that a degradation of fibre fractions could promote the digestive availability of protein, as has been observed following supplementations of β-glucanase and xylanase in rainbow trout (Dalsgaard *et al.*, 2012), as well as β-mannanase in swine (Lv *et al*., 2013; Ao *et al*., 2010a; Wang *et al*., 2009) and broilers (Li *et al*., 2010). Glencross *et al*. (2003) examined the influence of lupin oligosaccharides on nutrient digestibility in rainbow trout, concluding that removal of these components by α-galactosidase, or ethanol extraction, significantly improves N digestibility and that monosaccharide derivatives of this process are readily absorbed by the animal. Galactosidase has been reported as present following SSF of wheat bran by *A. niger* (Passos *et al.*, 2015), thus there is a strong possibility of such an occurrence during this study. Overall, improved growth and tendencies towards improved PER would be strongly supported by the theories of protein-sparing and increased digestibility of protein through carbohydrate degradation.

In the context of discussed results, it is of importance to consider and assess why improvements to performance appeared to be greatest following SYN supplementation in the yellow LKM diet. Yellow LKM typically contains a greater carbohydrate concentration than other narrow-leaf LKM. Bahr *et al.* (2014) reported that the dehulled seeds of the narrow-leaf lupin cultivars Probor, Borlu, Boregine and Vitabor contain 80, 61, 88 and 87 g/kg of carbohydrate, respectively, whilst the yellow lupin cultivar, Bornal, contains 104 g/kg of carbohyrdate. Additionally, yellow lupin contains around double the total concentration of oligosaccharides than other commercially cultivated lupins (Kasprowicz-Potocka *et al.*, 2013; Glencross *et al.*, 2004a); in particular, raffinose family oligosaccharides, including α-galactosides, are more predominant in yellow than narrow-leaf lupin (Pisarikova and Zraly, 2009; Gdala, 1997). Potential for carbohydrase activity is therefore greater in yellow LKM, which would correspond with why improvements were greatest following SYN partnership with this ingredient.

Lastly, it is worth considering that a supplementary influx of available minerals and trace elements could promote growth and development. Carcass mineral concentrations firstly indicate that concentrations of Ca, P, Mg and Zn were higher in fish fed narrow-leaf lupin, which cannot be explained by the nutrient concentrations within the diet. Supporting these observations, magnesium retention was significantly lowest in fish fed the yellow lupin diets, whilst Zn was numerically lower. Such was also the case with P in the YLC treatments compared to the Narrow-leaf diets. This would seem to suggest that availability of macro minerals and trace elements is greater from the narrow-leaf diets, regardless of SYN presence. Phytate concentration tends to be far higher in yellow lupin than narrow-leaf lupin (Rutkowski *et al.*, 2015) and likewise overall ash content is greater (Salini and Adams, 2014; Bahr *et al.*, 2014). The presence of this anti-nutrient causes P and other minerals to be bound in an unavailable state, which could explain the trends observed. The supplementation of SYN in the narrow-leaf LKM diet appeared to display negligible effects. However, following SYN supplementation in the yellow lupin diet, observational trends appear to show an increase in carcass Ca, P, Mg and Zn. A

SYN presence within the yellow lupin diet appeared to particularly influence the availability of P as retention of this mineral was noticeably augmented, to a level similar to that of the narrow-leaf lupin diets. An action of exogenous phytase, derived from SYN, could explain the discrepancies between the YLC and YLS diets; although it is perhaps surprising that the same trend was not observed in Mg and Zn, which is a commonly associated effect (Debnath *et al*., 2005c; Laining *et al*., 2012; Vandenberg *et al*., 2012). Assuming that differences in phytate concentration correspond with the scientific literature, there is likely to be a more restricted capacity for improvement with phytase action upon the narrow-leaf lupin nutrient complex. One may also consider that elevated NSP content could impair absorption of minerals so there may be a more restricted capacity in the yellow LKM diet. Interestingly, phytase has also been demonstrated to improve the degradation of lupin conglutin, which could inter-tie with previously described results (Moura *et al.*, 2013). Overall, despite indications of mineral liberation and absorption, this area remains unclear. In order to counteract difficulties in working with whole carcasses of teleost fish, this topic should be revisited using new approaches which can determine the bioavailability of minerals within the gut and deposition within individual tissues.

The intestine plays a critical role in digestion, nutrient assimilation and to some extent pathogen defence. Anti-nutritional factors may negatively impact its integrity, thus hindering the before mentioned processes; whilst increased nutrient profile may substantially aid in its morphology and function. For these reasons, integrity of the GIT is a good indicator of nutritional status and overall health. To date, a limited number of investigations have been conducted upon the influence of lupin meals on gut morphology and so their effects on this vital organ are yet to be clarified. Smulikowska *et al.*, (2014) reported that increasing levels of both yellow and narrow-leaf lupin caused detrimental effects to the potential absorptive area of the intestine in broilers. On the contrary, the inclusion of LKM in finfish diets has seemingly revealed little to no effect upon gastrointestinal histology, in a number of species (Omnes *et al*., 2015; Borquez *et al*.,

2011; Refstie *et al*., 2006; Glencross *et al*., 2006; Glencross *et al*., 2004b). This appears to also be the case when diets are experimentally dosed with lupin alkaloids (Serrano *et al*., 2012; Serrano *et al*., 2011). Although lupins appear to show no pathological deterioration of gut integrity in fish, to the author's knowledge, no known studies have examined tissue at an ultrastructural level. Furthermore, whether gut morphology can be modulated through exogenous fermentation sources in lupin-containing aquafeeds seems unexplored.

Time was identified to induce a significant change to EH. It appears that the dietary transition of the animals collectively from a commercial high protein/energy and low ANF diet to high-lupin diets increased EH after initial feeding. It is perhaps to be expected that such a dramatic dietary shift would display some form of alteration to gut structure, however numerous previous studies reported no marked differences between lupin and fishmeal-based diets (Omnes *et al*., 2015; Refstie *et al*., 2006). However distinct trends in EH, relating to the presence or absence of SYN, were identified; demonstrating that ubiquitous increases in EH were not common of all the dietary treatments throughout the time points and that SYN may have an influence on gut morphology. Whilst the control diets displayed no marked alteration to EH until day 26, significant enlargement of enterocytes was indicated thereafter. Most notably, the YLC treatment appeared to retain a near identical EH to fish sampled at the initial time point. This is perhaps surprising when we consider that until present, the YLC appears to perform at a lower standard than the other dietary treatments, yet EH was similar to that of fish fed a diet with very low ANF content and a nutrient profile which exceeds requirements. Meanwhile, the SYN-fed fish collectively displayed significant increases in EH, by approximately 5 µm, between day 0 and 26. However, this measurement was identified to return to a level which was not significantly distinguishable from those observed at day 0 by day 49. Histomorphometric appraisal of enterocytes within the scientific literature appears to show that enterocyte-related characteristics are highly susceptible to change, in response to nutritional status. Salmonids have been reported to display reductions in

enterocyte size following starvation (Shaibani *et al.*, 2013). This can similarly occur following more minor restrictions in nutrient profile, such as augmentation of soyabean meal inclusions (Peng *et al.*, 2013). Godlewski *et al*. (2006) demonstrated that soaking and boiling of soyabean meal, to reduce ANF content, increased the proliferation of enterocytes within the gut of rats. Further, this process was observed to mitigate the rate of programmed cell death under the presence of raw soya inclusions (Godlewski *et al.*, 2006). Functional feed additives have also been observed to modulate enterocyte size and proliferation, although they tend to become somewhat contradictory. For example, dietary nucleotides, which are recognised as highly beneficial to gut development and tissue repair in mammals, have been demonstrated to increase EH in red drum and turbot (Cheng *et al*., 2011; Peng *et al.*, 2013). Conversely, supplementary mannan-oligosaccharides (MOS), a widely acknowledged prebiotic, have been demonstrated to result in lower enterocyte heights in sharpsnout seabream (*Diplodus puntazzo*) (Ferrara *et al*., 2015). The production of oligosaccharides by SYN within the gut has already been discussed as a probability, thus results presented by Ferrara *et al*. (2014) seem to correspond with those recorded under the current investigation.

The present study provides a more in-depth quantitative appraisal than previous studies in the manner in which information on enterocyte height was collected prior to and during the feeding trial, which revealed developmental progressions, rather than a final suggested change. These fluctuations are proposed as a crude indication of dietary adaption; this could be due to nutrient provision and possibly establishment of equilibria within the gut microbiome. In terrestrial monogastrics, some studies have indicated that exogenous enzymes can liberate sufficient bioavailable nutrients to trigger enhanced performance without effect upon epithelial parameters (Amerah *et al*., 2008; Owens *et al*., 2008). Conversely, the results of this study indicate that exogenous facilitators of fermentation within the gut do elicit an effect upon epithelial cells and they demonstrate effectively that time is a critical factor in confidently identifying whether additives of this kind modulate morphology. Unfortunately, due to a lack of consistency over two time

points, it is not possible to confidently conclude at which level EH would be maintained in each treatment over time.

Perimeter ratios of the midgut (PR) were observed to change dramatically from the initial measurements in the first half of the investigation. This information indicates that the functional border of the midgut, in all treatments, increased in surface area after the feeding of the commercial diet was ceased and the animals were fed the plant protein based diets. This is of interest in itself as greater surface area is routinely considered to be a beneficial characteristic but it appears it may not be indicative of an optimal dietary formulation. By day 26, all treatments were almost indistinguishable in their relative gut surface area. This was maintained, by and large, until day 49 in the YLC, BLC and BLS treatments. However the continuation to day 49 saw YLS-fed fish display numerically greater surface area than the other treatments and a significant increase from the recorded surface area at day 26, suggesting an increasing occupation of functional brush border within the lumen. This seems to correspond with findings by Olkowski *et al*. (2010), who detected significant elongation of villi in the intestine of broilers fed NSPase-supplemented diets, based upon yellow lupin. Increases in absorptive area have similarly been observed through dietary inclusion of low molecular weight oligosaccharides intended as prebiotics (Dimitroglou *et al.*, 2009; Anguiano *et al*., 2013), which would correspond with speculation of the hydrolysis of NSPs in this study. The results are further supported by reports of increased intestinal surface area via multi-enzyme supplementations in poultry (Wu *et al*., 2004; Zhu *et al*., 2014). Within this study, the greater functional surface area following feeding of SYN supplemented yellow LKM is considered a positive improvement as it indicates a greater potential for the digestion and absorption of nutrients, corresponding with performance. Coupled with a reduction in enterocyte height, it is suggested that the observations of the YLS group could indicate accelerated proliferation and migration of epithelial cells. However, this trend is somewhat contradictory in the BLS group for reasons which remain unclear.

Lupin type appeared to have a significant influence upon microvilli diameter under the present conditions, whereby microvilli were observed to be thicker in the yellow lupin treatments. This suggests that the microvilli would be less densely packed within the fish fed yellow LKM diet, which to an extent is supported by the observed trends in microvilli counts (MVCT). Therefore, it appears that in this instance, SYN supplementation in the yellow LKM diet was not capable of eliciting a positive response in the density of microvilli. Microvilli height (MVH) was similarly affected by lupin type however this occurrence was only present when the control diets were isolated from their respective SYN diets. The yellow LKM resulted in a depreciation of positive ultrastructural morphology compared with the narrow-leaf LKM. Once again, considering that MVH within the YLS treatment had a tendency towards being similar to that of those expressed in the narrow-leaf treatments, there is strong indication that SYN within the yellow LKM diet exerted a beneficial effect upon ultrastructure whilst these effects were somewhat negligible in the narrow-leaf LKM diet. These results seemed to be expressed in trends towards improved absorption surface area (TAS) in the yellow LKM diet with SYN, with no noticeable difference with the narrow-leaf LKM. Promotion of this characteristic is once again highly associated with prebiotic oligosaccharides, such as MOS and β-glucans in aquatic species (Daniels *et al*., 2010; Dimitroglou *et al*., 2010; Kuhlwein *et al*., 2013). Overall, results of the present study appear to suggest that alterations to the gut content did occur, particularly with regards to the supplementation of SYN in the yellow LKM diet. Such changes are highly attributable to alterations in the environment within the lumen, specifically modulation of the bioavailable nutritional profile of digesta.

It is widely apparent that many of the histological observations noted in this study are common with effects observed following direct prebiotic supplementation or as a result of dietary additives which may hydrolyse polysaccharides into lower molecular weight carbohydrates. This being the case, modulation of the gut microbiome is highly

anticipated and should be visited for both confirmation and identification of possible health benefits.

From observed improvements particularly to animal performance and gut morphology, it can be concluded that SYN seems to be an effective promoter of nutritional profiles in finfish diets. However, much further work is required to determine exactly how SYN promotes performance. Although it is speculated that hydrolysis of carbohydrates is the most probable explanation, a lack of quantified nutrient digestibility and mineral bioavailabilities makes it difficult to unravel explanations regarding specifically which nutrient hydrolyses promoted the growth of SYN-fed fish. Furthermore, interactions between the additive and the animals digestive function remain unclear considering this comprises largely of three key components; morphology, enzymes and microbiome. To understand the processes occurring within the gastro-intestinal tract following the presence of SYN, a more in-depth appraisal of each of these factors is required. It is suggested that the use of yellow lupin is pursued, as this partnership appeared to produce an accentuated effect in the present study. Relating to commercial application, the higher anti-nutrient content of yellow LKM lends itself as particularly appropriate for bioremediation by functional feed additives, all whilst the higher protein content makes it more desirable to the feed industry. It is also proposed that the use of more dietetically-sensitive species, i.e. a carnivore, could facilitate the identification of contributing factors, as dietary intolerances become more pronounced and reversed. A species of this kind is also likely to be a more cost-effective candidate if application of the experimental ingredient is successful, as a higher market value allows added expenditure on feed additives. Continuation of this work will aim to explore these key considerations and hypotheses in Chapter 4.

CHAPTER 4. Application of Synergen™ in the diets of rainbow trout containing high yellow lupin inclusion

4.1 Introduction

4.1.1 Salmonid culture

Salmon, trout, char, grayling and freshwater whitefish (subfamily - Coregoninae), comprise the Salmonidae; a family of teleost fishes possessing a characteristic adipose fin and all breed in freshwater but extensively express anadromous life-cycles. There is uncertainty as to the true origins of salmonid culture but the crude widespread practice began to form at the turn of the 20th century (FAO, 2016; British Trout Association, 2015; Hardy *et al*., 2000). Being highly prized game animals, breeding programmes were initially developed for restocking or introduction to rivers for the purpose of sport fishing. These earliest reports of salmonid farming involved collection of wild seed and began to pave the way in understanding the general husbandry requirements of trout, salmon and char. Proceeding the 1950s, the first commercial trout farms, employing full life-cycle cultivation, became established and the popularity of these livestock as an accessible table fish soared (British Trout Association, 2015). Naturally, developments in genetic strains, feeding practices and production technologies quickly followed suit; this led to the complex nature of operating procedures and equipment which we see today.

Atlantic salmon (*Salmo salar*) production is undoubtedly the greatest development to date; global production was approximately 1 tonne in 1964, whilst by the end of 2013 this figure stood at over 2 million tonnes (FAO, 2016). Norway is the world's biggest producer of Atlantic salmon, followed by Chile and Scotland, all of which now depend greatly upon salmon farming for the overall stability and prosperity of their economies (FAO, 2016). Production of Atlantic salmon is also practiced in a number of other nations including Ireland, Iceland, Canada, the United States, the Faroe Islands and Tasmania. Research and development has been so rigorous in Atlantic salmon farming that a transgenic strain has recently been approved for human consumption by the U.S. Food and Drug Administration (FDA) (FDA, 2015); being the first GM animal to be granted

consent of this kind. This represents not only scientific achievement in salmonid farming but a significant milestone in commercial livestock production as a whole.

Rainbow trout (*Oncorhynchus mykiss*) farming began well before that of Atlantic salmon, with the species being exported over the globe for introduction as a sport fish. Nevertheless, commercial production continues to grow exponentially. Between the same years of 1964 and 2013, production of this species increased from approximately 31 thousand tonnes to approximately 814 thousand tonnes (FAO, 2016). Introduction and farming of rainbow trout has been so widespread that the species is now present on every continent except Antarctica (FAO, 2016). The importance of rainbow trout extends beyond that of the table market as it is still a highly-prized sport fish. In certain regions, for example within the U.K. and U.S.A., numerous rainbow trout hatcheries and grow-out farms are supported more so by restocking of natural river systems or dedicated angling waters than the table market itself. Farming of sport fish demands an equally healthy and robust animal. Moreover the pressure of producing aesthetically attractive animals is arguably more so than that of the table market; this is because blemishes and deformity, e.g. fin abrasions, shortened opercula and skeletal malformations, may not be disguised by processing techniques (e.g. filleting). Lastly, the species serves extensively within research as it is viewed as a general salmonid model; therefore the species is a centre pin to advancing knowledge in the culture of salmonids. Clearly, despite its more minor contribution as a food fish, compared to Atlantic salmon, rainbow trout represent a formidable pillar in salmonid aquaculture.

Other salmonid species, for example brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*), Arctic char (*Salvelinus alpinus*) and Chinook salmon (*Oncorhynchus tshawytscha*) are also cultured in various global locations. Albeit minor in terms of comparative current production volume, they still remain financially bountiful within niche markets and may continue to show promise for future development.

From the very true founding of the industry some 50 years ago, to one of the most lucrative forms of finfish farming today, salmonid production is one of the greatest success stories in modern farming. Nevertheless, despite huge scientific and technologically advances over the past decades, many issues threaten to mar the reputation and productivity of salmonid farming; not the least of which, nutrition.

4.1.2 Trends in salmonid diet formulation

Salmonids require energy dense diets consisting of high-grade protein and lipid; this means feeding costs are exceptionally high. Of course, each operation will vary according to its average performance, physical conditions and feed source but feed typically contributes well in excess of 60 % of the total operating expenses; perhaps unsurprising considering 1 tonne of salmonid grow-out diet currently retails between 1000 and 1250 GBP (personal observation).

The fishmeal (FM) and oil (FO) crisis has, and continues, to place tremendous pressure upon the salmonid industry but seen as the animals are naturally carnivorous, reducing dependency upon these ingredients has been a challenge, to say the least. These species require diets containing an amino acid profile similar to that of FM, as well as a correct balance of essential fatty acids (EFA), again similar to that of FO. Utilisation of carbohydrates is highly restricted in salmonids and their over inclusion may result in the increased utilisation of fat as an energy source (Skiba-Cassy *et al*., 2013).

Evidently recreating adequate salmonid diets with plant-derived products has been somewhat problematic. Nevertheless, successes have been achieved to date in reducing inclusion of marine-derived energy sources. Through ingredient manipulation, careful formulation and extensive nutrient supplementation, FM and FO inclusions are steadily reducing. In 1990 feeds used in Norwegian salmon production contained, on average, in excess of 65 % FM, by 2013 this figure stood at just over 18 % (Nofima, 2014). With regards to rainbow trout, this figure has been reduced to around 15 % in many diets.

Following a wealth of scientific research over the past few decades, soyabean products have predominantly filled the space previously occupied by FM, with more minor contributions from the likes of wheat, fababean, rapeseed, sunflower and guar. Bulk ingredient research has now begun to slow down. Attention is now increasingly being turned to solutions such as nutritional programming and genetic selection for carnivorous fish tolerance to plant proteins (Quinton *et al*., 2007a; Quinton *et al*., 2007b; Le Boucher *et al*., 2013; Guerden *et al*., 2013; Overturf *et al*., 2013; Yamamoto *et al*., 2015). This demonstrates that we are reaching a limit in what can naturally be achieved in ingredient selection but we still have not reached desired goals. Quite simply, we have attained much of what can be done with regards traditional feed manufacture and process technologies, now manipulation of the animal is required in order to optimise feed and cost efficiency. Although, a strain of GM Atlantic salmon has been approved, it is unrealistic to assume that advances in genetic manipulation and selection of salmonids will be made at a rate capable of keeping pace with the necessity for immediate dietary shifts. These are lengthy processes involving rigorous research, licencing, extension work and uptake from the sector. It is crucial to understand limitations and their species-specific potency when formulating future solutions.

4.1.3 Sustainability in salmonid nutrition

Thus far in this thesis, the 'three pillars of sustainability' (economics, environment and society) have featured in context of salmonid nutrition and are revised as follows so as to clarify the real necessity for innovative solutions.

To summarise and expand upon economic aspects:

1. Salmonid farming is a lucrative industry driven by a high-quality product, which equally requires high expenditure.
2. Fishmeal continues to decrease in its economic viability and more cost-effective approaches are required to support the increasing volume of feed required.

3. Supplements and additives aimed at combating nutritional risks (e.g. minerals and prebiotics) are costly yet endogenous counterparts are wasted through a lack of bioavailability.
4. Detriment to health and integrity of physiological, morphological and symbiotic characteristics may lead to a significant depreciation in 'economic FCR'.

The author proposes economic FCR to be defined as – *'the biological FCR of a stock (in monetary equivalent), minus the financial loss from individuals with depreciated value before market; due to disease, morphological defects, premature dispatch and death'.*

Summarising environmental aspects:

1. The drive for changes in dietary formulations is in part fuelled by the necessity for reducing dependence upon wild-capture fisheries for FM supply.
2. Excess nutrient loading of the environment has been shown to cause negative impacts upon surrounding ecosystems.
3. Sourcing micronutrients may cause severe detriment to the environment.

Lastly, summarising and expanding upon societal aspects includes:

1. Clients and consumers heavily scrutinise products thus they must be of highest quality.
2. Many welfare issues surround the risk of malnutrition.
3. Damage to recreational resources (e.g. rivers) surrounding salmonid operations mars consumer perception of the industry.
4. We must consider indirect social implications within the supply chain of mineral supplements.

Synergen™ has so far provided indications that many of these factors can be directly or indirectly improved. This investigation aims to test and quantifiable identify whether this SSF product may improve the availability of specific nutrients in rainbow trout feeds containing high dietary inclusion of yellow lupin. If effective, promotion of performance

and feed efficiency is to be expected and increased concentrations of specific nutrients will be present throughout the carcass if levels of these nutrients exceed maintenance requirements. The salmonid intestine is highly sensitive to numerous ANFs (Merrifield *et al*., 2009; Iwashita *et al*., 2009; Gu *et al*., 2014); therefore, degradation of such components will likely result in identifiable morphological differences in gut macro and/or ultrastructure. Furthermore, if the SSF product increases digestive capacity and thus changes the profile of nutrients in the posterior intestine, this will likely be reflected in a modulation of the associated microbial community within. Responses in the fish's own endogenous hydrolytic secretions may also occur. Supplementary availability of nutrients may also influence health and immunological parameters, outside of the intestine and thus these will also be explored. The relevance of such findings will be discussed in the context of the contemporary issues relating to salmonid culture which have been discussed in this introductory section.

4.2 Materials and methods

4.2.1 Experimental design

The feeding trial was designed to contain two phases; these were the initial 10 week nutritional phase (*N*-phase) leading on to a digestibility phase (*D*-phase). The *N*-phase was aimed at determination of performance, whilst the focus of the *D*-phase was quantification of nutrient availability. Sampling of tissues and digesta was conducted at the end of the *N*-phase as well as the *D*-phase. The time-points are specified herein but results will not be presented in chronological order of sampling; rather, in a format which is more lending to ease of data interpretation.

4.2.2 Experimental animals and housing

Both trial phases were conducted at Plymouth University's West Aquarium research facility, within the same system and utilising the same batch of fish throughout. Juvenile

XXX triploid rainbow trout (*Oncorhynchus mykiss*) (~ 20 g), of wild phenotype, were obtained from Exmoor Fisheries (Somerset, UK).

Upon entering the research facility, the fish were acclimatised for 2 hrs. Following this, the fish were stocked into circular 120 L tanks on a ~ 6200 L RAS, powered by a 1.50 hp pump (Certikin HPS150M; Oxfordshire, UK). Throughout conditioning, fish were fed BioMar Efico Enviro (BioMar; DK) at 1-2 % BW per day. Throughout conditioning and the trial, adequate water quality was maintained by biological, drum screen (Aquasonic DF100; AUS) and cartridge (HidroClean 105 µm; UK) filtration, supplied by a 0.75 hp pump (Certikin HPS575M; Oxfordshire, UK) at 20 m^3/hr. Temperature was maintained at 12.5 °C ± 1 via Optipac pool chillers (PSA; FR). Dissolved oxygen was maintained above 90 % with air supplied via low pressure side channel blower (Rietschle Ltd.; Hampshire, UK) to perforated piping below biological media and air stones within tanks. A 12 hrs light : 12 hrs dark photoperiod was implemented with AquaRay LED lights and timers (Tropical Marine Center; Hertfordshire, UK).

A 2 week elevation in salinity (max. 5 ppt), coupled with 2 weekly salt baths (35 ppt, 10 min) and 2 formalin baths (25 mg/l, 30 min), was performed following identification of *Gyrodactylus* sp.. Furthermore, a 7 day course of orally-administered florfenicol (Florocol, MSD Animal Health; Buckinghamshire, UK), at 10 mg/kg BW per day, was implemented as a routine precautionary measure. Clinical examination detected no ectoparasites, or clinical symptoms of any other pathogenic threats, for 10 days prior to *N*-phase commencement.

Following the conditioning period, the fish were graded by size and visual condition. For the *N*-phase, selected fish were stocked into quadruplicate tanks of 37 individuals (n = 4). Average initial fish weight was 43.58 g ± 0.41, corresponding to a stocking density of 13.44 kg/m^3 ± 0.13. Following the *N*-phase, the fish were restocked into triplicate tanks (remaining in their respective treatments) in groups of 28 individuals, for

commencement of the *D*-phase (n = 3). Average individual fish weight at this stage was 159.57 g ± 12.27, corresponding to 35.90 kg/m^3 ± 2.76.

4.2.3 Experimental ingredients and diets

Four experimental diets were formulated, using FeedSoft Pro™ (TX, USA), so as to satisfy all known nutrient requirements of rainbow trout (NRC, 2011) and be isonitrogenous, isolipidic and isocaloric (Table 4.1). The lupin control diet (LC) was formulated to contain 30 % yellow lupin (*L. luteus* cv. Pootalong), obtained from the same batch described in Chapter 3. Two inclusions of Synergen™ (SYN) were incorporated into the basal mix at the expense of corn starch. The SYN used was from the same batch as previously utilised and presented no notable difference in storage time prior to feed manufacture and feeding, compared with the previous tilapia diets. The experimental inclusion levels of SYN were 0.1 and 0.5 % (LS0.1 and LS0.5, respectively). A FM-based reference diet was also utilised.

4.2.4 Compositional analyses

Analyses were undertaken following methods described in Sec. 2.7 and 2.8.

Table 4.1. Feed formulations, proximate compositions and element concentrations of the experimental rainbow trout diets.

	Diets			
	LC	LS0.1	LS0.5	FMC
Ingredient (g / kg)				
Yellow Lupin	300.00	300.00	300.00	-
Herring Meal [1]	250.00	250.00	250.00	638.622
Soyabean meal [2]	180.71	180.71	180.71	-
Fish Oil [3]	137.15	137.15	137.15	119.97
Corn Starch [4]	70.14	69.14	65.14	229.40
Soya Protein Concentrate [5]	50.00	50.00	50.00	-
Carboxyl-methyl-cellulose [6]	5.00	5.00	5.00	5.00
Vitamin/Mineral Premix [7]	5.00	5.00	5.00	5.00
Ascorbyl-Phosphate [8]	1.00	1.00	1.00	1.00
Yttrium Oxide [9]	1.00	1.00	1.00	1.00
Synergen™	-	1.00	5.00	-
Proximate composition (%)				
Dry matter	99.30	99.41	99.41	99.23
Crude protein	43.07	43.23	43.49	44.01
Crude lipid	20.47	20.81	20.69	19.80
Crude fibre	2.70	2.72	2.77	-
Ash	6.60	6.68	7.05	6.70
Gross energy (MJ/kg)	21.66	21.68	21.63	23.21
Element concentrations				
Ca (g/kg)	78.01	78.31	77.99	157.61
P (g/kg)	80.26	83.79	81.37	136.17
K (g/kg)	104.30	103.98	106.32	64.42
Na (g/kg)	44.55	43.97	43.99	86.67
Mg (g/kg)	17.78	17.78	18.15	13.40
S (g/kg)	38.05	38.22	38.40	54.11
Fe (g/kg)	1.58	1.64	1.67	1.36

Zn (mg/kg)	644.74	639.60	641.12	777.46
Mn (mg/kg)	801.62	812.27	808.34	67.86
Cu (mg/kg)	133.56	122.65	114.89	73.42

[1] LT94 herring meal (CC Moore, UK); [2] HP100 (Hamlet, DK); [3] Epanoil (Seven Seas, UK); [4] (Sigma Aldrich, UK); [5] SPC 60 (BioMar, DK); [6] (Sigma Aldrich, UK); [7] 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); [8] Rovimix (DSM, UK); [9] (Sigma Aldrich, UK).

4.2.5 Feeding regimes

The experimental diets were fed in quadruplicate during the *N*-phase (a total of 10 weeks) and triplicate during the *D*-phase (12 days). Daily rations were determined via a predicted daily growth (PG), based on a standardised FCR of 1.00. Growth predictions were reset with actual weights following biomass sampling.

Predicted growth was estimated as follows:

$$Predicted\ Growth\ (g) = W + (((W / 100) \times FR) / FCR)$$

Whereby; *W* = tank weight (actual or predicted) of previous day (g), FR = feeding rate (% BW (g)) of previous day and FCR = standardised FCR of 1.0.

Feeding was performed by hand, 4 times per day, with fixed rations between 1.0 and 2.5 % BW. The fish were fed a reduced ration (0.0 - 1.0 % BW) on days prior to biomass sampling. The rationale behind fixed rations was to semi-restrict nutrient intake, due to the potential functional properties of SYN.

A specific feeding regime was implemented prior to sampling for endogenous protease activity; the details of this will be presented under Sec. 4.2.14.1.

A feeding rate of 1.5 % BW per day was employed throughout the *D*-phase. The day before *D*-phase sampling, fish were fed to satiation throughout the day in order to maximise extractable faecal quantity. Although it is recognised that this may affect feed efficiency, the rationale behind this regimen was eliminating the necessity for multiple sample collections which can cause substantial stress to the animals and damage intestinal morphology, subsequently impacting results.

4.2.6 Biomass sampling and control (*N*-phase)

The tanks were weighed in bulk on a bi-weekly basis (to 1 g). During week 6, stocking density was reduced from 37 to 25 fish per tank (ave. 28.80 kg/m^3 ± 1.18 to 22.87 kg/m^3 ± 1.04, respectively). This was undertaken on the basis of system carrying capacity constraints, so as to maintain optimum environmental conditions; performance calculations were adjusted accordingly.

4.2.7 Performance calculations

All performance calculations were undertaken as detailed in Sec. 2.9.

4.2.8 Faecal sampling (*D*-phase)

At the end of day 12 of the *D*-phase, the fish were anaesthetised in buffered MS222 (200 mg/L), until loss of equilibria and response to human contact was observed. Manual stripping of faeces was performed by hand, by lightly applying pressure to the hind portion of the abdomen. Faecal material was collected in aluminium trays over ice and pooled by tank. All of the fish were sampled and reintroduced to their respective tanks.

The faecal samples collected were freeze-dried, in accordance with Sec. 2.7.1. Following this, they were manually homogenised with a synthetic pestle and mortar.

Dry matter, crude protein, crude lipid, crude fibre, gross energy and mineral concentrations of feed and faeces were determined in accordance with methods described in Sec. 2.7.1, 2.7.2, 2.7.3, 2.7.4, 2.7.6 and 2.8.2, respectively.

Apparent digestibility/bioavailability coefficient (ADC/ABAC) calculations were performed as detailed in Sec. 2.11.

The FM- and lupin-based diets were not formulated on a digestible protein, lipid and energy basis, nor were they formulated to hold similar mineral profiles. Therefore, the

FMC-fed fish were not stripped of faeces, on ethical grounds, for analysis of macronutrient ADC and element ABAC, due to the foreseeable bias and limited scientific worth.

4.2.9 Whole carcass sampling

Sampling for whole carcass was performed at the end of the *D*-phase with approximately 400 g of pooled wet fish per replicate (n = 3). The whole fish were oven-dried according to procedures detailed in Sec. 2.7.1 and ground using a household blender.

4.2.10 Tissue mineral concentrations

After faecal collection at the end of the *D*-phase, 5 fish per tank were randomly selected and euthanised according to Sec. 2.5. Whole carcasses were frozen at -20 °C until subsequent processing and analyses. The carcasses were thawed prior to dissection. Entire fillets were lifted, skinned and excised below the last rib, of which the hind portion was retained; followed by rinsing in ultrapure water to wash away any blood and scales. Whole livers were separated from other visceral organs, residual connective tissue was removed and the specimens were briefly rinsed in ultrapure water to remove excess blood. The caudal fins were excised from the peduncle and again rinsed in ultrapure water. For samples of bone, vertebrae 31 to 40 (v31, v40) were selected. Flesh was cleared from the bone material with the use of a scalpel after blanching in boiling water.

All tissue samples were freeze-dried according to Sec. 2.7.1; after which, they were pooled by tank (n = 3) and manually ground using a synthetic coated pestle and mortar. The homogenising equipment was thoroughly rinsed in distilled water and dried between each use. ICP OES was implemented to determine element concentrations (Sec. 2.8).

Calcium : phosphorous ratio (Ca:P) was calculated for the vertebrae via the following:

$$Ca{:}P\ (AU) = Ca\ /\ P$$

Whereby; Ca = vertebral calcium concentration (mg/g) and P = vertebral phosphorous concentration (mg/g).

4.2.11 Vertebral histomorphometry

Following faecal collection at the end of the *D*-phase, 3 fish per tank were randomly selected and euthanised according to Sec. 2.5 (n = 3). The carcasses were dissected by removing fillets. The range in numbers of vertebrae, recorded within the population, was 60 to 61. The 47th (v47) and 48th (v48) vertebrae were located. These vertebrae lay within the caudal vertebral portion (i.e. the portion possessing neural and haemal spines), approximately two thirds of the way down, between the first haemal vertebrae and the adipose fin. The selected specimens were separated from the v46 and v49 at the intervertebral junctions with a scalpel.

The vertebral specimens were cleared of any remaining muscular tissues and neural and haemal

spines were excised. The specimens immediately underwent decalcification with 10% Ethylenediaminetetraacetic acid (EDTA), in phosphate-buffered saline (PBS), for 12 days at 4 °C, with continuous agitation (60 rpm). Fresh EDTA solution was replaced every 4 days.

Thereafter, the samples were processed in an identical manner to the methods described in Sec. 2.15. Sectioning was performed at 7 µm thicknesses using the same equipment detailed in Sec 2.15. Rehydration of bone specimens was performed in an automated manner, as previously described in Sec. 2.15; followed by manual staining with Mallory's trichrome (acid fuchsin, aniline blue/orange G) and haematoxylin (Table 7.3).

Screening was undertaken in accordance with methods detailed in Sec. 2.15. From the micrographs obtained, the vertebral bone area was measured in accordance with Nordvik *et al.* (2005) and Fox and Davies (2011). This area comprised of the lamellar compact bone of the amphicoel along with collagen fibres which sheath the notochord; ossified

areas outside of these layers were not included (as per Fox and Davies, 2011). Trabeculae (ossified protrusions from the autocentrum) were omitted from the study by deleting their presence during image analysis. This was performed to the level of their crypts. Consequently, all transverse measurements were taken from areas positioned at the crypt of two trabeculae. Gross anatomical descriptions and analytical measurements are displayed in Plate 4.1 (note: *pink* hue has been enhanced for the aid of centrum definition).

Centrum thickness index (CTI) was calculated as follows:

$$CTI\ (AU) = (CT / CD) \times 100$$

Whereby; CT = centrum thickness (μm) and CD = centrum diameter (μm)

Perimeter : area ratio (VPA) was calculated as follows:

$$VPA\ (AU) = (CP / CA) \times 1000$$

Whereby; CP = centrum perimeter (μm) and CD = centrum area (μm^2)

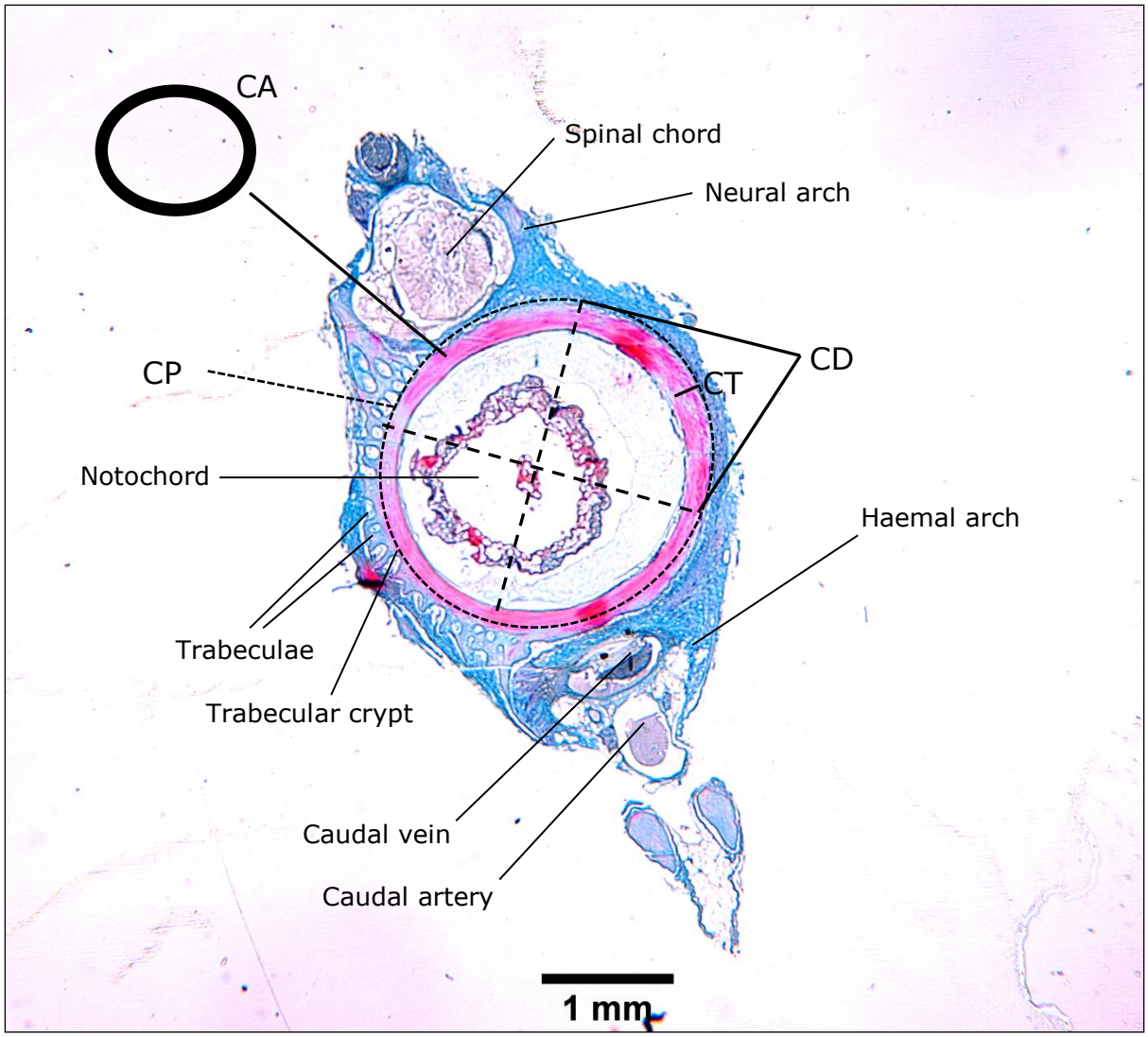

Plate 4.1 Transverse section of a 47/48th vertebrae of rainbow trout, stained with Mallory's trichrome with haematoxylin, displaying gross anatomical structure and appraised features.
Abbreviated features denote those quantified within this study. CA = centrum area. CP = centrum perimeter. CD = centrum diameter. CT = centrum thickness.

4.2.12 Haematological and serological parameters

At the end of the 10 week *N*-phase, following 12 hrs starvation, 2 fish per tank (n = 8) were heavily sedated (Sec. 2.6) and blood was collected from the caudal vein. Haemoglobin (Hb), haematocrit (Hct), serum glucose and serum lysozyme were analysed according to procedures detailed in Sec 2.14. Each fish was treated as an individual replicate throughout these parameters.

4.2.13 Intestinal histology

4.2.13.1 Sampling

Sampling of the posterior intestine (PI), for tissue, was scheduled immediately at the end of the 10 week *N*-phase. The fish from whom blood was obtained were sacrificed and utilised for these purposes (n = 8). Following euthanasia procedures described in Sec. 2.5; the IP cavity of fish was opened and the GIT was detached from the anus and intraperitoneal fat deposits were carefully separated from the gut tissue. The intestinal tract was excised below the stomach sack and at the anus. The PI was separated from the anterior intestine (AI) and flushed in PBS. Working from the anus, ~3 mm was discarded; the following ~3 mm was removed for scanning electron microscopy (SEM), followed by ~3 mm for transmission electron microscopy (TEM) and lastly ~7.5 mm for light microscopy (LM). These methods are exhibited in Plate 4.2. Each individual fish was treated as a replicate throughout histological appraisal (n = 8).

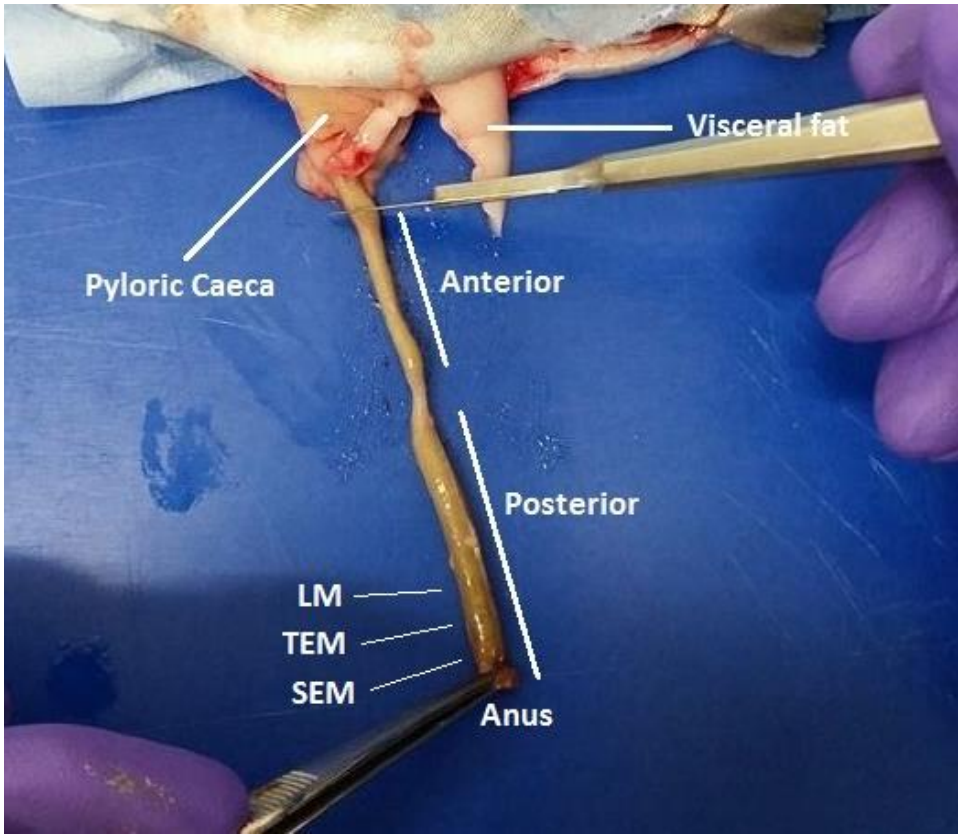

Plate 4.2. Rainbow trout dissection displaying intestinal morphological portions and sample site selections (not proportionally to scale).

4.2.13.2 Light Microscopy

Posterior intestinal samples were processed, sectioned and stained in accordance with the methods detailed in Sec. 2.15. The stains implemented were haematoxylin and eosin (H&E) and Periodic acid Schiff's (PAS), as detailed in Tables 7.1 and 7.2, respectively. Micrographs were collected using methods and equipment detailed in Sec. 2.15.

H&E-stained micrographs, at 20 X magnification, were appraised for enterocyte height (µm) (EH) through measurement of the cells at 50 intermittent locations around the intestinal folds of each fish (Plate 4.3A).

Lamina propria width (µm) (LPW) was measured in at least 30 locations per fish using H&E-stained micrographs at 20 X magnification (Plate 4.3A).

Goblet cell counts (no./mm) (GC) were performed Using PAS-stained micrographs at 20 X magnification. This was conducted in at least 20 intermittent locations, of varying distances (50 – 250 µm), around the epithelial layer of each fish (Plate 4.3B).

Calculation of surface area potential (perimeter ratio) could not be confidently performed due to depreciated specimen quality in a number of replicates.

Using PAS-stained micrographs at 10 X magnification, the thickness (µm) of the total muscularis, stratum longitudinale (SLG), stratum circulae (SCR) and stratum compactum (SCM) was measured at 12 locations around the intestinal cross-sections (Plate 4.3C). SLG, SCR and SCM are expressed as percentage of total muscularis thickness (%MT).

Muscularis thickness index (MTI) was calculated as follows:

$$MTI\ (AU) = MT / GP$$

Whereby; MT = total muscularis thickness (µm) and GP = total intestinal perimeter (µm).

All appraisals were conducted using ImageJ 1.45 (National Institutes of Health, USA).

4.2.13.3 Transmission electron microscopy

Specimens collected for TEM were processed, sectioned and screened in accordance with the materials and methods detailed in Sec. 2.16.

The micrographs were appraised for microvilli height (MVH) and diameter (MVD) as previously described in Sec. 3.2.8.1 (Plate 4.3D). All appraisals were conducted using ImageJ 1.45 (National Institutes of Health, USA).

4.2.13.4 Scanning electron microscopy

Intestinal specimens were suspended in a continuously stirring 1 % L-cysteine (Sigma, no. 168149) solution, for 30 sec, so as to clear epithelial mucus. They were subsequently fixed in 2.5 % glutaraldehyde containing 1 part 0.1 M sodium cacodylate buffer (pH 7.2). Storage of samples was conducted at 4 °C. Samples were rinsed twice, for 20 min, in 0.1 M sodium cacodylate buffer to remove fixative. Approximately 2 mm of fixed tissue was cut away from either end of the samples to remove areas likely to be damaged by dissection tools during previous steps and thus negate spurious observations of intestinal damage. Dehydration was conducted by exposing samples to graded ethanol solutions of 30, 50, 70, 90 and 100 % for 20 min at each stage. Samples were drained from their final ethanol solution and fresh 100 % ethanol was added. Following dehydration procedures, critical point drying of samples was performed (Quorum Tech. K850; Kent, UK). Samples were mounted on aluminium stubs using fine silver (Ag) paint (Ag in methyl isobutylkelone). Mounted samples were subsequently sputter-coated in gold, with an argon gas supply (Quorum Tech. K550X; Kent, UK). Samples were screened with JEOL JSM-6610 LV and JEOL JSM-7001 F scanning electron microscopes (Tokyo, Japan). Reference to microscope models used herein will be given as JSM-6610 or JSM-7001.

Micrographs obtained from 20,000 X magnification screening on the JSM—6610 were appraised for microvilli counts per μm^2 (MVCT), using an identical procedure to that used for TEM micrographs in Sec. 3.2.8.1. A total of 10 quadrants were analysed per fish and each individual was treated as a replicate (n = 8).

Microvilli surface area coverage (%) (MVCV) was estimated using micrographs obtained at 20,000 X magnification on the JSM-6610. This was performed by converting micrographs into binary form and adjusting threshold to a level determined as clearly depicting ultrastructural definition; resulting in microvilli displayed as black and space between in bright white (Plates 4.3F and 4.3G). This was performed using the original micrograph as a reference. MVCV was subsequently measured using an automated function and the known total surface area of the micrographs. Five micrographs were analysed per fish. Each individual was treated as a replicate (n = 8).

Using 2,500 X magnification micrographs obtained on the JSM-7001, enterocyte apical area (EAA) (μm^2) was measured (Plate 4.3E). At least 30 enterocytes were measured per animal and each individual was treated as a replicate (n = 8).

All quantitative appraisals were conducted using ImageJ 1.45 (National Institutes of Health, USA).

Total absorption surface area per enterocyte (ETAS) was estimated, using ultrastructural measurements obtained throughout electron microscopy. Each measurement was kept specific to the originating fish and each individual was treated as a replicate (n=8).

ETAS was calculated as follows:

$$ETAS\ (\mu m^2) = ((2 \times \pi \times 1/2\ MVD \times MVL) + (\pi \times 1/2\ MVD^2)) \times (MVCT) \times EAA$$

Whereby; π = Pi, MVD = mean microvilli diameter (μm), MVL = mean microvilli length (μm), MVCT = mean microvilli counts (no. / 1 μm^2) and EAA = enterocyte apical area (μm^2).

Extensive qualitative examinations were also performed on each fish, using magnifications between 100 and 20,000 X magnification. These examinations appraised the specimens for signs of necrosis, inflammation and overall structure.

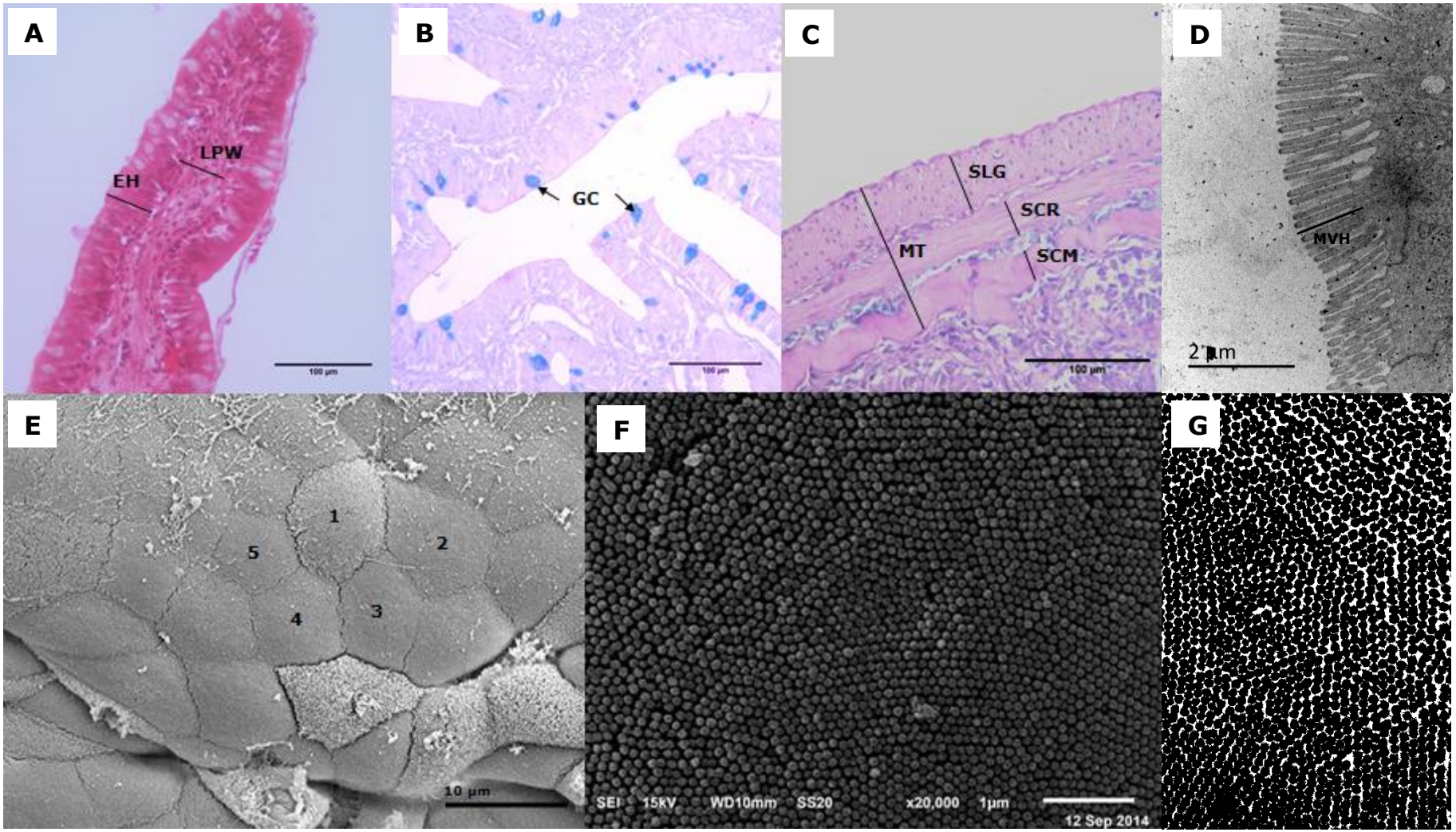

Plate 4.3 Light and electron micrographs of rainbow trout posterior intestine displaying appraisal methods

A = H&E-stained fold (20 X mag.): *EH* = enterocyte height, *LPW* = lamina propria width, scale bar = 100 µm; **B** = PAS-stained fold (20 X mag.): *GC* = goblet cell; **C** = PAS-stained muscularis (10 X mag.), MT = muscularis thickness, SLG = stratum longitudinale, SCR = stratum circulae, SCM = stratum compactum, scale bar = 100 µm: **D** = Brush border Epithelial (20,000 X mag. TEM), MVH = microvilli height, scale bar = 2 µm: **E** = Epithelial surface (2,500 X mag. SEM), *numbers* indicate individual enterocytes, scale bar = 10 µm. **F** = Epithelial surface (20, 000 X mag. SEM), scale bar = 1 µm. **G** = threshold-reversed epithelial layer displaying microvilli in *black* (20,000 X mag. SEM).

4.2.14 Endogenous protease activity

4.2.14.1 Sample collection

Sampling for intestinal proteolytic enzyme activities was performed at the end point of the *N*-phase. Following an overnight starvation period, tanks were fed four times to satiation, at 80 min intervals, over a period of 4 hrs. Exactly 80 min after their final feed, 2 fish per tank were manually euthanised and immediately immersed in ice. Following despatch, sampling was promptly undertaken on glass trays, over ice and under aseptic conditions. Aseptic conditions were implemented due to the selected animals also being used for microbiology; this process will be described in detail within Sec. 4.2.16.1.

The intraperitoneal cavity was opened and the hindmost portion of the intestine was clamped and detached at the anus. Fat was then cleared from the outer mucosal surface of the intestine. The anterior intestine (AI) was clamped at both ends and carefully removed away from the pyloric caeca and posterior intestine (PI). The clamps were removed and the AI was gently squeezed to remove digesta; remaining mucosa was rinsed with cold distilled water. Collected digesta and mucosa samples were immediately sealed in cryogenic tubes and immersed in liquid nitrogen. Samples were then stored at -80 °C until enzyme extraction. Each individual was treated as replicate (n=8).

4.2.14.2 Enzyme extraction

All crude enzyme extraction procedures were performed over ice, following direct removal from storage without a thaw period. Between 150.0 and 500.0 mg of sample was weighed into microcentrifuge tubes and homogenised in 2 volumes of ultra-pure water. The homogenate was sonicated in five, 3 sec bursts, taking care not to raise its temperature. Following sonication, homogenate was centrifuged at 20,000 X g for 20 min at 4 °C. The supernatant, containing enzymes, was then separated from lipid and solid residues via pipette and transferred to aliquots for storage at -80 °C.

4.2.14.3 Total alkaline protease

Total alkaline protease (TAP) activity was measured in the digesta of fish as an indicator of digestive capacity, following procedures described by Alarcón *et al.* (1998). Azocasein (Sigma-Aldrich, no. A2765) was dissolved, as a substrate, in ultrapure water at 1 % (w/v), to pH 9.00. Tris(hydroxymethyl)aminomethane (Tris) (100 mM) (Sigma-Aldrich, no. 252859) and $CaCl_2$ (10 mM) were dissolved at 1.2 % and 0.1 % (w/v), respectively, in ultra-pure water; followed by adjustment to pH 9.00 with 1 M HCl, as a buffer. Tris-$CaCl_2$-HCl buffer was then filtered through 11 µm filter paper. 500 µl of Azocasein substrate solution and 500 µl of Tris-$CaCl_2$-HCl buffer were added to 10 µl of enzyme extract, thoroughly mixed, and incubated for 30 min at 37 °C. The reaction was halted by addition of 500 µl 20 % (w/v) Trichloroacetic acid (TCA) (Sigma-Aldrich, no. T6399) in ultra-pure water. Blanks were prepared by addition of TCA-H_2O before addition of substrate. Mixtures were cooled by sealed immersion in ice and transferred to -20 °C conditions for a 15 min period. Mixtures were centrifuged at 10,500 X g for 15 min at 4 °C and supernatant was removed. Absorbance of supernatant was recorded at 366 nm in a spectrophotometer (Jenway 7315, Bibby Scientific; Staffs, UK). One unit of TAP activity (U) was defined as the amount of enzyme that released 1 µg of tyrosine per min, considering an extinction coefficient of 0.008 µg/ml/cm. Each sample was analysed in quadruplicate. Where data distribution was heavily skewed, denoising was performed by removal of samples with a value 2σ. Commonly occurring skews in enzyme activity between individuals are attributed to variable gut transit time dependent upon fish size (Pandian, 1967) and difficulties in ensuring consistent feed consumption between individuals, despite strict feeding regimes. All data sets were $n = \geq 6$. Values were expressed as U/g digesta.

4.2.14.4 Trypsin

Trypsin activity was measured in the digesta of fish as an indicator of digestive capacity following procedures described by Erlanger *et al*. (1961) and modified by Alarcón *et al*.

(1998). *N*-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) (Sigma-Aldrich, no. B3133) was dissolved in dimethyl sulfoxide (DMSO) (Sigma, no. 472301) to 0.5mM as a substrate. This substrate solution was dissolved at 1 % (v/v) in Tris-HCl 50 mM buffer, containing 20 mM Cl_2Ca (pH 8.5). Ten µl of enzyme extract and 190 µl of substrate-buffer solution were added to microplate wells, agitated for 20 sec and absorbance was measured at 20 sec intervals, for 10 min, at 405 nm (OPTImax microplate reader, Molecular Devices LLC; CA, USA). Each sample was analysed in quadruplicate. One unit of trypsin activity (U) was defined as the amount of enzyme that released 1 µmol of *p*-nitroanilide (*p*NA) per minute, considering an extinction coefficient of 8800 M/cm. Denoising was performed as previously described in Sec. 4.2.13.3. All data sets were $n = \geq 6$. Values were expressed as U/g digesta.

4.2.14.5 Chymotrypsin

Chymotrypsin activity was measured in the digesta of fish as an indicator of digestive capacity following procedures described by DelMar *et al*. (1979) and modified by Alarcón *et al*. (1998). *N*-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (SAPNA) (Sigma-Aldrich, no. S7388) was dissolved in DMSO (Sigma, no. 472301) to 0.2 mM, as a substrate. This substrate solution was dissolved at 1 % (v/v) in Tris-HCl 50 mM buffer, containing 20 mM Cl_2Ca (pH 8.5). Ten µl of enzyme extract and 190 µl of substrate-buffer solution were added to microplate wells, agitated for 20 sec and absorbance was measured at 20 sec intervals for 10 min at 405 nm (OPTImax microplate reader, Molecular Devices LLC; CA, USA). Each sample was analysed in quadruplicate. One unit of chymotrypsin activity (U) was defined as the amount of enzyme that released 1 µmol of *p*NA per minute, considering an extinction coefficient of 8800 M/cm. Denoising was performed as previously described in sec. 4.2.13.3. All data sets were $n = \geq 6$. Values were expressed as U/g digesta.

4.2.14.6 Leucine aminopeptidase

L-leucine aminopeptidase (LAP) activity was measured in the digesta and mucosa of fish as an indicator of absorptive capacity following procedures described by Pfeiderer (1970). L-Leucine-*p*-nitroanilide (Sigma, no. L9125) was dissolved in DMSO (Sigma, no. 472301) to 2 Mm, as a substrate. This substrate solution was dissolved at 1% (v/v) in Tris-HCl 100 mM buffer (pH 8.8). Ten µl of enzyme extract and 190 µl of substrate-buffer solution were added to microplate wells, agitated for 20 sec and absorbance was measured at 20 second intervals for 10 min at 405 nm (OPTImax microplate reader, Molecular Devices LLC; CA, USA). Each sample was analysed in quadruplicate. One unit of LAP activity (U) was defined as the amount of enzyme that released 1 µmol of *p*NA per minute, considering an extinction coefficient of 8800 M/cm. Denoising was performed as previously described in sec. 4.2.13.3. All data sets were $n = \geq 6$. Values were expressed as U/g digesta or U/g tissue.

4.2.14.7 Alkaline phosphatase

Alkaline phosphatase (ALP) activity was measured in the digesta and mucosa of fish as an indicator of absorptive capacity following procedures described by Bergmeyer (1974). As a substrate, *p*-nitrophenyl phosphate (405 mM) (Sigma, no. p4744) was dissolved in ultrapure water at a concentration of 0.17 % (w/v). Diethanolamine (4.8 ml) was dissolved in ultrapure water (40 ml) to 1 M. Following this, $MgCl_2 \cdot 6H_2O$ was dissolved in ultrapure water to 0.5 M and slowly added to the diethanolamine solution in drops. The diethanolamine-$MgCl_2$ was then adjusted to pH 9.8 using 1 M HCl and subsequently further diluted with 1 part of ultrapure water to create a 1 M diethanolamine buffer containing 1 mM $MgCl_2$. The *p*-nitrophenyl phosphate substrate solution was dissolved at 3.33 % (v/v) in diethanolamine-$MgCl_2$ buffer. All substrate preparation steps were performed under dark conditions where possible and substrate-buffer solution was utilised within 5 min of creation. Ten µl of enzyme extract and 290 µl of substrate-buffer solution were added to microplate wells, agitated for 20 sec and absorbance was

measured at 20 sec intervals for 10 min at 405 nm (OPTImax microplate reader, Molecular Devices LLC; CA, USA). Each sample was analysed in quadruplicate. One unit of ALP activity (U) was defined as the amount of enzyme that released 1 µg of nitrophenyl per minute, considering an extinction coefficient of 17,800 M/cm. Denoising was performed as previously described in sec. 4.2.13.3. All data sets were $n = \geq 6$. Values were expressed as U/g digesta or U/g tissue.

4.2.15 Statistical analysis

Statistical analyses of results obtained from methods described between Sec. 4.2.7 and 4.2.14 were undertaken using IBM SPSS Statistics 21. Data expressed as percentages were arcsine transformed prior to statistical analysis. All tests on normally-distributed data were conducted via ANOVA with post-hoc Fisher's LSD with significance accepted at $P \leq 0.05$. Non-parametric data were analysed via Kruskal-Wallis and Mann Whitney U tests with significance accepted at $P \leq 0.05$.

4.2.16 Intestinal microbiology

4.2.16.1 Sampling

As previously mentioned, the same fish selected for endogenous protease sampling were used for microbiological sampling. The aseptic conditions implemented involved constant work in front of a blue flame, gloves and work surface sterilisation with 70 % industrial methylated spirit (IMS) between each dissection and soaking of tools in 70 % IMS followed by lighting over a blue flame before each use. Prior to dissection, the fish were rinsed and wiped thoroughly with 70 % IMS, paying particular attention to the exterior of the peritoneal cavity where incisions were to made. Having cleared fat deposits from the outer mucosal surface and being separated from the anterior portion, the digesta found within the posterior intestine was collected into sterile microcentrifuge tubes (RNA/DNA Lobind; Eppendorf®, DE) by carefully applying pressure down the tissue, towards the

anus with the use of forceps. The samples were stored at -20 °C until use. Each individual was treated as replicate (*n* = 8).

4.2.16.2 DNA isolation and PCR

DNA isolations were conducted using a QIamp® Fast DNA Stool Mini Kit (Qiagen; NL), with minor modifications to the manufacturers protocol. In brief, the samples were homogenised and diluted into buffer supplied by the manufacturer in order to remove PCR inhibitors. The suspension was then incubated with lysozyme at 70 °C for 5 min and centrifuged at 20,000 X g for 1 min at room temperature. Forward of these stages, manufacturer guideline incubation steps were increased 3 fold. Lysate supernatant was subsequently introduced to a silica membrane to trap DNA and successive washes with provided reagents were performed to further remove inhibitors and contaminants. Again to note is that the final buffer addition was decreased 4 fold in order to concentrate the isolated DNA. DNA for further steps was thus eluted.

Further steps were performed according to Standen *et al*. (2015). DNA concentrations were firstly analysed using a Nanodrop™ 1000 (Thermo Scientific Ltd, DE, USA). Six fish per treatment were selected for further analysis (*n* = 6), based upon observations of those with the highest yield of DNA. PCR amplification of the 16S rRNA V1-V2 hypervariable regions was conducted according to Roeselers *et al.* (2011). Briefly, a 30 µL PCR reaction was performed with 4 µL of DNA template (diluted 1/10 in molecular-grade water), 1 µL (50 pmol) of primer 338R (GCW GCC WCC CGT AGG WGT), 1 µL (50 pmol) of primer 27F (5' - AGA GTT TGA TCM TGG CTC AG – 3'), 15 µL of MyTaq™ (Bioline, London, UK) and 9 µL of molecular-grade water. Thermal cycling was conducted using a Techne TC-512 (Thermal Cycler; Staffordshire, UK) under the following conditions: initial denaturation at 94 °C for 7 min, followed by 10 touchdown cycles of 94 °C for 30 sec, 62 °C for 30 sec and 72°C for 30 sec. A further 25 cycles were performed at 94 °C for 30 sec, 53 °C for 30 sec and 72 °C for 30 sec before a final extension for 7 min at 72 °C.

4.2.16.3 High-throughput sequence analyses

High-throughput sequence analysis was undertaken in accordance with Standen *et al*. (2015). In brief, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen; Crawley, UK) and DNA was quantified using a Qubit® 2.0 Fluorometer (Invitrogen; UK). Prior to sequencing, the concentration of the amplicons were adjusted to 26 pM after quantification with Ion Library Quantitation Kit. Amplicons were attached to Ion Sphere Particles (ISPs) using an Ion PGMTM Template OT2 400 kit according to the manufacturer's instructions. Multiplexed sequencing was conducted using Ion XpressTM barcode adapters and a 318TM chip, on an Ion Torrent® Personal Genome Machine (PGM) at the Systems Biology Centre at Plymouth University (UK). All the kits used were purchased from Life Technologies™ (USA). Sequences were binned by sample and filtered within the PGM software to remove low quality reads. Data were then exported as FastQ files.

Taxonomic analyses of sequence reads were performed after the removal of low quality scores (Q score <20) with FASTX-Toolkit (Hannon Lab, USA). Sequences were de-noised and analysed with QIIME (Caporaso *et al.*, 2010a). Briefly, OTU mapping was performed using default pipeline of QIIME with USEARH (Edgar, 2010), removing putatively erroneous reads (chimeras). Assignment of taxonomic classification for operational taxonomic units (OTUs) was based on the Greengenes database (DeSantis *et al.*, 2006) using the RDP classifier (Wang *et al.*, 2007), which clustered the sequences at 97% similarity with a 0.80 confidence threshold. PyNAST was employed to create a multiple alignment of the representative sequences for each OTU (Caporaso *et al.*, 2010b), with a minimum sequence length threshold of 150 base pairs (bp) and 95 % identification. Subsequently, the sequences were filtered to remove outliers and filter positions with gaps (0.95) and singletons. Highest homologous species, or genera (where possible) were identified considering >98 % similarity at 150 bp. This was performed using the 16S microbial Nucleotide BLAST-NCBI database.

Chao1, Shannon's diversity index and alpha diversity metrics were calculated through QIIME and Good's coverage was calculated from rarefied OTU tables, to assess sampling depth coverage through observed genera. Beta diversity metrics among samples were calculated using weighted, unique fraction metric (UniFrac) distances (Lozupone *et al.*, 2007) and Bray-Curtis similarity (Bray and Curtis, 1957). The distance matrices were represented by two-dimensional principal coordinates analysis (PCoA) plots.

Statistical analyses were conducted on sequences which represented >0.1 % of total sequences in one or more treatments. Statistical tests utilised were Kruskal-Wallis followed by Tukey-Kramer, these were performed using STAMP v2 0.8. Significance was accepted at $P \leq 0.05$.

4.3 Results

4.3.1 Fish and feed performance

At the end of the 10 week *N*-phase, the experimental animals had exceeded a 3-fold average increase in body weight. Significant differences in FW ($F = 24.39$, $P < 0.001$), WG ($F = 39.63$, $P < 0.001$), FCR ($F = 16.755$, $P < 0.001$), SGR ($F = 18.38$, $P < 0.001$) and PER ($F = 20.47$, $P < 0.001$) was observed between the dietary treatments (Table 4.2). Consistently, LC- and LS0.1-fed fish did not differ from one another, whilst significant improvement was observed in LS0.5 fed fish ($P < 0.05$). FMC fed fish performed significantly better than the lupin-based treatments in all instances ($P < 0.05$).

Table 4.3 Fish and feed performance values of the dietary treatments.

	Diet			
	LC	LS0.1	LS0.5	FMC
IW (g)	44.04 ± 0.44	43.26 ± 0.18	43.65 ± 0.34	43.38 ± 0.17
FW (g)	140.42 ± 5.71 [a]	135.44 ± 3.56 [a]	146.54 ± 2.33 [b]	159.56 ± 4.76 [c]
WG (g)	97.07 ± 4.63 [a]	93.33 ± 1.67 [a]	104.29 ± 1.14 [b]	117.02 ± 4.39 [c]
FCR	1.25 ± 0.47 [a]	1.22 ± 0.34 [a]	1.08 ± 0.20 [b]	0.99 ± 0.16 [c]
SGR	1.64 ± 0.76 [a]	1.65 ± 0.70 [a]	1.76 ± 0.58 [b]	1.87 ± 0.56 [c]
PER	1.73 ± 0.07 [a]	1.70 ± 0.03 [a]	1.86 ± 0.03 [b]	1.97± 0.06 [c]
Survival (%)	100	100	100	100
Somatic indices				
K-F	1.79 ± 0.11	1.75 ± 0.09	1.75 ± 0.04	1.85 ± 0.04
HIS	1.05 ± 0.06	0.99 ± 0.09	1.02 ± 0.05	0.94 ± 0.03

Abbreviations: LC = yellow lupin control diet; LS0.1 = yellow lupin basal + Synergen™ (0.1%); LS0.5 = yellow lupin basal + Synergen™ (0.5%); FMC = fishmeal reference/control diet; IW = initial fish weight; FW = final fish weight; WG = weight gain (g); FCR = feed conversion ratio; SGR = specific growth rate; PER = protein efficiency ratio; K-F = k-factor condition index; HIS = hepatosomatic index

[a, b, c] Diets possessing the same superscript in the same row are not significantly different (P≤0.05), no superscripts indicate no significant difference between any diets.

Values expressed as mean ± S.D. (n = 4). Statistical test: ANOVA + Fisher's LSD.

4.3.2 Nutrient digestibility and mineral bioavailability

Apparent digestibility and bioavailabity coefficients are displayed in Table 4.3. The apparent digestibility of total dry matter and crude lipid was unaffected by dietary treatment of SYN. Apparent digestibility of crude protein was significantly different between the lupin-based diets (F = 128.63, P < 0.001); significant incremental increases were observed between LC, LS0.1 and LS0.5. Crude fibre apparent digestibility was significantly different between treatments (F = 35.32, P < 0.001), being significantly higher in LS0.5 than LC and LS0.1. No significant difference was observed between LC and LS0.1. Gross enery apparent digestibility was significantly higher in LS0.1 than LC (P < 0.05), LS0.5 than LC (P < 0.05) and LS0.5 than LS0.1 (P < 0.05).

Calcium, phosphorous, potassium, magnesium and manganese Apparent Mineral Bioavailability Coefficients (ABAC) were significantly different between treatments (F = 155.95, P < 0.001; F = 447.19, P < 0.001; F = 630.82, P < 0.001; F = 1166.57, P < 0.001; F = 810.60, P <0.001, respectively). In all instances, ABAC was higher in LS0.1 than LC, LS0.5 than LC and LS0.5 than LS0.1. Sulfur ABAC was significantly different

between treatments (F = 196.52, P < 0.001). Apparent bioavailability was higher in LS0.1 than LC, LS0.5 than LC and LS0.5 than LS0.1.

Zinc ABAC was significantly different between treatments (F = 17.31, P < 0.005). Apparent bioavailability was higher in LS0.5 than LC and LS0.1.

Iron apparent bioavailaility was significantly different between treatments (F = 19.76, P < 0.005). Apparent bioavailability was higher in LS0.1 than LC and LS0.5.

Sodium apparent bioavailability coefficients yielded negative values in all treatments except FMC (37.93 % ± 0.48). A significant difference was observed between the lupin-based treatments (F = 325.54, P < 0.001). Significantly less Na was observed in LS0.1 than LC, LS0.5 than LC and LS0.5 than LS0.1.

Table 4.3 Apparent macronutrient digestibility coefficients (ADC) (%) and apparent mineral bioavailability coefficients (ABAC) (%) of the experimental lupin-based and fishmeal-based rainbow trout diets.

	Diet		
	LC	LS0.1	LS0.5
ADC (%)			
DM	96.97 ± 0.43	96.71 ± 0.21	97.08 ± 0.69
CP	83.65 ± 0.02 [a]	85.26 ± 0.03 [b]	85.55 ± 0.03 [c]
CL	89.11 ± 0.77	88.93 ± 0.10	90.43 ± 0.79
CF	33.99 ± 1.05 [a]	35.52 ± 1.60 [a]	44.69 ± 2.62 [b]
GE	66.29 ± 0.62 [a]	68.41 ± 0.83 [b]	71.76 ± 0.15 [c]
ABAC (%)			
Ca	0.67 ± 1.57 [a]	13.80 ± 1.62 [b]	20.20 ± 0.77 [c]
P	55.02 ± 0.72 [a]	63.86 ± 0.69 [b]	73.53 ± 0.47 [c]
Mg	33.43 ± 0.03 [a]	42.91 ± 0.64 [b]	51.27 ± 0.45 [c]
K	92.14 ± 0.05 [a]	93.30 ± 0.06 [b]	94.04 ± 0.11 [c]
S	53.82 ± 0.35 [a]	58.97 ± 0.37 [b]	62.00 ± 0.72 [c]
Fe	41.77 ± 2.19 [a]	48.34 ± 1.99 [b]	39.59 ± 0.83 [a]
Zn	29.39 ± 0.96 [a]	30.41 ± 2.68 [a]	41.59 ± 3.96 [b]
Mn	-0.93 ± 0.10 [a]	3.93 ± 0.98 [b]	17.16 ± 0.30 [c]
Na	-155.44 ± 1.42 [c]	-131.40 ± 2.59 [b]	-107.28 ± 2.70 [a]

Abbreviations: DM = dry matter; CP = crude protein; CL = crude lipid; CF = crude fibre; GE = gross energy; Ca = total calcium; P = total phosphorous; Mg = total magnesium; K = total potassium; S = total sulphur; Fe = total iron; Zn = total zinc; Mn = total manganese; Na = total sodium. Values expressed as mean ± S.D. (n=3). Statistical tests: ANOVA + Fisher's LSD (DM, CP, CL, CF, Ca, P, Mg, K, S, Fe, Zn, Mn, Na).

[a, b, c] Diets possessing the same superscript in the same row are not significantly different (P≤0.05), no superscripts indicate no significant difference between any diets

Limits of Detection (LOD): Ca = 0.39 mg/kg; P = 0.07 mg/kg; Mg = 0.03 mg/kg; K = 0.18 mg/kg; S = 0.09 mg/kg; Fe = 0.07 mg/kg; Zn = 0.08 mg/kg; Mn = 0.02 mg/kg; Na = 1.11 mg/kg

4.3.3 Carcass composition and tissue mineral concentrations

Carcass composition results are displayed in Table 4.4. Carcass moisture, crude lipid (CL) and ash content were unaffected by dietary treatment. However, crude protein (CP) content of whole carcasses was significantly different between fish fed the respective diets (F = 36.24, P < 0.001). Carcass CP was significantly higher in fish fed LS0.5 and FMC than LC and LS0.1; values for fish fed LS0.5 and FMC did not differ from one another. Increased availability of dietary protein in SYN diets (Table 4) is proposed as an explanation for observed results. Gross energy (GE) of whole carcass was found to be significantly affected by diet (F = 14.92, P = 0.001), being significantly higher in SYN-treated and FMC diets than LC.

Table 4.4 Whole carcass composition of fish fed the experimental diets.

	Diet			
	LC	LS0.1	LS0.5	FMC
Moisture (%)	70.22 ± 0.01	69.73 ± 0.01	69.79 ± 0.00	70.24± 0.01
CP (%)	15.24 ± 0.18 [a]	15.47 ± 0.24 [a]	16.34 ± 0.02 [b]	16.29 ± 0.11 [b]
CL (%)	11.06 ± 0.27	11.28 ± 0.48	11.41 ± 0.42	10.91 ± 0.53
Ash (%)	2.00 ± 0.01	2.10 ± 0.17	2.08 ± 0.17	-
GE (MJ/kg)	7.87 ± 0.04 [a]	8.01 ± 0.00 [b]	7.98 ± 0.03 [b]	7.99 ± 0.03 [b]

Abbreviations: CP = crude protein; CL = crude lipid; GE = gross energy. Values expressed as mean ± S.D. (n=3) of whole carcass (on wet basis). Statistical tests: ANOVA + Fisher's LSD.
[a, b, c] Diets possessing the same superscript in the same row are not significantly different (P≤0.05), no superscripts indicate no significant difference between any diets

Liver, muscle, vertebral and caudal fin mineral concentrations are presented in Table 4.5.

Liver Zn concentration was significantly higher in the FMC group compared to the lupin-fed fish (F = 9.129, P < 0.01), no effect was observed through SYN treatment. A significant difference in Mn liver concentration was observed (F = 4.654, P < 0.001). Mn concentration was significantly higher in LS0.5 than LC but not LS0.1, whilst LC and LS0.1 did not differ statistically. Mn concentration in fish fed the fishmeal-based diet was significantly lower than all lupin-fed fish.

Muscle of the fish fed the different diets contained significantly different Mn concentrations (F = 4.654, P < 0.05). Mn was significantly lower in FMC compared to LS0.1 and LS0.5 but not LC; the lupin-based diets did not significantly differ from one another. However, numerical trends towards increasing Mn concentration with SYN may be apparent, supported by statistical results close to significance between LC and LS0.5 (P = 0.06).

Vertebral Mn concentration again differed significantly between dietary treatments (F = 8.67, P < 0.01). The concentration was significantly lower in FMC fed fish compared to the fish fed the lupin-based diets. Similarly to muscle concentration, trends towards increased concentration with SYN are apparent. Although no significant differences in Ca and P concentrations were observed, the ratio of these elements (Ca:P) was significantly different among the treatments (F = 8.14, P <0.001). The LC group was found to differ from LS0.1 (P = 0.03), LS0.5 (P < 0.001) and FMC (P < 0.01). Likewise, the LS0.1 group differed from LS0.5 (P = 0.01) but not FMC (P > 0.05). The LS0.5 group did not differ from FMC although a close to significant result was observed (P = 0.058).

Caudal fin Na concentration was observed to differ significantly between treatments (F = 11.45, P < 0.005). Na was significantly higher in FMC than LS0.1 and LS0.5, and higher in LC than LS0.1. K caudal fin concentration was significantly different between treatments (F = 14.06, P = 0.001), being significantly higher in SYN treatments than LC and FMC. Caudal fin S concentration differed significantly between treatments (F = 8.64, P < 0.01), being significantly higher in FMC fed fish than in the fish fed the lupin-based diets. Mg concentration was significantly different between treatments (F = 4.64, P < 0.05). Concentration of Mg in the caudal fin of FMC-fed fish was significantly lower than those of SYN-fed fish. Lupin-fed fish did not differ significantly from one another, nor did LC and FMC. Mn concentration differed significantly between treatments (F = 14.33, P = 0.001). The concentration of this element was significantly lowest in FMC fed fish. The concentration of Mn in fins of LS0.5-fed fish was significantly higher than that of LC, whilst LC and LS0.1 did not differ from one another.

Table 4.5 Tissue mineral concentrations of liver, muscle, vertebrae and caudal fin in experimental fish

	Diet				
	LC	LS0.1	LS0.5	FMC	LOD
Liver					
P (mg/g)	11.72 ± 0.27	11.68 ± 0.45	11.40 ± 0.54	11.64 ± 0.20	0.007
K (mg/g)	11.54 ± 1.13	11.56 ± 0.46	11.70 ± 0.31	11.85 ± 0.59	0.005
S (mg/g)	8.43 ± 0.56	8.59 ± 0.23	8.58 ± 0.57	8.84 ± 0.34	0.028
Na (mg/g)	4.23 ± 0.51	4.29 ± 0.09	4.32 ± 0.31	4.69 ± 0.16	0.053
Mg (mg/g)	0.74 ± 0.05	0.77 ± 0.02	0.75 ± 0.04	0.76 ± 0.04	0.018
Ca (mg/g)	0.38 ± 0.10	0.46 ± 0.12	0.44 ± 0.29	0.45 ± 0.08	0.144
Cu (mg/g)	0.30 ± 0.02	0.28 ± 0.02	0.27 ± 0.02	0.26 ± 0.07	0.001
Fe (mg/g)	0.19 ± 0.05	0.17 ± 0.03	0.18 ± 0.02	0.15 ± 0.03	0.003
Zn (mg/g)	0.15 ± 0.01 [a]	0.16 ± 0.02 [a]	0.16 ± 0.01 [a]	0.20 ± 0.01 [b]	0.003
Mn (µg/g)	4.12 ± 0.12 [a]	4.39 ± 0.49 [ab]	4.90 ± 0.34 [b]	2.70 ± 0.27 [c]	0.309
Muscle					
K (mg/g)	14.73 ± 0.80	14.59 ± 0.30	14.13 ± 0.65	14.97 ± 0.09	0.005
P (mg/g)	8.59 ± 0.27	8.59 ± 0.29	8.43 ± 0.17	8.77 ± 0.15	0.002
S (mg/g)	7.64 ± 0.05	7.71 ± 0.11	7.64 ± 0.25	8.00 ± 0.27	0.037
Na (mg/g)	2.42 ± 0.06	2.38 ± 0.06	2.38 ± 0.09	2.19 ± 0.14	0.022
Mg (mg/g)	0.99 ± 0.04	0.98 ± 0.03	0.94 ± 0.02	0.96 ± 0.01	0.002
Ca (mg/g)	0.81 ± 0.11	0.98 ± 0.09	1.05 ± 0.24	0.83 ± 0.12	0.005
Zn (µg/g)	16.27 ± 0.18	17.41 ± 1.00	17.33 ± 0.68	17.51 ± 0.77	0.001
Fe (µg/g)	12.03 ± 1.78	11.98 ± 1.39	11.89 ± 1.82	11.94 ± 1.63	1.485
Mn (µg/g)	0.61 ± 0.20 [ab]	0.77 ± 0.23 [a]	0.93 ± 0.07 [a]	0.40 ± 0.18 [b]	0.140
Vertebrae					
Ca (mg/g)	93.81 ± 9.01	91.29 ± 6.02	87.50 ± 3.99	90.68 ± 7.73	0.010
P (mg/g)	56.69 ± 4.44	55.99 ± 3.00	54.66 ± 2.09	55.88 ± 4.06	0.015
Ca:P	1.65 ± 0.03 [a]	1.63 ± 0.02 [b]	1.60 ± 0.02 [c]	1.62 ± 0.02 [bc]	N/A
Na (mg/g)	15.99 ± 0.75	16.11 ± 0.47	16.91 ± 0.13	15.74 ± 0.62	0.011
K (mg/g)	7.38 ± 0.85	7.38 ± 0.47	8.06 ± 0.25	7.96 ± 0.69	0.003
S (mg/g)	3.30 ± 0.06	3.32 ± 0.08	3.43 ± 0.15	3.44 ± 0.09	0.003
Mg (mg/g)	2.11 ± 0.18	2.09 ± 0.07	2.08 ± 0.05	2.02 ± 0.12	<0.001
Zn (µg/g)	76.68 ± 7.82	85.33 ± 0.72	85.79 ± 12.72	93.95 ± 8.80	0.580
Mn (µg/g)	12.30 ± 3.32 [a]	13.14 ± 0.70 [a]	14.21 ± 2.25 [a]	6.23 ± 1.06 [b]	1.509
Caudal fin					
Ca (mg/g)	106.90 ± 5.08	103.90 ± 4.67	107.32 ± 2.58	98.90 ± 2.63	0.006
P (mg/g)	62.07 ± 4.36	59.73 ± 7.46	58.97 ± 3.73	51.91 ± 4.13	0.003
Na (mg/g)	44.62 ± 1.14 [ab]	33.16 ± 5.51 [c]	39.14 ± 2.54 [bc]	48.82 ± 3.21 [a]	0.012
K (mg/g)	5.30 ± 0.28 [a]	7.41 ± 0.38 [b]	6.81 ± 0.81 [b]	5.39 ± 0.24 [a]	0.003
S (mg/g)	5.40 ± 0.06 [a]	5.32 ± 0.22 [a]	5.42 ± 0.10 [a]	5.80 ± 0.05 [b]	0.001
Mg (mg/g)	2.25 ± 0.10 [ab]	2.36 ± 0.18 [a]	2.36 ± 0.08 [a]	2.05 ± 0.05 [b]	<0.001
Zn (µg/g)	95.78 ± 7.11	95.15 ± 12.54	104.29 ± 3.86	102.57 ± 6.40	1.320
Mn (µg/g)	15.37 ± 3.21 [a]	16.38 ± 1.21 [ab]	19.71 ± 2.40 [b]	8.30 ± 1.33 [c]	0.001
Co (µg/g)	5.64 ± 2.15	6.83 ± 1.59	4.93 ± 1.59	7.40 ± 1.93	2.708
Cr (µg/g)	2.61 ± 0.17	2.02 ± 0.36	1.63 ± 0.50	2.52 ± 0.68	1.046

Abbreviations: Ca = total calcium; P = total phosphorous; Mg = total magnesium; K = total potassium; S = total sulphur; Fe = total iron; Zn = total zinc; Mn = total manganese; Na = total sodium; Cu = total copper; Co = total cobalt; Cr = total chromium; LOD = limit of detection (3*S.D. of Blank + Blank) Values expressed as mean ± S.D. (n=3) on DM basis. Statistical test: ANOVA + Fisher's LSD. – (*continued on next page*)

[a, b, c] Diets possessing the same superscript in the same row are not significantly different (P≤0.05), no superscripts indicate no significant difference between any diets

4.3.4 Vertebral histomorphometry

No significant difference in centrum thickness index (CTI) was observed between treatments (F = 2.49, P > 0.05) (Table 4.7). Similarly, no significant difference in vertebrae perimeter:centrum area ratio (VPA) was observed (F = 0.834, P > 0.05) (Table 4.7). Representative micrographs are exhibited in Plate 4.4.

Table 4.6 Bone histomorphometric indices of the 47th-48th vertebrae of rainbow trout fed the experimental diets.

	Diet			
	LC	LS0.1	LS0.5	FMC
CTI	7.04 ± 1.31	7.32 ± 0.43	6.76 ± 0.83	7.88 ± 0.79
VPA	6.72 ± 1.44	6.23 ± 0.87	6.57 ± 0.74	5.99 ± 1.14

Abbreviations: CTI = centrum thickness index; VPA = vertebrae perimeter : centrum area ratio.
Values expressed as mean ± S.D. Statistical tests: ANOVA (n = 9).

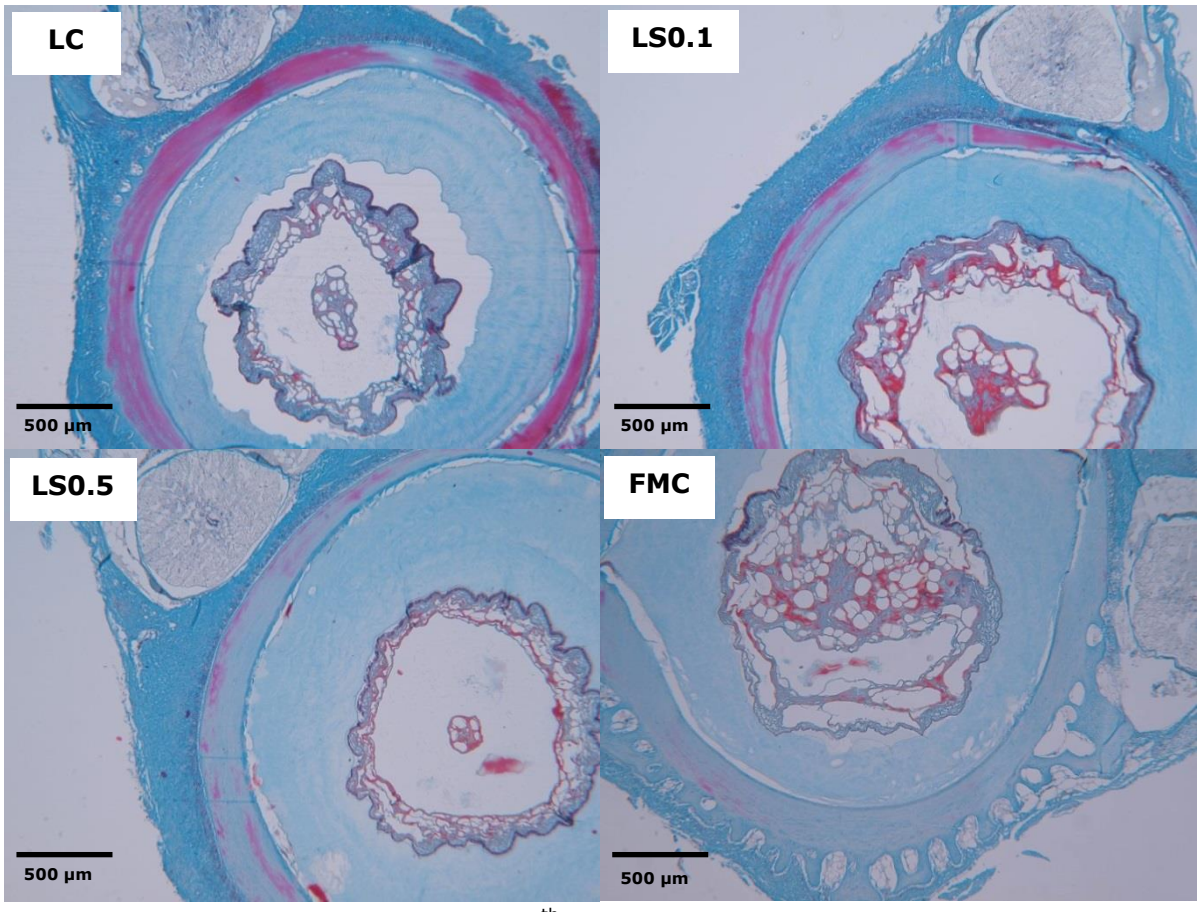

Plate 4.4 Histological sections of 47-48th vertebrae of fish fed the experimental diets.

4.3.5 Haematology and serology

Haematological and serological indices of health and immune status are displayed in Table 4.7.

No significant differences in hemaglobin (Hb) concentrations were observed among dietary treatments. Hematocrit (Hct) concentration appeared significantly different between treatments (F = 7.81, P = 0.001). Fish fed the 0.1 % SYN inclusion exhibited significantly higher Hct concentration than all other treatments. Significantly higher Hct was observed in the blood of LC fish compared to that of FMC fed fish, whilst values for fish fed LS0.5 did not differ significantly from those fed LC or FMC.

Serum glucose levels were significantly affected by dietary treatment (F = 6.71, P < 0.005). Significantly higher serum glucose levels were observed in FMC fed fish compared to fish fed LC and LS0.1, whilst elevation was also observed in LS0.5 over LC and LS0.1. No significant difference between LC and LS0.1 fed fish was observed, whilst LS0.5 and FMC fed fish did not differ significantly from one another.

Serum lysozyme activity was significantly different between treatments (F = 3.65, P < 0.05). Activity was significantly higher in SYN treatments than control diets; no difference in activity was observed between LS0.1 and LS0.5 or LC and FMC.

Table 4.7 Haematological and serological parameters of rainbow trout fed the experimental diets.

	Diet			
	LC	LS0.1	LS0.5	FMC
Hb (g/dl)	0.18 ± 0.03	0.19 ± 0.02	0.18 ± 0.02	0.17 ± 0.01
Hct (%PCV)	36.50 ± 4.75 [a]	40.50 ± 2.78 [b]	36.00 ± 3.02 [ac]	33.14 ± 1.68 [c]
Glc (mg/dl)	44.19 ± 8.47 [a]	47.26 ± 7.25 [a]	65.65 ± 7.38 [b]	72.90 ± 12.45 [b]
Lyz (U/ml)	1156.46 ± 116.03 [a]	1427.98 ± 279.75 [b]	1450.71 ± 235.75 [b]	1167.31 ± 109.42 [a]

Abbreviations: Hb = haemoglobin; Hct = haematocrit; Glc = serum glucose; LYZ = serum lysozyme; PCV = packed cell volume

Values expressed as mean ± S.D. Statistical test: ANOVA + Fisher's LSD. Hb n=8; Hct n=8; Glc n=8 (LC, FMC) and n=7 (LS0.1, LS0.5); LYZ n=8.

[a, b] Diets possessing the same superscript in the same row are not significantly different (P≤0.05), no superscripts indicate no significant difference between any diets

4.3.6 Intestinal morphology

Results of the quantitative appraisal of posterior intestinal morphology, by light and electron microscopy techniques, are displayed in Table 4.9.

Enterocyte height (EH) was observed to differ significantly between treatments ($P < 0.05$). Median EH of LS0.1 and LS0.5 fed fish did not differ from one another but were both significantly greater than LC fed fish.

Globlet cell counts (GC) were significantly different between dietary treatments ($F = 10.15$, $P = 0.001$). Fish fed 0.5 % SYN were observed to have a significant reduction in GC compared to those fed LC and LS0.1. No significant difference was observed in GC between LC and LS0.1 fed fish. Lamina propria width was unaffected by dietary treatment ($P > 0.05$).

Muscularis thickness, corrected to total intestinal diameter (MTI), did not differ among dietary treatments, nor did proportional contributions of muscle layers ($P > 0.05$).

Mean microvilli length (MVL), diameter (MVD) and enterocyte apical area (EAA) did not differ significantly between dietary treatments ($P > 0.05$). Microvilli counts (MVC) were significantly different between treatments ($F = 9.481$, $P = 0.001$), due to significantly lower counts in LS0.1 than other treatments. Estimated total absorption surface areas per enterocyte were significantly different between treatments ($F = 5.69$, $P < 0.05$). Reflective of numerically lower MVL, and significantly reduced MVCT, LS0.1-fed fish displayed significantly reduced ETAS compared to LC and LS0.5 fed fish. Mean microvillar percentage coverage (MVCV) at the brush border was significantly affected by dietary treatment ($F = 4.97$, $P < 0.05$). Fish fed 0.5 % SYN displayed a higher coverage of microvilli compared to fish fed LC and LS0.1. Qualitative appraisal observed a reduction in areas of conformational irregularity or where denuding of microvilli had occurred in LS0.5 (Plate 4.6B). Further, qualitative assessment appeared to show tighter assembly of enterocytes and a higher degree of regularity in the structure of the brush border within the LS0.5 group, compared with both the LC and LS0.1 groups (see Plates

4.5 to 4.7). Qualitative appraisal did appear to show greater spaces between enterocytes and a generally more irregular ultrastructure within the LS0.1 group (see plates 4.5 to 4.7).

Table 4.8 Morphological parameters of the posterior intestine of the lupin-fed fish, with and without Synergen™ inclusion.

	Diet		
	LC	LS0.1	LS0.5
Macrostructure			
EH (µm)	38.55 ± 2.24 [a]	41.24 ± 1.04 [b]	41.36 ± 0.31 [b]
GC (no./mm)	224.06 ± 26.95 [a]	226.57 ± 18.45 [a]	182.63 ± 19.33 [b]
LPW (µm)	16.57 ± 3.53	16.44 ± 2.08	16.02 ± 2.31
Muscularis			
MTI	1.65 ± 0.13	1.49 ± 0.37	1.79 ± 0.29
SLON (%MT)	34.83 ± 5.63	34.84 ± 7.07	34.97 ± 3.80
SCR (%MT)	25.28 ± 2.55	26.09 ± 2.81	25.98 ± 5.36
SCM (%MT)	16.07 ± 1.56	17.84 ± 3.63	17.49 ± 3.10
Ultrastructure			
MVL (µm)	1.40 ± 0.34	1.22 ± 0.20	1.48 ± 0.19
MVD (µm)	0.13 ± 0.02	0.12 ± 0.00	0.12 ± 0.01
MVCT (/µm^2)	82.51 ± 9.40 [a]	69.90 ± 6.19 [b]	85.74 ± 7.32 [a]
EAA (µm^2)	41.22 ± 2.11	41.39 ± 2.35	41.29 ± 2.31
ETAS (µm^2)	1833.75 ± 400.06 [a]	1365.51 ± 216.40 [b]	1877.63 ± 396.65 [a]
MVCV (%)	89.92 ± 2.18 [a]	88.07 ± 1.65 [a]	92.04 ± 1.58 [b]

Abbreviations: EH = enterocyte height; GC = globlet cell counts; LPW = lamina propria width; MT = total muscularis thickness; MTI = muscularis thickness index; SLON = % stratum longitudinale of MT; SCR = % stratum circulae of MT; SCM = % stratum compactum of MT; MVL = microvilli length; MVD = microvilli diameter; MVCT = microvilli counts; EAA = enterocyte apical area; ETAS = estimated total absorptive surface area per enterocyte; MVCV = microvilli coverage.

Values expressed as mean ± S.D. Statistical tests: ANOVA + Fisher's LSD (LPW, MTI, SLON, SCR, SCM, MVL, MVD, MVCT, EAA, ETAS, MVCV); Kruskal-Wallis + Mann-Whitney U (EH). *n* = 8.

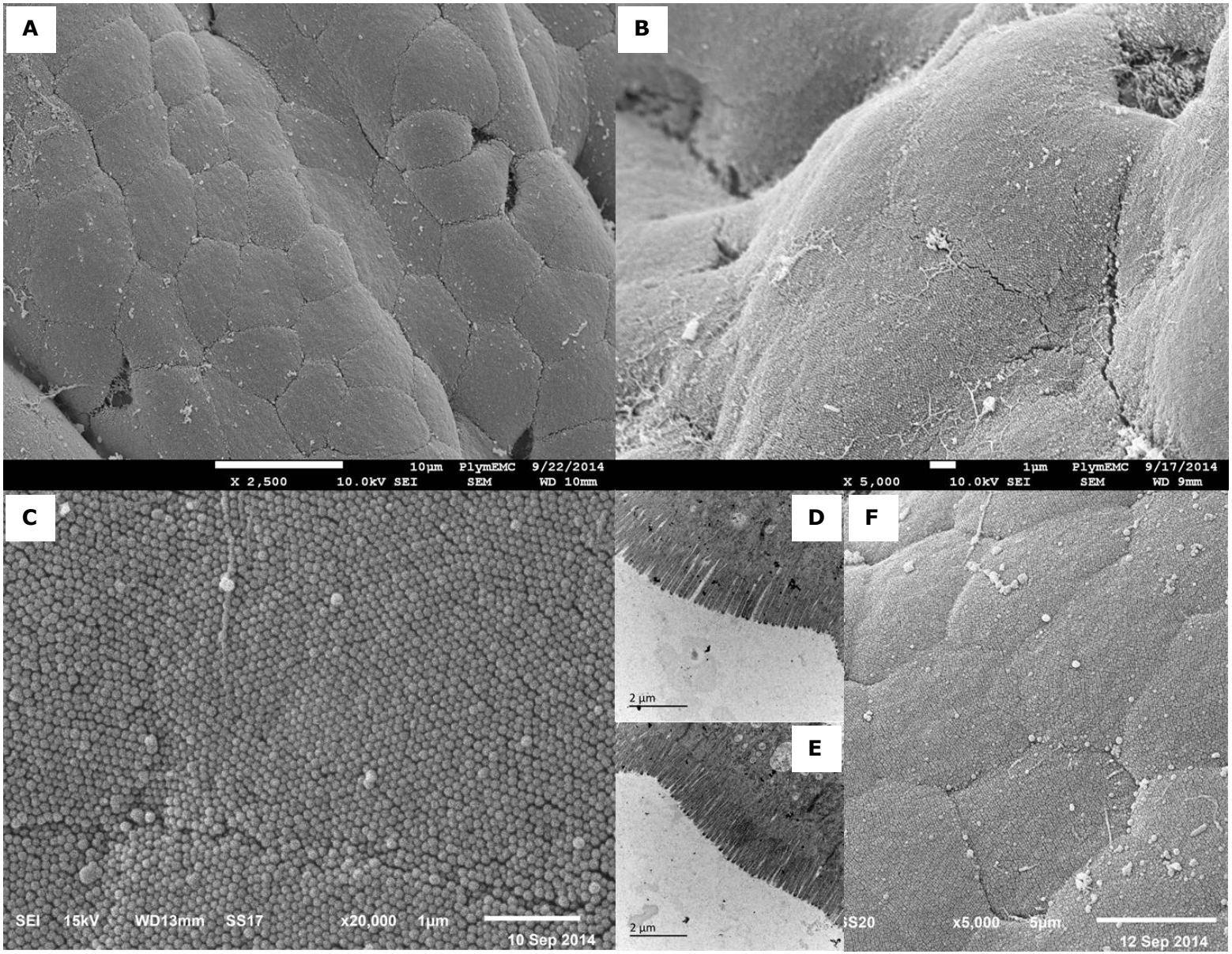

Plate 4.5 Intestinal ultrastructure of fish fed the basal lupin diet (LC) after 10 weeks.
A = 2,500 X mag. SEM, **B** = 5,000 X mag. SEM, **C** = 20,000 X mag. SEM, **D** & **E** = 20,000 X mag. TEM, **F** = 5,000 X mag. SEM

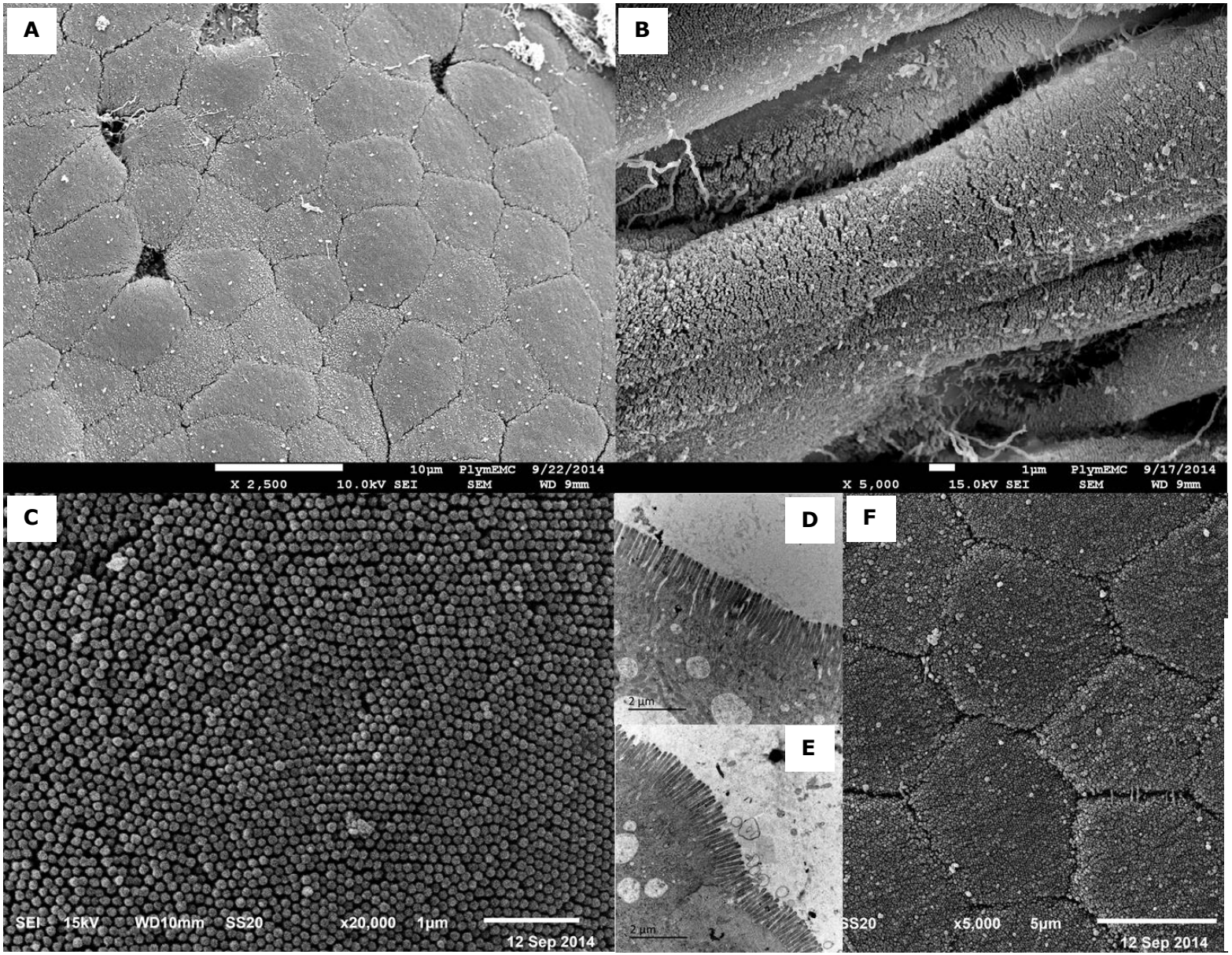

Plate 4.6 Intestinal ultrastructure of fish fed a 0.1 % SYN inclusion (LS0.1) after 10 weeks.
A = 2,500 X mag. SEM, **B** = 5,000 X mag. SEM, **C** = 20,000 X mag. SEM, **D** & **E** = 20,000 X mag. TEM, **F** = 5,000 X mag. SEM

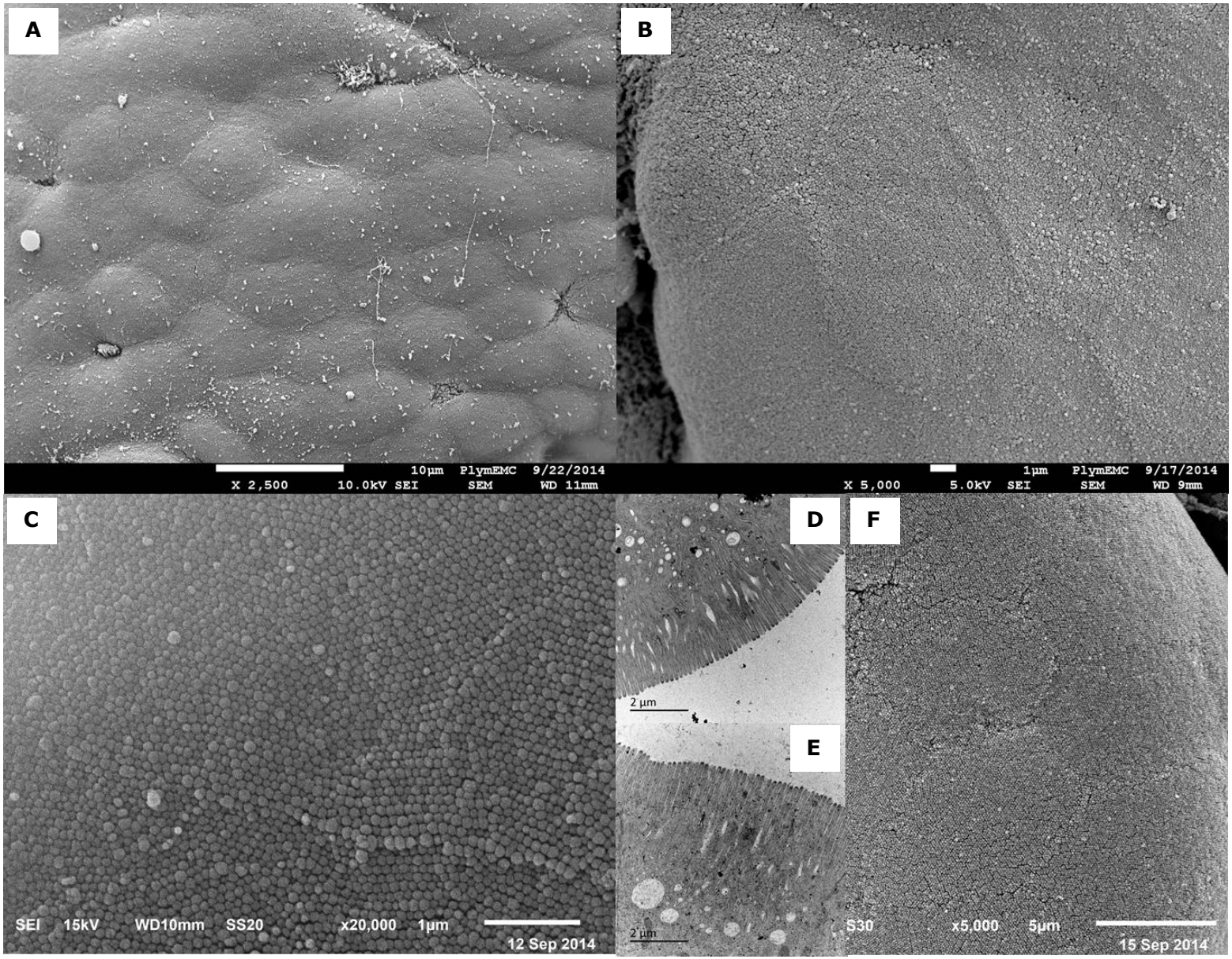

Plate 4.7 Intestinal ultrastructure of fish fed a 0.5 % SYN inclusion (LS0.5) after 10 weeks.
A = 2,500 X mag. SEM, **B** = 5,000 X mag. SEM, **C** = 20,000 X mag. SEM, **D** & **E** = 20,000 X mag. TEM, **F** = 5,000 X mag. SEM

4.3.7 Intestinal proteolytic enzyme activity

Anterior intestinal protease activity results are displayed in Table 4.9.

Total alkaline protease (TAP), trypsin and chymotrypsin activities in the digesta of fish were unaffected by dietary treatment ($P > 0.05$).

Alkaline phosphatase (ALP) activity in digesta was significantly different between treatments ($F = 3.76$, $P < 0.05$). Activity of ALP in digesta was significantly higher in SYN treatments than LC. No significant difference in ALP activity was present between SYN treatments. L-leucine aminopeptidase (LAP) activity in digesta was indicated to be significantly affected by dietary treatment ($F = 6.04$, $P = 0.01$). Activity of LAP was slightly significantly ($P = 0.045$) higher in LS0.1 fed fish than LC fed fish. Activity of LAP was also significantly higher in LS0.5 fed fish than LC fed fish. No significant difference was observed between SYN treatments.

No significant effects of dietary treatment were observed in ALP and LAP activities in anterior intestinal mucosa samples, although numerical tendencies towards decreased activity with SYN supplementation may be present.

Table 4.9 Proteolytic enzyme activities in digesta and mucosa of fish fed the lupin based diet, with and without SYN inclusion.

	Diet		
	LC	LS0.1	LS0.5
Digesta			
TAP* (U/g)	17.50 ± 3.75	16.03 ± 1.26	17.71 ± 1.32
Trypsin (U/g)	68.18 ± 21.31	66.97 ± 14.02	65.03 ± 13.89
Chymotrypsin (U/g)	325.50 ± 129.00	290.29 ± 27.89	368.57 ± 98.38
ALP (U/g)	51.64 ± 8.94 [a]	78.92 ± 17.14 [b]	79.37 ± 20.56 [b]
LAP (U/g)	5.91 ± 1.27 [a]	7.61 ± 2.85 [b]	8.50 ± 1.06 [b]
Mucosa			
ALP (U/g)	216.73 ± 56.31	203.24 ± 57.98	169.73 ± 68.43
LAP (U/mg)	837.87 ± 225.36	737.07 ± 113.59	692.20 ± 102.33

Abbreviations: TAP = total alkaline protease; ALP = alkaline phosphatase; LAP = L-leucine aminopeptidase. $*10^{-3}$.

Values expressed as mean ± S.D. Statistical tests: ANOVA + Fisher's LSD.

4.3.8 Intestinal microbiota

A total of 1,734,497 raw reads were obtained from the intestinal samples. After low-quality reads (Q score <20) were excluded, 68,494 ± 11,487, 63,726 ± 8,718 and 55,838 ± 7,477 sequences were obtained for the LC, LS0.1 and LS0.5 treatments, respectively. Good's coverage estimates plateaued at ≥0.99 within all treatments (Table 4.11), showing adequate sequence coverage was present in order to assume identified OTUs were representative of the population. Species richness and diversity were observed to be highest in the LS0.1 group, followed by the LC and the LS0.5 groups, as demonstrated by Chao1, observed species and Shannon's diversity (Table 4.10).

Table 4.10 Alpha diversity metrics of allochthonous microbial populations within the dietary treatments

	Treatment		
	LC	LS0.1	LS0.5
Good's coverage	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00
Chao1	108.28 ± 29.88	130.85 ± 13.15	108.49 ± 12.63
Shannon[1]	3.99 ± 1.11	4.84 ± 0.56	3.85 ± 0.40
Observed species	80.96 ± 24.36	101.15 ± 11.11	86.17 ± 9.28
PD[2]	4.40 ± 0.79	4.60 ± 0.32	4.09 ± 0.37

[1] Shannon's diversity index, [2] phylogenetic distances. (n = 6)

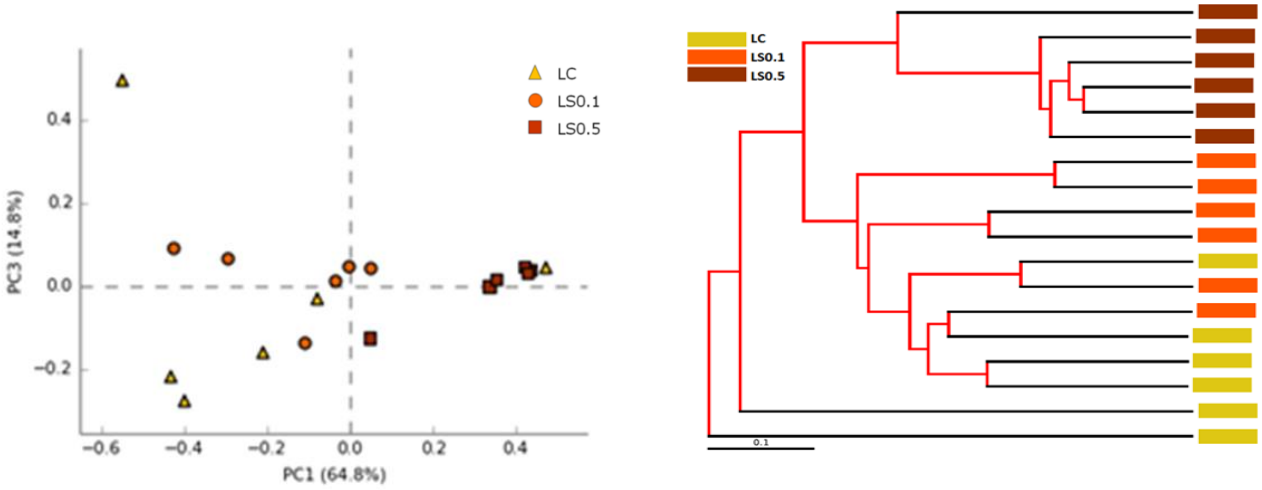

Figure 4.1 Allochthonous microbiome relatedness of fish fed a lupin-based diet, with and without inclusions of SYN.
Left = Principle coordinates analysis (PCoA) of phyla within the lupin-based diets. ***Right*** = Bray Curtis jackknife UPGMA showing hierarchical clustering of microbiota from lupin-based dietary treatments; bootstrap values are indicated by red branches (75-100%).

PCoA plots appear to show some degree of separation between the dietary treatments, with the highest clustering observed within the LS0.5 group (Fig. 4.1). This was strongly supported by the UPGMA which clearly demonstrates clustering of the LS0.5, distinctly separated from the LC and LS0.1 groups, which express some overlapping similarities between sampled individuals (Fig 4.1).

A total of six OTUs, at a phylum level, were found to each account for over 0.1 % of the total microbiome reads; these being, Firmicutes, Fusobacteria, Actinobacteria, Bacteroidetes, Spirochaetes and Proteobacteria (Fig. 4.2). Other identified phyla (<0.1 %) were Acidobacteria, Planctomycetes, Nitrospirae and the candidate phyla GN02 and OP3 (Fig 4.2). The proportional abundance of reads assigned to Fusobacteria were found to be close to significance between treatments (P = 0.09), qualitative assessment indicated proportions to decrease with SYN inclusion (Fig. 4.3A). The proportional abundance of reads assigned to Firmicutes was found to differ significantly between treatments (P < 0.05). Proportions were significantly higher in LS0.1 (40.20 % of total sequences) than LC (1.60 % total) and LS0.5 (3.80 % total) (Fig. 4.3B). A significant difference in the abundance of reads assigned to Proteobacteria was indicated between treatments (P < 0.05) (Fig. 4.4A). Proportional contribution of Proteobacteria was significantly higher in LS0.5 fish (87.80 % total) than LC (56.10 % total) and LS0.1 (36.60 % total). The abundance of reads assigned to Spirochaetes was significantly different between treatments (P < 0.05) (Fig. 4.4B); it was observed that OTUs within this phyla contributed 22.10, 0.30 and 0.1 % of sequences in LC, LS0.1 and LS0.5, respectively.

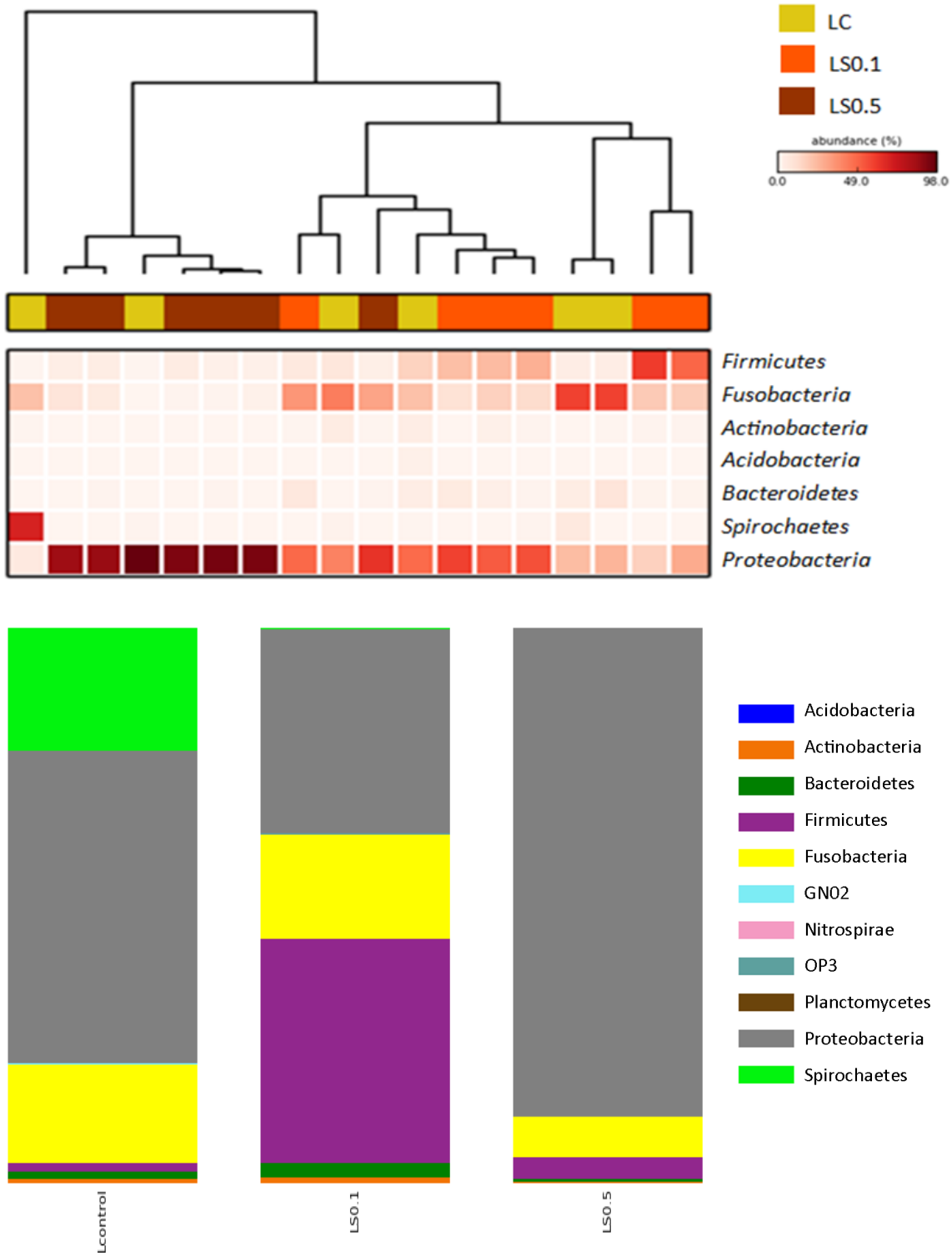

Figure 4.2 Abundance and proportional contributions of phyla within the allochthonous microbiomes of the dietary groups.
Top = Heatmap displaying the abundance of phyla (>0.1 % contribution) within individual fish (*columns*) and their respective dietary group (*colours*). Intensity of *red squares* denotes abundance (%). ***Bottom*** = proportional contributions of all phyla identified within the dietary groups.

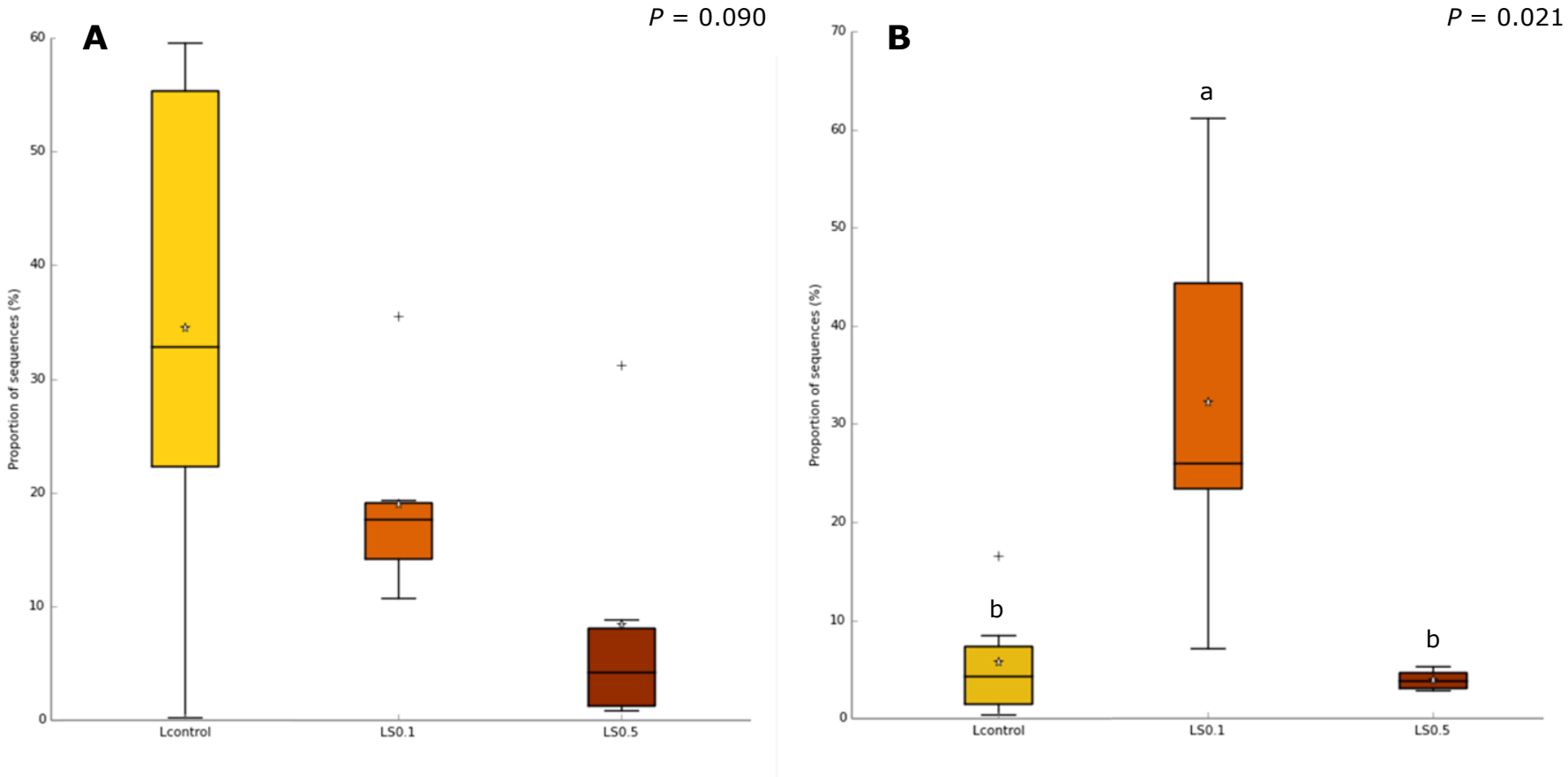

Figure 4.3 Proportional contribution of selected bacterial phyla within the digesta of the dietary groups.
A = Fusobacteria. **B** = Firmicutes.
Boxes sharing the same superscript are not significantly different (P≤0.05).

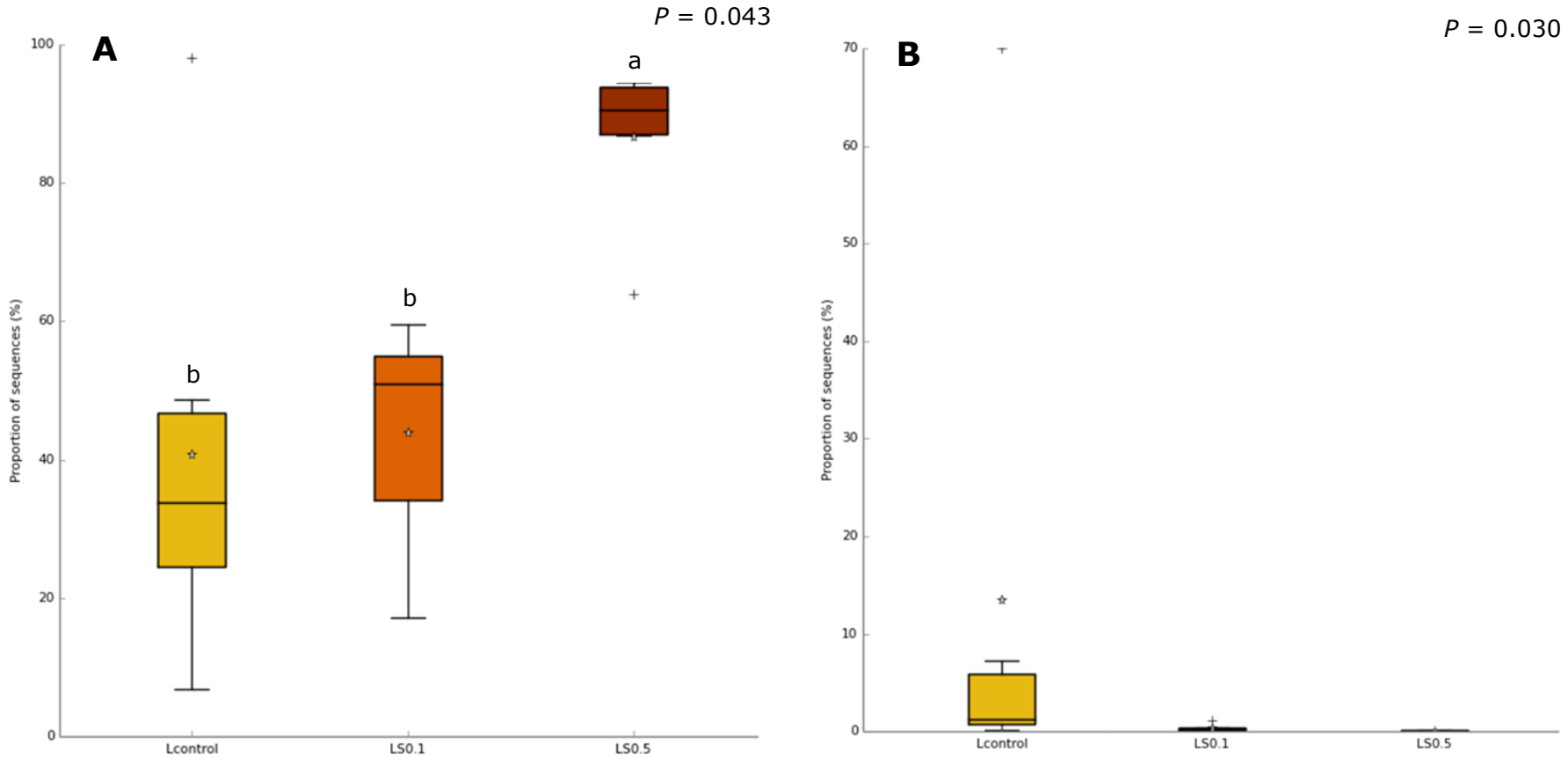

Figure 4.4 Proportional contribution of selected bacterial phyla within the digesta of the dietary groups.
A = Proteobacteria. **B** = Spirochaetes.
Boxes sharing the same superscript are not significantly different (P≤0.05) (**A**). (**B**) LS0.5 expressed 0.00% proportion of sequences, thus post-hoc analysis could not be performed.

The *core microbiome* of the experimental fish was found to comprise of 17 genera, whilst 2, 21 and 4 unique genera were identified within the LC, LS0.1 and LS0.5 treatments, respectively (Fig. 4.5). A total of 7 genera were shared only between LC and LS0.1, whilst LS0.1 and LS0.5 exclusively shared 4 genera. No genera exclusively shared between the LC and LS0.5 treatments were identified, reaffirming previously discussed observations of divergence between these groups.

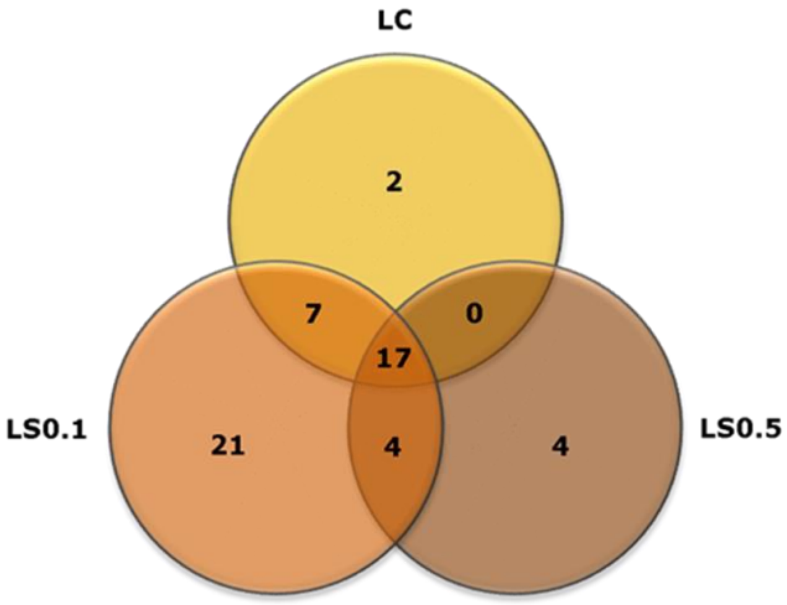

Figure 4.5 Venn diagram displaying the numbers of shared and exclusive genera within the allochthonous microbial population of the dietary groups.

Graphical presentation of all genera accounting for over 0.1 % of sequences in each fish is displayed in Fig 4.6.

The proportions of sequences assigned to three genera within the family Enterobacteriaceae were found to differ significantly between treatments. Firstly, an unidentified genus was found to differ significantly ($P < 0.5$), being greater in LS0.5 than LC and LS0.1 (Fig. 4.7A). This unidentified genus contributed 67.70 % of sequences in LS0.5 compared to 7.50 % in LS0.1 and 0.30 % in LC. Further attempts to identify genus and species returned similarity in sequence to both *Enterobacter* spp. and *Klebsiella* spp. both of which frequently expressing >98 % similarity. It is of note that

similarity to *Enterobacter cloaceae* and *E. aerogenes* was repeatedly observed, with these species being most probable for presence within the salmonid microbiota. Secondly, sequences assigned to *Erwinia* were found to differ significantly ($P < 0.05$) between treatments, due to higher abundance in LS0.5 compared to LC and LS0.1 (Fig. 4.7B). Species identification results were inconclusive. Lastly, *Cronobacter* was found to follow the previous trend as the previously discussed Enterobacteriaceae genera ($P < 0.05$) (Fig. 4.7C). Mean contribution in the LS0.5 group was 3.90 % of reads, compared to 0.00 and 0.10 % in the LC and LS0.1 groups, respectively. Again, species identification results were inconclusive. Overall, Enterobacteriaceae dominated sequence reads assigned to the Proteobacteria fraction of the LS0.5 treatment, with 72.40 % contribution compared with 0.70 and 8.40 % in the LC and LS0.1 groups, respectively. The overall Proteobacteria proportions observed in the LC group were comprised of a range of classes, orders and families with relatively low proportional contribution, once one individual which expressed very high proportions of *Deefgea* was omitted. The LS0.1 group similarly exhibited no clear dominating genera within the phyla of Proteobacteria, with *Pseudomonas*, *Moritella*, an identified genus belonging to Comamonadaceae and the believed *Enterobacter* contributing 4.10, 7.40, 3.10 and 7.50 % of reads, respectively, alongside 30 other genera contributing between 0.10 and 3.00 % of sequences.

Bacillus proportions differed significantly between treatments ($P < 0.05$); the contribution to total microbiome was significantly greater in LS0.1 (33.30 %) than LC (0.50 %) and LS0.5 (1.00 %) (Fig. 4.8A). No significant difference was observed between LC and LS0.5. Species identification revealed a high degree of relatedness (>99 %) to *Bacillus cereus*, *Bacillus mycoides*, *Bacillus subtillis* and *Bacillus thuringiensis*. This result explains previously observed results of increased Firmicutes in LS0.1.

Janthinobacterium proportions differed significantly between treatments ($P < 0.05$) (Fig. 4.8B). No treatment exhibited on average over 1 % contribution of this genera; but a complete absence in the LS0.5 group rendered a significant different between this treatment and LC and LS0.1, whilst presence appeared to decrease with SYN inclusion.

A significant difference in the contribution of an unidentified genus, belonging to the order Aeromonadales, was detected between treatments ($P < 0.05$), being significantly greater in LC than LS0.5 (Fig. 4.8C). The contribution was approximately 0.1 % of sequences observed in LC, <0.1 % in LS0.1 and negligible in LS0.5. This sequence showed >99 % similarity to *Aeromonas salmonicida*.

A significant difference in an unidentified genus belonging to the family Brevinemataceae was observed between treatments ($P < 0.05$) (Fig. 4.8D); the proportional contribution was numerically greater in LC (22.10 %) than LS0.1 (0.30 %) and LS0.5 (0.10 %). Species identification revealed 98 % similarity to *Photobacterium piscicola*; however, identification of sequences frequently returned no conclusive results leaving a degree of uncertainty to the species identity.

Although no significant differences were observed, a number of OTUs appeared higher in the LS0.1 group and must be noted (Fig 4.6). *Moritella* contribution was 7.40 % of reads in the LS0.1 group compared with 2.10 % in both other analysed treatments. Identification returned 100 % similarity to *Moritella viscosa. Flavobacterium* presence was doubled in the LS0.1 group's collective microbiome compared with the other two treatments (0.2 and 0.1 %, respectively); identification to species level was inconclusive. *Pseudomonas* represented 4.10 % of the sequences within the LS0.1 group, compared with 0.40 and 0.90 % in the LC and LS0.5 treatments, respectively. Species identification was inconclusive. Other genera which increased within the LS0.1 group include 0.7 % *Clostridium* (0.2 and 0 % in LC and LS0.5), 0.4 % *Rudanella* (0 % in LC and LS0.5), 0.8 % *Shewanella* (0.3 and 0 % in LC and LS0.5), 0.7 % *Pleisomonas* (0.4 and 0.2 % in LC and LS0.5), 0.6 % *Staphylococcus* (0.3 and 0 % in LC and LS0.5), 0.8 % *Sphingomonas* (0 % in LC and LS0.5), 0.6 % *Collimonas* (0% in LC and LS0.5), 0.3 % *Microbacterium* (0 % in LC and LS0.5) and 0.7 % of an unidentified genus belonging to Streptococcaceae (0 % in LC and LS0.5).

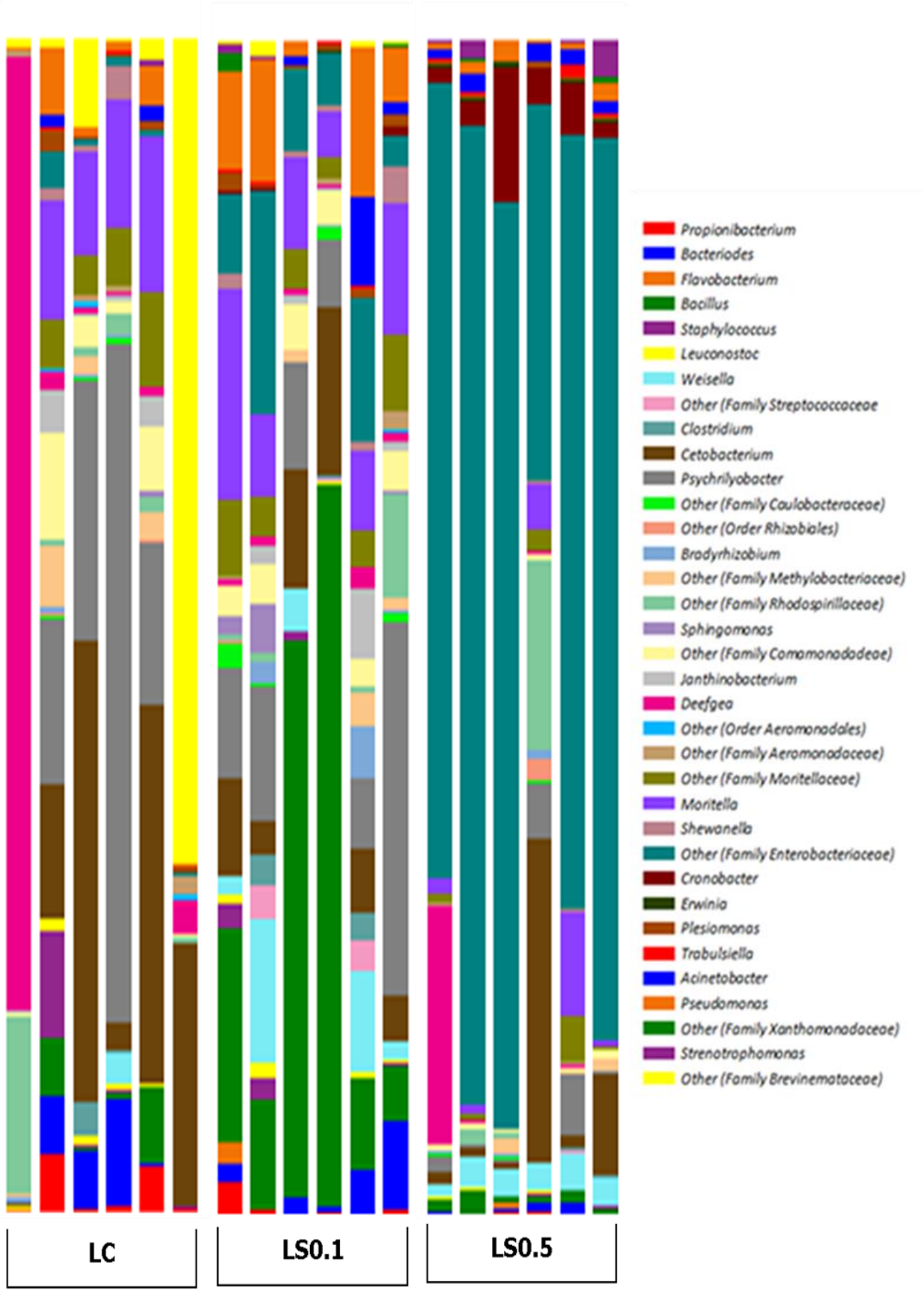

Figure 4.6 Proportional contributions of identified genera within the microbiomes of sampled individuals. OTUs presented are those which express >0.1 % contribution towards the total number of sequences identified.

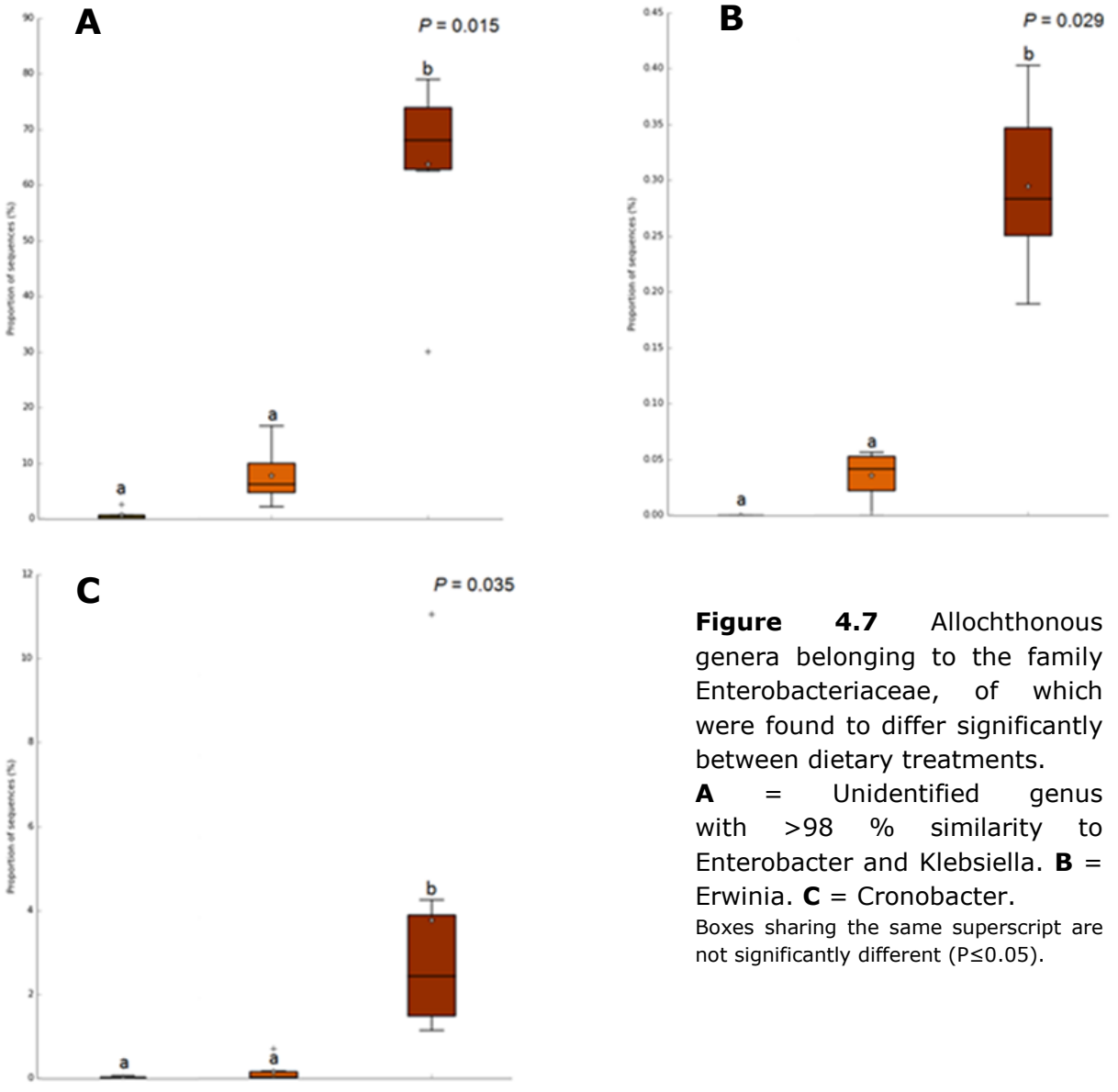

Figure 4.7 Allochthonous genera belonging to the family Enterobacteriaceae, of which were found to differ significantly between dietary treatments. **A** = Unidentified genus with >98 % similarity to Enterobacter and Klebsiella. **B** = Erwinia. **C** = Cronobacter.
Boxes sharing the same superscript are not significantly different (P≤0.05).

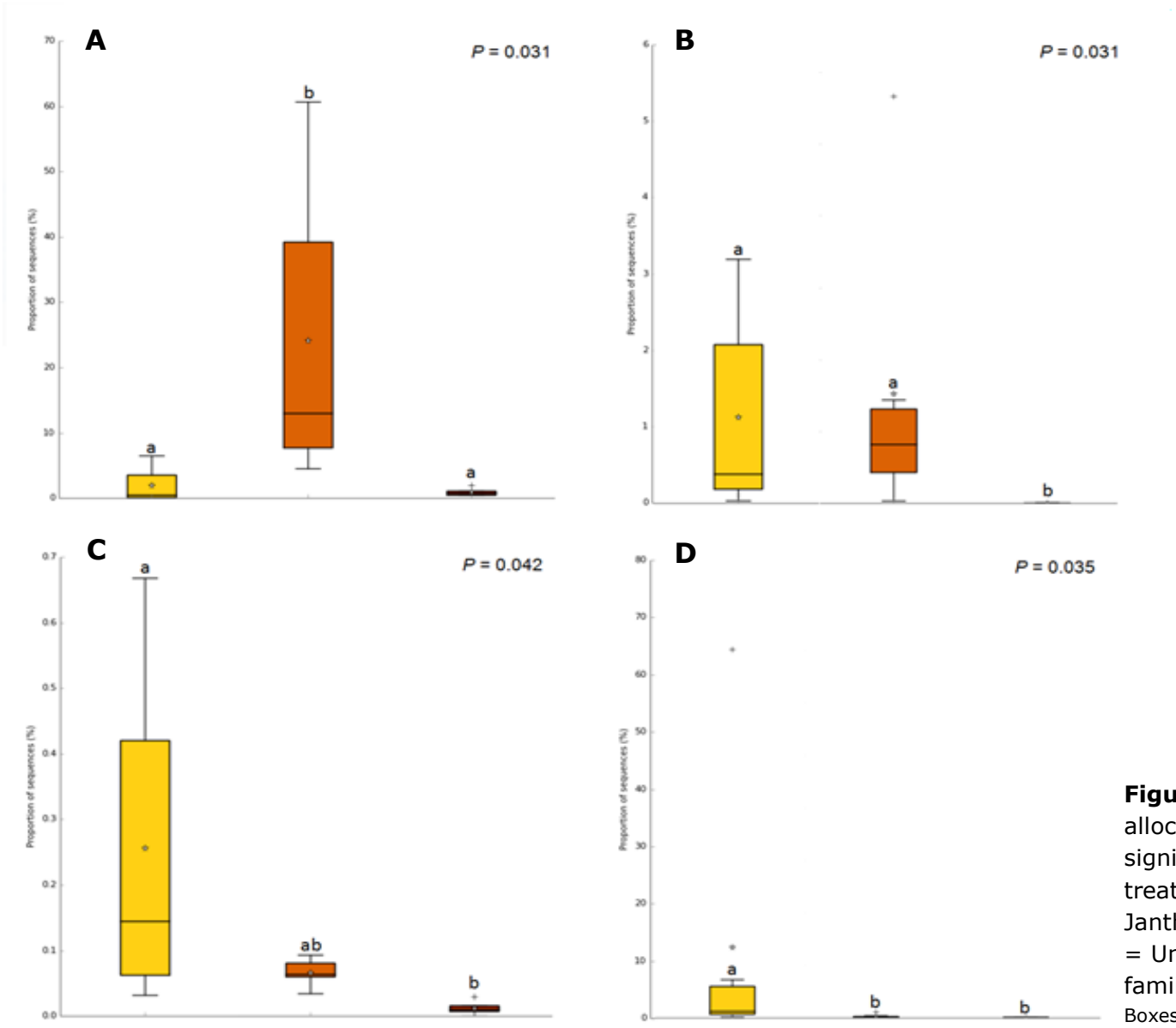

Figure 4.8 Miscellaneous allochthonous genera found to differ significantly between dietary treatments. **A** = Bacillus. **B** = Janthinobacterium **C** = Aeromonas. **D** = Unidentified genus belonging to the family Brevinemataceae.

Boxes sharing the same superscript are not significantly different (P≤0.05).

4.4 Discussion

4.4.1 Fish performance and feed efficiency

The performance enhancing benefits of bioactive feed components are well regarded in terrestrial monogastrics and to some extent in warm-water aquatic species. However, their application in farm livestock generally becomes less successful as the core temperature of the animal drops. Clearly, the temperate salmonid species are likely to be a great challenge. In comparison with most other aquacultured species, salmonids require low water temperatures throughout their production. Eggs require particularly cool water, with death of eyed eggs and deformity of hatching alevins becoming economically crippling and unethical above 12 °C; thereafter, optimum rearing temperatures for growth are dependent upon species but can generally be considered to be between 12 and 16 °C. Moreover, temperatures throughout the year will seldom remain optimal for grow-out sites, with seasonal fluctuations regularly reducing water temperatures to well below 12 °C whilst still maintaining good performance. That being said, bioactive components (be it enzymes or microbes) are not ubiquitous in their optimal or effective range of temperatures so the application of multi-component ingredients such as Synergen™ (SYN) are highly attractive and theoretically hold more potential for retaining some form of beneficial activity. The inclusion of SYN in yellow lupin-based diets for rainbow trout returned solid evidence of performance enhancement at 12.5 °C within this investigation; however, this was only apparent following an inclusion rate of 0.5 %, with the 0.1 % inclusion returning negligible effects. Previously, the 0.1 % inclusion was deemed effective in lupin-based diets for Nile tilapia but it is evident that functionality of SYN was reduced following transition to a salmonid species, in this case rainbow trout. Lower rearing temperature will inevitably lead to a reduction in the activation energy available for bioactive components to work effectively under *in vivo* conditions. Although perhaps crude in appearance, simply increasing inclusion rate has been demonstrated as an effective means of maintaining the functionality of phytase applications in salmonid diets (Vandenberg *et al*., 2012). Indeed in this case, a five-fold

increase was highly effective in promoting growth performance and general feed utilisation. Most promisingly, the LS0.5-fed fish were closer in performance to those fed a high-grade fishmeal (FM) diet, which could be considered cost-ineffective due its ingredient matrix. However, in this study the FM-diet served as a reference, semi-purified diet for achieving maximum allowable performance under the experimental conditions. Rainbow trout fed phytase supplemented diets have previously been reported to express performance characteristics closer to a FM-based diet than the original basal diet (Vandenberg *et al.*, 2011).

4.4.2 Macronutrient availability and status

Quantification of nutrient availabilities revealed many improvements in both SYN inclusion levels for rainbow trout fed the lupin-based diets. The increase in apparent digestibility of crude protein, by 2.27 % between the LC and LS0.5 groups, serves a likely contributor to enhanced growth, protein efficiency and also an increase in carcass crude protein content. In support of these findings, augmented protein efficiency, leading to increased performance has been observed in broilers fed commercial diets supplemented with a similar product, Allzyme®SSF (Yadava *et al*., 2009). Improved availability of protein is also extensively regarded as one of the main benefits of phytase additions, with observations across many species (Kumar *et al*., 2012). Gross energy digestibility was similarly improved following SYN supplementation, increasing by 8.25 % between the LC and LS0.5 groups. Once again, increased gross energy was expressed within the carcasses of sampled fish. Improved gross energy availability has been extensively observed following supplementation of a range of purified exogenous enzyme preparations in monogastrics; including endo-β-glucanase and endo-β-xylanase in pigs (O'Connell *et al.*, 2006), multi-enzymes and xylanase in poultry (Danicke *et al.*, 2000; Zhang *et al.*, 2012) and multi-enzymes in tilapia (de Oliveira *et al.*, 2007; Guimaraes *et al.*, 2009). Increased gross energy provision has also been reported as possible following phytase supplementation in rainbow trout diets (Cheng and Hardy, 2002). The mode of

action in this instance is widely regarded to consist of degradation of chelates and reduction of intestinal viscosity which augments enzyme-substrate interactions. With the latter of the explanations, one would expect to see some form of improved carbohydrate degradation.

Although the LS0.1 diet also induced significant improvements in digestible protein and energy, this was not observed in the digestibility of crude fibre. Only the LS0.5 dietary treatment induced an effect on this carbohydrate parameter, with an increase of approximately 31.5 %. It is recognised that limitations exist in determination of crude fibre, considering it is not comprehensive of all non-starch polysaccharides. Its analytical quantification does lose substantial quantities of soluble hemicelluloses during the procedure. However, it is fair to suggest that some exogenous carbohydrase enzymes, derived from SYN, particularly cellulase, expressed efficacy within the luminal environment of the GIT. Hemicellulase action is also likely since industrial fermentation processes with *A. niger* are regarded to produce potent activities of xylanases, mannanases and galactosidases (Laerke *et al.*, 2015; Inoue *et al.*, 2015; Manzanares *et al.*, 1998; Magalhaes *et al.*, 2014). Consequently, polysaccharide chain length would have been reduced to a level which caused monosaccharides to be more available to the animals, or to a level capable of significantly increasing short-chain oligosaccharides which could be fermented by the intestinal microbiota. Either way, a protein sparing effect is highly likely to have occurred, considering the significant decrease of carbohydrate fractions from the diet.

Serum glucose levels were significantly elevated in the LS0.5 and FMC compared with LC and LS0.1. Elevated blood glucose in salmonids is often regarded as a stress response (Benfey and Biron, 2000) or volatile glucose homeostasis but clearly in this case there are no grounds on which to attribute stress or poor regulation as factors since the elevation was observed in the two highest performing treatments, including one which was fed a relatively optimal dietary formulation. Therefore, attribution to dietary factors is most likely. The first, and simplest, explanation is that of increased provision of

dietary glucose. It may perhaps be surprising that the FMC fed fish expressed the highest levels but it is important to remember that although FM-based, this semi-purified diet contained appreciable quantities of starch, which can be considered a relatively non-complex carbohydrate matrix. The anterior intestine of salmonids is known to contain activity of α-amylase as well as mucosal maltase, sucrase and lactase (Korgdahl *et al*., 2004; Furne *et al*., 2005; Furne *et al*., 2008; Geurden *et al*., 2007; Santigosa *et al*., 2008) so glucose can be obtained from the hydrolysis of dietary starch (Krogdahl *et al*., 2004). Furthermore, oligosaccharides have been shown to impair the intestinal uptake of glucose in rats (Sone *et al*., 1992) whilst fibre in general was observed to do so in fish (Shiau *et al*., 1989); thus possible degradation of long- and short-chain polysaccharides in LS0.5 may have facilitated sugar absorption. All things considered, the higher serum glucose levels observed may be a result of improved availability due to reduced substrate complexity in the FMC group and exogenous carbohydrase activity in LS0.5. Indeed, in the case of LS0.5, increased crude fibre digestibility indicates that a significant absorption of cellulose-derived glucose was achieved. Conversely to these suggestions, chickens and quails supplemented with exogenous enzymes have been reported to display no effect on blood glucose levels (Moharrery, 2006; Jozefiak *et al*., 2011; Sahin *et al*., 2007). However one must consider the slow glucose turnover time and metabolic assimilation of glucose in carnivorous fish, compared to birds and mammals (Polakof et al., 2012); as well as the fact that digesta was still present in the intestine of sampled fish showing that digestion and assimilation of nutrients was on going at the time of measurement.

It is also worth considering that proteinaceous dietary factors may have influenced the observed results. In a recent study on blunt snout bream (*Megalobrama amblycephala*), dietary leucine levels were observed to increase plasma glucose (Ren *et al.*, 2015), indicating an important role of this amino acid in glucose homeostasis. The variations in crude protein digestibility which are likely to alter leucine provision may have played a role in glucose homeostasis but further work is required considering the novelty of Ren

et al.'s (2015) findings. Lastly, the presence of hypoglycaemic-stimulating lupin γ-conglutin must be considered, as discussed in Chapter 3; although it is largely unclear whether SYN has any capability of degrading this protein. It would be of great benefit for future work on the dietary application of lupins to explore the effect of γ-conglutin on glycaemia in fish.

4.4.3 Micronutrient availability, status and osteology

Vast improvements to mineral and trace element availabilities were observed throughout the SYN-supplemented diets. Perhaps of most significance was a 55 to 74 % increase in phosphorous uptake between the LC and LS0.5 diets. Phytase supplementation of plant protein diets for rainbow trout have previously reported results that are very much in line with those observed under the current study. Riche and Brown (1996) reported increases in P availability from 48 to 75 %, whilst Carter and Sajjadi (2011) observed increases from 45 to 80 % and Verlhac-Trichet *et al.* (2014) reported an increase from 49 to 71 % with 2000 FTU. The slightly higher baseline ABAC of P in this study could be largely attributed to the non-conservative mineral premix addition during dietary formulation, allowing good initial P availability. Overall, in terms of the vital criteria of P availability, SYN appears to produce comparable efficacy to dedicated phytate-degrading products. Furthermore, in correspondence with other research, all element bioavailabilities which increased throughout the SYN inclusions in the present study have been previously reported as improved following phytase inclusions in finfish diets (Liebert and Portz, 2005; Liebert and Portz, 2007; Laining *et al.*, 2012; Cheng *et al.*, 2004; Vandenberg *et al.*, 2012; Debnath *et al.*, 2005c; Baruah *et al.*, 2005; Hussain *et al.*, 2015a; Hussain *et al.*, 2015b; Hung *et al.*, 2015; Liu *et al.*, 2014). It is highly likely that the increased mineral bioavailabilities observed in this study have some, or all, of their origin in a degradation of phytate-mineral chelates. Since the basal diet contained both soyabean products and lupin kernel meal, it is probable that the phytate degraded in this process was from both ingredient types. Phytate is known to complex with the

proteins of both of the plants utilised in the diets (Hartman, 1979; Hidvegi and Lasztity, 2002). Theoretically, considerable reductions in inorganic excretions could be achieved with dietary SYN inclusions and may wish to be explored in future studies. Furthermore, there exists possibility for allowing savings in mineral supplementations by compensating the available levels with liberation from bulk ingredient sources. The use of more marginal diet formulations is encouraged in future, so as to increase the likelihood of identifying the extent to which ABAC and excretion of minerals may be improved.

Despite following the same trends in increased bioavailability of elements highly associated with phytate chelation, S is set aside in that it is not typically involved in such complexes. The increased availability, thus disappearance of S from the lumen is more likely to be attributed to increased digestibility of protein since it forms an integral part of abundant sulfur-bridge amino acids (i.e. methionine and cysteine).

The limited availability of Zn has regularly been associated with phytate chelation (Satoh *et al*., 1993) and targeted degradation of the compound has regularly been observed as successful in increasing the provision of this essential trace element (Cheng *et al*., 2004; Debnath *et al*., 2005c; Baruah *et al*., 2007b; Kumar *et al*., 2012; Vandenberg *et al*., 2012). This study did reveal a significant increase in Zn availability following SYN inclusions however the trends were not as strong as was observed in many other elements since no significant difference was found between LC and LS0.1. Elements will most certainly not be ubiquitous in their distribution throughout the gross seed structure, nor will they be in their distribution amongst binding agents within. Porres *et al.* (2007) identified this occurrence in all three major lupin species, indicating that the proportions of the total content of elements varies between the embryo, cotyledon and hull and that association with α-galactosides similarly differs. Considering that Porres *et al.* (2007) reported that partitioning of Zn throughout lupin seed layers is not in line with that of P, as well as Zn concentration increasing and P concentration decreasing following removal of α-galactosides, it is fair to say that a different enzymatic action may have contributed

significantly to the release of Zn. Being somewhat in line with the trends in CF ADC, this may have partially occurred through liberation from polysaccharides.

Fe did not appear to follow an increase in availability throughout the SYN inclusions, since LS0.1 displayed the greatest bioavailability coefficient. The conditions for intestinal Fe uptake in fish are not well understood, making it difficult to elucidate why this may have been the case. Furthermore, endogenous Fe losses can be high (Bury and Grosell, 2003; Bury *et al*., 2003), thus possibly masking true effects of dietary availability. Interestingly, haematocrit was also increased in the LS0.1 treatment but not the LS0.5, indicating a higher Fe status in the former; despite, haemoglobin not expressing such strong trends. Ultimately, it is unclear whether there was indeed a relationship between Fe uptake/loss and erythrocyte-related characteristics. The supplementation of tilapia diets, with a mixture of pepsin, papain and α-amylase, has also been observed to increase red blood cell counts, haematocrit and haemoglobin (Goda *et al*., 2012), although a scientific explanation as to why this occurred was similarly not identified. The possibility of the LC and the LS0.5 actually expressing a drop in haematocrit levels would be typical of pathogenic infection in salmonids (Snow *et al*., 2001) but it is highly unlikely considering the performance and the apparent health of the animals.

Overall, the increase in digestibility of multiple macronutrients as well as bioavailability of minerals demonstrated a multi-faceted efficacy in improving nutrient profile; with much similarity to results observed in both phytase (see Kumar *et al*., 2012) and carbohydrase (see Castillo and Gatlin, 2015) inclusions in finfish diets.

The bioavailability of Na is most distinctly isolated from those discussed previously due to the behaviours and roles of this element in the digestive process. These largely comprise of participation in various active transport systems; e.g. Na^+/K^+ pump and Na glucose transporter. Absorption of Na occurs largely in the oesophagus and gastric portions of salmonids, with significant excretions beginning in the anterior intestine (Bucking *et al*., 2011). Its absorption depends less so upon the digestive mechanisms

critical for assimilation of other dietary elements. All three lupin-based diets expressed negative coefficients, showing loss of Na was greater than dietary intake. However the quantity of Na found within the stripped faecal samples was indicated to decrease with SYN inclusion. The faecal excretion of Na in Atlantic salmon has previously been reported as elevated following high plant protein diets, this has been inclusive of soyabean, maize, sunflower, rapeseed and pea inclusions but interestingly, it has been reported as less pronounced in lupin (Storebakken *et al*., 1998). Storebakken *et al*. (1998) suggested that NSPs could be the root-cause since they have a high affinity for cationic elements. However, Aslaksen *et al*. (2007) provided evidence that this may not be the case as dehulled beans yielded the same result as those which were fully hulled. The same authors also demonstrated that the level of Na excretion was not correlated with phytic acid. Instead, attribution was given to the disruption of the epithelial layer by other ANFs causing excessive endogenous loss.

Quantification of element concentrations within specific tissues suggested little variation in most instances between dietary treatments but it is important to consider that a greater size of fish could result in the more uniform concentrations observed, due to greater tissue mass accretion. After all, this is a factor which could well be a contributor to the promotion of growth, in the first instance. Homeostasis of minerals is also very tightly regulated (Lall, 2002) and without deficiency, the chance of observing significant effects is greatly reduced. Nonetheless, some significant differences were identified, indicating greater, or indeed lesser, mineral status between the dietary treatments.

Zn was identified as being present in a significantly higher concentration in the liver of FMC-fed fish, whilst numerical trends of this sort were carried throughout all identified tissues. This could largely be explained by the discrepancies of original dietary Zn levels, with approximately 640 to 645 mg/kg within the lupin-based diets and approximately 780 mg/kg in the FMC. Furthermore, following SYN inclusion numerical trends in increasing Zn concentrations, which somewhat followed ABAC trends, also appeared to be present. These results are somewhat surprising since the liver of finfish has

previously been reported to not respond noticeably to dietary zinc levels or sources, since zinc metallothioneins do not appear to be stored in this organ, as is the case in mammals (Leeming, 2013 p. 13). Nonetheless, other tissues, including the caudal fin of rainbow trout, have been identified as good indicators of Zn status (Leeming, 2013).

Mn appeared to be the most prominent trace element which was subject to change in its concentration within the analysed tissues. This is perhaps unsurprising considering the great differences in dietary Mn concentration between the lupin- and FM-based diets. However, this does serve to demonstrate the points made in Sec. 4.1.4, regarding ingredient-specific micronutrient provision. This may be surprising considering the ABAC of Mn in the LC group was negative; however, one must consider the very high concentration and endogenous losses through the intestinal tract which could equate to an equal turnover, i.e. an ABAC value close to zero. Nevertheless, SYN inclusion appeared to increase the concentration of Mn in all analysed tissues, either significantly or quantitatively. The role of Mn and its essentiality is well established in animal nutrition. Its participation includes acting as an enzyme cofactor, comprising integral parts of metalloenzymes involved in carbohydrate and lipid metabolism and it is also known to interchangeably activate numerous enzymes with Mg (Lall, 2002). The application of yellow lupin alone appears to be capable of providing ample Mn, which could have profound effects upon metabolic functions and structural formations (Knox *et al*., 1981). With such high concentrations, concern may arise due to potential toxicity; however most animals exhibit good resistance to dietary toxicosis of Mn due to their excretory pathways (Keen *et al*., 1999), which would support the previous suggestion of Mn turnover. It has been noted that elevated dietary Mn levels may be especially attractive in broodstock nutrition (Lall, 2002). Increasing Mn supplementation in laying hens has indeed been observed to improve a number of egg quality parameters as well as overall productivity (Hossain and Rezendle, 1996; Hossain and Bertechini, 1998; Venglovska *et al*., 2014; Zhu *et al*., 2015). These results are a strong incentive for considering lupin

inclusions in salmonid diets, more so considering the Mn requirement of salmonids appears to be higher than many other fish (Lall, 2002).

Despite no significant modulation of vertebral Ca and P concentrations between treatments, the ratios of these elements was found to differ as a consequence of diet, although all treatments expressed typical salmonid ratios, judging by the work Helland *et al*., 2005. The significant incremental reduction in Ca:P ratio following SYN inclusion indicates that proportional contribution of P increased slightly in these dietary treatments. This seems to contradict the findings of (Helland *et al*., 2005), whom reported whole body Ca:P ratio of Atlantic salmon to increase between a low and high P diets; probably with similar trends in vertebral Ca:P, judging by reported Ca and P concentrations. On the other hand, inclusion of graded levels of phytic acid in Atlantic salmon diets was suggested to also raise Ca:P within the body, alongside coupled numerical trends within vertebrae, which was ultimately caused by P deprivation (Helland *et al*., 2006). Interestingly, Helland *et al*. (2006) also identified an increase in the occurrence of hyper-dense vertebrae within fish fed a higher phytic acid diet, suggesting a greater mineralisation. This could perhaps intertie with the observation of overall reductions in vertebral Ca and P concentrations following SYN inclusion, which is believed to have exerted phytic acid-degrading properties upon the diets. Overall, deficiency of P has been implicated in structural deformities, reduced bone hardness and a reduction in bone size (Baeverfjord *et al*., 1998; Vielma and Lall, 1998; Roberts *et al*., 2001), so a greater contribution could display amelioration of these pathologies. This study did not identify any alteration to the centrum size, thus potential for improved for structural integrity could not be suggested on a morphological level. Supplementing broiler diets with Allzyme®SSF was observed to show no effect upon bone strength (Gentilini *et al.*, 2009); however the authors did not quantify mineral bioavailability, leading to uncertainty as to whether these findings were in correspondence with one another. It must be noted that the size of fish utilised in the present study was one which had already undergone a vast proportion of its ontogenetic developments and was long-past life-stages where

ossification is most critical. Nevertheless, seen as the mineral release by SYN appeared to modulate bone mineralisation even in advanced juveniles, future work should consider younger developmental stages in order to reassess whether mineral release by SYN may improve structural integrity.

4.4.4 Intestinal morphology, microbiota and proteolytic activity

The high degree of modulation observed between the gut microbiomes of the fish displayed the extent to which SYN altered substrate profile within the intestine. The shared similarities between the LC and LS0.1 groups, leading to high differentiation with the LS0.5 treatment reflected trends previously observed with regards to nutrient availabilities. Overall, supplementation of 0.1 % SYN appeared to drastically reduce the proportion of Spirochaete reads in the microbial community with a dramatic increase of Firmicute reads and to some extent those assigned to Bacteroidetes. Increasing this inclusion 5-fold, reduced Spirochaetes to undetectable levels and the vast majority of the environment became dominated by Proteobacteria; in turn, the proportional abundance of reads assigned to Fusobacteria, Firmicutes and Bacteroidetes appeared to decrease. It appears that at a phylum level, the microbial composition of rainbow trout fed typical contemporary commercial diets lays somewhere in between the expressed populations of the LS0.1 and LS0.5 groups; considering demonstration of the dominance of Proteobacteria, with considerable contribution of both Fusobacteria and Firmicutes in roughly equal proportions (Lyons *et al*., 2015).

Exploring further into the taxonomic levels of the identified microbiota, revealed even more drastic changes to the microbiome. For example, the Proteobacteria contributions were vastly different in their comprising OTUs at a lower level, with dominance of Deefgea (40 %) in the LC group, a diverse number of genera in the LS0.1 group and a striking dominance of Enterobacteriaceae in the LS0.5 group. Enterobacteriaceae are highly regarded microbes within industrial biofuel production; they exhibit an attractive and characteristic efficiency in their proliferation within environments which are rich in

glucose, and to some extent xylose, (Li *et al*., 2015; Ji *et al*., 2011). This is a very good indication of substantial lignocellulolytic activity following a 0.5 % SYN inclusion; supporting a theory of substrate degradation to a monosaccharide or short-chain oligosaccharide level, prior to digesta arriving within the posterior intestine where microbial fermentation is most prominent. It would also correlate with the CF ADC results which are a good indicator of cellulose to glucose hydrolysis. This occurrence would appear to contradict the findings of Olkowski *et al*. (2010), where broilers fed yellow-lupin based diets treated with a commercial multi-enzyme product (Ronozyme® VP) had significantly reduced numbers of Enterobacteriaceae within their caeca and excreta; although, as highlighted, this seems highly dependent on inclusion rate of the bioactive product.

The strong indication of *E. cloacae* (or very closely related species) contributing almost 70 % of microbiota reads within the LS0.5 could be particularly promising considering reports that it may be potential probiotic against *Yersinia ruckeri* in rainbow trout (Capkin and Altinok, 2009). *Y. ruckeri* can be a devastating pathogen to salmonid operations, particularly rainbow trout, as the cause of enteric redmouth disease (ERM). This pathogen regularly results in high mortality if inadequately managed and costly, pre-emptive vaccinations and therefore routinely implemented.

A number of OTUs were observed to increase more so in the LS0.1 group than others, of which some are of particular concern and should be highlighted. The identification of *Moritella viscosa* is of concern due to the fact that this species of bacteria is a potent pathogen to salmonids, causing 'winter ulcer disease' (WUD) which may induce heavy losses, particularly at lower temperatures. Higher presence of *Flavobacterium* should also be discussed as species from this genus are the cause of 'bacterial coldwater disease' (BCWD), which is of similar great concern to the salmonid industry. Somewhat conversely to these findings, channel catfish (*Ictalurus punctatus*), supplemented with Allzyme®SSF at 0.05 % have been demonstrated to display longer resistance time to *Flavobacterium collumnare*; although after 8 days, the study did not indicate any

significant improvement to survival (Zhao *et al*., 2015). Lastly, *Pseudomonas* reads were approximately 4-fold higher in the LS0.1 group and similarly of concern due to the pathogenicity of some species within this genus to fish. This is all somewhat surprising considering increases in a number of other OTUs found within this treatment. Firstly, *Bacillus* spp. proliferated much more in this treatment. *Bacillus* spp. are heavily documented as probiotics, including within the salmonid GIT; for a detailed review of these, readers are referred to the comprehensive review by Merrifield *et al.* (2010). Furthermore, lactic acid bacteria (LAB), which are generally considered to be of benefit to aquatic hosts, appeared to display a greater presence in the LS0.1 group, with genera such as *Enterococcus*, *Streptococcus*, *Leuconstoc* and *Weisella*. Contrariwise to the LS0.1 group but in accordance with the LS0.5 fish, broilers fed xylanase have been observed to display reduced counts of LAB (Owens *et al*., 2008; Yang *et al*., 2008). This has also been demonstrated in pigs fed β-glucanase and β-xylanase (Smith *et al*., 2010). However, it must be considered that certain species within the genera *Enterococcus*, *Streptococcus* and *Weisella* are pathogenic threats to salmonids in practice.

It is also of note that the LS0.5 group generally displayed the least amount of variation between sampled individuals. This suggests a SYN-induced effect of greater population consistency and predictability within the intestinal microflora of fish receiving a 0.5 % dietary dose; all whilst a 0.1 % inclusion tended to suggest quite the opposite effect.

The vastly different microbial profile characteristics in the LS0.1 is proposed as possibly attributable to a more partial hydrolysis of indigestible dietary fractions by an inclusion rate which was slightly limited in activity under the current conditions. If correct, a flood of various types and molecular weights of oligosaccharides may have occurred; causing bacterial fermentation in the hindgut to thrive and high competition for substrates and niches to occur, resulting in microbial imbalance. Simply, a large and diverse quantity of nutrient sources available to microbes but not their host may stimulate a diverse microbial population; which from taxonomic identification in this study may not be necessarily favourable. This theory, which is highly dependent on functional additive

inclusion rate, should be investigated further. If correct, this could serve to further validate the strategy of superdosing bioactive feed supplements which is frequently becoming practiced in terrestrial monogastric animal production.

Goblet cell numbers appeared greatly reduced in the LS0.5 treatment, suggesting a decreased level of mucus secretion. Elevated fibre content, including soyabean hulls, has been demonstrated to augment goblet cell abundances in the duodenum of piglets (Pascoal *et al*., 2015). Similarly, high fibre dietary components have been observed to stimulate goblet cell proliferation and activity in rodents (Lundin *et al*., 1993; Schmidtwittig *et al*., 1996; Ito *et al*., 2009; Hino *et al*., 2012, Hino *et al*., 2013). Such occurrences have also been associated with the increase in digesta viscosity by soluble NSPs (Piel *et al*., 2005). Furthermore, fibre-induced goblet cell proliferation has been demonstrated to occur independently of microflora, in germ-free rats, but effects may be attenuated by colonising bacteria (McCullough *et al*., 1998). Lectins are another causative agent behind goblet cell proliferation due to the manner in which they bind to oligosaccharides and the mucosal surface (Menghi *et al*., 1989); therefore a reduction in carbohydrates could reduce their potency. It is of interest that goblet cell numbers appear to follow the same trend as those observed in the digestibility of fibre. Considering existing evidence that cellulosic and hemicellulosic polymers increase goblet cell proliferation, be it directly or indirectly, it is suggested that increased hydrolysis of such compounds in LS0.5, as indicated by crude fibre digestibility and perhaps also the microbiota, could contribute to the morphological results observed. Alternatively, it is also worthy to note that goblet cell proliferation and thus mucus production has been suggested as a response to sloughing off pathogenic bacteria within the gut of fish species, such as Arctic charr (Lodemel *et al*., 2001). So the reduced abundance of goblet cells in LS0.5 may also be linked to its microbiome characteristics. In any case, the reduced presence of goblet cells in the LS0.5 group appears to indicate a reduced investment in mitigating the effects of stressors within the lumen.

Mucosal duodenal layers have been observed to become thicker following probiotic application in fish (Batista *et al.*, 2015) and there is theoretical potential for these muscular tissues to exhibit morphological responses if digesta viscosity, thus ease of passage, is drastically altered. However no such observations were noted under the current study.

The LC group generally exhibited healthy gut ultrastructure, in correspondence with previous findings regarding the application of lupins in salmonid diets (Borquez *et al*., 2011; Serrano *et al*., 2011; Serrano *et al*., 2012). However, microvilli lengths were numerically lower, whilst counts were significantly lower in the LS0.1 group, which resulted in a significantly reduced estimated total absorption area per enterocyte. It is therefore apparent that some form of intermediary product or process of nutrient digestion by SYN prompted the depression of beneficial morphology, as the LS0.5-fed fish showed similar, even perhaps improved morphology compared with those fed the basal diet. Out of the macronutrients which were altered, no intermediary products of protein hydrolysis are likely to cause such effects, again leaving carbohydrate fractions to be scrutinised. Oligosaccharides are generally reputed to achieve the opposite effect to the one in question, by fuelling probiotic species in a controlled manner but in this instance an unfavourable community appeared to proliferate containing numerous pathogenic species. In an *in vitro* study of the Atlantic salmon foregut, the pathogenic bacteria *Aeromonas salmonicida* and *Vibrio anguillarum* were observed to result in damage to epithelial cells, including disorganisation of microvilli (Ringo *et al*., 2007). *Vibrio vulnificus* was similarly observed to negatively impact upon the brush border in gilthead seabream (*Sparus aurata*) (Khemiss *et al.*, 2009). When the substantially higher diversity of microbial species within the LS0.1 group is considered, the theory of a microbial influence upon the observed microvilli-related results cannot be discounted. This could only be aggravated with the apparent increase in spatial area between both microvilli and enterocytes, allowing greater potential for infiltration and adhesion of pathogenic microbes.

Exogenous-endogenous enzyme interactions in animal nutrition are poorly understood and sparsely investigated. Although the introduction of exogenous sources usually aims to supplement the digestive system with previously absent components, it has been highlighted that interactions are a possibility, reducing the cost of hydrolytic investment by the animal (Bedford and Partridge, 2010). The activity of trypsin and chymotrypsin (the indicators of digestive capacity) appeared not to be altered by the SYN inclusions, suggesting no endo-exogenous interactions or any noticeable reduction in inhibitors. This is somewhat supported by Vandenberg *et al*.'s (2011) findings, where phytase-supplemented rainbow trout were effective in reducing protein-limiting phytate yet they did not affect trypsin activity within the intestine of exposed fish. However, ALP and LAP activity were indicated to be elevated following SYN inclusion within the digesta; whilst activity within their predominant site, the mucosa, appeared slightly reduced. Few reports exist but xylanase and phospholipase have been demonstrated to have no effect upon ileal LAP activity in pigs (Sileikiene *et al.,* 2006). Considering significant increases of ALP and LAP activity within the digesta may be coupled with numerical decreases in expression within the mucosa, it cannot be discounted that the results observed are simply a consequence of secretion into the lumen; despite highly rigorous efforts to standardise feeding, intestinal transit and sampling times. However, a number of causative agents must still be considered for explaining the elevated LAP and ALP activity within the lumen.

Firstly, nutrient profiles within the lumen may influence expression levels of ALP. Of particular note, high luminal Ca concentrations have been demonstrated to increase the expression of ALP in the intestine of rats (Brun *et al*., 2012). Meanwhile, intestinal Na has been reported to decrease mucosal aminopeptidase in broilers (Zdunczyk *et al.*, 2012). Mineral interactions of this kind would appear to follow trends in the recorded mineral bioavailabilities within the gut. Specific ingredients, such as algae derived proteins, have also increased LAP and ALP activities in finfish although the mechanisms for such occurrences remain largely unclear (Vizcaino *et al*., 2014). There is also

indication that mannan-oligosaccharides (with antibiotics) can similarly stimulate ALP secretion in poultry intestines (Yang *et al.*, 2007).

Alternatively, the activity of ALP could be viewed from a standpoint of a response to the microbial community, taking into account its role in pathogen defence. ALP serves to detoxify lipopolysaccharides (LPS) derived from the cell wall components of Gram-negative bacteria (Chen *et al*., 2010), ultimately acting as a vital anti-inflammatory enzyme (Kaliannan *et al*., 2013). Supplementing rainbow trout diets with LPS has indeed shown stimulation of intestinal ALP secretion (Nya and Austin, 2011). A number of the common genera observed within this study are theoretically capable of eliciting a greater expression of ALP, due to their Gram-negative cell wall and thus LPS coating. These include Proteobacteria, Fusobacteria and Bacteroidetes. However, there appears to be no grounds on which to suggest bacterial cell wall type triggered a release of ALP, since the proportional contributions of sequences from Gram-negative bacteria did not follow ALP trends and total cell levels were not investigated. Besides, this would similarly provide no explanation to the elevated LAP activities.

Although these assays were aimed at identifying the activity of endogenous proteases, the significant results observed in LAP and ALP activity within the lumen may be scrutinised as to whether they were in fact endogenous. Certainly, the intestinal tracts of the experimental fish possessed sources of exogenous bioactivity, from the colonising microbiota as well as *A. niger* (Gomez-Guinan, 2004). A number of bacteria which extracellularly produce protease have been isolated from the GIT of fish. Those previously reported that were also present in the microbiome of the presently studied fish are *Enterobacter* spp. *Pseudomonas* spp. *Aeromonas* spp. and *Bacillus* spp. (Hoshino *et al*., 1997; Morita *et al*., 1998; Esakkiraj *et al*., 2009; Ray *et al*., 2010; Askarian *et al*., 2012; Das *et al*., 2014). Although it is recognised that many other genera and species are likely to secrete LAP and ALP, collective contribution of these four genera was recorded as 2.10, 45.30 and 69.70 % within the LC, LS0.1 and LS0.5 groups, respectively; which may be a contributing factor to the activity of LAP and ALP being

higher in the fish receiving a higher dose of SYN. These considerations, along with the fact that LAP and ALP activity differences were in small quantities within the lumen, leads to strong questioning whether the results may have been influenced by isozymes of microbial origin, rather than those expressed endogenously. All things considered, whether SYN elicits responses in the secretion of endogenous proteases remains unclear. However, judging by the results of this study any influences are realistically unlikely to provoke limiting or promoting effects upon the animal's absorptive capacity of amino acids.

4.4.5 Modulation of serum lysozyme

The LS0.1 and LS0.5 exhibited lysozyme activity modulation within the blood. Lysozyme is an integral part of the innate immune system, being involved in the hydrolysis of predominantly Gram-positive bacterial cell walls. Studies have examined the stimulation of lysozyme activity in fish following dietary supplementation of Gram-positive probiotics such as *Lactobacillus acidophilus* (Talpur *et al*., 2014) and *Enterococcus faecium* (Kim *et al*., 2012), confirming substantial elevation in activity when doing so. Similarly, prebiotics may stimulate lysozyme activity through associated microbial proliferation, as has been observed with galacto-oligosaccharides, mannan-oligosaccharides and most extensively, β-glucans (Kim *et al*., 2012; Aramli *et al*., 2015; Dawood *et al*., 2015; Ghaedi *et al*., 2015). There is possibility that the increased lysozyme activity within the SYN groups could be attributed to a greater proliferation or activity of Gram-positive bacteria. However, with the methods employed, it is unfortunately not possible to quantifiably evaluate this possibility with confidence. If this is the case, then it is fair to say that the effect observed was a response to two highly distinct microbiomes.

Although lysozyme is typically associated with defence against bacteria, it can also catalyse the breakdown of 1,4-β-*N*-acetylglucosaminyl oligosaccharides (Dixon and Webb, 1979), which are found within chitodextrin of fungal cell walls. Residual *A. niger* from the SSF procedure will inevitably be present within the diet, so theoretically lysozyme

activity could respond to the presence of these fungal polysaccharides, regardless of their non-pathogenic threat. Indeed, lysozyme derived from insects and plants alike is believed to play a pivotal role in defence against common fungal infections (Javar *et al*., 2015; Manikandan *et al*., 2015) and its use in fungicidal drugs is of interest in human pharmacology (Woods *et al*., 2012). Furthermore, a recent study which supplemented polysaccharides from the mycelia of caterpillar fungus (*Cordyceps sinensis*), into the diets of white shrimp (*Litopenaeus vannamei*), stimulated a prolific increase in haemato-lysozyme activity (Deng *et al.*, 2015). This theoretical potential is of worthy of consideration. Future studies should consider whether lysozyme activity can be modulated by fungal residues from fermentation procedures, to determine whether this possibility is indeed correct. A functionality of this kind could be highly beneficial in priming the immune system for possible pathological threats. Efforts have already been made to investigate the benefits of bacterial cell wall derivatives in salmonid diets, with pronounced immunomodulation observed in doing so (Skalli *et al.*, 2013). However, efficacy through a product such as SYN would present a more multi-faceted and cost effective approach.

4.5 Conclusion

Yellow lupin-based diets provided adequate performance and nutrient availability to juvenile rainbow trout and may hold certain benefits over other plant proteins, notably a high Mn content which could contribute to reducing inorganic supplementation. However, it appears that to resourcefully acquire this mineral, as well as others, supplementary abilities in the digestion of the feed would be highly beneficial. Synergen™ inclusion, at 0.5 %, was highly effective in augmenting the macro and micronutrient profile of the diet containing 30 % yellow lupin, attributable to its likely residual bioactive components. This evidently led to substantial improvements in production and feed efficiency parameters, equating to performance which was more comparable with a high-grade FM diet than the original lupin-based counterpart. It is also recognised that the observed results are highly attractive for improving environmental impact, due to the likely

reduction of both organic and inorganic waste. Although technically effective at increasing nutrient provision, the recommended Synergen™ dose, of 0.1 %, only presented marginal increases in performance leading to questions as to why growth was not higher. In depth quantification of intestinal morphology revealed signs of detriment to the epithelial ultrastructure of the fish fed a 0.1 % Synergen™, along with a concerning microbial community due to the proportional presence of known microbes of high pathogenicity to salmonids. The relatively poorer gut health of fish fed 0.1 % Synergen™ is a probable explanation to the minimal improvements to performance which were observed. Meanwhile the fish fed a 0.5 % inclusion displayed some marked evidence of improved morphological health, as well as reduced proportional presence of salmonid pathogens and little variation between individuals. It was also noted that Synergen™ may hold potential for immunomodulation, perhaps from its non-active residual fungal mass. Overall, an inclusion of 0.1 % Synergen™ appears to produce volatility in health criterion, whilst a 0.5% inclusion of the product resulted in highly promising results from production, animal health and environmental standpoints alike. From these conceptual results, the application of high Synergen™ inclusions in contemporary salmonid diets therefore appears a promising avenue for investigation.

CHAPTER 5. Pilot-scale investigation of Synergen™ efficacy in salmonid culture under commercial conditions: from feed manufacture to fish rearing

5.1 Introduction

5.1.1 Formulations and physical criteria for aquafeeds

The introduction of a compound feed to an aquatic environment strongly distinguishes the manufacturing strategies and physical properties required for a finfish diet, compared with those utilised for ruminant, pig or poultry production, so as to ensure that maximal nutrient levels are delivered effectively to the livestock. In producing a terrestrial animal diet, constituent ingredients must adhere physically to a point where intensive grazing does not cause significant loss in 'fines' (particles or pieces of a physical size which cannot be efficiently consumed by the animal under natural feeding habits). Minimising fines is also somewhat consistent with regards to fish feeds, whereby disintegration must be curtailed after ingestion to prevent losses back through the mouth and the opercula; particularly in species possessing pharyngeal mills. However, most importantly, water causes rapid physical disintegration and leaching of nutrients from a feed lacking a suitable structure, prior to contact with the animal (Watson *et al*., 2015). As previously explored, the cost of feeding many finfish is exceptionally high, requiring costly and nutritionally valuable ingredients. Therefore, any loss of constituent nutrients to the surrounding water can be seen as a greatly limiting factor to animal performance, minimising pollution and maximising economic efficiency. This is of particular importance in younger life-stages, when pellets are smaller and relative leaching rate becomes augmented (Asuncion *et al*., 2009).

In order to achieve a feed which meets physical specifications, formulations are integral and as such, are carefully composed, since each raw ingredient may bring desirable or unfavourable traits to the production of a compound diet. Although, experimental work in the application of bulk ingredients lay the foundations for the inclusion of products in

commercial formulations, they may well be met with restrictions at the manufacturing stage due to negative properties they impart upon either the processing stages (e.g. exerting stress upon machinery, poor hydrophilia) or the end pellet (e.g. inadequate hardness, water stability etc.). In the case of the previous two chapters, high-lupin diets were manufactured under cold-press conditions, which allow great flexibility in feed formulation not only because of the mechanics of the manufacturing technique but also in the hand feeding methods utilised thereafter, which can be monitored and instantaneously adjusted to a level where potential loss of nutritional value is minimised. Such flexibilities are not possible in commercial practice. Lupins have been demonstrated to exert alterations to the properties of feed mixes and pellets when included in salmonid rations. Up to 30 % inclusion of lupin kernel meal was reported to increase bulk density, sink rate, shear and crush strength of pellets, whilst reducing oil uptake during vacuum coating and limiting pellet expansion, compared with soyabean meal (Glencross *et al*., 2010). Therefore, it is of importance to identify the potential of SYN in a contemporary formulation, known to provide a basal mixture which can be processed with ease into a stable pellet formation; inevitably, this will also alter available substrates.

5.1.2 Aquafeed manufacture

Although very much integral to producing a physically robust compound diet, careful formulation is futile in achieving the desired physical characteristics of aquafeeds without the implementation of the modern pellet manufacturing techniques and conditions which the industry employs; namely, extrusion.

Following homogenisation of the ingredients, mixes immediately begin to be subjected to harsh treatment. Preconditioning begins to heat the feed mixture, which serves to create uniform hydration and initiate cooking which decreases potential mechanical stress in the following steps as well as improving the subsequent digestibility of ingredients *in vivo*; this process can be expected to exert temperatures of around 85 to 95 °C (Young and Forte, 2016a).

Following conditioning, the dietary mixture enters the extruder barrel where steam is injected to further raise the moisture content (Young and Forte, 2016b). During its transit, the extrudate is greatly exposed to increasing thermal energy which can be expected to reach temperatures between 100 and 140 °C; this causes a further, greater, degree of cooking to occur (Young and Forte, 2016b). The final products are capable of withstanding the physical stressors of being introduced to an aqueous environment, contain little bacterial contamination and can be manufactured to a density which is species or purpose appropriate (i.e. sinking, slow-sinking, floating pellet). This technology has been instrumental in increasing feed efficiency and reducing environmental impact; however, with growing interest in functional, bioactive feed additives, such as Synergen™, difficulties in application are faced.

5.1.3 Retention of bioactive functionality under feed processing conditions

Over the past couple of decades, the common consensus has very much been that bioactive feed components will be rendered ineffective if fully subjected to the harsh processing conditions which are practiced within the aquafeed manufacturing industry. This has undoubtedly been one of the greatest limiting factors in the pre-extrusion application of exogenous enzymes, which indeed tend to be very much thermolabile.

Instead of including additives within dietary mixtures prior to extrusion, pre-treatments of ingredients and post-extrusion approaches have mostly been researched and applied to date but we are yet to see a widespread uptake into commercial finfish diets, as has been observed with pre-manufacture application in poultry and pigs. Pre-treatment and post-extrusion additions of bioactive components have undeniably yielded successes in improving nutrient provision in salmonids under experimental conditions (Verlhac-Trichet *et al*., 2014; Denstadli *et al*., 2011; Wang *et al*., 2009; Denstadli *et al*., 2007). However, applications of this sort would require additional investment in labour and hardware so practicalities and cost-effectiveness become complicated. Some have suggested that application methods such as liquid spray-coating and vacuum-coating with lipid, do not

present an overly costly investment of time, finances and resources (Verlhac-Trichet *et al*., 2014; Kumar *et al*., 2012); as much as this may be true of large-scale feed mills, accessibility and operation in small-scale facilities is debateable. Furthermore, functionality of the additives may still be limited when these methods are employed. For example, external coating of bioactive components may restrict contact with substrates. Such occurrences may be visible in the scientific literature whereby discrepancies in the success of the same additive have been observed with application method seemingly being the only true variable; for example the studies conducted by Stone *et al*. (2003) and Tachibana *et al*. (2010) upon the commercial multi-enzyme (Natugrain Blend®). Top-dressing of products may also increase contact with external factors which could reduce their efficacy either during storage or use. This could include a loss of the additive to aqueous surroundings upon feeding, familiar to the previously discussed issues of nutrient leaching. Therefore, one returns to investigating whether pre-pelleting introductions may be successful, as a cost-effective approach which is accessible to small-scale feed producers and the world's aquafeed giants alike.

Despite the numerous statements in the scientific literature regarding the denaturation of bioactive feed additives during extrusion cooking, considerably few reports exist which have truly quantified the extent of such occurrences, let alone subsequently fed such diets to animals. The optimum temperature for microbe-derived bioactivity varies tremendously between sources; for example, the activity of phytase from common bacterial and fungal species utilised in biotechnological processes appears to range between 40 to 80 °C (Greiner and Konietzny, 2006). Similarly, one must also consider that exposure beyond the upper limits of optimum conditions, will not return consistent rates or levels of denaturation between enzymatic sources. Even within the kingdom of fungi, industrially cultured species and strains express highly variable thermostabilities between their digestive enzymes (Wyss *et al.*, 1998).

It is the chemical structures expressed by the enzymes which strongly dictate their thermostability, influencing complete resistance to denaturation or refolding to an active

conformation following exposure to extreme temperatures (i.e. > 80 °C) (Wyss *et al*., 1998; Danson *et al*., 1996; Xiang *et al*., 2004). Homologues of enzymes which are routinely used in animal feed preparations, for example phytases and xylanases, are well known to exhibit such characteristics (Yin *et al*., 2014; Ullah *et al*., 2008; Xiang *et al*., 2004). Targeted pressures such as directed evolution, or mutagenesis, have been very successful in improving the thermotolerance of fungal extra-cellular enzymes, even with *A. niger* (Wang *et al*., 2006; McDaniel *et al*., 2008; Zhang *et al*., 2007; Liao *et al*., 2013; Sriprang *et al*., 2006; Xie *et al*., 2011), which is considered to secrete particularly thermolabile enzymes.

An investigation by Slominski *et al*. (2007) into the effect of extrusion at 60 and 70 °C upon microbial phytase activity reported FTU losses between 50 and 60 %, which very much seemed dependent upon the source of the product. Eeckhout (2000) observed that 40 and 60 % phytase (Natuphos® G) activity could be recovered following ingredient conditioning at 82 °C and recovery was over 32 % following pelleting at temperatures in excess of 92 °C. More surprisingly, it has been reported that 'heat-stable' phytases can retain considerable activity (> 60 %) following pelleting in excess of 93 °C, which subsequently improved bird performance during an *in vivo* trial (Timmons *et al*., 2008). One of the phytase products investigated by Timmons et al. (2008) (Phyzyme® XP-TPT) retained activity in the magnitude of approximately 64, 70 and 80 % when inclusion was incrementally doubled from 0.5 to 2.0 times the manufacturer's recommended dosage, respectively. The work of Eeckhout (2000) and Timmons *et al*. (2008) strongly support opposition against the paradigm of phytase products losing all beneficial bioactive potential post-pelleting; serving to show that such claims are often broad and have a tendency to border upon unsubstantiated.

The Synergen™ strain of *A. niger* has not been directly subjected to targeted pressures to improve thermostability but it is continually, naturally selected for overexpression of phytate-degrading properties, which could be advantageous under aquafeed processing conditions. Moreover, considering the results of Timmons *et al*.'s (2008) study,

counteraction of activity loss could be partially mitigated by amplifying traditional inclusion rates. Prior to investigation, it is also important to consider that Synergen™ is not a refined product, thus it contains a multitude of different bioactive components which are likely to express a range of thermo-tolerances. Subsequently, even if loss of the more sensitive constituents (e.g. phytase) occurs, other enzymes may remain more architecturally intact leaving potential for hydrolytic functionality elsewhere. Furthermore, beyond bioactivity potential, the product could provide the animals with nutrients capable of promoting health and performance, in the form of metabolites and semi-fermented hemicellulosic compounds remaining from the fermentation process.

This investigation therefore aims to determine whether Synergen™ can continue to promote nutrient availability and production performance following exposure to a simulation of industrial feed manufacture and under a commercial rearing setting. If the SSF product retains efficacy, under the experimental conditions, it is to be expected that fish performance, feed efficiency and nutrient availability will be improved.

5.2 Materials and methods

5.2.1 Experimental design

The study was designed to assess production performance over an 8 week period, during which performance was tracked. Faecal collection, via a non-invasive method, was integrated into the latter stages of the feeding period, for determination of macro-nutrient and trace element availabilities. In order to support and expand *in vivo* results, the pre- and post-extruded experimental diets were tested for phosphate-liberating potential, via an *in vitro* method, at various temperatures.

5.2.2 Experimental system and animals

The trial was conducted at Exmoor Fisheries (Brompton Regis, UK), in an indoor hatchery. All animals were XXX triploid genotype and wild phenotype. Fish were graded by size and visual condition from concrete production raceways into square 100 L tanks, in groups of 240 fish. Average initial fish weight was 6.07 g ± 0.03; corresponding to an initial stocking density of 14.57 kg/m^3 ± 0.06. Water supply was flow-through, virgin spring water which was filtered through synthetic wool before introduction to tanks. Flow rate in the tanks was ~6 L/min. Throughout the trial, the water temperature was 10.5 °C ± 0.5 and dissolved oxygen was in excess of 85.00 %. An 18 hrs light: 6 hrs dark photoperiod was implemented with fluorescent lights and timers. The fish were exposed to *N*-chloro tosylamide (Chloramine T) (Halamid® Aqua, Axcentive, FR) at 8 mg/L on day 0 as a precautionary method, followed by treatment at days 13 and 15 following early detection of *Icthyobodo necatrix*. No clinical signs of infection were apparent post treatment.

5.2.3 Diet formulation

The experimental diets utilised in this study were formulated and manufactured in partnership with Life Bioencapsulation (Almeria, ES). Formulations were conducted to a specification of satisfying nutrient requirements (NRC, 2011) but in a conservative manner to simulate a least cost formulation. Emphasis was placed upon the use of wheat-derived products, where possible, to maximise potential substrate specificity. Plant protein ingredients accounted for approximately 61 % of the dietary dry weight, providing 75 % of dietary protein. The inclusion rates of Synergen™ were 0 % (Con), 0.5 % (S0.5), 1.0 % (S1.0) and 1.5 % (S1.5). Inclusions of the additive were performed at the expense of unfermented wheat bran, to negate bias in the eventuality of complete inefficacy. Yttrium oxide (YO) (Sigma-Aldrich, ES) was added as a straight dilution into the entire dry mix, at a rate of 0.1 %. Dietary formulations and nutrient compositions of the feeds fed during the nutritional phase of the study are displayed in Table 5.1.

Table 5.1 Formulations, proximate compositions and element concentrations of the experimental, extruded rainbow trout diets.

	Diets			
	Con	S0.5	S1.0	S1.5
Ingredient (g / kg)				
Soyabean protein concentrate [1]	170.0	170.0	170.0	170.0
Wheat gluten [2]	170.0	170.0	170.0	170.0
Fish/rapeseed oil (40/60) [3]	163.0	163.0	163.0	163.0
Fishmeal LT 95 [4]	161.0	161.0	161.0	161.0
Soyabean meal [5]	90.0	90.0	90.0	90.0
Rapeseed meal [6]	90.0	90.0	90.0	90.0
Sunflower meal [7]	90.0	90.0	90.0	90.0
Wheat meal [8]	20.0	20.0	20.0	20.0
Sodium alginate [9]	20.0	20.0	20.0	20.0
Wheat bran [10]	15.0	10.0	5.0	0.0
Synergen™	0.0	5.0	10.0	15.00
Vitamin/mineral premix ¥	11.0	11.0	11.0	11.0
Proximate composition (%)				
Dry matter	99.50	99.46	99.49	99.48
Crude protein	49.46	49.47	49.53	49.50
Crude lipid	22.10	22.20	22.25	22.19
Crude fibre	5.29	5.30	5.30	5.28
Ash	6.51	6.55	6.53	6.56
NFE	16.14	15.94	15.88	15.95
***Element concentrations** *				
Ca (g/kg)	8.18	8.12	8.15	7.98
P (g/kg)	7.68	7.61	7.58	7.30
K (g/kg)	96.30	93.85	94.09	88.90
Na (g/kg)	4.74	4.72	4.59	4.21
Mg (g/kg)	2.29	2.27	2.26	2.14
S (g/kg)	5.22	5.18	5.09	4.76
Fe (mg/kg)	218.43	225.33	218.21	241.54
Zn (mg/kg)	237.45	233.02	230.50	221.83
Mn (mg/kg)	64.60	64.13	63.62	63.54
Cu (mg/kg)	20.79	20.40	20.27	20.11

[1] Soycomil (Andres Pintaluba, ES); [2] (Marti SA, ES); [3] (Piensos Cartagena, ES); [4] LT steam dried (Suysegala, ES); [5] (Rafael Ruiz, ES); [6] (Piensos Cartagena, ES); [7] (Piensos Cartagena, ES); [8] (Piensos Cartagena, ES); [9] (Sigma, ES); [10] (Piensos Cartagena, ES); ¥ Skretting España (ES) (values are g kg^{-1} except to those in parenthesis): 25; Choline. 10; DL-α-tocoferol. 5; ascorbic acid. 5; $(PO_4)2Ca_3$. 5. Premix composition: retinol acetate. 1000000 IU kg^{-1}; calciferol. 500 IU kg^{-1}; DL-α-tocoferol. 10; menadione sodium bisulphite. 0.8; thiamin hydrochloride. 2.3; riboflavin. 2.3; pyridoxine hydrochloride. 15; cianocobalamin. 25; nicotinamide. 15; pantothenic acid. 6; folic acid. 0.65; biotin. 0.07; ascorbic acid. 75; inositol. 15; betaine. 100; polypeptides. 12; Zn. 5; Se. 0.02; I. 0.5; Fe. 0.2; CuO. 15; Mg. 5.75; Co. 0.02; Met. 1.2; Cys. 0.8; Lys. 1.3; Arg. 0.6; Phe. 0.4; Tryp. 0.7. * Limits of Detection (LOD): Ca = 0.39 mg/kg; P = 0.07 mg/kg; Mg = 0.03 mg/kg; K = 0.18 mg/kg; S = 0.09 mg/kg; Fe = 0.07 mg/kg; Zn = 0.08 mg/kg; Mn = 0.02 mg/kg; Na = 1.11 mg/kg; Cu = 0.01 mg/kg.

5.2.4 Diet manufacture

The feed ingredients were finely ground in a Grinders & Graters HP II (CHP, ES) and mixed in a vertical helix ribbon mixer (Sammic BE-40, 40 L capacity, Sammic S.A., ES) for 20 min prior to the addition of SYN. Upon addition of SYN to the basal mix, all of the ingredients were further combined for a 15 min period; following which water (up to 300 mL/kg) was added to the mixture to form homogeneous dough. The dough was subsequently introduced to a single screw laboratory extruder (Miltenz 51SP, JSConwell Ltd, NZ).

The temperature reached during conditioning was 75 °C with a total residence time of 5 min. At this stage, a further 300 mL of water was added per kg of dry mix, along with the steam introduced by the generator which operated at a producing rate of 15 kg dry steam per hour. Following conditioning, the dietary mix entered the extrusion barrel.

The main motor of the extruder worked at 20 Hz, which allowed a feed production rate of 25 kg per hour. Meanwhile, the cutter operated at 30 Hz. The extruder was composed of four barrel sections with temperature probes situated within each. The following temperatures were reached in each barrel section: 80, 95, 100, and 105 °C (from feeding chamber to exit, respectively). The extruder was not fitted with a pressure probe, however this is assumed to have been high due to the low diameter of the die plate holes which produced an average pellet size of 1.5 mm. Temperature of pellets at the exit of the die plate was near to 100 ºC. Upon exit, the pellets were vacuum-coated with oil to reach the desired lipid level. The extruded pellets were subsequently dried using a laboratory drying and cooling system (Airs 1070i, Air-frío, ES) at 18 °C for 12 hrs.

The diets were stored at -20 °C for a maximum of 2 weeks prior to shipping. During shipping, which took approximately 5 days, exposure temperatures were unknown but are estimated to have not exceeded 30 °C. Upon arrival at the trial facility, the diets were kept in sealed containers throughout the experimental period with temperature exposure ranging between -3 °C and 18 °C, during a 10 week period.

5.2.5 Feeding regimes

Daily rations were determined via a predicted daily growth (PG), based on a standardised FCR of 1.20 during hand feeding and 1.00 during automatic feeding. The prediction utilised was identical to the one described in Sec. 4.2.5. Growth predictions were reset with actual weights following biomass sampling.

The fish were conditioned to the diets for a period of 7 days after being stocked into the experimental tanks, being fed rations between 0.5-1.5 % BW per day. This period was not included within the performance data herein. During weeks 1 and 2, after stocking, the fish were fed a 2 % BW per day ration, four times per day. From weeks 4 to 8, the fish were fed for approximately 10 hours per day via clockwork belt feeders (FIAP GmbH; DE). Feeders were rigorously cleaned and dried on a daily basis, before feed was loaded and tanks were monitored for excess feeding multiple times per day. Rations during weeks 4 to 8 were between 3 and 2 % BW per day, respectively. The fish were fed reduced rations (0.5-1.2 % BW) on days prior to biomass sampling. The rationale behind fixed rations was to semi-restrict nutrient intake, due to the potential functional properties of Synergen™. Digestibility diets containing YO (0.1 %) were fed for a total of 9 days, commencing 2 days before the start of week 7.

5.2.6 Sampling

Before commencement of the trial, approximately 60 representative fish were taken from the source stock and sacrificed. Whole carcasses were frozen (-20 °C) prior to preparation for carcass nutrient and trace element composition.

Faecal material was collected from traps for a total of 7 days, during week 7; this was conducted at 06:00, 12:00, 18:00 and 00:00 each day. Throughout collection, vessels were completely surrounded by ice-salt slurry (approx. 35 ppt), to minimise continued fermentation, and housed in polystyrene insulating jackets. The slurry was replaced when deemed appropriate (every 6-12 hrs). Collection was performed by draining the vessels and manually removing faecal matter into bags submerged within ice-salt slurry.

The samples were frozen at -20 °C until use. Each vessel was examined for signs of uneaten pellets prior to collection; any uncertainty was dealt with by complete sample discard.

At the end of week 8, 30 fish per tank were sacrificed and frozen at -20 °C for later determination of nutrient and trace element composition.

Blood was obtained from the caudal vein of 5 fish per tank at the end of week 8, after which the specimens were prepared and analysed for serum lysozyme activity in accordance with Sec. 2.14.4.

5.2.7 Proximate composition and element concentrations

Chemical composition of diets, fish and faeces was performed in accordance with methods detailed under Sec 2.7; whilst element concentrations were determined following the protocols described under Sec 2.8.

5.2.8 Calculations

All calculations utilised within this investigation followed methods described in Sec. 2.9, 2.10, 2.11, 2.12 and 2.13, bar apparent net protein utilisation (ANPU). ANPU was calculated as follows:

$$\textit{Apparent net protein utilisation}\ (\text{AU}) = 100 \times [(CP_F - CP_I) / (FP / 100) \times \text{ADC}]$$

Whereby; CP_F = final total carcass protein (g/kg) per tank, CP_I = initial total carcass protein (g/kg) per tank, FP = protein fed (g) per tank [week 0 – 8] and ADC = crude protein apparent digestibility coefficient (%) of diet fed.

5.2.9 *In vitro* free phosphate release

In vitro free phosphate release was determined in accordance with the digestion step methods described by Ao *et al*. (2010b), which were slightly modified, in terms of dilution factors to accommodate the physical characteristics of the salmonid feed samples. All samples were run in duplicate. Both pre and post-extruded feed samples

were analysed at 40 °C. The values obtained from post-extruded (pelleted) feed samples were corrected for their higher lipid content, since oil vacuum-coating was performed after extrusion. Additionally, the pre-extruded feed material was analysed at 15 and 10 °C to determine the effect of rearing temperature upon free phosphate release. Multiple attempts to analyse post-extruded samples at 15 and 10 °C were aborted due to a lack of confidence in results, attributed to observations of discrepancies in lipid viscosity within the samples between 40 °C and ≤15 °C.

Prior to digestion, pelleted feed samples were finely ground using a pestle and mortar. Following this, 2.5 g of feed sample and 14 mL of de-ionised water were added to centrifuge tubes and conditioned at 40, 15 or 10 °C in a water bath for 30 min. After conditioning, 0.5 mL of pepsin (Sigma no. P7125) solution (15,000 IU/mL, pH 3.00) was added to the tubes, to simulate gastric digestion. The samples were incubated for a further 45 min at their respective temperate, with intermittent mixing via a vortex every 10 min. Next, the samples entered an anterior intestinal phase whereby 2 ml of porcine pancreatin (Sigma no. P3292) solution (4.63 mg per 2 mL) was added to each tube, along with approximately 1 mL of 1M sodium bicarbonate to achieve a digesta pH of 6.50. Incubating at respective temperatures was undertaken for a total of 60 min; again, with intermittent mixing via a vortex every 10 min. Hydrolytic activity within the digesta was halted via submersion in an ice bath for 5 min. The digesta samples were subsequently centrifuged at 14,000 X g for 20 min after which the supernatant was filtered through a 0.2 µm pore syringe filter.

Free phosphate was determined in accordance with methods presented by Kim and Lei (2005). In brief, into fresh centrifuge tubes, 1 mL of filtrate plus 1 mL of 15 % TCA solution was added. The filtrates were subsequently centrifuged at 2,000 X g for 10 min, following which the supernatant was diluted (1:10) in nanopure water (18 MΩ·cm). Phosphate standards were prepared in an identical, 1:10 dilution manner. Into each tube, 2 mL of colour reagent (3 volumes of 1 M sulfuric acid; 1 volume 2.5 % [w/v] ammonium molybdate tetrahydrate; 1 volume 10 % [w/v] ascorbic acid) was added and

mixed thoroughly. The samples were subsequently incubated in a water bath at 50 °C for 15 min for colouration development. Following incubation, the samples were cooled for 5 min in a water bath at room temperature (approx. 18 °C). Absorbance was read at 820 nm (Beckman Coulter Du730®, USA). Free phosphate release (mg/kg) was calculated against a standardised curve.

5.2.10 Statistical analysis

All statistical analyses were performed using SigmaPlot 13.0 (Systat Software Inc., USA). Arcsine transformation was performed upon all parameters expressed as percentages herein. In the case of normally-distributed data, ANOVA was implemented upon means. Data exhibiting skewed distribution was analysed based upon medians via Kruskal-Wallis, followed by multiple pairwise comparison via Dunn's Method, where significant differences were identified ($P \leq 0.05$).

5.3 Results

5.3.1 Fish and feed performance

In each tank, of every treatment, the fish attained more than a three-fold average growth, over the 8 week period. Animal and general feed performance results are presented in Table 5.2. No significant differences were observed in any of the analysed parameters at the end of 8 weeks feeding ($P > 0.05$). However, marginal tendencies towards improved performance with SYN inclusion may be apparent. Overall, performance values appeared to reflect a sub-optimal dietary formulation. To note, survival includes fish which were euthanised according to criterion which would be expected in a commercial production scenario, e.g. extreme growth retardation or animals deemed to represent a biological health hazard to others. Incidences of this sort ranged between 2 and 6 fish per tank but showed no apparent trends between treatments and as such, are not presented within this study as a meaningful result.

Table 5.2 Fish and feed performance fingerling rainbow trout fed experimental extruded diets, with and without Synergen inclusion, over a 8 week period.

	Diet			
	Con	S0.5	S1.0	S1.5
IW (g)	6.06 ± 0.05	6.07 ± 0.03	6.06 ± 0.01	6.08 ± 0.03
FW (g)	21.45 ± 1.06	21.50 ± 1.10	21.59 ± 0.49	22.25 ± 0.90
WG (g)	15.38 ±1.11	15.43 ± 1.07	15.53 ± 0.48	16.17 ± 0.92
FCR	1.04 ± 0.05	1.03 ± 0.05	1.01 ± 0.03	1.00 ± 0.03
SGR	2.41 ± 0.01	2.43 ± 0.12	2.45 ± 0.04	2.51 ± 0.08
PER	1.94 ± 0.07	1.96 ± 0.13	1.98 ± 0.09	1.98 ± 0.12
ANPU	24.49 ± 1.92	24.44 ± 3.03	24.05 ± 4.17	27.12 ± 4.29
Survival (%)	97.92 ± 0.42	96.11 ± 1.73	95.97 ± 1.73	97.78 ± 0.96

Values expressed as mean ± S.D. (n = 3).

5.3.2 Somatic indices

No significant differences in k-factor (K-F) or hepatosomatic index (HIS) were observed between the fish fed the dietary treatments (P > 0.05) (Fig. 5.1).

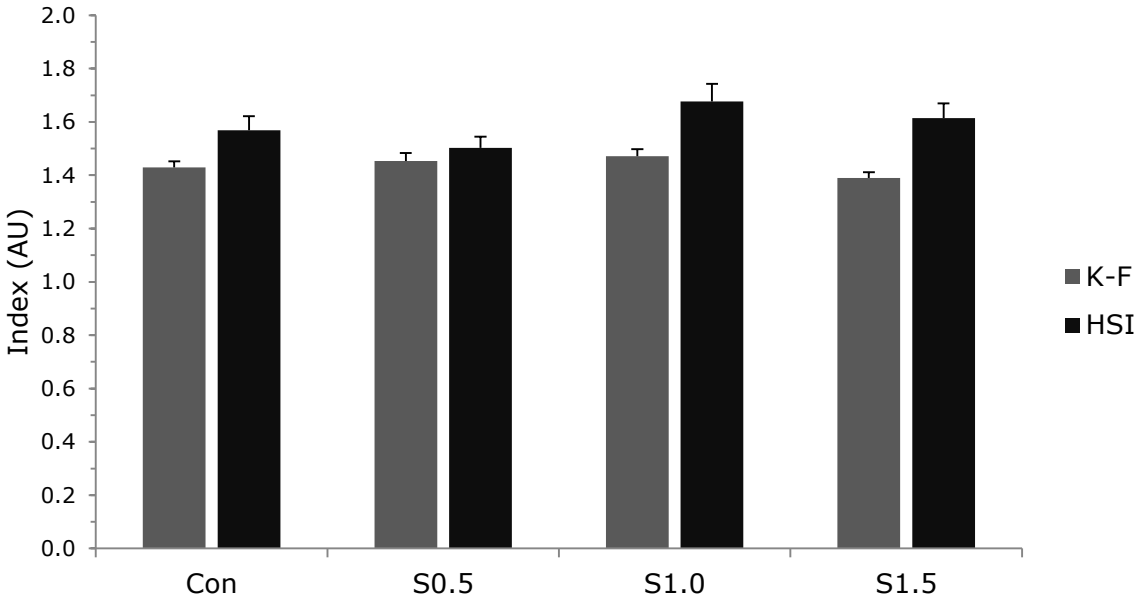

Figure 5.1 Somatic indices (AU) of fingerling rainbow trout after 8 weeks feeding with experimental extruded diets, with and without Synergen™ inclusions.
K-F = k factor; HIS = hepatosomatic index. Values expressed as mean + S.E. (n = 12)

5.3.3 Nutrient and trace element availability and retention

Apparent digestibility coefficient (ADC) and apparent element bioavailability coefficients (ABAC) are displayed in Table 5.3. Total diet ADC was lower than what would be expected of a commercial diet aimed at maximising performance, which was in line with

specifications of the formulation. However, no significant influence of SYN upon this parameter was observed ($P > 0.05$). Crude protein apparent digestibility (CP ADC) was significantly different between treatments ($F = 8.91$, $P < 0.01$); this was identified to be a consequence of significant higher values in all SYN-treated diets compared with the control group. No significant difference was observed between the different SYN inclusion rates.

No significant differences were observed between the dietary treatments in terms of ABAC of calcium (Ca), phosphorous (P), magnesium (Mg), potassium (K), sulphur (S), zinc (Zn), manganese (Mn) or sodium (Na).

Table 5.3 Apparent digestibility and bioavailability coefficients of nutrients obtained via faecal collection of fingerling rainbow trout fed extruded diets with and without Synergen™ inclusions.

	Diet			
	Con	S0.5	S1.0	S1.5
ADC (%)				
Diet	69.55 ± 1.88	73.18 ± 2.14	71.81 ± 2.61	72.29 ± 3.37
CP	91.89 ± 0.33 [a]	93.26 ± 0.40 [b]	93.50 ± 0.76 [b]	93.75 ± 0.29 [b]
ABAC (%)				
Ca	-3.04 ± 2.88	-2.49 ± 8.80	-8.60 ± 2.34	-9.84 ± 18.66
P	44.39 ± 1.86	44.72 ± 4.58	41.20 ± 1.43	43.29 ± 3.14
Mg	58.57 ± 1.05	57.36 ± 3.43	56.01 ± 1.45	54.52 ± 1.68
K	99.66 ± 0.03	99.62 ± 0.08	99.73 ± 0.01	99.68 ± 0.04
S	88.52 ± 0.24	90.22 ± 1.10	90.20 ± 0.37	88.78 ± 1.66
Zn	19.03 ± 2.78	22.26 ± 2.57	14.42 ± 9.51	19.51 ± 1.45
Mn	3.15 ± 4.96	6.35 ± 3.15	0.34 ± 2.33	-1.75 ± 7.53
Na	77.87 ± 11.05	79.01 ± 4.88	82.14 ± 0.31	74.18 ± 3.00

Values expressed as mean ± S.D. (n = 3). Values containing the same superscript in the same row are not significantly different ($P > 0.05$). Absence of superscripts denotes no

significant difference between treatments.

At the end of the 8 week feeding period, there were no significant difference between the dietary groups in terms of calcium, phosphorous, magnesium or zinc retention within the whole carcass of the animals (P > 0.05) (Fig. 5.2). However, marginal tendencies towards increasing retention of phosphorous and magnesium may be present.

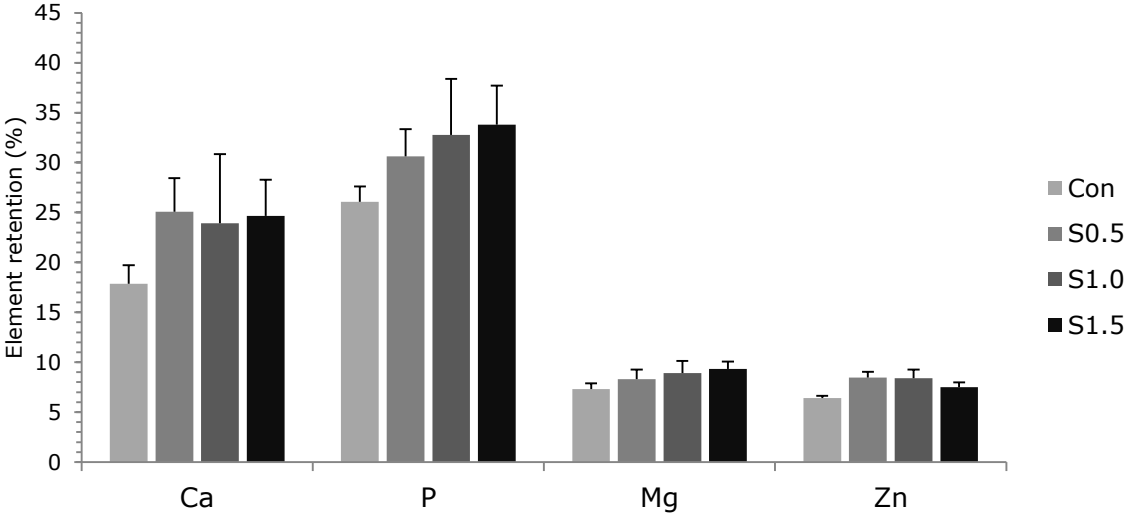

Figure 5.2 Element retentions (%) of fingerling rainbow trout fed experimental extruded diets, with and without Synergen™ inclusions, after 9 weeks.
Values expressed as mean + S.E. (n = 3).

5.3.4 Serum lysozyme

Serum lysozyme activity results are displayed in Fig 5.3. Serum lysozyme activity was found to differ significantly between the dietary treatments (H = 11.285, P = 0.01). Significantly higher activity was identified in the fish fed a 0.5 % SYN inclusion than those fed 1.0 and 1.5 % inclusions.

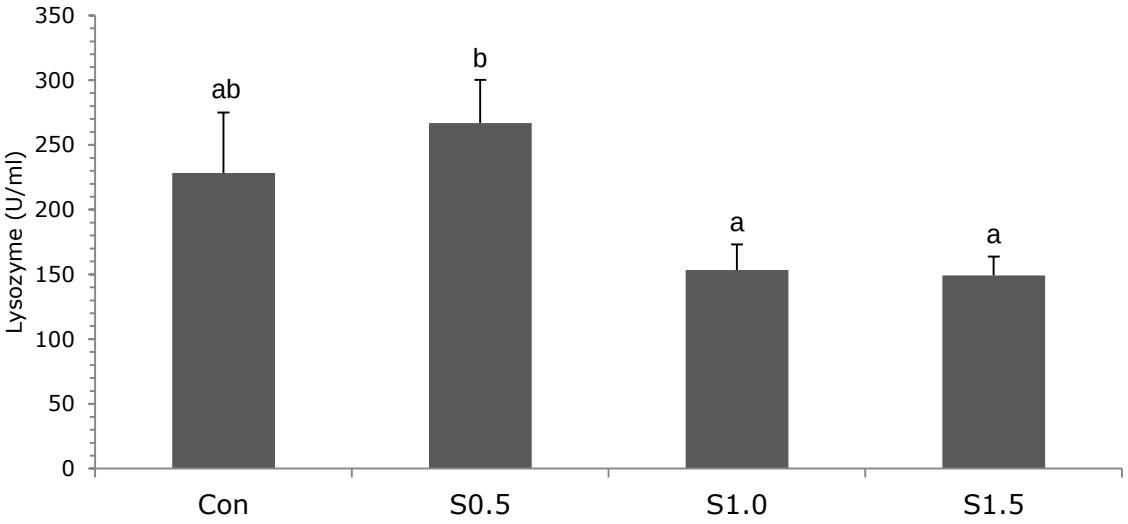

Figure 5.3 Serum lysozyme activity (U/ml) of fingerling rainbow trout fed experimental extruded diets, after 8 weeks.
Values expressed as mean + S.E. (*n* = 11, 13, 15, 12 in Con, S0.5, S1.0 and S1.5, respectively).

5.3.5 *In vitro* free phosphate release

As determined by the *in vitro* assay, SYN appears to be capable of releasing over 43 % more free phosphate than the feed samples intrinsic capabilities before extrusion and at a 40 °C ambient temperature. This was dampened by approximately 50 % following exposure to the extrusion conditions detailed in Sec. 5.2.3. The capabilities of releasing free phosphate by both the tested SYN inclusion rates and the intrinsic activity were comparable between an extruded feed at 40 °C and an un-extruded feed at 15 °C. The greatest total reduction across all treatments, as well as reduction on the basis of SYN inclusion was observed under an ambient temperature of 10 °C (un-extruded).

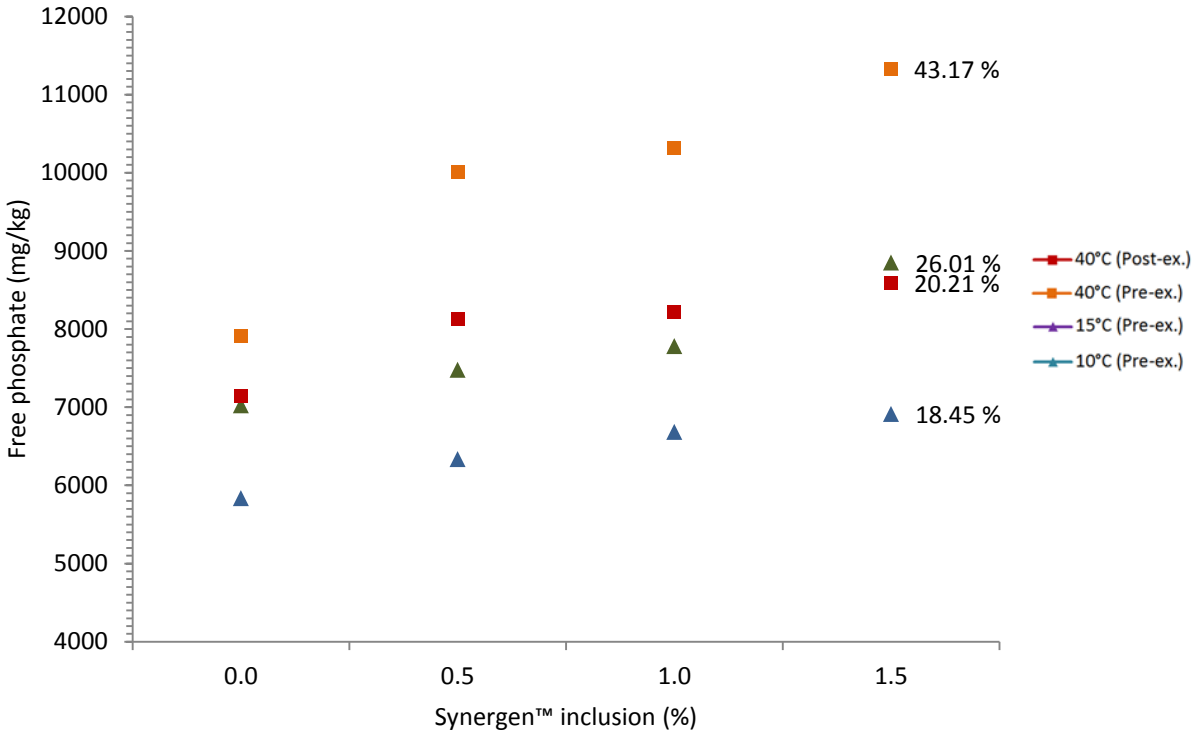

Figure 5.4 *In vitro* free phosphate release of pre- and post-extruded feed samples used within the feeding study, at varying ambient temperatures.
Values in *brackets* denote percentage increase in free phosphate liberation between the feeds containing 0.0 and 1.5 % inclusion of Synergen™.

5.4 Discussion and conclusion

After 8 weeks of feeding the experimental diets to fingerling rainbow trout, which were reared at 10.5 °C, Synergen™ (SYN) was observed to not provide any substantial benefit to production performance. However, marginal tendencies towards increase performance may have been apparent. Furthermore, the availability of protein, in terms of its apparent digestibility was recorded as significantly heightened, by approximately 2 %, following SYN inclusion. This would seem to suggest that a very low level of functionality was preserved, despite the extrusion process and low water temperature.

Upon interpretation of the bioavailability of elements, neither significant effects nor trends were decipherable from the data obtained, which would typically provide good indication of the mode of action of the previously described results. It is recognised that

the method utilised in the collection of faeces within this study possesses a major drawback, this being the potential for nutrient leaching from the faeces between sampling points. Upon scrutiny of the results, out of all of the elements analysed, those which have a higher solubility in water (notably, Ca) did appear to generally express higher variation. The ABAC of Na, a particularly soluble element in its salt forms, appeared unusually high considering the diet type where one would expect to see a negative coefficient (Storebakken *et al*., 1998; see Chapter 4), suggesting a high disappearance of this element. It is questioned whether a high degree of leaching disguised the true ABAC value of Na. When working with fish of the size utilised within the present study, direct collection from the animals would be seen to be highly open to criticism on moral and ethical grounds, so stripping and dissection methods were avoided. A more regular collection of faecal matter could indeed be implemented to minimise leaching. However, under the current conditions, where the animals were only accustomed to one brief visual contact with humans each day (cleaning and loading feeders), this could have induced a heightened risk of stress and unacceptance of feed, with work undertaken at regular intervals in close proximity to the tanks; this would only impeded further upon the rigidity of the sampling method. Future studies may wish to revisit this analysis by using an improved collection trap design, or preferentially a manual collection from larger fish since stripping is recognised to be more reliable than settlement methods (Blyth *et al*., 2015). Instead, readers are encouraged to consider the nutrient retention values presented as more confident identification of the true biological findings. These values, under the current experimental conditions, appeared to show tendencies towards higher retention of particularly P and Mg within fish receiving a dietary dose of SYN.

Using an *in vitro* method, targeted at modelling the potential for phytase sources to release free phosphate in diets, insight was gained towards explaining why SYN appeared greatly ineffective under the current conditions. The first, arguably most major, point of note are that, neither SYN's bioactive capabilities of releasing free phosphate,

nor those intrinsic to the diet, appeared completely eliminated following exposure to extrusion conditions in excess of 100 °C. The retention of bioactivity from SYN is evidenced by the SYN-treated diets releasing more free phosphate than the control diet even after extrusion. With regards to intrinsic bioactivity, the free phosphate value obtained from the post-extruded control feed at 40 °C was higher than that of the unextruded control feed at 10 and 15 °C. These findings strongly contradict statements in the latest review of phytase application in finfish diets, which include "[…] the thermal process during feed manufacture would completely destroy indigenous phytase enzymatic activity." (Kumar *et al*., 2012). The results are particularly intriguing considering *A. niger* phytase is considered to be particularly thermolabile and the strain used in the manufacturing of SYN has not be subjected to any deliberate genetic or directed evolutionary pressures to improve this somewhat negative trait. These findings could be of strong consideration to those who wish to apply SYN to warmwater finfish diets, in an uncomplicated inclusion manner. Most interestingly, the greatest loss of activity was observed upon lowering the ambient temperature from 40 to 10 °C, not by extrusion. Nevertheless, there still appears to be some potential for beneficial quantities of P to be released at this lower temperature, if the diet is not extruded, which correlates with previous demonstrations of phytase efficacy at temperatures of 10 to 11 °C (Vandenberg *et al*., 2011; Dalsgaard *et al*., 2009; Forster *et al*., 1999). The *in vitro* results appear to correlate closely with *in vivo* findings of a study which identified positive improvement to growth and P availability in rainbow trout fed *A. niger*-derived phytase at 15 °C but at 10 °C effects became substantially subdued (Rodehutscord *et al*., 1995). A temperature of 15 °C seemed to confirm that potential for P-liberating activity was still relatively high, which is supported by the majority of other studies which have investigated phytase supplementations in rainbow trout culture at this temperature (e.g. Verlhac-Trichet *et al.*, 2014; Vandenberg *et al*., 2011; Cheng and Hardy, 2002; Sugiura *et al*., 2001). Overall, none of the tested variables are believed to restrict SYN-derived phytase to a level whereby functionality of the additive would be negligible, be it by denaturation or reduction of activation energy. It seems that it is more a combination of

the two extremes (i.e. extrusion and low rearing temperature) which limit the ingredients capabilities to work at a level which would produce attractive performance whilst being cost-effective, in a commercial setting.

In the previous investigation (Chapter 4), serum lysozyme activity was observed to increase within fish receiving a dose of SYN; speculation surrounding this occurrence suggested a possible involvement of chitodextrin derived from the cell wall of *A. niger*. In order to further investigate this possibility, serum lysozyme was analysed under the present experimental conditions, which were deemed to have had limited effects upon the modification of nutritional substrates within the digestive tract of the animals and undoubtedly increased the quantity of fungal-derived polysaccharides within the feed rations. The effect extrusion may have upon the structure of chitin has been investigated to a minor extent and is briefly discussed in a review of extrusion technology upon polysaccharides by Wolf (2010). Most relevantly, the deformation of chitin under pressure and shear energies was investigated by Mogilevskaya *et al*. (2006), concluding that chrystalinity could be modified but the presence of water restores the original conformation of the polymer. Besides, extrusion processing of chitin, to form chitosan, appears to simply deacetylate the polysaccharide (Wolf, 2010). This is not the site of hydrolysis for lysozyme (Dixon and Webb, 1979). Indeed, should deacetylation of chitin occur, the hydrolytic functionality of lysozyme is not inhibited (Pangburn *et al*., 1982; Sashiwa *et al*., 1990). This understanding would further support speculation that, should the previous hypothesis be correct, a modulation was to be anticipated even within an extruded diet. Compared to the control, SYN treatments did not affect the serum lysozyme activity in this study. Due to clear differences in variables between the previous study, such as animal size, age, rearing temperature and dietary formulation, it is difficult to speculate reasons as to why the results were as observed. This is considering these factors may all have an influence upon lysozyme activity, as reviewed by Saurabh and Sahoo (2008). Furthermore, there are variables which remain technically unknown but are most likely to differ, such as the gut microbiomes and

degree of stress under which the animals were reared (Saurabh and Sahoo, 2008). Reasons as to why lysozyme activity appeared higher in S0.5 group compared with the two higher inclusion levels remains unclear. Further work is still required to investigate whether SYN may modulate lysozyme activity and if so, identify the mechanisms behind these occurrences.

In conclusion, strong insight was gained into the retention of phosphorous-liberating activity by Synergen™ under simulated commercial feed extrusion and rearing conditions. *In vitro* analysis indicated that neither temperatures of 105 °C or 10 °C were capable of reducing the additive's capacity to release theoretically bioavailable P at a level which could be of some benefit to finfish production, using a contemporary formulation. However, a combination of the two extreme temperatures was deemed to have subdued activity to a level where minimal significant differences were apparent when the diets were fed to fingerling rainbow trout. Nevertheless, the results demonstrate strong opposition to the common belief that phytase containing products are completely incapacitated under modern aquafeed processing techniques, showing great promise for future work.

CHAPTER 6. Solid-state fermentation product application in aquafeeds: present knowledge and future direction

6.1 Overview of programme of research

This programme of research, comprising of a sequence of investigations, explored the potential of incorporating selected cultivars of lupins and other plant-protein sources with the addition of a solid-state fermentation (SSF) product (Synergen™, Alltech Inc.), in formulated diets of two species of finfish. These were rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*), representing species with well-established but continuously growing value in the farmed seafood sector. Prior to commencement of this work, many questions relating to the area of research needed to be addressed. These can be summarised as follows:

1. Can SSF products improve the availability of nutrients within plant by-products destined for finfish diets?
2. If effective, how may macronutrients and trace elements be released and mobilised? Further, do these impart any notable beneficial characteristics upon fish health, welfare and production efficiency, in terms of growth and feed performance?
3. How may SSF products influence the intestinal environment with regards to its structural topography and cellular organisation, microbiota and endogenous bioactivity?
4. To what extent may SSF products retain bioactivity under low rearing temperatures and the harsh environment of commercial feed extrusion?

These questions will be succinctly re-addressed under the following chapter, identifying and discussing what has been learnt, what remains unclear and where future research efforts should be directed.

6.1.1 Improving nutrient profiles

It is grossly evident that if global livestock production is to continue its production in a sustainable, prosperous manner, feed protein-security must be addressed. An uptake of lupins (*Lupinus* spp.) into feed rations has been proposed as, possibly, the only solution to reducing dependency upon the considerable quantities of imported soya (*Glycine max*) which is fed to livestock throughout Europe (Soya UK, 2015; Mercedes *et al*., 2015). With numerous species of lupin available for commercial cultivation, thereafter providing potential choices for feed manufacturers, selecting a species which is fit for purpose is of great consideration. However, one cannot simply ignore that a number of nutritional limitations, notably non-starch polysaccharide profile, may not truly place any lupins on par with soya to begin with.

The poultry and pig industries have a diverse arsenal of licenced and scientifically-proven digestive aids at their disposal (Bedford and Partridge, 2010). Therefore, uptake of novel bulk ingredients, including cereals and plant by-products, could be easily facilitated by exogenous enzymatic mitigation of their anti-nutrients. Meanwhile, with regards to the aquaculture industry, the situation is vastly more challenging. This is due to a distinct absence of routinely implemented functional feed additives which are capable of degrading anti-nutritional factors and enhancing nutrient availability. Therefore, a licenced, multi-faceted source of exogenous bioactive components presents an attractive solution for the aquaculture industry.

In this thesis, Chapters 3 and 4 explored the application of the SSF product in association with high lupin (*Lupinus* spp.) inclusions in diets for Nile tilapia and rainbow trout, respectively. These two species of fish have a profound economic importance across the globe and their production is set to continue expanding, which will in turn greatly contribute towards the increasing demand for high-quality aquafeed and global feed security. The choice of both tilapia and rainbow trout extended the ability to evaluate the application of the experimental ingredients ingredients at a wider

temperature spectrum, for optimum assessment of a feed product likely sensitive to the varying ambient rearing temperatures found in the aquaculture sector. Additionally, these species represent differences in trophic feeding levels and nutritional sensitivities, with tilapia being omnivorous and rainbow trout carnivorous. Therefore biological responses to exogenous degradation of dietary components could be examined in greater depth.

Part of the objective of Chapter 3 was to determine which species of lupin, narrow-leaf lupin (*Lupinus angustifolius*) or yellow lupin (*Lupinus luteus*), is more suitable to application in Nile tilapia diets. Overall, considering growth performance, mineral retention and gut morphology, it appeared that the narrow-leaf lupin may have been marginally superior to yellow lupin. However, one cannot ignore that yellow lupin has a higher, thus more attractive, overall crude protein content (Sipsas, 2003). This also meant that soyabean was included at a higher percentage in the narrow-leaf diet which could have masked some of the true effects. Furthermore when the observed responses of including the SSF product were considered, definitively deciding which species of lupin was more favourable became complicated; examples of which are discussed further in this chapter.

Through the application of SYN in diets containing yellow or narrow-leaf lupin for Nile tilapia (Chapter 3), and a yellow lupin-based diet for rainbow trout (Chapter 4), significant improvements to fish performance and feed efficiency were observed throughout. This provided strong indication of the benefits of the SSF product in optimising nutrient availability to the fish. Within Chapter 4, it was even observed that a SYN-supplemented diet could achieve performance closer to a purely fish-derived protein diet, compared to the original 30 % lupin diet which was nonetheless formulated to satisfy all known nutrient requirements for rainbow trout (NRC, 2011). Attempts were made during the tilapia trial to give indication of specifically which modes of action were the causes of improvement, following application of the SSF product. However, nutrient retention, blood glucose and carcass composition results returned marginal differences

between the experimental treatments, making it difficult to confidently draw conclusions. Nevertheless, an in-depth evaluation of nutrient availability, within rainbow trout, appeared to display augmented degradation of protein and carbohydrate, as well as the liberation of a suite of minerals following SSF product inclusion in the high lupin diet. There was also evidence of elevated glucose levels within the blood of fish fed a diet dosed with the SSF product, which was believed to have been derived from dietary monosaccharide uptake from the facilitated digestion of carbohydrates. Overall it appeared that supplementation of an SSF product provided a multi-faceted action upon degrading dietary components, with liberation of nutrients commonly facilitated through inclusions of carbohydrases, phytase and to some extent proteases (Castillo and Gatlin 2014; Kumar, 2012; Moura *et al*., 2012). However, it was not possible to quantify the contribution of each of these possible enzymes to the results observed.

Nevertheless, these observations could be of profound importance by presenting a number of possible formulation strategies for future applications in the aquafeed sector. Firstly, SSF products could be applied as has been investigated until now, as a means of extending beyond satisfying basic nutritional requirements. This is an emerging paradigm in finfish nutrition, which does have great merit considering the necessity to improve animal health and welfare as well as ensuring optimum growth is achieved (Li and Gatlin, 2004). Alternatively, SSF products could potentially be included in order to achieve bioavailable nutrient requirements within sub-optimal formulations. This approach will require a great deal more work to be conducted as inherent risks are greater when considering the complexity and interactions of substrates encountered in modern diets. Simply, liberation of essential nutrients from the feed matrix must be guaranteed to a level which meets at least minimal requirements for whichever species in question. Lastly, the application of SSF products could be utilised as a tool for allowing flexibility in feed formulation, be it based upon ingredient cost, temporal availability or sustainability credentials. The case of lupins within this body of research very much

supports this option, with evidence of both increasing nutrient availability and potential mitigation of associated anti-nutritional factors, e.g. phytate and NSPs.

No matter which strategy is adopted, movement towards improved sustainability could be attained using these approaches. This could include optimising health and welfare, maximising economic efficiency and implementing a wider-range of protein sources considered to be more environmentally sound than traditional ingredients. With greater feed efficiency, a further advantage could be that of reducing nutrient discharge from intensive fish production systems, resulting in obvious benefits to environmental impact. This could be particularly notable with regards to inorganic pollutants, notably phosphorous, which is routinely over supplied in aquatic feed formulations; contributing to eutrophication of water bodies located downstream of aquacultural operations. However, this is yet to be quantified confidently.

In the final experiment (Chapter 5), increased nutrient availability became somewhat supressed when the SSF product was supplemented into a practical, extruded diet for fingerling rainbow trout, under typical farm conditions. Nonetheless, protein digestibility was increased and tendencies towards elevated mineral retention and growth performance were still apparent but again, which potential component of the SSF product caused this is unclear. This trial did utilise a more contemporary formulation, without inclusion of lupins, but the dampened intensity of differences present between dietary treatments appeared largely attributable to environmental and feed manufacturing constraints, rather than the efficacy of SYN upon the substrates contained within the feed nutrient matrix. These will be discussed later in this chapter. Nevertheless, *in vitro* assessment confirmed that SYN's bioactivity, which can release phosphorous, is retained over a wide range of conditions, applicable to most commercial finfish which are reared under different water temperatures.

Assessing the mobilisation and deposition of the increased influx of available nutrients produced results which were perhaps to be expected. An increase in protein digestibility

did equate to an increase in carcass protein in rainbow trout in Chapter 4, whilst a tendency towards the same may have been apparent in tilapia. These results are highly attractive for maximising the end, consumable product quality in farming practice. Evaluations of element retentions and concentrations were not as distinct; however, the pathways behind deposition of these micronutrients are much more tightly regulated (Orriss *et al*., 2012). Due to uncertainty of the SSF product's efficacy under the experimental conditions, vitamin and mineral supplementation was not performed in a conservative manner, thus potential to observe differences was most probably lessened. This would seem to support a formulation strategy of improving sub-optimal diets with regards to micronutrients.

Manganese was an element of high interest when assessing mobilisation and deposition of nutrients in diets containing lupins. The manganese content of lupins is typically high but judging by the bioavailability of this element in Chapters 4, this is not to say it is particularly available to finfish. Indeed, this seems to be the case with plant-derived manganese as a whole (Chapter 5; Antony Jesu Prabhu *et al*., 2016). However, inclusion of the SSF product released significant quantities of this element which was subsequently absorbed by the intestine and higher concentrations were then expressed throughout the liver, muscle, fin and vertebrae. Based upon this, it is suggested that SSF products could be useful tools for harnessing the individual nutritional traits of bulk ingredients. This could open further potential for bulk ingredients to feature in formulations not only based upon their availability, cost, overall protein content or amino-acid profile but in a manner where they are purposely included for their micronutrient profile.

6.1.2 SSF products and the intestinal environment

Within farm animal nutrition as a whole, the application of exogenous enzyme sources is well researched, particularly in poultry and swine. However, changes which may occur within the intestinal tract following application of such ingredients have seldom been

investigated and are therefore poorly understood (Bedford and Partridge, 2010). Without critical evaluations of the effects that exogenous enzymes may have upon physiological functioning and digestive characteristics, at the sites where these additives express their mode of action, it is questionable whether scientific advancements can be made at the optimal rate. The intestine has a profound importance as an integral site for efficient nutrient acquisition and pathogen defence but it is of a very sensitive nature. This organ may respond in a multitude of ways dependent upon the compounds which transit through its lumen; from its physical topography and cellular organisation to its exocrine secretions and lastly, its characteristics as a microbial ecosystem (Merrifield *et al*., 2010). Experimental trials utilising exogenous enzyme sources should therefore begin to encompass assessments of gut morphology, integrity, endogenous digestive enzyme secretions and activity, as well as associated microbiota. This was undertaken in this series of investigations, not only for the benefit of research upon the specific ingredient or finfish species but for monogastric research as a whole.

Within the individual trials of this body of work, it was perhaps to be anticipated that the characteristics of the intestine would be modulated, considering the extensive indications of substrate modifications within the digesta. This was indeed shown to be the case. Tilapia exposed to the SSF product in the yellow lupin diet, in particular, firstly gave indication of an amelioration of morphological characteristics which are desirable to optimising nutritional efficiency and also pose as a good indicator of digestive health; this was particularly visible at an ultrastructural level. There was also indication that, over time, the epithelial surface begins to respond distinctively to the degradation and liberation of nutrients found within the lumen. Within rainbow trout (Chapter 4), differences in intestinal morphology were also observed, with observations considered to be positive improvements, particularly when a 0.5 % dose of SYN was administered. The most distinctive difference at this point was perhaps the abundance of goblet cells, which were dramatically reduced following this higher inclusion of SYN. However, questions arose after the lower inclusion rate (0.1 %) returned observations of reduced functional

surface area within the posterior intestine, at an ultrastructural level. Assessment of the intestinal environment progressed to explore the associated allochthonous microbial communities, which revealed differences between the high and low inclusion rates of SYN. Whilst the 0.1 % group displayed a high level of taxonomic diversity with relatively great variation between individuals, the 0.5 % group displayed a consistent domination of a select few microbial genera which were most probably beneficial since performance of these animals was substantially improved. In the group receiving a 0.1 % dose of the SSF product, it was also observed that numerous potent salmonid pathogens were present in higher proportional contributions to the microbiome, which was undoubtedly concerning. Ultimately, it was concluded that the 0.1 % group exhibited a degree of imbalance and unpredictability which could be associated with the morphological depreciation of the epithelial surface, whilst the polar opposite appeared the case with the higher inclusion. Evidently, had an extensive evaluation of the intestine not been performed, it would have been grossly unclear why the lower inclusion of SYN did not elicit a higher degree of performance promotion considering nutrient availabilities were, by and large, improved. A number of finfish studies investigating the application of exogenous carbohydrases have reported improved nutrient availability but no truly correlating promotion of performance. For example, xylanase in rainbow trout (Dalsgaard *et al.*, 2012) and a mixture of cellulase, hemicellulase and pectinase in lupin-based rainbow trout diets (Farhangi and Carter, 2007). Other studies investigating dietary carbohydrase additions have observed depreciation of animal performance and feed efficiency. Kazerani and Shahsavani (2011) attributed such observations to intolerance of monomeric residues in a study on common carp; despite the fact that this species naturally consumes significant quantities of plant material and commercial formulations include relatively high concentrations of carbohydrate. Meanwhile, with multi-enzyme pre-treated soyabean and sunflower cake fed to rainbow trout, Denstadli *et al*. (2011b) recognised the possibility that the implemented process may have produced an abundance of anti-nutritional oligosaccharides. Judging by the results of this investigation, questions arise as to whether microbial and gut morphological modulations

may have influenced previous studies, including the tilapia trial in Chapter 3, going undetected and extensively unconsidered as a contributing factor in said studies.

With regards to exogenous carbohydrase sources, it is essential to consider that degradation of polysaccharides will typically pass a stage whereby nutrient sources are more available to microbes than the animal itself. Through such considerations, the possibility of manufacturing prebiotics *in vivo* could be a particularly attractive objective for carbohydrase applications. This could be more resourceful than current efforts to reduce carbohydrate levels in feeds, whilst they are partially reinstated as low molecular weight oligosaccharides as potential prebiotics. Evidently, substrates and enzyme sources will need to be extensively studied and inclusion rates thoroughly calculated but theoretical potential for such approaches is feasible. Alternatively, this work may suggest that 'superdosing' is a more preferable option, so as to degrade the maximum quantity of substrates during intestinal transit; favouring elimination over amelioration of ANFs, including oligosaccharides. So far, superdosing has only been implemented with phytase and it is encouraged that work examines this approach with other exogenous enzyme classes. What can be concluded is that an appropriate inclusion of SSF products can improve intestinal morphology and allow a predictable balance of microbes within the lumen but it is imperative that adequate bioactivity is provided to ensure deleterious effects do not occur.

6.2 Future work

Our knowledge of SSF product applications in finfish diets does still remain in its infancy, with many questions yet to be answered. From the information gathered in this series of investigations, there are a number of identifiable areas which warrant specific attention in future investigations, so that progress and viable applications are maximised.

6.2.1 The gastrointestinal environment

Chapters 3 and 4 of this investigation aimed to gain insight into the interactions of SSF products on the animal's own digestive characteristics, with success in doing so. However for a more comprehensive insight, a number of future analyses may wish to be considered since the gastrointestinal tract and its associated functions comprises such a complex network of cells, physical structures and physiological processes.

Although, histological appraisals of the intestine can provide invaluable information on the nutritional health of animals, genomic, transcriptomic and proteomic methods may be considered in future for supplementary information.

With regards to the intestinal microbiota, Chapter 4 comprehensively analysed the allochthonous microbial community but determination of the autochthonous community is yet to be undertaken. Lyons *et al*. (2015) identified that the autochthonous community of farmed rainbow trout tends to display a more even representation of different bacterial classes than the allochthonous microbiota. It was also identified that a number of dominating taxa within the allochthonous community, e.g. Enterobacteriaceae, which dominated by approximately 90 % in the high SYN diet in Chapter 4, become much less prevalent at the mucosal surface (Lyons *et al*., 2015). It is predicted that a high degree of modulation would occur within the autochthonous community following effective inclusion of SSF products, which would be of interest to study in detail. It is also important to note that bulk ingredient formulations will influence the microbial taxa which populate and proliferate within the gut; therefore it would be of interest to assess the microbiome of fish fed diets supplemented with SSF products which are based upon commonly implemented ingredients, e.g. soyabean, wheat and rapeseed. Additionally, assessment of abundances and activities of communities may wish to be assessed; further to relative abundance of 16S rRNA. Overall, it is suggested that future studies on exogenous enzymes and SSF products scrutinise the microbiome more regularly. This will allow conclusions to be drawn with more confidence, since, as discussed in Sec.

6.1.2, many studies to date have explained their results on the basis of nutrient availabilities and/or tolerances alone.

Future work should also begin to consider quantifying the extent of substrate degradation within the intestinal lumen, so as to allow a better understanding of the responses observed within the organ. Moura *et al*. (2012) provided excellent information in this regard, where monomeric and dimeric residues of polysaccharide degradation were analysed in the chyme of Nile tilapia receiving a dietary dose of a solid-state fermentation product; unfortunately no assessment of the intestinal structure, endogenous enzyme activities or microflora were conducted in parallel. On the other hand, the investigations conducted in this body of work did not analyse potential residues of carbohydrate degradation in the intestine. It is therefore proposed that a highly attractive experimental design for future studies would be an even more advanced holistic perspective, encompassing the analyses conducted in Chapter 4 and those of Moura *et al*. (2012). Analysis of intestinal viscosity would also be highly beneficial, as a somewhat routine procedure in poultry studies but under-regarded in finfish; despite predictable importance within this sector, in coming years, as sustainable and efficient production is optimised.

All things considered, it is suggested that future studies should attempt to pursue appraisals of the finfish intestine, in the most holistic manner feasible, given the apparent complexity of interactive effects when products containing multiple bioactive components are added to finfish diets.

6.2.2 Ingredients and diets

This series of investigations dedicated much focus to the evaluation of SSF product inclusions in diets containing lupins, as a means of improving their nutritional value. Although lupins have seen periodic success in animal feed rations, much information is still lacking, in terms of their nutritional profile. Extensively quantifying the nutritional profile of each available species and cultivar is imperative if work is to continue

promoting their use. It was highlighted previously that lupins are competitive with soya in terms of protein content; however, existing information on amino acid profiles of lupins is somewhat sparse, in the scientific literature. Similarly, NSP profiles of the various commercially available cultivars is distinctly difficult to access, which will make it challenging for enzymologists and feed formulators to carefully conduct inclusions of exogenous enzymes in animal feed rations. Revisiting chemical compositions of modern lupin varieties is therefore warranted.

Chapter 5 aimed in part to identify whether the SSF product could improve the nutritional value of a salmonid diet which did not focus upon lupin. However, due to processing constraints, it remained unclear how effective this could be. Future studies using SSF products should consider to begin systematically evaluating the efficacy of their inclusion in diets containing contemporary bulk ingredients in aquafeeds; e.g. soya, maize, rapeseed and wheat.

Advancing our understanding of the effects of including SSF products in finfish diets would benefit greatly from the scientific assessment of more nutritionally marginal diets than have been observed in the present studies. Although it was observed that the SSF product was capable of releasing nutrients and promoting performance in experimental diets which satisfied known nutrient requirements, within this series of investigations, it would be of great worth to explore whether the product's inclusion may maintain health and performance in sub-optimal diets, or even those which are deficient on a digestible nutrient basis. Care must be taken in these approaches to ensure ethical justification but positive results are foreseeable which could contribute to advancing feed sustainability criterion.

The ingredients utilised in the actual SSF process may also be a topic of consideration. The studies presented in this body of research utilised a product of the SSF of a wheat substrate which did achieve success in promoting nutrient availability and subsequently, animal performance and health when included in diets with low, if any, inclusion of

wheat-based bulk ingredients. Nevertheless, substrate specificity cannot be ignored. Different plant proteins vary considerably in their nutrient profiles and associated ANFs (Francis, 2001). Therefore, those involved in the production of SSF products destined for use in animal feeds may wish to offer products where the fermentation substrates reflect contemporary bulk diet formulations, e.g. soya, maize and rapeseed, as well as wheat, so that exogenous hydrolytic action can be optimised. It is suggested that this could take the form of single ingredient SSF products, e.g. the SSF of soya by-products, or alternatively, the SSF of ingredient cocktails which mirror formulations, e.g. soya, maize, rapeseed and wheat mixes.

6.2.3 Life-stages

The mode of action of SYN appears to be almost entirely based upon the liberation of bound, unavailable, nutrients. Such releases of additional nutrients would undoubtedly present a benefit to livestock during grow-out stages by promoting growth, health and reducing effluent nutrient loads. However, their sensitivity to nutritional pathology typically becomes reduced with age and attention often begins to focus upon minimising feed cost. For this reason, supplementing diets with functional feed products must be highly cost-effective in advanced life-stages. Following the studies detailed within this work, it is suggested that the application of SSF products be investigated in more sensitive life-stages, where scope for incorporating additional functional products, thus expense, becomes greater.

Juvenile life-stages (e.g. fry and fingerlings) require very careful nutritional approaches to their culture whilst gastrointestinal and structural conformation of the animals undergoes crucial development and maturation (Rust, 2002). In achieving optimal development in this way, the precedent is set for healthy, valuable stock at the grow-out phase. The application of SSF products to juvenile life-stages is therefore one which is particularly attractive. Having observed improved availability of macronutrients and minerals, as well as signs of reduced nutritional stressors, even in larger fish, it is

encouraged that work continues in younger fish to reassess performance and development. It is encouraged that evaluations of this kind continue to pursue the evaluation of contemporary nutritional pathologies, such as monitoring incidences of skeletal malformations and depreciated gut health; since it is believed that the probability of achieving improvements in these characteristics will be heightened under such circumstances.

The application of SSF products to broodstock diets may also wish to be considered. Taking salmonids as an example, the commercial value of hen and cock-fish is their harvestable production of viable gametes, not the animals per se. In order to ensure a sustainable and equitable practice, those producing eggs must ensure that quality is upheld to the highest degree, since many fish producers purchase fertilised seed for their operations. Protein, essential amino acid, lipid, fatty acid and vitamin provision at the gonadal development stage of female fish is well recognised to affect fecundity and subsequent hatchability and survival of offspring (Izquierdo *et al*., 2001). However, broodstock nutrition is rather sparsely investigated in aquaculture. This is surprising since producing healthy future livestock generations depends upon this investment. On the other hand, the sparse efforts in this field may be unsurprising considering the costs involved with such work (Migaud *et al*., 2013). It remains uncertain whether the drive to reduce marine-derived ingredients will fall as heavily upon specialist broodstock diets, as it has upon the other life-stages where feed manufacture is highly dictated by mass volumetric requirements. Indeed, broodstock diets are a minor volume in the aquafeed industry and they already command an established premium price (Migaud *et al*., 2013). However, should the drive for higher plant inclusions occur, there would be great scope for investigating the application of SSF products, having witnessed the results observed in these studies. It is possible that a facilitation of nutrient acquisition and sparing, by SSF product inclusion, could impart a promotion of egg and ovarian fluid quality in heavily plant-based broodstock diets. Furthermore, improving nutrient availability could potentially promote the health and survival of the broodstock themselves; especially

salmonids, which can be susceptible to severe weakening prior to and post spawning. This is equally important for tilapia where much emphasis is placed upon broodstock management, particularly considering this species expresses high fecundity and broodstock are sequentially spawned in practice.

6.2.3 Overcoming thermal constraints

In chapter 5, the effect of feed extrusion was investigated upon the potential for free phosphate release by the focus SSF product. This study identified that extrusion did not reduce potential as much as might have been expected, if one is to regard the common consensus upon the topic (Kumar, 2012). In fact, a lowering of rearing temperature, to approximately 10 °C, was identified to dampen activity most greatly. This came as a great surprise considering applications of phytase in temperate species, notably salmonids, have generally been successful (Vandenberg *et al*., 2011; Dalsgaard *et al*., 2009; Forster *et al*., 1999). This also includes the work detailed within Chapter 4, where the SSF product was highly effective at 12.5 °C, with the lowest inclusion rate tested in the subsequent trial (0.5 %). It was concluded that neither low rearing temperature nor high extrusion temperature (105 °C) were capable of singularly reducing potential, to a level which could not be of some benefit to the animal. However, with both conditions present, constraints emerge and they must be overcome.

The simplest approach to overcoming processing constraints would be for future work to investigate, *in vivo*, whether SSF products can remain effective in extruded diets for warmwater species, e.g. tilapia, carp and catfish. However, to allow a versatile product which can still remain efficient in temperate species, biotechnological efforts should attempt to implement methods which can improve stability or safeguard the functional bioactive components from heat-related stress. The two most feasible options are that of bulk ingredient pre-treatment and extraction of functional components for post-pelleting application, which would both avoid the extrusion process entirely. On the other hand,

more novel technologies may emerge in the future as potential means of protecting bioactivity during feed processing such as encapsulation.

Microencapsulation continues to gather attention as a means of protecting the viability of probiotics from various stressors, including temperature (Liu et al., 2015; Wang et al., 2015). This technology has been highlighted as an attractive tool for the animal feed processing industry (Shahrulzaman et al., 2015) and it may present an avenue for retaining functionality within SSF products. These efforts will indeed require significant cross-disciplinary work but objectives should be very much achievable.

6.3 Overall conclusions

When applied to the diets of both warmwater and temperate fish species, SSF products appear capable of releasing previously unavailable nutrients from feed. The sources of nutrients which are degraded appear to include those which can be categorised as anti-nutritional factors, such as phytate and a variety of non-starch polysaccharides. The degradation of previously indigestible dietary fractions and increased nutrient provision can subsequently enhance animal and feed performance which would be of great benefit to production operations. Furthermore, through modulation of the nutrient profile, SSF products can be capable of causing downstream effects upon intestinal health which are likely to be a contributing factor to improved performance and could be of significant benefit to health in general. However, it was brought to light that inclusions of low bioactivity may eliminate or reverse the benefits to be gained by such applications, by negatively affecting intestinal health. Nevertheless, if intestinal health is upheld, the release of bioavailable macro and trace elements by SSF product inclusions is particularly attractive considering how this could theoretically increase the nutrient budget for physiological processes and morphological development alike, as well as reducing nutrient effluent loads. Through application in diets containing lupins, it became apparent that SSF products hold potential as very useful tools in increasing the acceptability of novel alternative ingredients by allowing more flexibility in feed formulations.

The uptake of bioactive feed ingredients in the aquafeed sectors has been restricted by their thermosensitivity; however, even without selected pressures to increase thermostability, the potential of SSF products to release nutrients after commercial feed extrusion conditions can remain surprisingly high. Similarly, low rearing temperatures can still produce increased nutrient liberation. However, a combination of the two temperature extremes can seriously subdue the benefits to be gained from SSF product inclusions when fed to salmonids, in particular, under commercial conditions. Therefore, these conditions are likely to be the major limiting factor in uptake by the commercial sector. Nevertheless, overcoming restraints is anticipated to be highly manageable with a number of established and novel technologies available. In doing so, the multi-faceted benefits of SSF products could be a key to unlocking the nutritional potential of modern, plant-based aquafeeds.

7 APPENDICES

7.1 Experimental systems

Plate 7.1 Warmwater RAS utilised for experimental work under Chapter 3, using Nile tilapia.

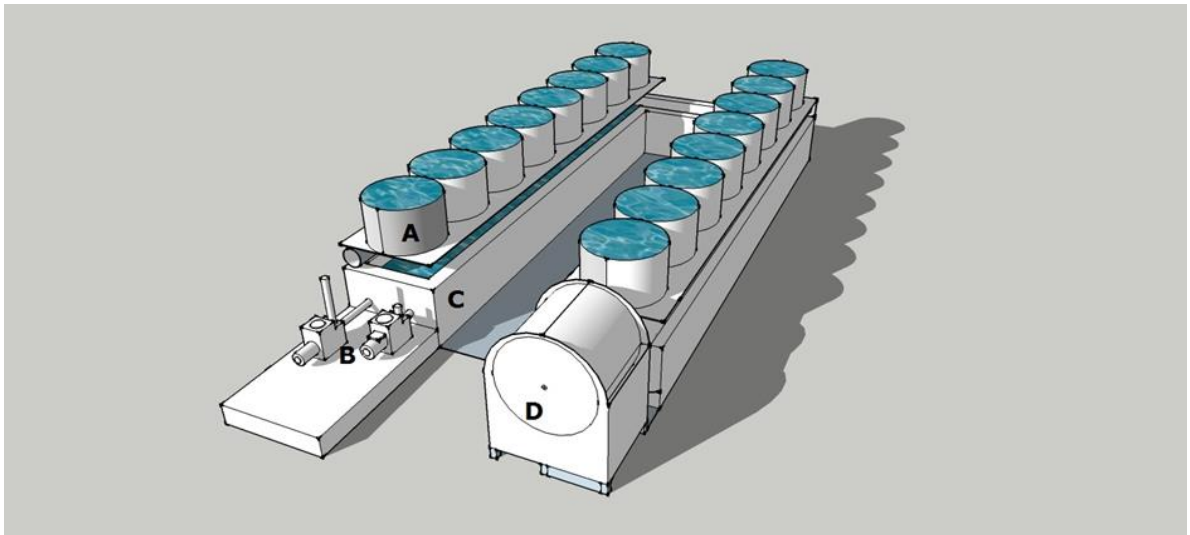

Plate 7.2 Temperate RAS utilised for experimental work with rainbow trout under Chapter 4.
A = tanks, B = pumps, C = sump/bio-filter, D = drum filter
Design courtesy of Ben Eynon.

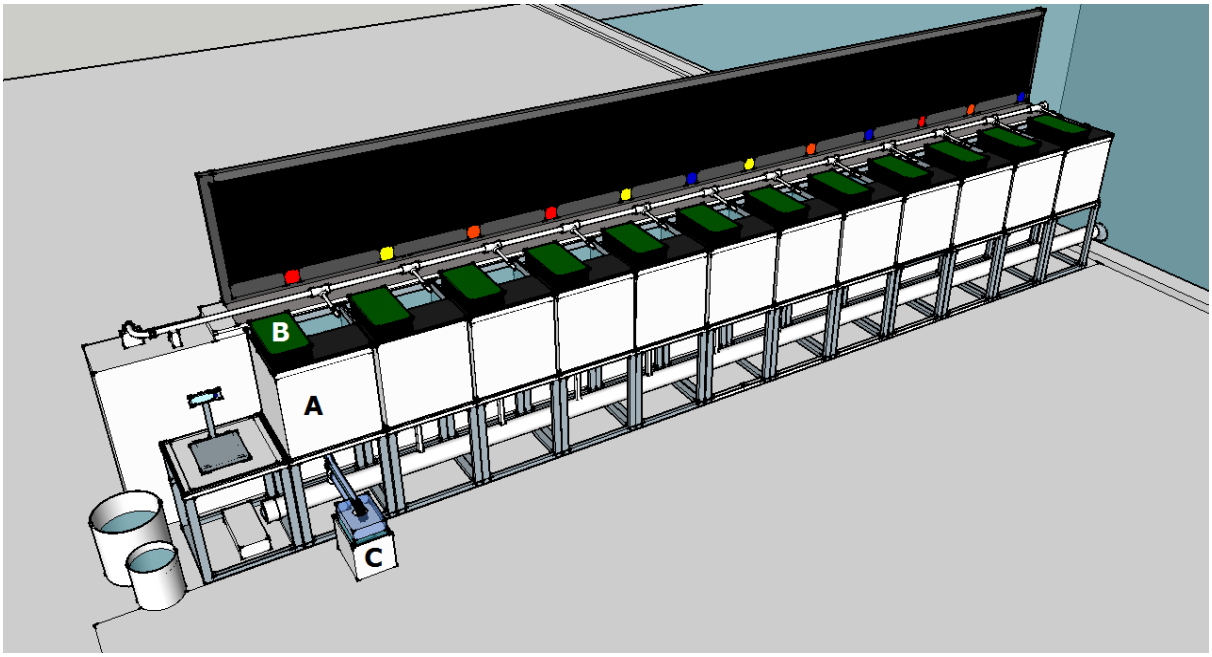

Plate 7.3 Temperate flow-through system utilised for experimental work conducted with rainbow trout under Chapter 5.
A = tank, B = automatic feeder, C = faecal trap

7.2 Histological staining procedures

Table 7.1 Haematoxylin and eosin staining procedure

Reagent	Time (min : sec)
Histolene	02:00
Histolene	02:00
Absolute Alcohol	02:00
Absolute Alcohol	02:00
90 % Alcohol	02:00
70 % Alcohol	02:00
50 % Alcohol	02:00
Mayer's Haematoxylin	60:00
Tap Water	05:00
Saturated Lithium Carbonate	00:04
Tap Water	02:00
Acid Alcohol	00:02
Lithium Carbonate	00:04
Tap Water	01:00
Eosin	05:00
Tap Water	00:30
90 % Alcohol	00:05
Absolute Alcohol	02:00
Absolute Alcohol	02:00
Histolene	02:00
Histolene	02:00
Histolene	02:00

Table 7.2 Periodic acid Schiff's staining procedure

Reagent	Time (min : sec)
Histolene	02:00
Histolene	02:00
Absolute Alcohol	02:00
Absolute Alcohol	02:00
90 % Alcohol	02:00
70 % Alcohol	02:00
50 % Alcohol	02:00
Periodic Acid	05:00
Tap Water	03:00
Schiff's	15:00
Tap Water	05:00
Mayer's Haematoxylin	01:00
Tap Water	05:00
90 % Alcohol	00:30
Absolute Alcohol	01:00
Absolute Alcohol	02:00
Histolene	02:00
Histolene	02:00
Histolene	02:00

Table 7.3 Mallory's Trichrome with haematoxylin staining procedure

Reagent	Time (min : sec)
Histolene	02:00
Histolene	02:00
Absolute Alcohol	02:00
Absolute Alcohol	02:00
90 % Alcohol	02:00
Distilled Water	02:00
Mayer's Haematoxylin	30:00
Tap Water	05:00
Saturated Lithium Carbonate	00:01
Tap Water	02:00
Acid Alcohol	02:00
Saturated Lithium Carbonate	00:01
Tap Water	02:00
Acid Fuchsin (1 %)	00:08
Tap Water	05:00
Acid Phosphomolybdic Acid (1 %)	01:30
Distilled Water	02:00
Mallory's	00:10
Distilled Water	05:00
90 % Alcohol	00:05
Absolute Alcohol	00:10
Absolute Alcohol	02:00
Histolene	02:00
Histolene	02:00
Histolene	02:00

8. REFERENCES

Abro, R., Sundell, K., Sandbiom, E., Sundh, H., Brannas, E., Kiessling, A., Lindberg, J. E. and Lundh, T. (2014). Evaluation of chitinolytic activities and membrane integrity in gut tissues of Arctic charr (*Salvelinus alpinus*) fed fish meal and zygomycete biomass. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, 175, pp. 1-8.

AB Vista (2013). *Superdosing*. [online] Available at: http://phytate.info/superdosing-phytases (Accessed 8 March 2016).

Acamovic, T. (2001). Commercial application of enzyme technology for poultry production. *Worlds Poultry Science Journal,* 57 (3), pp. 225-245.

Adeola, O. and Bedford, M. R. (2004). Exogenous dietary xylanase ameliorates viscosity-induced anti-nutritional effects in wheat-based diets for White Pekin ducks (*Anas platyrinchos domesticus*). *British Journal of Nutrition,* 92 (1), pp. 87-94.

Adeola, O., Nyachoti, C. M. and Ragland, D. (2007). Energy and nutrient utilization responses of ducks to enzyme supplementation of soybean meal and wheat. *Canadian Journal of Animal Science,* 87 (2), pp. 199-205.

Ai, Q., Mai, K., Zhang, W., Xu, W., Tan, B., Zhang, C. and Li, H. (2007). Effects of exogenous enzymes (phytase, non-starch polysaccharide enzyme) in diets on growth, feed utilization, nitrogen and phosphorus excretion of Japanese seabass, *Lateolabrax japonicus*. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* 147 (2), pp. 502-508.

Al-Amery, M., Fukushige, H. and Hildebrand, D. (2015). Single seed selection for low phytate lines. *Journal of the American Oil Chemists Society*, 92 (8), pp. 1119-1123.

Alarcon, F. J., Diaz, M., Moyano, F. J. and Abellan, E. (1998). Characterization and functional properties of digestive proteases in two sparids; gilthead seabream (*Sparus*

aurata) and common dentex (*Dentex dentex*). *Fish Physiology and Biochemistry*, 19 (3), pp. 257-267.

Albrektsen, S., Hope, B. and Aksnes, A. (2009). Phosphorous (P) deficiency due to low P availability in fishmeal produced from blue whiting (*Micromesistius poutassou*) in feed for under-yearling Atlantic salmon (*Salmo salar*) smolt. *Aquaculture*, 296 (3-4), pp. 318-328.

Amerah, A. M., Ravindran, V., Lentle, R. G. and Thomas, D. G. (2008). Influence of particle size and xylanase supplementation on the performance, energy utilisation, digestive tract parameters and digesta viscosity of broiler starters. *British Poultry Science*, 49 (4), pp. 455-462.

Amerah, A. M., Gilbert, C., Simmins, P. H. and Ravindran, V. (2011). Influence of feed processing on the efficacy of exogenous enzymes in broiler diets. *Worlds Poultry Science Journal,* 67 (1), pp. 29-46.

Amin, M. N., Katersky Barnes, R. and Adams, L. R. (2014). Effects of different protein and carbohydrate levels on growth performance and feed utilisation of brook trout, *Salvenlinus fontinalis* (Mitchill, 1814), at two temperatures. *Journal of Applied Ichthyology*, 30 (2), pp. 340-349.

Amirkolaie, A. K., Leenhouwers, J. I., Verreth, J. A. J. and Schrama, J. W. (2005). Type of dietary fibre (soluble versus insoluble) influences digestion, faeces characteristics and faecal waste production in Nile tilapia (*Oreochromis niloticus* L.). *Aquaculture Research*, 36 (12), pp. 1157-1166.

Amirkolaie, A. K. (2011). Reduction in the environmental impact of waste discharged by fish farms through feed and feeding. *Reviews in Aquaculture*, 3 (1), pp. 19-26.

Anguiano, M., Pohlenz, C., Buentello, A. and Gatlin, D. M. III. (2013). The effects of prebiotics on the digestive enzymes and gut histomorphology of red drum (*Sciaenops*

ocellatus) and hybrid striped bass (*Morone chrysops* x *M. saxatilis*). *British Journal of Nutrition*, 109 (4), 623-629.

Ao, X., Meng, Q. W., Yan, L., Kim, H. J., Hong, S. M., Cho, J. H. and Kim, I. H. (2010a). Effects of non-starch polysaccharide-degrading enzymes on nutrient digestibility, growth performance and blood profiles of growing pigs fed a diet based on corn and soybean meal. *Asian-Australian Journal of Animal Science*, 23 (1), pp. 1632-1638.

Ao, T., Cantor, A. H., Prescatore, A. J., Pierce, J. L. and Dawson, K. A. (2010b). Effects of citric acid, alpha-galactosidase and protease inclusion on in vitro nutrient release from soybean meal and trypsin inhibitor content in raw whole soybeans. *Animal Feed Science and Technology*, 162 (1-2), pp. 58-65.

AOAC (2016). *Official methods of analysis* (20th edition). Washington DC, USA: AOAC International.

Aramli, M. S., Kamangar, B. and Nazari, R. M. (2015). Effects of dietary beta-glucan on the growth and innate immune response of juvenile Persian sturgeon, *Acipenser persicus*. *Fish & Shellfish Immunology*, 47 (1), pp. 606-610.

Askarian, F., Zhou, Z., Olsen, R. E., Sperstad, S. and Ringo, E. (2012). Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogens. *Aquaculture*, 326-329, pp. 1-8.

Aslaksen, M. A., Kraugerud, O. F., Penn, M., Svihus, B., Denstadli, V., Jorgensen, H. Y., Hilestad, M., Krogdahl, A. and Storebakken, T. (2007). Screening of nutrient digestibilities and intestinal pathologies in Atlantic salmon, *Salmo salar*, fed diets with legumes, oilseeds, or cereals. *Aquaculture*, 272 (1-2), pp. 541-555.

Asuncion, P. M., Aguado-Gimenez, F., Garcia-Garcia, B., Ballester, G. and Telfer, T. (2009). Settling velocity and total ammonia nitrogen leaching from commercial feed and

faecal pellets of gilthead seabream (*Sparus aurata* L. 1758) and seabass (*Dicentrarchus labrax* L. 1758). *Aquaculture Research*, 40 (15), pp. 1703-1714.

Azaza, M. S., Khiari, N., Dhraief, M. N., Aloui, N., Kraiem, M. M. and Elfeki, A. (2015). Growth performance, oxidative stress indices and hepatic carbohydrate metabolic enzymes activities of juvenile Nile tilapia, *Oreochromis niloticus* L., in response to dietary starch and protein ratios. *Aquaculture Research*, 46 (1), pp. 14-27.

Baeverfjord, G., Asgard, T. And Shearer, K. D. (1998). Development and detection of phosphorus deficiency in Atlantic salmon, *Salmo salar* L., parr and post-smolts. *Aquaculture Nutrition*, 4 (1), pp. 1-11.

Bahr, M., Fecher, A., Hasenkopf, K., Mittermaier, S. and Jahreis, G. (2014). Chemical composition of dehulled seeds of selected lupin cultivars in comparison to pea and soya bean. *LWT – Food Science and Technology*, 59 (1), pp. 587-590.

Barekatain, M. R., Antipatis, C., Choct, M. and Iji, P. A. (2013). Interaction between protease and xylanase in broiler chicken diets containing sorghum distiller's dried grains with solubles. *Animal Feed Science and Technology*, 182 (1-4), pp. 71-81.

Barreto, R. E., Carvalho, G. G. A. and Volpato, G. L. (2011). The aggressive behaviour of Nile tilapia introduced into novel environments with variation in enrichment. *Zoology*, 114 (1), pp. 53-57.

Bartoli, M., Nizzoli, D., Longhi, D., Laini, A. and Viaroli, P. (2007). Impact of a trout farm on the water quality of an Apennine creek from daily budgets of nutrients. *Chemistry and Ecology*, 23 (1), pp. 1-11.

Baruah, K., Pal, A. K., Sahu, N. P., Jain, K. K., Mukherjee, S. C. and Debnath, D. (2005). Dietary protein level, microbial phytase, citric acid and their interactions on bone mineralization of *Labeo rohita* (Hamilton) juveniles. *Aquaculture Research*, 36 (8), pp. 803-812.

Baruah, K., Sahu, N. P., Pal, A. K., Jain, K. K., Debnath, D. and Mukherjee, S. C. (2007a). Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. *Aquaculture Research*, 38 (2), pp. 109-120.

Baruah, K., Sahu, N. P., Pal, A. K., Debnath, D., Yengkokpam, S. and Mukherjee, S. C. (2007b). Interactions of dietary microbial phytase, citric acid and crude protein level on mineral utilization by rohu, *Labeo rohita* (Hamilton), juveniles. *Journal of the World Aquaculture Society*, 38 (2), pp. 238-249.

Baruah, K., Pal, A. K., Sahu, N. P., Debnath, D., Yengkokpam, S., Norouzitallab, P. and Sorgeloos, P. (2009). Dietary Crude Protein, Citric Acid and Microbial Phytase Interacts to Influence the Hemato-Immunological Parameters of Rohu, *Labeo Rohita*, Juveniles. *Journal of the World Aquaculture Society*, 40 (6), pp. 824-831.

Batista, S., Ramos, M. A., Cunha, S., Barros, R., Cristovao, B., Rema, P., Pires, M. A., Valente, L. M. P. and Ozorio, R. O. A. (2015). Immune responses and gut morphology of Senegalese sole (*Solea senegalensis*, Kaup 1858) fed monospecies and multispecies probiotics. *Aquaculture Nutrition*, 21 (5), pp. 625-634.

Bedford, M. R. and Schulze, H. (1998). Exogenous enzymes for pigs and poultry. *Nutrition Research Reviews*, 11 (1), pp. 91-114.

Bedford, M. R. (2003). New enzyme technologies for poultry feeds. *British Poultry Science*, 44 (1), pp. 14-16.

Bedford, M. R. and Partridge, G. G. (eds.) (2010). *Enzymes in farm animal nutrition*. Oxford, UK: CAB International, pp. 1-270

Benfey, T. J. and Biron, M. (2000). Acute stress response in triploid rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). *Aquaculture*, 184 (1-2), pp. 167-176.

Berge, G. M., Witten, P. E., Baeverfjord, G., Vegusdal, A., Wadsworth, S. and Ruyter, B. (2009). Diets with different n-6/n-3 fatty acid ratio in diets for juvenile Atlantic salmon, effects on growth, body composition, bone development and eicosanoid production. *Aquaculture*, 296 (3-4), pp. 299-304.

Bergmeyer, H. U. (1974) *Methods of Enzymatic Analysis*, 2nd ed., Volume II. New York, USA: Academic Press Inc., pp. 860-864

Bian, W., Wang, L., Zhang, H., Wang, J., Tian, Z. and Chen, X. (2012). Study on phosphorus pollution load of aquaculture in Baiyangdian Lake. In *Advances in Environmental Science and Engineering, Pts 1-6* (Iranpour, R., Zhao, J., Wang, A., Yang, F. L. & Li, X., eds.), pp. 1406-1411.

Bigi, A., Foresti, E., Gregorini, R., Ripamonti, A., Roveri, N. and Shah, J. S. (1992). The role of magnesium on the structure of biological apatites. *Calcified tissue international*, 50 (5), pp. 439-444.

Binh Van, V., Dien, P. B., Huy, Q. N. and Fotedar, R. (2015). Optimized fermented lupin (*Lupinus angustifolius*) inclusion in juvenile barramundi (*Lates calcarifer*) diets. *Aquaculture*, 444, pp. 62-69.

Blyth, D., Tabrett, S., Bourne, N. and Glencross, B. (2015). Comparison of faecal collection methods and diet acclimation times for the measurement of digestibility coefficients in barramundi (*Lates calcarifer*). *Aquaculture Nutrition*, 21 (2), pp. 248-245.

Bokori, J. and Kosa, E. (2001). The use of exogenous enzymes in animal nutrition 3. Practical results of enzyme supplementation to diets in feeding of ruminants. *Magyar Allatorvosok Lapja*, 123 (3), pp. 164-172.

Booth, M. A., Anderson, A. J. and Allan, G. L. (2006). Investigation of the nutritional requirements of Australian snapper *Pagrus auratus* (Schneider 1801): digestibility of

gelatinized wheat starch and clearance of an intra- peritoneal injection of D- glucose. *Aquaculture Research,* 37 (4), pp. 975-985.

Booth, M. A., Moses, M. D. and Allan, G. L. (2013). Utilisation of carbohydrate by yellowtail kingfish *Seriola lalandi*. *Aquaculture*, 376-379, pp. 151-161.

Borquez, A., Serrano, E., Dantagnan, P., Carrasco, J. and Hernandez A. (2011). Feeding high inclusion of whole grain white lupin (*Lupinus albus*) to rainbow trout: effects on growth, nutrient digestibility, liver and intestine histology, and muscle fatty acid composition. *Aquaculture Research*, 42 (8), pp. 1067–1078.

Bray, R. J. and Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs*, 27 (1), pp. 325-349.

British Trout Association (2015). *History of trout farming*. [online] Available at: http://britishtrout.co.uk/trout/farming-trout/history-of-trout-farming/ (Accessed 8 March 2016).

Brown, B. A. (ed.) (1988). *Routine hematology procedures*. Philadelphia, PA, USA: Leo and Febiger, pp. 7-122.

Brown, R. M. (2004). Cellulose structure and biosynthesis: What is in store for the 21st century? *Journal of Polymer Science Part A: Polymer Chemistry,* 42 (3), pp. 487-495.

Brufau, J., Francesch, M. and Perez-Vendrell, A. M. (2006). The use of enzymes to improve cereal diets for animal feeding. *Journal of the Science of Food and Agriculture*, 86 (11), pp. 1705-1713.

Brun, L. R., Brance, M. L. and Rigalli, A. (2012). Luminal calcium concentration controls intestinal calcium absorption by modification of intestinal alkaline phosphatase activity. *British Journal of Nutrition*, 108 (2), pp. 229-233.

Bucking, C., Fitzpatrick, J. L., Nadella, S. R., McGaw, I. J. and Wood, C. M. (2011). Assimilation of water and dietary ions by the gastrointestinal tract during digestion in seawater-acclimated rainbow trout. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, 181 (5), pp. 615-630.

Bureau, D. P., Harris, A. M. and Cho, C. Y. (1998). The effects of purified alcohol extracts from soy products on feed intake and growth of chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 161 (1-4), pp. 27-43.

Burr, G. S., Wolters, W. R., Barrows, F. T. and Hardy, R. W. (2012). Replacing fishmeal with blends of alternative proteins on growth performance of rainbow trout (*Oncorhynchus mykiss*), and early or late stage juvenile Atlantic salmon (*Salmo salar*). *Aquaculture*, 334-337, pp. 110-116.

Bury, N. R. and Grosell, M. (2003). *Iron acquisition by teleost fish*. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology*, 135 (2), pp. 97-105.

Bury, N. R., Walker, P. A. and Glover, C. N. (2003). Nutritive metal uptake in teleost fish. *Journal of Experimental Biology*, 206 (1), 11-23.

Buttle, L. G., Burrells, A. C., Good, J. E., Williams, P. D., Southgate, P. J. and Burrells, C. (2001). The binding of soybean agglutinin (SBA) to the intestinal epithelium of Atlantic salmon, *Salmo salar* and Rainbow trout, *Oncorhynchus mykiss*, fed high levels of soybean meal. *Veterinary Immunology and Immunopathology*, 80 (3-4), pp. 237-244.

Caine, W. R., Verstegen, M. W. A., Sauer, W. C., Tamminga, S. and Schulze, H. (1998). Effect of protease treatment of soybean meal on content of total soluble matter and crude protein and level of soybean trypsin inhibitors. *Animal Feed Science and Technology*, 71 (1-2), pp. 177-183.

Camargo, J. A., Gonzalo, C. and Alonso, A. (2011). Assessing trout farm pollution by biological metrics and indices based on aquatic macrophytes and benthic macroinvertebrates: a case study. *Ecological Indicators*, 11 (3), pp. 911-917.

Campbell, G. L. and Bedford, M. R. (1992). Enzyme applications for monogastric feeds – a review. *Canadian Journal of Animal Science*, 72 (3), pp. 449-466.

Capkin, E. and Altinok, I. (2009). Effects of dietary probiotic supplementations on prevention/treatment of yersiniosis disease. *Journal of Applied Microbiology*, 106 (4), pp. 1147-1153.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. and Knight, R. (2010a). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7 (5), pp. 335-336.

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., Knight, R. (2010b). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*, 26 (2), pp. 266–267

Capraro, J., Magni, C., Scarafoni, A. and Duranti, M. (2009). Susceptibility of lupin gamma-conglutin, the plasma glucose-lowering protein of lupin seeds, to proteolytic enzymes. *Journal of Agricultural and Food Chemistry*, 57 (18), pp. 8612-8616.

Carter, C. G. and Sajjadi, M. (2011). Low fishmeal diets for Atlantic salmon, *Salmo salar* L., using soy protein concentrate treated with graded levels of phytase. *Aquaculture International*, 19 (3), pp. 431-444.

Cashman, K. D. and Flynn, A. (1999). Optimal nutrition: calcium, magnesium and phosphorus. *Proceedings of the Nutrition Society*, 58 (2), pp. 477-487.

Castillo, S. and Gatlin, D. M. III. (2015). Dietary supplementation of exogenous carbohydrase enzymes in fish nutrition: a review. *Aquaculture*, 435 (1-4), pp. 286-292.

Castro, C. A. C., Hernandez, L. H. H., Araiza, M. A. F., Perez, T. R. and Lopez, O. A. (2011). Effects of diets with soybean meal on the growth, digestibility, Phosphorus and Nitrogen excretion of juvenile rainbow trout *Onchorhynchus mykiss*. *Hidrobiologica*, 21 (2), pp. 118-125.

Chakrabarti, I., Gani, M. A., Chaki, K. K., Sur, R. and Misra, K. K. (1995). Digestive enzymes in 11 freshwater teleost fish species in relation to food habit and niche segregation. *Comparative Biochemistry and Physiology Part A: Physiology*, 112 (1), pp. 167-177.

Chartchai, K., Chatchai, S. N. and Saisamorn, L. (2006). Nutritive Quality of β-Mannanase Treated Copra Meal in Broiler Diets and Effectiveness on Some Fecal Bacteria. *International Journal of Poultry Science*, 5 (11), pp. 1087.

Chegeni, A., Torki, M. and Kamyab, A. (2011). Effects of β- mannanase-based enzyme in corn-soy and corn-soy-canola diets on broiler performance. *Journal of Applied Animal Research* 39 (3), pp. 261-268.

Chen, K. T., Malo, M. S., Moss, A. K., Zeller, S., Johnson, P., Ebrahimi, F., Mostafa, G., Alam, S. N., Ramasamy, S., Warren, H. S., Hohmann, E. L and Hodin, R. A. (2010). Identification of specific targets for the gut mucosal defense factor intestinal alkaline phosphatase. *American Journal of Physiology - Gastronintestinal and Liver Physiology*, 299 (2), pp. 467-475.

Cheng Z. J. J. and Hardy, R. W. (2004). Effects of microbial phytase supplementation in corn distiller's dried grains with solubles on nutrient digestibility and growth performance of rainbow trout, *Oncorhynchus mykiss*. *Journal of Applied Aquaculture*, 15 (3-4), pp. 83-100.

Cheng, Z. J. J., Hardy, R. W., Verlhac, V. and Gabaudan, J. (2004). Effects of microbial phytase supplementation and dosage on apparent digestibility coefficients of nutrients and dry matter in soybean product-based diets for rainbow trout *Oncorhynchus mykiss*. *Journal of the World Aquaculture Society*, 35 (1), pp. 1-15.

Cheng, Z., Buentello, A. and Gatlin, D. M. III. (2011). Dietary nucleotides influence immune responses and intestinal morphology of red drum Sciaenops ocellatus. *Fish and Shellfish Immunology*, 30 (1), pp. 143-147.

Chien, Y. H. and Chiu, Y. H. (2003). Replacement of soybean (*Glycine max* (L.) Merrill) meal by lupin (*Lupinus angustifolius*) seed meal in diet for juvenile tilapia (*Oreochromis niloticus* x *O. aureus*) reared indoors. Aquaculture Research, 34 (14), pp. 1261-1268.

Chikwati, E. M., Sahlmann, C., Holm, H., Penn, H., Krogdahl, A. and Bakke, A. M. (2013). Alterations in digestive enzyme activities during the development of diet-induced enteritis in Atlantic salmon, *Salmo salar* L.. *Aquaculture*, 402 (1-4), pp. 28-37.

Chimits, P. (1957). The tilapia and their culture*. FAO Fish Bulletin*, 10, pp. 1-24.

Cowey, C. B., Knox, D., Walton, M. J. and Adron, J. W. (1977). The regulation of gluconeogenesis by diet and insulin in rainbow trout (*Salmo gairdneri*). *British Journal of Nutrition*, 38 (3), pp. 463-470.

Cowieson, A. J., Hruby, M. and Pierson, E. E. M. (2006). Evolving enzyme technology: impact on commercial poultry nutrition. *Nutrition Research Reviews*, 19 (1), pp. 90-103.

Czubinski, J., Dwiecki, K., Siger, A., Neunert, G. and Lampart-Szczapa, E. (2014). Characterisation of different digestion susceptibility of lupin seed globulins. *Food Chemistry*, 143 (1), pp. 418-426.

Dabrowski, K. and Guderley, H. (2002). *Intermediary metabolism*. In: J. E. Halver and R. W. Hardy, ed., *Fish Nutrition*, 3rd ed. San Diego, USA: Academic Press, pp. 310-360.

Dalsgaard, J., Ekmann, K. S., Pedersen, P. B. and Verlhac, V. (2009). Effect of supplemented fungal phytase on performance and phosphorus availability by phosphorus-depleted juvenile rainbow trout (*Oncorhynchus mykiss*), and on the magnitude and composition of phosphorus waste output. *Aquaculture*, 286 (1-2), pp. 105-112.

Dalsgaard, J., Verlhac, V., Hjermitslev, N. H., Ekmann, K. S., Fischer, M., Klausen, M. and Pedersen, P. B. (2012). Effects of exogenous enzymes on apparent nutrient digestibility in rainbow trout (*Oncorhynchus mykiss*) fed diets with high inclusion of plant-based protein. *Animal Feed Science and Technology*, 171 (2-4), pp. 181-191.

Danicke, S., Jeroch, H., Bottcher, W. and Simon, O. (2000). Interactions between dietary fat type and enzyme supplementation in broiler dietts with high pentosan contents: effects on precaecal and total tract digestibility of fatty acids, metabolizability of gross energy, digesta viscosity and weights of small intestine. *Animal Feed Science and Technology*, 84 (3-4), pp. 279-294.

Daniels, C. L., Merrifield, D. L., Boothroyd, D. P., Davies, S. J., Factor, J. R. and Arnold, K. E. (2010). Effect of *Bacillus* spp. and mannan oligosaccharides (MOS) on European lobster (*Homarus gammarus* L.) larvae growth performance, gut morphology and gut microbiota. *Aquaculture*, 304 (1-4), pp. 49-57.

Danson, M. J., Hough, D. W., Russell, R. J. M., Taylor, G. L. and Pearl, L. (1996). Enzyme thermostability and thermoactivity. *Protein Engineering*, 9 (8), pp. 629-630.

Das, K. M. and Tripathi, S. D. (1991). Studies on the digestive enzymes of grass carp, *Ctenopharyngodon idella* (Val.). *Aquaculture*, 92, pp. 21-32.

Das, P., Mandal, S., Khan, A., Manna, S. K. and Ghosh, K. (2014). Distribution of extracellular enzyme-producing bacteria in the digestive tracts of 4 brackish water fish species. *Turkish Journal of Zoology*, 38 (1), pp. 79-88.

Dato-Cajegas, C. R. S. and Yakupitiyage, A. (1996). The need for dietary mineral supplementation for Nile tilapia, *Oreochromis niloticus*, cultured in semi-intensive system. *Aquaculture*, 144 (1-3), pp. 227-237.

Davis, A. and Gatlin, D. M. III. (1996). Dietary mineral requirements of fish and marine crustaceans. *Reviews in Fisheries Science*, 4 (1), pp. 75-99.

Dawood, M. A. O., Koshio, S., Ishikawa, M. and Yokoyama, s. (2015). Interaction effects of dietary supplementation of heat-killed *Lactobacillus plantarum* and beta-glucan on growth performance, digestibility and immune response of juvenile red sea bream, *Pagrus major*. *Fish & Shellfish Immunology*, 45 (1), pp. 33-42.

Debnath, D., Sahu, N. P., Pal, A. K., Baruah, K., Yengkokpam, S. and Mukherjee, S. C. (2005a). Present scenario and future prospects of phytase in aquafeed - Review. *Asian-Australasian Journal of Animal Sciences*, 18 (12), pp. 1800-1812.

Debnath, D., Pal, A. K., Sahu, N. P., Jain, K. K., Yengkokpam, S. and Mukherjee, S. C. (2005b). Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. *Aquaculture Research*, 36 (2), pp. 180-187.

Debnath, D., Sahu, N. P., Pal, A. K., Jain, K. K., Yengkokpam, S. and Mukherjee, S. C. (2005c). Mineral status of *Pangasius pangasius* (Hamilton) fingerlings in relation to supplemental phytase: absorption, whole-body and bone mineral content. *Aquaculture Research*, 36 (4), 326-335.

DelMar, E. G., Largman, C., Brodrick, J. W. and Geokas, M. C. (1979). A sensitive new substrate for chymotrypsin. *Analytical Biochemistry*, 99 (2), pp. 316-320.

Demers, N. E. and Bayne, C. J. (1997). The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. *Developmental and Comparative Immunology*, 21 (4), pp. 363-373.

Deng, B., Wang, Z. P., Tao, W. J., Li, W. F., Wang, C., Wang, M. Q., Ye, S. S., Du, Y. J., Wu, X. X. and Wu, D. (2015). Effects of polysaccharides from mycelia of *Cordyceps sinensis* on growth performance, immunity and antioxidant indicators of the white shrimp *Litopenaeus vannamei*. *Aquaculture Nutrition*, 21 (2), 173-179.

Denstadli, V., Skrede, A., Krogdahl, A., Sahlstrom, S. and Storebakken, T. (2006). Feed intake, growth, feed conversion, digestibility, enzyme activities and intestinal structure in Atlantic salmon (*Salmo salar* L.) fed graded levels of phytic acid. *Aquaculture*, 256 (1-4), pp. 365-376.

Denstadli, V., Storebakken, T., Svihus, B. and Skrede, A. (2007). A comparison of online phytase pre-treatment of vegetable feed ingredients and phytase coating in diets for Atlantic salmon (*Salmo salar* L.) reared in cold water. *Aquaculture*, 269 (1-4), pp. 414-426.

Denstadli, V., Hillestad, M., Verlhac, V., Klausen, M. and Overland, M. (2011). Enzyme pretreatment of fibrous ingredients for carnivorous fish: Effects on nutrient utilisation and technical feed quality in rainbow trout (*Oncurhynchus mykiss*). *Aquaculture*, 319 (3-4), pp. 391-397.

de Oliveira, G. R., Logato, P. V. R., de Freitas, R. T. F., Rodrigues, P. B., Fialho, E. T. and Diodatti, F. C. (2007). Nutrient digestibility in diets with enzyme complex for Nile tilapia. *Revista Brasileira De Zootecnia-Brazilian Journal of Animal Science*, 36 (6), pp. 1945-1952.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P. and Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA

gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, 72 (7), pp. 5069–5072.

Deschamps, M. H., Girondot, M., Labbe, L. and Sire, J. Y. (2009). Changes in vertebral structure during growth of reared rainbow trout, Oncorhynchus mykiss (Walbaum): a new approach using modelling of vertebral bone profiles. *Journal of Fish Diseases*, 32 (3), pp. 233-246.

Deutsch, L., Graslund, S., Folke, C., Troell, M., Huitric, M., Kautsky, N. and Lebel, L. (2007). Feeding aquaculture growth through globalization: Exploitation of marine ecosystems for fishmeal. *Global Environmental Change-Human and Policy Dimensions*, 17 (2), pp. 238-249.

Dimitroglou, A., Merrifield, D. L., Moate, R., Davies, S. J., Spring, P., Sweetman, J. and Bradley, G. (2009). Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Animal Science*, 87 (10), pp. 3226-3234.

Dimitroglou, A., Merrifield, D. L., Spring, P., Sweetman, J., Moate, R. and Davies, S. J. (2010a). Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). *Aquaculture*, 300 (1-4), pp. 182-188.

Dimitroglou, A. (2010b). *Effects of dietary mannan oligosaccharide (MOS) supplementation in relation to intestinal integrity, microbiota, health and production of cultured fish species.* PhD. University of Plymouth.

Dixon, M. and Webb, E. C. (1979). *Enzymes*, 3rd ed.. London, UK: Longman Group Ltd., pp. 309-3013.

Duranti, M., Gius, C., Sessa, F. And Vecchio, G. (1995). The saccharide chain of lupin seed conglutin γ is not responsible for the protection of the native protein from

degradation by trypsin, but facilitates the refolding of the acid-treated protein to the ressistant conformation. *European Journal of Biochemistry*, 230, pp. 886-891.

Earth Policy Institute (2013). *Farmed fish production overtakes beef*. [online] Available at: http://www.earth-policy.org/plan_b_updates/2013/update114 (Accessed 7 March 2016).

Eaton-Mordas, C. A. and Moore, K. G. (1978). Seed glycoproteins of lupinus angustifolius. *Phytochemistry*, 17 (4), pp. 619-621.

Edgar, R. C. (2010), Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, 26 (19), pp. 2460–2461.

Eeckhout, I. M. (2000). Phytase quality suffers from steam pelleting and storage temperature. *Feed Technology*, 4 (2), pp. 18-21.

Ellestad, L. E., Dahl, G., Angel, R. and Soares, J. H. (2003). The effect of exogenously administered recombinant bovine somatotropin on intestinal phytase activity and in vivo phytate hydrolysis in hybrid striped bass *Morone chrysops* x *M. saxatilis*. *Aquaculture Nutrition*, 9 (5), pp. 327-336.

Enes, P., Panserat, S., Kaushik, S. and Oliva-Teles, A. (2011). Dietary Carbohydrate Utilization by European Sea Bass (*Dicentrarchus labrax* L.) and Gilthead Sea Bream (*Sparus aurata* L.) Juveniles. *Reviews in Fisheries Science*, 19 (3), pp. 201-215.

Esakkiraj, P., Immanuel, G., Sowmya, S. M., Iyapparaj, P. and Palavesam, A. (2009). Evaluation of protease-producing ability of fish gut isolate *Bacillus cereus* for aqua feed. *Food and Bioprocess Technology*, 2 (4), pp. 383-390.

Espinoza, A. J. and Giraud-Heraud, E. (2012). *International market for soybeans coexistence of GM and non-GM products*. [online] Available at: http://fdir.idei.fr/wp-content/uploads/2012/newsletter7.pdf (Accessed 14 June 2016).

FAO (1992). Technology of production of edible flours and protein products from soybeans. [online] Available at: http://www.fao.org/docrep/t0532e/t0532e00.HTM (Accessed 7 March 2016).

FAO (2009). *Meat and meat products*. [online] Available at: http://www.fao.org/docrep/012/ak341e/ak341e09.htm (Accessed 7 March 2016).

FAO (2010). *World aquaculture 2010*. [online] Available at: http://www.fao.org/docrep/014/ba0132e/ba0132e00.htm (Accessed 19 Feb 2016).

FAO (2014a). *The state of world fisheries and aquaculture*. [online] Available at: www.fao.org/3/a-i3720e.pdf (Accessed 19 Feb. 2016).

FAO (2014b). *Cultured aquatic species information programme – Oreochromis niloticus (Linnaeus, 1758)*. [online] Available at: http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/en (Accessed 7 Feb. 2016).

FAO (2016). *Cultured aquatic species information programme – Oncorhynchus mykiss (Walbaum, 1792)*. [online] Available at: http://www.fao.org/fishery/culturedspecies/Oncorhynchus_mykiss/en (Accessed 8 March. 2016).

FAOSTAT (2016). [online] Available at: http://faostat3.fao.org/browse/Q/QC/E (Accessed 7 March 2016).

Farhangi, M. and Carter, C. (2007). Effect of enzyme supplementation to dehulled lupin-based diets on growth, feed efficiency, nutrient digestibility and carcass composition of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*, 38 (12), pp. 1274-1282.

FDA (2015). *AquAdvantage salmon.* [online] Available at: http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/ucm280853.htm (Accessed 8 March 2016).

Ferrara, E., Gustinelli, A., Fioravanti, M. L., Restucci, B., Quaglio, F. and Marono, S. (2015). Histological and micro-/macro-morphological evaluation of intestine in sharpsnout seabream (*Diplodus puntazzo*) fed soybean meal-based diets added with MOS and inulin as prebiotics. *Aquaculture International*, 23 (6), pp. 1525-1537.

Ferreira, R. B., Melo, T. S. and Teixeira, A. N. (1995). Catabolism of the seed storage proteins from Lupinus albus: fate of globulins during germination and seedling growth. *Australian Journal of Plant Physiology*, 22 (3), pp. 373-381.

Fines, B. C. and Holt, G. J. (2010). Chitinase and apparent digestibility of chitin in the digestive tract of juvenile cobia, Rachycentron canadum. *Aquaculture*, 303 (1-4), pp. 34-39.

Fjelldal, P. G., Hansen, T., Breck, O., Sandvik, R., Waagbo, R., Berg, A. and Ornsrud, R. (2009). Supplementation of dietary minerals during the early seawater phase increase vertebral strength and reduce the prevalence of vertebral deformities in fast-growing under-yearling Atlantic salmon (*Salmo salar* L.) smolt. *Aquaculture Nutrition*, 15 (4), pp. 366-378.

Fjelldal, P. G., Hansen, T. M., Lock, E.-J., Wargelius, A., Fraser, T. W. K., Sambraus, F., El-Mowafi, A., Albrektsen, S., Waagbo, R. and Ornsrud, R. (2015). Increased dietary phosphorous prevents vertebral deformities in triploid Atlantic salmon (*Salmo salar* L.). *Aquaculture Nutrition*, 22 (1), pp. 72-90.

Foley, R. C., Jimenez-Lopez, J. C., Kamphuis, L. G., Hane, J. K., Melser, S. and Singh, K. B. (2015). Analysis of conglutin seed storage proteins across lupin species using transcriptomic, protein and comparative genomic approaches. *BMC Plant Biology*, 19 (15), 106.

Fontagne, S., Silva, N., Bazin, D., Ramos, A., Aguirre, P., Surget, A., Abrantes, A., Kaushik, S. and Power, D. M. (2009). Effects of dietary phosphorus and calcium level on growth and skeletal development in rainbow trout (*Oncorhynchus mykiss*) fry. *Aquaculture*, 297 (1-4), pp. 141-150.

Forster, I., Higgs, D. A., Bakhshish, S. D., Mahmoud, R. and Parr, J. (1999). Potential for dietary phytase to improve the nutritive value of canola protein concentrate and decrease phosphorus output in rainbow trout (*Oncorhynchus mykiss*) held in 11 °C fresh water. *Aquaculture*, 179 (1-4), pp. 109-125.

Fox, D. J. and Davies, S. J. (2011). Corn-expressed dietary phytase augments vertebral and scale mineral content in juvenile rainbow trout (Oncorhynchus mykiss). *Aquaculture Nutrition*, 17 (4), pp. 840-852.

Francis, G., Makkar, H. P .S. and Becker, K. (2001). Antinutritioal factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, 199 (3-4), pp. 197-227.

Furne, M., Hidalgo, M. C., Lopez, A., Garcia-Gallego, M., Morales, A. E., Domezain, A., Domezaine, J. and Sanz, A. (2005). Digestive enzyme activities in Adriatic sturgeon *Acipenser naccarii* and rainbow trout *Oncorhynchus mykiss*. A comparative study. *Aquaculture*, 250 (1-2), pp. 391-398.

Furne, M., Garcia-Gallego, M., Hidalgo, M. C., Morales, A. E., Domezain, A., Domezain, J. and Sanz, A. (2008). Effect of starvation and refeeding on digestive enzyme activities in sturgeon (*Acipenser naccarii*) and trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, 149 (4), pp. 420-425.

Garcia, F., Romera, D. M., Gozi, K. S., Onaka, E. M., Fonseca, F. S., Schalch, S. H. C., Candeira, P. G., Guerra, L. O. M., Carmo, F. J., Carneiro, D. J., Martins, M. I. E. G. and Portella, M. C. (2013). Stocking density of Nile tilapia in cages placed in a hydroelectric reservoir. *Aquaculture*, 410 (1-4), pp. 51-56.

Gdala, J., Jansman, A. J. M., Buraczewska, L., Huisman, J. and Van Leeuwen, P. (1997). The influence of α-galactosidase supplementation on the ileal digestibility of lupine seed carbohydrates and dietary protein in young pigs. *Animal Feed Science and Technology*, 67 (1), pp. 115-125.

Gentilini, F. P., Silva, R. A. G. da, Nunes, P. M., Gonçalves, F. M., Kuhn, C., Anciuti, M. A. and Rutz, F. (2009). Performance and bone resistance of laying hens supplemented with Allzyme® SSF in the diets. *Archivos de Zootecnia*, 58 (224), pp. 645-653.

Geurden, I., Aramendi, M., Zambonino-Infante, J. and Panserat, S. (2007). Early feeding of carnivorous rainbow trout (*Oncorhynchus mykiss*) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, 292 (6), R2275-R2283.

Geurden, I., Borchert, P., Balasubramanian, M. N., Schrama, J. W., Dupont-Nivet, M., Quillet, E., Kaushik, S. J., Panserat, S. and Medale, F. (2013). The positive impact of the early-feeding of a plant-based diet on its future acceptance and utilisation in rainbow trout. *PLoS One*, 8 (12), e83162.

Ghaedi, G., Kyvanshokooh, S., Azam, H. M. and Akhlaghi, M. (2015). Effects of dietary beta-glucan on maternal immunity and fry quality of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 441 (1-4), pp. 78-83.

Glencross, B. D., Boujard, T. and Kaushik, S. J. (2003). Influence of oligosaccharides on the digestibility of lupin meals when fed to rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, 219 (1-4), pp. 703-713.

Glencross, B. D., Carter, C. G., Duijster, N., Evans, D. R., Dods, K., McCafferty, P., Hawkins, W. E., Maas, R. and Sipsas, S. (2004a). A comparison of the digestibility of a range of lupin and soybean protein products when fed to either Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 237 (1-4), pp. 333-346.

Glencross, B., Evans, D., Hawkins, W. and Jones, B. (2004b). Evaluation of dietary inclusion of yellow lupin (*Lupinus luteus*) kernel meal on the growth, feed utilisation and tissue histology of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 235 (1-4), pp. 411-422.

Glencross, B., Hawkins, W., Evans, D., Rutherford, N., Dods, K., Maas, R., McCafferty, P. and Sipsas, S. (2006). Evaluation of the nutritional value of prototype lupin protein concentrates when fed to rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 251 (1), pp. 66-77.

Glencross, B., Hawkins, W., Evans, D., Rutherford, N., McCafferty, P., Dods, K., Karopoulos, M., Veitch, C., Sipsas, S. and Buirchell, B. (2008). Variability in the composition of lupin (*Lupinus angustifolius*) meals influences their digestible nutrient and energy value when fed to rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 277 (3-4), pp. 220-230.

Glencross, B., Hawkins, W., Maas, R., Karopoulos, M. and Hauler, R. (2010). Evaluation of the influence of different species and cultivars of lupin kernel meal on the extrusion process, pellet properties and viscosity parameters of salmonid feeds. *Aquaculture Nutrition*, 16 (1), pp. 13-24

Glencross, B., Rutherford, N. and Hawkins, W. (2011). A comparison of the growth performance of rainbow trout (*Oncorhynchus mykiss*) when fed soybean, narrow-leaf or yellow lupin meals in extruded diets. *Aquaculture Nutrition*, 17 (2), pp. 317-325.

Goda, A. M. A., Mabrouk, H. A.-H. H., Wafa, M. A. E.-H. And El-Affi, T. M. (2012). Effect of using baker's yeast and exogenous digestive enzymes as growth promoters on growth, feed utilization and hematological indices of Nile tilapia, *Oreochromis niloticus* fingerlings. *Journal of Agricultural Science & Technology B*, 2 (1b), pp. 15.

Godlewski, M. M., Slazak, P., Zabielski, R., Piastowska, A. and Gralak, M. A. (2006). Quantitative study of soybean-induced changes in proliferation and programmed cell

death in the intestinal mucosa of young rats. *Journal of Physiology and Pharmacology*, 57 (7), 125-133.

Gomez-Guinan, Y. (2004). Activity of acid and alkaline phosphatases (intracellular and extracellular) in rhizosphere fungi from *Arachis hypogaea* (Papiloneaceae). *Revista De Biologia Tropical*, 52 (1), pp. 287-295.

Goncalves, G. S., Pezzato, L. E., Padilha, P. D. and Barros, M. M. (2007). Apparent phosphorus availability in vegetable feedstuffs and supplementation of phytase enzyme for Nile tilapia *Oreochromis niloticus*. *Revisita Brasileira de Zootecnia*, 36 (5), pp. 1473-1480.

Government of Western Australia (2015). *Western Australian lupin industry*. [online] Available at: https://www.agric.wa.gov.au/grains-research-development/western-australian-lupin-industry (Accessed 7 March 2015).

Green, T. J., Smullen, R. and Barnes, A. C. (2013). Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, *Salmo salar* is associated with alterations in gut microbiota. *Veterinary Microbiology*, 166 (1-2), pp. 286-292.

Greiner, R. and Konietzny U. (2006). Phytase for food application. *Food Technology and Biotechnology*, 44 (2), pp. 125-140.

Gu, M., Kortner, TM., Penn, M., Hansen, A. K. and Krogdahl, A. (2014). Effects of dietary plant meal and soya-saponin supplementation on intestinal and hepatic lipid droplet accumulation and lipoprotein and sterol metabolism in Atlantic salmon (*Salmo salar* L.). *British Journal of Nutrition*, 111 (3), pp. 432-444.

Guerreiro, I., Peres, H., Castro, C., Perez-Jimenez, A., Castro-Cunha, M. and Oliva-Teles, A. (2012). Water temperature does not affect protein sparing by dietary carbohydrate in Senegalese sole (*Solea senegalensis*) juveniles. *Aquaculture Research*, 45 (2), pp. 289-298.

Guimaraes, I. G., Falcon, D. R., Schich, D., Barros, M. M. and Pezzato, L. E. (2009). Apparent digestibility of diets containing enzyme supplement for Nile tilapia. Arquivo *Brasileiro De Medicina Veterinaria E Zootecnia*, 61 (6), pp. 1397-1402.

Hardy, R. W., Fornshell, G. C. G. and Brannon, E. L. (2000). *Rainbow trout culture*. In: R Stickney, ed., *Fish Culture*. New York, USA: John Wiley & Sons, pp. 716-722.

Hardy, R. W. and Tacon, A. G. J. (2002). *Fish meal: Historical uses, production trends and future outlook for sustainable supplies.* In: R. R. Stickney and J. P. McVey, ed., *Responsible Marine Aquaculture*, 1st ed. New York, USA: CABI Publishing, pp. 311-325.

Hardy, R. W. (2010). Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Aquaculture Research*, 41 (1), pp. 770-776.

Harholt, J., Suttangkakul, A. and Scheller, H. V. (2010). Biosynthesis of Pectin. *Plant Physiology*, 153 (2), pp. 384-395.

Hartman, G. (1979). Removal of phytate from soy protein. *Journal of the American Oil Chemists' Society*, 56 (8), pp. 731-735.

Hartviksen, M., Vecino, J. L. G., Ringo, E., Bakke, A. M., Wadsworth, S., Krogdahl, A., Ruohonen, K. and Kettunen, A. (2014). Alternative dietary protein sources for Atlantic salmon (*Salmo salar* L.) effect on intestinal microbiota, intestinal and liver histology and growth. *Aquaculture Nutrition*, 20 (4), pp. 381-398.

Hastings, W. H. (1946). Enzyme supplements to poultry feeds. *Poultry Science*, 25 (6), pp. 548-586.

Hefnawy, T. H. (2011). Effect of processing methods on nutritional composition and anti-nutritional factors in lentils (Lens culinaris). *Annals of Agricultural Sciences*, 56 (2), pp. 57-61.

Helland, S., Refstie, S., Espmark, A., Hjelde, K. and Baeverfjord, G. (2005). Mineral balance and bone formation in fast-growing Atlantic salmon parr (*Salmo salar*) in response to dissolved metabolic carbon dioxide and restricted dietary phosphorus supply. *Aquaculture*, 250 (1-2), pp. 364-376.

Helland, S., Denstadli, V., Witten, P. E., Hjelde, K., Storebakken, T., Skrede, A., Asgaard, T. and Baeverfjord, G. (2006). Hyper dense vertebrae and mineral content in Atlantic salmon (*Salmo salar* L.) fed diets with graded levels of phytic acid. *Aquaculture*, 261 (2), pp. 603-614.

Hemre, G.-I., Mommsen, T. P. and Krogdahl, A. (2002). Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquaculture Nutrition*, 8 (3), pp. 175-194.

Hidalgo, M. C., Urea, E. and Sanz, A. (1999). Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture*, 170 (3-4), pp. 267-283.

Hidvegi, M. and Lasztity R. (2002). Phytic acid content of cereals and legumes and interactions with proteins. *Periodica Polytechnica, Chemical Engineering*, 46 (1), pp. 59-64.

Hino, S., Takemura, N., Sonoyama, K., Morita, A., Kawagishi, H., Aoe, S. and Morita, T. (2012). Small Intestinal Goblet Cell Proliferation Induced by Ingestion of Soluble and Insoluble Dietary Fiber Is Characterized by An Increase in Sialylated Mucins in Rats. *Journal of Nutrition*, 142 (8), pp. 1429-1436.

Hino, S., Saita, H. and Morita, T. (2013). Non-effect of an antibiotic treatment on dietary fiber-induced goblet cell proliferation in the ileum of rats. *Bioscience Biotechnology and Biochemistry*, 77 (8), pp. 1782-1784.

Hlavac, D., Adamek, Z., Hartman, P. and Masilko, J. (2014). Effects of supplementary feeding in carp ponds on discharge water quality: a review. *Aquaculture International*, 22 (1), pp. 299-320.

Hlophe, S. N., Moyo, N. A. G. and Ncube, I. (2013). Postprandial changes in pH and enzyme activity from the stomach and intestines of *Tilapia rendalli* (Boulenger, 1897) *Oreochromis mossambicus* (Peters, 1852) and *Clarias gariepinus* (Burchell, 1822). *Journal of Applied Ichthyology*, 30 (1), pp. 35-41.

Hooge, D. M., Pierce, J. l., Mcbride, K. W. and Rigolin, P. J. (2010). Meta- analysis of Broiler Chicken Trials Using Diets With or Without Allzyme® SSF Enzyme Complex. *International Journal of Poultry Science*, 9 (9), pp. 819-823.

Hoshino, T., Ishizaki, K., Sakamoto, T., Kumeta, H., Yumoto, I., Matsuyama, H. and Ohgiya, S. (1997). Isolation of a Pseudomonas species from fish intestine that produces a protease active at low temperature. *Letters in Applied Microbiology*, 25 (1), pp. 70-72.

Hossain, S. M. and Rezende, M. J. M. (1996). Effect of several levels of manganese and available phosphorus on egg production and egg shell quality. *Arquivo Brasileiro De Medicina Veterinaria E Zootecnia*, 48 (5), pp. 567-573.

Hossain, S. M. and Bertechini, A. G. (1998). Effect of varying manganese and available phosphorus levels in the diet on egg production and eggshell quality of layers. *Animal Feed Science and Technology*, 71 (3-4), pp. 303-308.

Hossain, M. A., Focken, U. and Becker, K. (2001). Galactomannan-rich endosperm of Sesbania (*Sesbania aculeata*) seeds responsible for retardation of growth and feed utilisation in common carp, *Cyprinus carpio* L.. *Aquaculture*, 203 (1-2), pp. 121-132.

Hung, T. V., Handsom, P. D., Amenta, V. C., Kyle, W. S. A. and Yu, R. S. T. (1988). Mineral-composition and distribution in lupin seeds and in flour, spray-dried powder and

protein isolate produced from the seeds. *Journal of the Science of Food and Agriculture*, 45 (2), pp. 145-154.

Igbasan, F. A., Guenter, W. and Slominski, B. A. (1997). The effect of pectinase and α-galactosidase supplementation on the nutritive value of peas for broiler chickens. *Canadian Journal of Animal Science*, 77 (3), pp. 537-539.

Ingerslev, H.-C., Strube, M. L., Jorgensen, L. v. G., Dalsgaard, I., Boye, M. and Madsen, L. (2014). Diet type dictates the gut microbiota and the immune response against Yersinia ruckeri in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology*, 40 (2), pp. 624-633.

Inoue, H., Yano, S. and Sawayama, S. (2015). Effect of beta-mannanase and beta-mannosidase supplementation on the total hydrolysis of softwood polysaccharides by the *Talaromyces cellulolyticus* cellulase system. *Applied Biochemistry and Biotechnology*, 176 (6), pp. 1673-1686.

Ito, H., Satsukawa, M., Arai, E., Sugiyama, K., Sonoyama, K., Kiriyama, S. and Morita, T. (2009). Soluble fiber viscosity affects both goblet cell number and small intestine secretion in rats. *Journal of Nutrition*, 139 (9), pp. 1640-1647.

Iwashita, Y., Yamamoto, T., Furuita, H., Sugita, T. and Suzuki, N. (2008). Influence of certain soybean antinutritional factors supplemented to a casein-based semipurified diet on intestinal and liver morphology in fingerling rainbow trout *Oncorhynchus mykiss. Fisheries Science*, 74 (5), pp. 1075-1082.

Iwashita, Y., Suzuki, N., Matsunari, H., Sugita, T. and Yamamoto, T. (2009). Influence of soya saponin, soya lectin, and cholyltaurine supplemented to a casein-based semipurified diet on intestinal morphology and biliary bile status in fingerling rainbow trout *Oncorhynchus mykiss*. *Fisheries Science*, 75 (5), pp. 1307-1315.

Izquierdo, M. S., Fernandez-Palacios, H. and Tacon, A. G. J. (2001). Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture*, 197 (1-4), pp. 25-42.

Jacob, H. E. (1944). *Six thousand years of bread – its holy and unholy history*. New York, USA: Doubleday, Doran and Company Inc., pp. 18-34

Jarvis, M. C., Forsyth, W. and Duncan, H. J. (1988). A survey of the pectic content of nonlignified monocot cell walls. *Plant physiology*, 88 (2), pp. 309-314.

Javar, S., Mohamed, R., Sajap, A. S. and Lau, W. H. (2015). Expression profiles of lysozyme- and prophenoloxidase-encoding genes in Spodoptera species challenged with entomopathogenic fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin using qRT-PCR. *Invertebrate Reproduction & Development*, 59 (4), pp. 230-236.

Jervis, J., Kastl, C., Hildreth, S. B., Biyashev, R., Grabau, E. A., Sagai-Maroof, M. A. and Helm, R. F. (2015). Metabolite profiling of soybean seed extracts from near-isogenic low and normal phytate lines using orthogonal separation strategies. *Journal of Agricultural and Food Chemistry*, 63 (44), pp. 9879-9887.

Ji, XJ, Huang, H. and Ouyang, PK. (2011). Microbial 2,3-butanediol production: a state-of-the-art review. *Biotechnology Advances*, 29 (3), pp. 351-364.

Jozefiak, D., Rutkowski, A., Kaczmarek, S., Jensen, B. B., Engberg, R. M. and Hojberg, O. (2010). Effect of β-glucanase and xylanase supplementation of barley- and rye-based diets on caecal microbiota of broiler chickens. *British Poultry Science*, 51 (4), pp. 546-557.

Jozefiak, D., Ptak, A., Kaczmarek, S., Mackowiak, P. and Engberg, R. M. (2011). Exogenous supplementation of carbohydrases lowers serum insulin and cholesterol and improves the nutritive value of full-fat rapeseed in chickens. *Journal of Animal and Feed Science*, 20 (1), 107-117.

Kacem, A., Baglinere, J. L. and Meunier, F. J. (2013). Resorption of scales in Atlantic salmon (*Salmo salar*) during its anadromous migration: a quantitative study. *Cybium*, 37 (3), pp. 199-206.

Kasprowicz-Potocka, M., Walachowska, E., Zaworska, A. and Frankiewicz, A. (2013). The assessment of influence of different nitrogen compounds and time on germination of *Lupinus angustifolius* seeds and chemical composition of final products. *Acta Societatis Botanicorum Poloniae*, 82 (3), pp. 199-206.

Kazerani, H. R. and Shahsavani, D. (2011). The effect of supplementation of feed with exogenous enzymes on the growth of common carp (*Cyprinus carpio*). *Iranian Journal of Veterinary Research*, 12 (2), pp. 127-132.

Keen, C. L., Ensunsa, J. L., Watson, M. H., Baly, D. L., Donovan, S. M., Monaco, M. H. and Clegg, M. S. (1999). Nutritional aspects of manganese from experimental studies. *Nuerotoxicology*, 20 (2-3), pp. 213-223.

Khajepour, F. and Hosseini, S. A. (2012). Citric acid improves growth performance and phosphorus digestibility in Beluga (*Huso huso*) fed diets where soybean meal partly replaced fish meal. *Animal Feed Science and Technology*, 171 (1), pp. 68-73.

Khemiss, F., Ahmadi, S., Massoudi, R., Ghoul-Mazgar, S., Safta, S., Moshtaghie, A. A. and Saidane, D. (2009). Effect of in vitro exposure to *Vibrio vulnificus* on hydroelectrolytic transport and structural changes of sea bream (*Sparus aurata* L.) intestine. *Fish Physiology and Biochemistry*, 35 (3), pp. 541-549.

Kim, TW. and Lei, XG. (2005). An improved method for a rapid determination of phytase activity in animal feed. *Journal of Animal Science*, 83 (5), pp. 1062-1067.

Kim, J. C., Pluske, J. R. and Mullan, B. P. (2008). Nutritive value of yellow lupins (*Lupinus luteus* L.) for weaner pigs. *Australian Journal of Experimental Agriculture*, 48 (9), pp. 1225-1231.

Kim, J. C., Mullan, B. P., Nicholls, R. R. and Pluske, J. R. (2011). Effect of Australian sweet lupin (*Lupinus angustifolius* L.) inclusion levels and enzyme supplementation on the performance, carcass composition and meat quality of grower/finisher pigs. *Animal Production Science*, 51 (1), pp. 37-43.

Kim, Y.-R., Kim, E.-Y., Choi, S.-Y., Hossain, M. T., Oh, R., Heo, W.-S., Lee, J. M., Cho, Y. C. and Kong, I.-S. (2012). Effect of a Probiotic Strain, *Enterococcus faecium*, on the Immune Responses of Olive Flounder (*Paralichthys olivaceus*). *Journal of Microbiology and Biotechnology*, 22 (4), pp. 526-529.

King, R. H., Dunshea, F. R., Morrish, L., Eason, P. J., van Barneveld, R. J., Mullan, B. P. and Campbell, R. G. (2000). The energy value of *Lupinus angustifolius* and *Lupinus albus* for growing pigs. *Animal Feed Science and Technology*, 83 (1), pp. 17-30.

Kleman-Leyer, K. M., Siika-Aho, M., Teeri, T. T. and Kirk, T. K. (1996). The cellulases endoglucanase I and cellobiohydrolase II of *Trichoderma reesei* act synergistically to solubilize native cotton cellulose but not to decrease its molecular size. *Applied and Environmental Microbiology*, 62 (8), pp. 2883-2887.

Kleywegt, G. J., Zou, J. Y., Divne, C., Davies, G. J., Sinning, I., Stahlberg, J., Reinikainen, T., Srisodsuk, M., Teeri, T. T. and Jones, T. A. (1997). The crystal structure of the catalytic core domain of endoglucanase I from *Trichoderma reesei* at 3.6 angstrom resolution, and a comparison with related enzymes. *Journal of Molecular Biology*, 272 (3), pp. 383-397.

Knott, B. C., Momeni, M. H., Crowley, M. F., Mackenzie, L. F., Gotz, A. W., Sandgren, M., Withers, S. G., Stahlberg, J. and Beckham, G. T. (2014). Mechanism of cellulose hydrolysis by a two-step, retaining cellobiohydrolase elucidated by structural and transition path sampling studies. *Journal of the American Chemical Society*, 136 (1), pp. 321-329.

Knox, D., Cowey, C. B. and Adron, J. W. (1981). The effect of low dietary manganese intake on rainbow-trout (*Salmo-gairdneri*). *British Journal of Nutrition*, 46 (3), pp. 495-501.

Knox, D., Cowey, C. B. and Adron, J. W. (1983). Studies on the nutrition of rainbow-trout (*Salmo-gairdneri*) magnesium-deficiency – the effect of feeding with a Mg-supplemented diet. *British Journal of Nutrition*, 50 (1), pp. 121-127.

Knudsen, D., Ron, D., Baarsden, G., Smedsgaard, J., Koppe, W. and Frokiaer, H. (2006). Soyasapponins resist extrusion cooking and are not degraded during gut passage in Atlantic salmon (*Salmo salar* L.). *Journal of Agricultural and Food Chemistry*, 54 (17), pp. 6428-6435.

Knudsen, D., Jutfelt, F., Sundh, H., Sundell, K., Koppe, W. And Frokiaer, H. (2008). Dietary soya saponins increase gut permeability and play a key role in the onset of soyabean-induced enteritis in Atlantic salmon (*Salmo salar* L.). *British Journal of Nutrition*, 100 (1), pp. 120-129.

Kortner, T. M., Skugor, S., Penn, M. H., Mydland, L. T., Djordjevic, B., Hillestad, M., Krasnov, A. and Krogdahl, A. (2012). Dietary soyasapponin supplementation to pea protein concentrate reveals nutrigenomic interactions underlying enteropathy in Atlantic salmon (*Salmo salar*). *Bmc Veterinary Research*, 8 (1), pp. 101

Krawczyk, M., Mikulski, D., Przywitowski, M. and Jankowski, J. (2015). The effect of dietary yellow lupine (*L. Luteus* cv. Baryt) on growth performance, carcass characteristics, meat quality and selected serum parameters of turkeys. *Journal of Animal and Feed Sciences*, 24 (1), 61-70.

Krogdahl, A., Sundby, A. and Olli, J. J. (2004). Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) digest and metabolize nutrients differently. Effects of water salinity and dietary starch level. *Aquaculture*, 229 (1-4), pp. 335-360.

Krogdahl, A., Hemre, G. I. and Mommsen, T. P. (2005). Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. *Aquaculture Nutrition*, 11 (2), pp. 103-122.

Krogdahl, A., Penn, M., Thorsen, J., Refstie, S. and Bakke, A. M. (2010). Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. *Aquaculture Research*, 41 (3), pp. 333-344.

Kuhlwein, H., Emery, M. J., Rawling, M. D., Harper, G. M., Merrifield, D. L. and Davies, S. J. (2013). Effects of a dietary β-(1,3)(1,6)-ɒ-glucan supplementation on intestinal microbial communities and intestinal ultrastructure of mirror carp (*Cyprinus carpio* L.). *Journal of Applied Microbiology*, 115 (5), pp. 1091-1106.

Kumar, V., Sinha, A. K., Makkar, H. P. S., De Boeck, G. and Becker, K. (2012). Phytate and phytase in fish nutrition. *Journal of Animal Physiology and Animal Nutrition*, 96 (3), pp. 335-364.

Kutovic, I., Marshall, S. N. and Simpson, B. K. (2006). Isolation and characterization of a trypsin fraction from the pyloric ceca of chinook salmon (*Oncorhynchus tshawytscha*). *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, 143 (4), pp. 432-440.

Laerke, H. N., Arent, S., Dalsgaard, S. and Knudsen, K. E. B. (2015). Effect of xylanases on ileal viscosity, intestinal fiber modification, and apparent ileal fiber and nutrient digestibility of rye and wheat in growing pigs. *Journal of Animal Science*, 93 (9), pp. 4323-4335.

Laining, A., Ishikawa, M., Koshio, S., Lideman and Yokoyama, S. (2012). Dietary inorganic phosphorus or microbial phytase supplementation improves growth, nutrient utilization and phosphorus mineralization of juvenile red sea bream, *Pagrus major*, fed soybean-based diets. *Aquaculture Nutrition*, 18 (5), pp. 502-511.

Lall, S. P. (2002). *The Minerals*. In: J. E. Halver and R. W. Hardy, ed., *Fish Nutrition*, 3rd ed. San Diego, USA: Academic Press, pp. 260-300.

Le Boucher, R., Vandeputte, M., Dupont-Nivet, M., Quillet, E., Ruelle, F. and Vergnet, A. (2013). Genotype by diet interactions in European sea bass (*Dicentrarchus labrax* L.): nutritional challenge with totally plant-based diets. *Journal of Animal Science*, 91 (1), pp. 44–56.

Leeming, A. (2013). *The bioavailability and assimilation of dietary zinc in rainbow trout (Oncorhynchus mykiss).* PhD. University of Plymouth.

Leenhouwers, J. I., Adjei-Boateng, D., Verreth, J. A. J. and Schrama, J. W. (2006). Digesta viscosity, nutrient digestibility and organ weights in African catfish (*Clarias gariepinus*) fed diets supplemented with different levels of a soluble non-starch polysaccharide. *Aquaculture Nutrition*, 12 (2), pp. 111-116.

Leenhouwers, J. I., Ortega, R. C., Verreth, J. A. J. and Schrama, J. W. (2007a). Digesta characteristics in relation to nutrient digestibility and mineral absorption in Nile tilapia (*Oreochromis niloticus* L.) fed cereal grains of increasing viscosity. *Aquaculture*, 273 (4), pp. 556-565.

Leenhouwers, J. I., ter Veld, M., Verreth, J. A. J. and Schrama, J. W. (2007b). Digesta characteristiscs and performance of African catfish (*Clarias gariepinus*) fed cereal grains that differ in viscosity. *Aquaculture*, 264, pp. 330-341.

Le Luyer, J., Deschamps, M. H., Prolux, E., Stewart, N. P., Robert, C. and Vandenberg, G. (2014). Responses of different body compartments to acute dietary phosphorus deficiency in juvenile triploid rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Journal of Applied Ichthyology*, 30 (4), pp. 825-832.

Li, P. and Gatlin, D. M. (2004). Dietary brewers yeast and the prebiotic Grobiotic (TM) AE influence growth performance, immune responses and resistance of hybrid striped

bass (*Morone chrysops* x *M. saxatilis*) to *Streptococcus iniae* infection. *Aquaculture*, 231 (1-4), pp. 445-456.

Li, J., Li, J. and Wu T. (2006). Ontogeny of protease, amylase and lipase in the alimentary tract of hybrid Juvenile tilapia (*Oreochromis niloticus* × *Oreochromis aureus*). *Fish Physiology and Biochemistry*, 32 (4), pp. 295-303.

Li, J. S., Li, J. L. and Wu, T. T. (2009). Effects of non-starch polysaccharides enzyme, phytase and citric acid on activities of endogenous digestive enzymes of tilapia (*Oreochromis niloticus* x *Oreochromis aureus*). *Aquaculture Nutrition*, 15 (4), pp. 415-420.

Li, Y., Chen, X., Chen, Y., Li, Z. And Cao, Y. (2010). Effects of β-mannanase experessed by *Pichia pastroris* in corn-soybean meal diets on broiler performance, nutrient digestibility, energy utilization and immunoglobulin levels. *Animal Feed Science and Technology*, 159 (1), pp. 59-67.

Li, LX., Li, K., Wang, Y., Chen, C., Xu YQ., Zhang, LJ., Han BB., Gao, C., Tao, F., Ma, CQ and Xu, P. (2015). Metabolic engineering of *Enterobacter cloacae* for high-yield production of enantiopure (2R,3R)-2,3-butanediol from lignocellulose-derived sugars. *Metabolic Engineering*, 28 (1), 19-27.

Liao, Y., Li, C.-M., Chen, H., Wu, Q., Shan, Z. and Han, X.-Y. (2013). Site-directed mutagenesis improves the thermostability and catalytic efficiency of *Aspergillus niger* N25 phytase mutated by I44E and T252R. *Applied Biochemistry and Biotechnology*, 171 (4), pp. 900-915.

Liebert, F. and Portz, L. (2005). Nutrient utilization of Nile tilapia *Oreochromis niloticus* fed plant based low phosphorus diets supplemented with graded levels of different sources of microbial phytase. *Aquaculture*, 248 (1-4), pp. 111-119.

Liebert, F. and Portz, L. (2007). Different sources of microbial phytase in plant based low phosphorus diets for Nile tilapia Oreochromis niloticus may provide different effects on phytate degradation. *Aquaculture*, 267 (1-4), pp. 292-299.

Lin, J.-H., Cubi, Y., Hung, S. S. O. and Shiau, S.-Y. (1997). Effect of feeding strategy and carbohydrate source on carbohydrate utilization by white sturgeon (*Acipenser transmontanus*) and hybrid tilapia (*Oreochromis niloticus* X *O. aureus*). *Aquaculture*, 148 (2-3), 201-211.

Lin, S., Mai, K. and Tan, B. (2007). Effects of exogenous enzyme supplementation in diets on growth and feed utilization in tilapia, *Oreochromis niloticus* x *O-aureus*. *Aquaculture Research*, 38 (15), pp. 1645-1653.

Liu, L. W., Su, J. M. and Luo, Y. L. (2012). Effect of partial replacement of dietary monocalcium phosphate with neutral phytase on growth performance and phosphorus digestibility in gibel carp, *Carassius auratus gibelio* (Bloch). *Aquaculture Research*, 43 (9), pp. 1404-1413.

Liu, H., Gong, J., Chabot, D., Miller, S. S., Cui, S. W., Ma, J., Zhong, F. and Wang, Q. (2015). Protection of heat-sensitive probiotic bacteria during spray-drying by sodium caseinate stabilized fat particles. *Food Hydrocolloids*, 51 (1), pp. 459-467.

Lizarazo, C. I., Yli-Halla, M. and Stoddard, F. L. (2015). Pre-crop effects on the nutrient composition and utilization efficiency of faba bean (*Vicia faba* L.) and narrow-leafed lupin (*Lupinus angustifolius* L.). *Nutrient Cycling in Agroecosystems*, 103 (3), 311-327.

Lodemel, J. B., Mayhew, T. M., Myklebust, R., Olsen, R. E., Espelid, S. and Ringo, E. (2001). Effect of three dietary oils on disease susceptibility in Arctic charr (*Salvelinus alpinus* L.) during cohabitant challenge with *Aeromonas salmonicida* ssp *salmonicida*. *Aquaculture Research*, 32 (12), pp. 935-945.

Lozupone, C. A., Hamady, M., Kelley, S. T. and Knight, R. (2007). Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology*, 73 (5), pp. 1576-1585.

Lu, C.-H., and Ku, C.-C. (2013). Effects of shrimp waste meal on growth performance and chitinase activity in juvenile cobia (*Rachycentron canadum*). *Aquaculture Research*, 44 (8), pp. 1190-1195.

Lucas, M. M., Stoddard, F. L., Annicchiarico, P., Frias, J., Martinez-Villaluenga, C., Sussman, D., Duranti, M., Seger, A., Zander, P. M. and Pueyo, J. J. (2015). The future of lupin as a protein crop in Europe. *Frontiers in Plant Science*, 6, pp. 705.

Lucht, J. M. (2015). Public acceptance of plant biotechnology and GM crops. *Viruses-Basel*, 7 (8), pp. 4254-4281.

Lundin, E., Zhang, J. X., Huang, C. B., Reuterving, C. O., Hallmans, G., Nygren, C. and Stenling, R. (1993). Oat bran, rye bran, and soybean hull increase goblet cell-volume density in the small-intestine of the golden-hamster – a histochemical and stereologic light-microscopic study. *Scandanavian Journal of Gastroenterology*, 28 (1), pp. 15-22.

Lupatsch, I., Kissil, G. W., Skalan, D. And Pfeffer, E. (1997). Apparent digestibility coefficients of feed ingredients and their predictability in compound diets for gilthead seabream, *Sparus aurata* L.. *Aquaculture Nutrition*, 3 (1), pp. 81-89.

Lv, J. N., Chen, Y. Q., Guo, X. J., Piao, X. S., Cao, Y. H. and Dong, B. (2013). Effects of supplementation of β-mannanase in corn-soybean meal diets on performance and nutrient digestibility in growing pigs. *Asian-Australasian Journal of Animal Science*, 26 (4), pp. 579-587.

Lyons, P. P., Turnbull, J. F., Dawson, K. A. and Crumlish, M. (2015). Exploring the microbial diversity of the distal intestinal lumen and mucosa of farmed rainbow trout

Oncorhynchus mykiss (Walbaum) using next generation sequencing (NGS). *Aquaculture Research*, doi:10.1111/are.12863.

Magalhaes, F., Aguiar, T. Q., Oliveira, C. and Domingues, L. (2014). High-level expression of *Aspergillus niger* beta-galactosidase in *Ashbya gossypii*. *Biotechnology Progress*, 30 (2), pp. 261-268.

Manikandan, M., Balasubramaniam, R. and Chun, S. C. (2015). A single-step purification of cauliflower lysozyme and its dual role against bacterial and fungal plant pathogens. *Applied Biochemistry and Biotechnology*, 177 (2), pp. 556-566.

Manzanares, P., de Graaff, L. H. and Visser, J. (1998). Characterization of galactosidases from *Aspergillus niger*: purification of a novel alpha-galactosidase activity. *Enzyme and Microbial Technology*, 22 (5), pp. 383-390.

Mathlouthi, N., Lalles, J. P., Lepercq, P., Juste, C. and Larbier, M. (2002). Xylanase and beta-glucanase supplementation improve conjugated bile acid fraction in intestinal contents and increase villus size of small intestine wall in broiler chickens fed a rye-based diet. *Journal of Animal Science*, 80 (11), pp. 2773-2279.

Mayor, D. J. and Solan, M. (2011). Complex interactions mediate the effects of fish farming on benthic chemistry within a region of Scotland. *Environmental Research*, 111 (5), pp. 635-642.

McCullough, J. S., Ratcliffe, B., Mandir, N., Carr, K. E. and Goodlad, R. A. (1998). Dietary fibre and intestinal microflora: effects on intestinal morphometry and crypt branching. *GUT*, 42 (6), pp. 799-806.

McDaniel, A., Fuchs, E., Liu, Y. And Ford, C. (2008). Directed evolution of *Aspergillus niger* glucoamylase to increase thermostability. *Microbial Biotechnology*, 1 (6), pp. 523-531.

Mehri, M., Adibmoradi, M., Samie, A. and Shivazad, M. (2010). Effects of beta-Mannanase on broiler performance, gut morphology and immune system. *African Journal of Biotechnology*, 9 (37), pp. 6221-6228.

Meng, X., Slominski, B. A., Nyachoti, C. M., Campbell, L. D. and Guenter, W. (2005). Degradation of cell wall polysaccharides by combinations of carbohydrase enzymes and their effect on nutrient utilization and broiler chicken performance. *Poultry science*, 84 (1), pp. 37-47.

Menghi, G., Accili, D., Bondi, A. M. and Gabriella, M. G. (1989). Enzymatic degradation and quantitative lectin labelling for characterizing gycoconjugates which act as lectin acceptors in cat submandibular-gland. *Histochemistry*, 90 (5), pp. 331-338.

Merrifield, D. L., Dimitroglou, A., Foey, A., Davies, S. J., Baker, R. T. M., Bogwald, J., Castex, M. and Ringo, E. (2010). The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture*, 302 (1-2), pp. 1-18.

Michael, I. and Ely, M. (2011). Effect of cellulose structure on enzymatic hydrolysis. *BioResources*, 6 (3), 2818.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herraez, M. P. and Carillo, M. (2013). Gamete quality and broodstock management in temperate fish. *Reviews in Aquaculture*, 5 (1), pp. 5194-5223.

Mogilevskaya, E. L., Akopova, T. A., Zelenetskii, A. N. and Ozerin, A. N. (2006). The crystal structure of chitin and chitosan. *Polymer Science Series A*, 48 (2), PP. 116-123.

Mohammadbeygi, M., Imanpour, M. R., Taghizadeh, V. and Shabani, A. (2012). Endo beta 1-3(4) glucanase supplementation of barley based diet and its effects on some hematological parameteres of common carp (*Cyprinus carpio*). *African Journal of Agricultural Research*, 7 (49), pp. 6624-6629.

Moharrery, A. (2006). Comparison of performance and digestibility characteristics of broilers fed diets containing treated hulled barley or hulless barley. *Czech Journal of Animal Science*, 51 (3), pp. 122-131.

Morales, G. A. and Moyano, F. J. (2010). Application of an in vitro gastrointestinal model to evaluate nitrogen and phosphorus bioaccessibility and bioavailability in fish feed ingredients. *Aquaculture*, 306 (1-4), pp. 244-251.

Morales, G. A., Moyano, F. J. and Marquez, L. (2011). In vitro assessment of the effects of phytate and phytase on nitrogen and phosphorus bioaccessibility within fish digestive tract. *Animal Feed Science and Technology*, 170 (3-4), pp. 209-221.

Morita, Y., Hasan, Q., Sakaguchi, T., Murakami, Y., Yokoyama, K. and Tamiya, E. (1998). Properties of a cold-active protease from psychrotrophic *Flavobacterium balustinum* P104. *Applied Microbiology and Biotechnology*, 50 (6), pp. 669-675.

Mosse, J. (1990). Nitrogen to protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content. *Journal of the Science of Food and Agriculture*, 38 (1), pp. 18-24.

Moura, G. D., Lanna, E. A. T., Filer, K., Falkoski, D. L., Donzele, J. L., Oliveira, M. G. D. and de Rezende, S. T. (2012). Effects of enzyme complex SSF (solid state fermentation) in pellet diets for Nile tilapia. *Revista Brasileira de Zootecnia*, 41 (10), pp. 2139-2143.

Mudd, G. M. (2010). The environmental sustainability of mining in Australia: key mega-trends and looming constraints. *Resources Policy*, 35 (2), pp. 98-115.

Mullaney, E. J., Daly, C. B. and Ullah, A. H. J. (2000). Advances in phytase research. *Advances in Applied Microbiology,* 47, pp. 157-199.

Ng, W. K., Lim, H. A., Lim, S. l. and Ibrahim, C. O. (2002). Nutritive value of palm kernel meal pretreated with enzyme or fermented with *Trichoderma koningii* (Oudemans)

as a dietary ingredient for red hybrid tilapia (*Oreochromis* sp.). *Aquaculture Research*, 33 (15), pp. 1199-1207.

Nijiru, M., Okeyo-Owuor, J. B., Muchiri, M. and Cowx, I. G. (2004) Shifts in the food of Nile tilapia, *Oreochromis niloticus* (L.) in Lake Victoria, Kenya. *African Journal of Ecology*, 42 (3), pp. 163-170.

Nofima (2014). *Resource utilisation of Norwegian salmon farming in 2012 and 2013*. [online] Available at: http://nofima.no/wp-content/uploads/2014/11/Nofima_report_resource_utilisation_Oct_2014.pdf (Accessed 7 March 2016).

Nordvik, K., Kryvi, H., Totland, G. K. and Grotmol, S. (2005). The salmon vertebral body develops through mineralization of two preformed tissues that are encompassed by two layers of bone. *Journal of Anatomy*, 206 (2), pp. 103-114.

NRC (1998). *Nutritional Requirements of Swine*. Washington, USA: National Academies Press.

NRC (2011). *Nutritional Requirements of Fish*. Washington, USA: National Academies Press.

Nunes, A. J. P., Sa, M. V. C., Browdy, C. L. and Vazquez-Anon, M. (2014). Practical supplementation of shrimp and fish feeds with crystalline amino acids. *Aquaculture*, 431 (1), pp. 20-27.

Nya, E. J. and Austin, B. (2011). Dietary modulation of digestive enzymes by the administration of feed additives to rainbow trout, *Oncorhynchus mykiss* Walbaum. *Aquaculture Nutrition*, 17 (2), E459-E466.

O'Connell, J. M., Callan, J. J. and O'Doherty, J. V. (2006). The effect of dietary crude protein level, cereal type and exogenous enzyme supplementation on nutrient

digestibility, nitrogen excretion, faecal volatile fatty acid concentration and ammonia emissions from pigs. *Animal Feed Science and Technology*, 127 (1-2), pp. 73-88.

Odetallah, N. H., Parks, C. W. and Ferket, P. R. (2002). Effect of wheat enzyme preparation on the performance characteristics of Tom turkeys fed wheat- based rations. *Poultry science*, 81 (7), pp. 987-994.

Ogunkoya, A. E., Page, G. I., Adewolu, M. A. and Bureau, D. P. (2006). Dietary incorporation of soybean meal and exogenous enzyme cocktail can affect physical characteristics of faecal material egested by rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 254 (1-4), 466-475.

Ogura, T., Hernandez, A., Aizawa, T., Ogihara, J., Sunairi, M., Alcaino, J., Salvo-Garrido, H. and Maureira-Butler, I. J. (2013). Identification of a low digestibility delta-conglutin in yellow lupin (*Lupinus luteus* L.) seed meal for Atlantic salmon (*Salmo salar* L.) by coupling 2D-PAGE and mass spectroscopy. *PLoS One*, 8 (11), e80369.

Olkowski, B., Janiuk, I. and Jakubczak, A. (2010). Effect of enzyme preparation with activity directed towards degradation of non-starch polysaccharides on yellow lupine seed based diet for young broilers. *Acta Veterinaria Bmo*, 79 (2), pp. 395-402.

Olkowski B. (2011). Lupin as primary protein source in young broiler chicken diets: Effect of enzymes preparations catalyzing degradation of non-starch polysaccharides or phytates. *World Journal of Microbiology & Biotechnology*, 27 (2), pp. 341-347.

Omnes, M. H., Silva, F. C. P., Moriceau, J., Aguirre, P., Kaushik, S. and Gatesoupe, F. J. (2015). Influence of lupin and rapeseed meals on the integrity of digestive tract and organs in gilthead seabream (*Sparus aurata* L.) and goldfish (*Carassius auratus* L.) juveniles. *Aquaculture Nutrition*, 21 (2), pp. 223-233.

Overturf, K., Barrows, F. T. and Hardy, R. W. (2013). Effect and interaction of rainbow trout strain (*Oncorhynchus mykiss*) and diet type on growth and nutrient retention. *Aquaculture Research*, 44 (4), pp. 604-611.

Owen, M. A. G., Eynon, B., Woodgate, S., Davies, S. J. and Fox, S. (2012). Increased water current induces micro-architectural changes to the vertebral bone of juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 344 (1-4), pp. 141-146.

Owens, B., Tucker, L., Collins, M. A. and McCracken, K. J. (2008). Effects of different feed additives alone or in combination on broiler performance, gut microflora and ileal histology. *British Poultry Science*, 49 (2), pp. 202-212.

Pangburn, S. H., Trescony, P. V. and Heller, J. (1982). Lysozyme degradation of partially deacetylated chitin, its films and hydrogels. *Biomaterials*, 3 (2), pp. 105-108.

Pascoal, L. A. F., Thomaz, M. C., Watanabe, P. H., Ruiz, U. d.-S., Amorim, A. B., Everton, D. and da Silva, S. Z. (2015). Purified cellulose, soybean hulls and citrus pulp as a source of fiber for weaned piglets. *Scientia Agricola*, 72 (5), pp. 400-410.

Passos, A. A., Andrade, C., Phillips, C. E., Coffey, M. T. and Kim, S. W. (2015). Nutrient value of spray field forages fed to pigs and the use of feed enzymes to enhance nutrient digestibility. *Journal of Animal Science*, 93 (4), pp. 1721-1728.

Peng, M., Xu, W., Ai, Q. and Zhang, K. (2013). Effects of nucleotide supplementation on growth, immune responses and intestinal morphology in juvenile turbot fed diets with graded levels of soybean meal (*Scopthalmus maximus* L.). *Aquaculture*, 392-395, pp. 51-58.

Perez-Jimenez, A., Abelian, E., Arzicun, M., Cardenete, G., Morales, A. E. and Hidalgo, M. C. (2015). Nutritional and metabolic responses in common dentex (*Dentex dentex*) fed on different types and levels of carbohydrates. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, 184 (1), pp. 56-64.

Pfeider, G. (1970). *Methods in Enzymology*. XIX, pp. 514-521.

Pisarikova, B. and Zraly, Z. (2009). Nutritional value of lupine in diets for pigs (a review). *Acta Veterinaria Brno*, 78 (1), pp. 399-409.

Polakof, S., Panserat, S., Soengas, J. L. And Moon, T. W. (2012). Glucose metabolism in fish: a review. *Journal of Comparative Physiology. B, Biochemical, Systemic and Environmental Physiology*, 182 (8), pp. 1015-1045.

Pontoppidan, K., Glitsoe, V., Guggenbuhl, P., Quintana, A. P., Nunes, C. S., Pettersson, D. and Sandberg, A. S. (2012). In vitro and in vivo degradation of myo-inositol hexakisphosphate by a phytase from Citrobacter Braakii. *Archives of Animal Nutrition*, 66 (6), pp. 431-444.

Porres, J. M., Aranda, P., Lopez-Jurado, M. and Urbano, G. (2007). Nitrogen fractions and mineral content in different lupin species (*Lupinus albus*, *Lupinus angustifolius*, and *Lupinus luteus*). changes induced by the alpha-galactoside extraction process. *Journal of Agricultural and Food Chemistry*, 55 (18), pp. 7445-7452.

Potter, S. M., Jimenezflores, R., Pollack, J. A., Lone, T. A. and Berberjimenez, M. D. (1993). Protein saponin interaction and its influence on blood-lipids. *Journal of Agricultural and Food Chemistry*, 41 (8), pp. 1287-1291.

Prabhu, A. J. P., Geurden, I., Fontagne-Dicharry, S., Veron, V., Larroquet, L., Mariojoulis, C., Schrama, J. W. and Kaushik, S. J. (2016). Responses in micro-mineral metabolism in rainbow trout to change in dietary ingredient composition and inclusion of a micro-mineral premix. *PLoS One*, 11 (2), e0149378.

Premier Nutrition (2005). *Ingredients Matrix*. Rugeley, UK: Premier Nutrition Products Ltd.

Quinton, C. D., Kause, A., Ruohonen, K. and Koskela, J. (2007a). Genetic relationships of body composition and feed utilization traits in European whitefish (*Coregonus*

lavaretus L.) and implications for selective breeding in fishmeal- and soybean meal-based diet environments. *Journal of Animal Science*, 85 (12), pp. 3198-3208.

Quinton, C. D., Kause, A., Koskela, J. and Ritola, O. (2007b). Breeding salmonids for feed efficiency in current fishmeal and future plant-based diet environments. *Genetics Selection Evolution*, 39 (4), pp. 431-446.

Rawling, M. D., Merrifield, D. L., Snellgrove, D. L., Kuehlwein, H., Adams, A. and Davies, S. J, (2012). Haemato-immunological and growth response of mirror carp (*Cyprinus carpio*) fed a tropical earthworm meal in experimental diets. *Fish and Shellfish Immunology*, 32 (6), pp. 1002-1007.

Ray, A. K., Roy, T., Mondal, S. and Ringo, E. (2010). Identification of gut-associated amylase, cellulase and protease-producing bacteria in three species of Indian major carps. *Aquaculture Research*, 41 (10), pp. 1462-1469.

Read, E. S., Barrows, F. T., Gaylord, T. G., Paterson, J., Petersen, M. K. and Sealey, W. M. (2014). Investigation of the effects of dietary protein source on copper and zinc bioavailability in fishmeal and plant-based diets for rainbow trout. *Aquaculture*, 432 (1-4), pp. 97-105.

Refstie, S., Svihus, B., Shearer, K. D. and Storebakken, T. (1999). Nutrient digestibility in Atlantic salmon and broiler chickens related to viscosity and non-starch polysaccharide content in different soyabean products. *Animal Feed Science and Technology*, 79 (4), pp. 331-345.

Refstie, S., Glencross, B., Landsverk, T., Sorensen, M., Lilleeng, E., Hawkins, W. and Krogdahl, A. (2006). Digestive function and intestinal integrity in Atlantic salmon (*Salmo salar*) fed kernel meals and protein concentrates made from yellow or narrow-leafed lupins. *Aquaculture*, 261 (4), pp. 1382-1395.

Ren, M., Habte-Tsion, H.-M., Liu, B., Miao, L., Ge, X., Xie, J., Liang, H., Zhou, Q. and Pan, L. (2015). Dietary leucine level affects growth performance, whole body

composition, plasma parameters and relative expression of TOR and TNF-alpha in juvenile blunt snout bream, *Megalobrama amblycephala. Aquaculture*, 448 (1-4), pp. 162-168.

Richardson, N. L., Higgs, D. A., Beames, R. M. and McBride, J. R. (1985). Influence of dietary calcium, phosphorus, zinc and sodium phytate level on cataract incidence, growth and histopathology in juvenile Chinook salmon (*Oncorhynchus-tshawytscha*). *Journal of Nutrition*, 115 (5), pp. 553-567.

Riche, M. and Brown, P. B. (1996). Availability of phosphorus from feedstuffs fed to rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, 142 (3-4), pp. 269-282.

Roberts, R. J., Hardy, R. W. and Sugiura, S. H. (2001). Screamer disease in Atlantic salmon, *Salmo salar* L., in Chile. *Journal of Fish Diseases*, 24 (9), pp. 543-549.

Rodehutscord, M., Becker, A. and Pfeffer, E. (1995). Effect of a supplemental Aspergillus niger-phytase on the utilization of phosphorus of plant-origin by rainbow-trout (*Oncorhynchus mykiss*). *Archives of Animal Nutrition – Archiv fur Tierernahrung*, 48 (1-2), pp. 211-219.

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Colleen, M. C., Guillemin, K. and Rawls, J. F. (2011). Evidence for a core gut microbiota in the zebrafish. *The ISME Journal*, 5 (1), pp. 1595-1608.

Rust, M. B. (2002). *Nutritional physiology*. In: J. E. Halver and R. W. Hardy, ed., *Fish Nutrition*, 3rd ed. San Diego, USA: Academic Press, pp. 260-300.

Rutkowski, A., Kaczmarek, S. A., Mehdysz, M., Nowaczewski, S. and Jamroz, D. (2015). Concentrates made from legume seeds (*Lupinus angustifolius, Lupinus luteus and Pisum sativum*) and rapeseed meal as protein sources in laying hen diets. *Annals of Animal Science*, 15 (1), pp. 129-142.

Saha, S., Roy, R. N., Sen, S. K. and Ray, A. K. (2006). Characterization of cellulase-producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (Peters) and grass carp, *Ctenopharyngodon idella* (Valenciennes). *Aquaculture Research*, 37 (3), pp. 380-388.

Sahin, T., Kaya, I., Unal, Y., Elmali, D. K. and Atakisi, E. (2007). Effects of enzyme supplementation wheat and barley based quail diets on growth, carcass quality and some blood parameters. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 13 (2), 115-120.

Salini, M. J. and Adams, L. R. (2014). Growth performance, nutrient utilisation and digestibility by Atlantic salmon (*Salmo salar* L.) fed Tasmanian grown white (*Lupinus albus*) and narrow- leafed (*L. angustifolius*) lupins. *Aquaculture*, 426 (1-4), pp. 296-303.

Santigosa, E., Sanchez, J., Medale, F., Kaushik, S., Perez-Sanchez, J. and Gallardo, M. A. (2008). Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. *Aquaculture*, 282 (1-4), 68-74.

Santos, F., Wegkamp, A., de Vos, W. M., Smid, E. J. and Hugenholtz, J. (2008). High-level folate production in fermented foods by the B12 producer *Lactobacillus reuteri* JCM1112. *Applied and Environmental Microbiology,* 74 (10), pp. 3291–3294.

Sashiwa, H. Saimoto, H. Shigemasa, Y. Ogawa, R. and Tokura, S. (1990). Lysozyme susceptibility of partially deacetylated chitin. *International Journal of Biological Macromolecules*, 12 (5), pp. 295-296.

Satoh, S., Porn-Ngam, N., Takeuchi, T. & Watanabe, T. (1993). Effect of various types of phosphates on zinc availability to rainbow trout. *Nippon Suisan Gakkaishi*, 59 (1), pp. 1395-1400.

Satoh, S., Yamamoto, T., Takeuchi, T. and Watanabe, T. (1983). Effects on growth and mineral-composition of rainbow-trout of deletion of trace-elements or magnesium from

fish-meal diet. *Bulletin of the Japanese Society of Scientific Fisheries*, 49 (3), pp. 425-429.

Saurabh, S. and Sahoo, P. K. (2008). Lysozyme: an important defence molecule of fish innate immune system. *Aquaculture Research*, 39 (3), pp. 223-239.

Schiarea, S., Arnold, L. F. R., De Combarieu, E. and Chiabrando, C. (2013). In-depth glycoproteomic characterization of γ-conglutin by high-resolution accurate mass spectrometry. *PLoS One*, 8 (9), e73906.

SchmidtWittig, U., Enss, M. L., Coenen, M., Gartner, K. and Hedrich, H. J. (1996). Response of rat colonic mucosa to high fiber diet. *Annals of Nutrition and Metabolism*, 40 (6), pp. 343-350.

Selle, P. H. and Ravindran, V. (2007). Microbial phytase in poultry nutrition. *Animal Feed Science and Technology*, 135 (1-2), pp. 1-41.

Seo, H.-J., Cho, Y.-E., Kim, T., Shin, H.-I. and Kwun, I.-S. (2010). Zinc may increase bone formation through stimulating cell proliferation, alkaline phosphatase activity and collagen synthesis in osteoblastic MC3T3-E1 cells. *Nutrition Research and Practice*, 4 (5), pp. 356-361.

Serrano, E., Storebakken, T., Penn, M., Overland, M., Hansen, J. O. and Mydland, L. T. (2011). Responses in rainbow trout (*Oncorhynchus mykiss*) to increasing dietary doses of lupinine, the main quinolizidine alkaloid found in yellow lupins (*Lupinus luteus*). *Aquaculture*, 318 (1), pp. 122-127.

Serrano, E., Storebakken, T., Borquez, A., Penn, M., Shearer, K. D., Dantagnan, P. and Mydland, L. T. (2012). Histology and growth performance in rainbow trout (*Oncorhynchus mykiss*) in response to increasing dietary concentration of sparteine, a common alkaloid in lupins. *Aquaculture Nutrition*, 18 (2), pp. 313-320.

Shahrulzaman, S. and Muhamad, I. I. (2015). Microencapsulation of alginate-immobilized bagasse with *Lactobacillus rhamnosus* NRRL 442: enhancement of survivability and thermotolerance. *Carbohydrate Polymers*, 119 (1), pp. 173-181.

Shaibani, M. E., Amiri, B. M. and Khodabandeh, S. (2013). Starvation and refeeding effects on pyloric caeca structure of Caspian salmon (*Salmo trutta caspius*, Kessler 1877) juvenile. *Tissue Cell*, 45 (3), pp. 204-210.

Shiau, S. Y., Kwok, C. C., Chen, C. J., Tong, H. T. and Hsieh, H. B. (1989). Effects of dietary fibre on the intestinal absorption of dextrin, blood sugar level and growth of tilapia, *Oreochromis niloticm* x *O. aureus*. *Journal of Fish Biology*, 34 (6), pp. 929-935.

Shiau, S. Y. (1997). Utilization of carbohydrates in warmwater fish – with particular reference to tilapia, *Oreochromis niloticus* X *O. aureus*. *Aquaculture*, 151 (1-4), pp. 79-96.

Sileikiene, V., Diebold, G., Tafaj, M. and Mosenthin, R. (2006). Effects of supplementation of xylanase, phospholipase or combination of both to a wheat based diet on digestive function in early-weaned piglets. *Journal of Animal and Feed Sciences*, 15 (1), pp. 47-55.

Sinha, A. K., Kumar, V., Makkar, H. P. S., De Boeck, G. and Becker, K. (2011). Non-starch polysaccharides and their role in fish nutrition - a review. *Food Chemistry*, 127 (4), pp. 1409-1426.

Sipsas, S. (2003). *Protein concentrates and isolates*. Grains in Aquaculture Feeds - 3 May 2003. Fisheries Occasional Publications, Department of Fisheries, Western Australia, pp 7.

Skalli, A., Castillo, M., Andree, K. B., Tort, L., Furones, D. and Gisbert, E. (2013). The LPS derived from the cell walls of the Gram-negative bacteria *Pantoea agglomerans*

stimulates growth and immune status of rainbow trout (*Oncorhynchus mykiss*) juveniles. *Aquaculture*, 416 (1-4), pp. 272-279.

Skiba-Cassy, S., Panserat, S., Larquier, M., Dias, K., Surget, A., Plagnes-Juan, E., Kaushik, S. and Seliez, I. (2013). Apparent low ability of liver and muscle to adapt to variation of dietary carbohydrate:protein ratio in rainbow trout (*Oncorhynchus mykiss*). *British Journal of Nutrition*, 109 (8), pp. 1359-1372.

Slominski, B. A, Davie, T., Nyachoti, M. C. and Jones, O. (2007). Heat stability of endogenous and microbial phytase during feed pelleting. *Livestock Science*, 109 (1-3), 244-246.

Slominski, B. A. (2011). Recent advances in research on enzymes for poultry diets. *Poultry Science*, 90 (9), pp. 2013-2023.

Smith, L. S. (2002). In: J. E. Halver, ed., *Fish Nutrition*, 2nd ed. San Diego, USA: Academic Press, pp. 332.

Smith, D. M., Tabrett, S. J. and Glencross, B. D. (2007). Growth response of the black tiger shrimp, *Penaeus monodon* fed diets containing different lupin cultivars. *Aquaculture*, 269 (1-4), pp. 436-446.

Smith, A. G., Reilly, P., Sweeney, T., Pierce, K. M., Gahan, D. A., Callan, J. J. and O'Doherty, J. V. (2010). The effect of cereal type and exogenous enzyme supplementation on intestinal microbiota and nutrient digestibility in finisher pigs. *Livestock Science*, 133 (1-3), pp. 148-150.

Smulikowska, S., Konieczka, P., Czerwinski, J., Mieczkowska, A. and Jankowiak, J. (2014). Feeding broiler chickens with practical diets containing lupin seeds (*L. angustifolius* or *L. luteus*): effects of incorporation level and mannanase supplementation on growth performance, digesta viscosity, microbial fermentation and gut morphology. *Journal of Animal and Feed Sciences*, 23 (1), pp. 64-72.

Snow, M., Raynard, R. S. and Bruno, D. W. (2001). Comparative susceptibility of Arctic char (*Salvelinus alpinus*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) to the Scottish isolate of infectious salmon anaemia virus. *Aquaculture*, 196 (1-2), pp. 47-54.

Sone, Y., Makino, C. and Misaki, A. (1992). Inhibitory effect of oligosaccharides derived from plant xyloglucan on intestinal glucose-absorption in rat. *Journal of Nutritional Science and Vitaminology*, 38 (4), pp. 391-395.

Sorensen, M., Penn, M., El-Mowafi, A., Storebakken, T., Cai, C., Overland, M. and Krogdahl, A. (2011). Effect of stachyose, raffinose and soya-saponins supplementation on nutrient digestibility, digestive enzymes, gut morphology and growth performance in Atlantic salmon (*Salmo salar*, L). *Aquaculture*, 314 (1-4), pp. 145-152.

Soya UK (2015). *Lupins in UK agriculture and aquaculture* (LUKAA). Shropshire, UK [20 Jan 2015].

Sriprang, R., Asano, K., Gobsuk, J., Tanapongpipat, S., Champreda, V. and Eurwilaichitr, L. (2006). Improvement of thermostability of fungal xylanase using site-directed mutagenesis. *Journal of Biotechnology*, 126 (4), 454-462.

Standen, B. T., Rodiles, A., Peggs, D. L., Davies, S. J., Santos, G. A. and Merrifield, D. L. (2015). Modulation of the intestinal microbiota and morphology of tilapia, Oreochromis niloticus, following the application of a multi-species probiotic. *Applied Microbiology and Biotechnology*, 99 (20), pp. 8403-8417.

Stellwag, E. J., Smith, T. D. and Luczkovich, J. J. (1995). Characterization and ecology of carboxymethylcellulase-producing anaerobic bacterial communities associated with the intestinal tract of the pinfish, *Lagodon rhomboides*. *Applied and Environmental Microbiology*, 61 (2), pp. 813-816.

Stone, D. A. J. (2003a). Dietary Carbohydrate Utilization by Fish. *Reviews in Fisheries Science*, 11 (4), pp. 337-369.

Stone, D. A. J., Allan, G. L. and Anderson, A. J. (2003b). Carbohydrate utilization by juvenile silver perch, *Bidyanus bidyanus* (Mitchell). IV. Can dietary enzymes increase digestible energy from wheat starch, wheat and dehulled lupin? *Aquaculture Research*, 34 (2), 135-147.

Storebakken, T., Shearer, K. D. and Roem, A. J. (1998). Availability of protein, phosphorus and other elements in fish meal, soy-protein concentrate and phytase-treated soy-protein-concentrate-based diets to Atlantic salmon, *Salmo salar*. *Aquaculture*, 161 (1-4), 365-379.

Strause, L. and Saltman, P. (1987). Role of manganese in bone metabolism. *Acs Symposium Series*, 354 (1), pp. 46-55.

Sugiura, S. H., Gabaudan, J., Dong, F. M. and Hardy, R. W. (2001). Dietary microbial phytase supplementation and the utilization of phosphorus, trace minerals and protein by rainbow trout *Oncorhynchus mykiss* (Walbaum) fed soybean meal-based diets. *Aquaculture Research*, 32 (7), pp. 583-592.

Sulivan, M., Hammond, G., Roberts, R. J. and Manchester, N. J. (2007a). Spinal deformation in commercially cultured Atlantic salmon, *Salmo salar* L.: a clinical and radiological study. *Journal of Fish Diseases*, 30 (12), pp. 745-752.

Sulivan, M., Reid, S. W. J., Terment, H., Manchester, N. J., Roberts, R. J., Stone, D. A. J. and Hardy, R. W. (2007b). The aetiology of spinal deformity in Atlantic salmon, *Salmo salar* L.: influence of different commercial diets on the incidence and severity of the preclinical condition in salmon parr under two contrasting husbandry regimes. *Journal of Fish Diseases*, 30 (12), pp. 759-767.

Sundu, B., Kumar, A. and Dingle, J. (2006). Palm kernel meal in broiler diets: effect on chicken performance and health. *Worlds Poultry Science Journal*, 62 (2), pp. 316-325.

Tachibana, L., Pinto, L. G. Q., Goncalves, G. S. and Pezzato, L. E. (2010). Xylanase and beta-glucanase on nutrient aparent digestibility of triticale by Nile tilapia. *Arquivo Brasileiro de Medicina Vetinaria e Zootecnia*, 62 (2), pp. 445-452.

Tacon, A. G. J. and Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture*, 285 (1-4), pp. 146-158.

Tahoun, A.-A. M., Mabroke, R. S., El-Haroun, E. R. and Suloma, A. (2011). Effect of exogenous enzyme supplementation on reproductive performance of broodstock Nile tilapia reared in a hapa-in-pond hatchery system. *Egyptian Journal of Aquatic Biology and Fish*, 15 (3), pp. 61-73.

Talpur, A. D., Munir, M. B., Mary, A. and Hashim, R. (2014). Dietary probiotics and prebiotics improved food acceptability, growth performance, haematology and immunological parameters and disease resistance against *Aeromonas hydrophila* in snakehead (*Channa striata*) fingerlings. *Aquaculture*, 426 (1-4), pp. 14-20.

Tang, Q., Wang, C., Xie, C., Jin, J. and Huang, Y. (2012). Dietary available phosphorus affected growth performance, body composition, and hepatic antioxidant property of juvenile yellow catfish *Pelteobagrus fulvidraco*. *The Scientific World Journal*, 2012: 987570.

Tibbetts, S. M., Milley, J. E. and Lall, S. P. (2014). Chemical composition and nutritional properties of freshwater and marine microalgal biomass cultured in photobioreactors. *Journal of Applied Phycology*, 27 (3), pp. 1109-1116.

Timmons, J. R., Angel, R., Harter-Dennis, J. M., Saylor, W. W. and Ward, N. E. (2008). Evaluation of heat-stable phytases in pelleted diets fed to broilers from day zero to thirty-five during the summer months. *Journal of Applied Poultry Research*, 17 (4), pp. 482-489.

UK Governement (2015). Latest animal feed production statistics (GB animal feed – dataset). [online] Available at: https://www.gov.uk/government/statistics/animal-feed-production (Accessed 7 March 2016).

Ullah, A. H. J., Sethumadhavan, K. and Mullaney, E. J. (2008). Unfolding and refolding of *Aspergillus niger* PhyB phytase: role of disulfide bridges. *Journal of Agricultural and Food Chemistry*, 56 (17), pp. 8179-8183.

United Nations (2015). *World population prospects – The 2015 revision*. [online] Available at: http://esa.un.org/unpd/wpp/publications/files/key_findings_wpp_2015.pdf (Accessed 7 March 2016).

Uscanga, A., Moyano, F. J. and Alvarez, C. A. (2010). Assessment of enzymatic efficiency on protein digestion in the tilapia *Oreochromis niloticus*. *Fish Physiology and Biochemistry*, 36 (4), pp. 1079-1085.

van Barneveld, R. J. (1999). Understanding the nutritional chemistry of lupin (Lupinus spp.) seeds to improve livestock production efficiency. *Nutrition Research Reviews*, 12 (2), pp. 203-230.

Vandenberg, G. W., Scott, S. L., Sarker, P. K., Dallaire, V. and de la Nouee, J. (2011). Encapsulation of microbial phytase: Effects on phosphorus bioavailability in rainbow trout (*Oncorhynchus mykiss*). *Animal Feed Science and Technology*, 169 (3-4), pp. 230-243.

Vandenberg, G. W., Scott, S. L. and de la Noue, J. (2012). Factors affecting nutrient digestibility in rainbow trout (Oncorhynchus mykiss) fed a plant protein-based diet supplemented with microbial phytase. *Aquaculture Nutrition*, 18 (4), pp. 369-379.

van Zyl, W. H., Rose, S. H., Trollope, K. and Görgens, J. F. (2010). Fungal β-mannanases: Mannan hydrolysis, heterologous production and biotechnological applications. *Process Biochemistry*, 45 (8), pp. 1203-1213.

Venglovska, K., Gresakova, L., Placha, I., Ryzner, M. and Cobanova, K. (2014). Effects of feed supplementation with manganese from its different sources on performance and egg parameters of laying hens. *Czech Journal of Animal Science*, 59 (4), pp. 147-155.

Verlhac-Trichet, V., Vielma, J., Dias, J., Rema, P., Santigosa, E., Wahli, T. And Vogel, K. (2014). The efficacy of a novel microbial 6-phytase expressed in *Aspergillus oryzae* on the performance and phosphorus utilization of cold- and warm-water fish: rainbow trout, *Oncorhynchus mykiss*, and Nile tilapia, *Oreochromis niloticus*. *Journal of the World Aquaculture Society*, 45 (4), pp. 367-379.

Vielma, J. and Lall, S. P. (1998). Phosphorus utilization by Atlantic salmon (*Salmo salar*) reared in freshwater is not influenced by higher dietary calcium intake. *Aquaculture*, 160 (1-2), pp. 117-128.

Viviers, S. and Boudler, J. M. (2010). Sustainability reporting in the mining sector: identifying critical issues. *Tydskrif Vir Geesteswetenskappe*, 50 (1), pp. 66-86.

Vizcaino, A. J., Lopez, G., Saez, M. I., Jimenez, J. A., Barros, A., Hidalgo, L., Camacho, Rodriguez, J., Martinez, T. F., Ceron-Garcia, M. C. and Alarcon, F. J. (2014). Effects of the microalga *Scenedesmus almeriensis* as fishmeal alternative in diets for gilthead sea bream, *Sparus aurata*, juveniles. *Aquaculture*, 413 (1-4), pp. 34-43.

Walk, C. L., Santos, T. T. and Bedford, M. D. (2014). Influence of superdoses of a novel microbial phytase on growth performance, tibia ash, and gizzard phytate and inositol in young broilers. *Poultry Science*, 93 (5), pp. 1172-1177.

Wang, Y., Fuchs, E., da Silva, R., McDaniel, A., Seibel, J. and Ford, C. (2006). Improvement of *Aspergillus niger* glucoamylase thermostability by directed evolution. *Starch – Starke*, 58 (10), pp. 501-508.

Wang, Q., Garrity, G. M., Tiedje, J. M. and Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, 73 (16), pp. 5261-5267.

Wang, J. P., Hong, S. M., Yan, L., Yoo, J. S., Lee, J. H., Jang, H. D., Kim, H. J. and Kim, I. H. (2009). Effects of single or carbohydrase cocktail in low-nutrient-density diets on growth performance, nutrient digestibility, blood characteristics, and carcass traits in growing-finishing pigs. *Livestock Science*, 126 (1-3), pp. 215-220.

Wang, S.-Y., Ho, Y.-F., Chen, Y.-P. and Chen, M.-J. (2015). Effects of a novel encapsulating technique on the temperature tolerance and anti-colitis activity of the probiotic bacterium *Lactobacillus kefiranofaciens* M1. *Food Microbiology*, 46 (1), pp. 494-500.

Watanabe, T., Kiron, V. and Satoh, S. (1997). Trace minerals in fish nutrition. *Aquaculture*, 151 (1-4), pp. 185-207.

Watson, A. M., Barrows, F. T. and Place, A. R. (2015). Leaching of taurine from commercial type aquaculture feeds. *Aquaculture Research*, 46 (6), pp. 1510-1517.

Widyaratne, G. P., Patience, J. F. and Zijlstra, R. T. (2009). Effect of xylanase supplementation of diets containing wheat distiller's dried grains with solubles on energy, amino acid and phosphorus digestibility and growth performance of grower-finisher pigs. *Canadian Journal of Animal Science*, 89 (4), pp. 91-95.

Wilson, R. P. (2002). *Amino acids and proteins*. In: J. E. Halver and R. W. Hardy, ed., *Fish Nutrition*, 3rd ed. San Diego, USA: Academic Press, pp. 144-175.

Witten, P. E. and Hall, B. K. (2003). Seasonal changes in the lower jaw skeleton in male Atlantic salmon (*Salmo salar* L.): remodelling and regression of the kype after spawning. *Journal of Anatomy*, 203 (5), pp. 435-450.

Wolf, B. (2010). Polysaccharide functionality through extrusion processing. *Current Opinion in Colloid & Interface Science*, 15 (1-2), pp. 50-54.

Woods, C. H., Hooper, D. N., Ooi, E. H., Tan, L.-W. and Carney, A. S. (2012) Fungicidal activity of lysozyme is inhibited in vitro by commercial sinus irrigation solutions. *American Journal of Rhinology & Allergy*, 26 (4), pp. 298-301.

Woyengo, T. A., Slominski, B. A. and Jones, R. O. (2010). Growth performance and nutrient utilization of broiler chickens fed diets supplemented with phytase alone or in combination with citric acid and multicarbohydrase. *Poultry Science*, 89 (10), pp. 2221-2229.

Wu, Y. B., Ravindran, V., Thomas, D. G., Birtles, M. J. and Hendriks, W. H. (2004). Influence of phytase and xylanase, individually or in combination, on performance, apparent metabolisable energy, digestive tract measurements and gut morphology in broilers fed wheat-based diets containing adequate level of phosphorus. *British Poultry Science*, 45 (1), pp. 76-84.

Wyss, M., Pasamontes, L., Remy, R., Kohler, J., Kusznir, E., Gadient, M., Muller, F. and van Loon, A. P. G. M. (1998). Comparison of the thermostability properties of three acid phosphatases from molds: *Aspergillus fumigatus* phytase, *A-niger* phytase, and *A-niger* pH 2.5 acid phosphatase. *Applied and Environmental Microbiology*, 64 (11), pp. 4446-4451.

Xavier, B., Sahu, N. P., Pal, A. K., Jain, K. K., Misra, S., Dalvi, R. S. and Baruah, K. (2012). Water soaking and exogenous enzyme treatment of plant-based diets: effect on growth performance, whole-body composition, and digestive enzyme activities of rohu, *Labeo rohita* (Hamilton), fingerlings. *Fish Physiology and Biochemistry*, 38 (2), pp. 341-353.

Xiang, T., Liu, Q., Deacon, A. M., Koshy, M., Kriksunov, I. A., Lei, X. G., Hao, Q., Thiel, D. J. (2004). Crystal structure of a heat-resilient phytase from *Aspergillus fumigatus* carrying a phosphorylated histidine. *Journal of Molecular Biology*, 339 (2), pp. 437-445.

Xie, J., Song, L., Li, XR., Yi, XR, Yi, XL., Xu, H., Li, J., Qiao, D. and Cao, Y. (2011). Site-directed mutagenesis and thermostability of xylanase XYNB from *Aspergillus niger* 400264. *Current Microbiology*, 62 (1), pp. 242-248.

Yadava, P. K., Niranjan, P. S., Udebeyir, S. K. and Verma, D. N. (2009). Performance of broiler chicken as affected by varying levels of multi enzyme supplementation. *Animal Nutrition and Feed Technology*, 9 (1), pp. 103-109.

Yamamoto, T., Satoh, S., Takeuchi, T. and Watanabe, T. (1983). Mineral requirements .13. effects on rainbow-trout of deletion of manganese or trace-elements from fish-meal diet. *Bulletin of the Japanese Society of Scientific Fisheries*, 49 (2), pp. 287-293.

Yamamoto, T., Iwashita, Y., Matsunari, H., Sugita, T., Furuita, H., Akimoto, A., Okamatsu, K. and Suzuki, N. (2010). Influence of fermentation conditions for soybean meal in a non-fish meal diet on the growth performance and physiological condition of rainbow trout *Oncorhynchus mykiss*. *Aquaculture*, 309 (1-4), 173-180.

Yamamoto, T., Murashita, K., Matsunari, H., Sugita, T., Furuita, H., Iwashita, Y., Amano, S. and Suzuki, N. (2012). Influence of dietary soy protein and peptide products on bile acid status and distal intestinal morphology of rainbow trout *Oncorhynchus mykiss*. *Fisheries Science*. 78 (6), pp. 1273-1283.

Yamamoto, T., Murashita, K., Matsunari, H., Oku, H., Furuita, H., Okamoto, H., Amano, S. and Suzuki, N. (2015). Selectively bred juvenile F2 amago salmon *Oncorhynchus masou ishikawae* fed a low fishmeal diet exhibit growth comparable to unselected juveniles fed a fishmeal-based diet. *Fisheries Science*, 81 (1), pp. 83-89.

Yang, Y., Iji, P. A.m, Kocher, A., Mikkelsen, L. L. And Choct, M. (2007). Effects of mannanoligosaccharide on growth performance, the development of gut microflora, and gut function of broiler chickens raised on new litter. *Journal of Applied Poultry Research*, 16 (2), pp. 280-288.

Yang, Y., Iji, P. A., Kocher, A., Mikkelsen, L. L. And Choct, M. (2008). Effects of xylanase on growth and gut development of broiler chickens given a wheat-based diet. *Asian-Australasian Journal of Animal Sciences*, 21 (11), pp. 1659-1664.

Yeheyis, L., Kijora, C., van Santen, E., Wink, M., Danier, J. and Peters, K. J. (2012). Crude protein, amino acid and alkaloid contents of annual sweet lupin (*Lupinus* spp. L.) forages and seeds grown in Ethiopia. *Experimental Agriculture*, 48 (3), pp. 414-427.

Yigit, N. O. and Olmez, M. (2011). Effects of cellulase addition to canola meal in tilapia (*Oreochromis niloticus* L.) diets. *Aquaculture Nutrition*, 17 (2), pp. 494-E500.

Yin, X., Yao, Y., Wu, M. C., Zhu, T. D., Zeng, Y. and Pang, Q. F. (2014). A unique disulfide bridge of the thermophilic xylanase syxyn11 plays a key role in its thermostability. *Biochemistry – Moscow*, 79 (6), pp. 531-537.

Young, G. and Forte, D. (2016a). *Preconditioning for extrusion of aquafeed – Part 2*. [online] Available at: http://www.allaboutfeed.net/Equipment/Articles/2016/3/Preconditioning-for-extrusion-of-aquafeed---Part-2-2759213W/ (Accessed 13 June. 2016).

Young, G. and Forte, D. (2016b). *Aquafeed Extrusion Technology*. Presented at the Centre for Food Technology (Fôrtek), Ås, Norway (9 May, 2016).

Yu, S., Cowieson, A., Gilbert, C., Plumstead, P. and Dalsgaard, S. (2012). Interactions of phytate and myo-inositol phosphate esters (IP1–5) including IP5 isomers with dietary protein and iron and inhibition of pepsin. *Journal of Animal Science*. 90, pp. 1824–1832

Zamini, A. A., Kanani, H. G., Esmaeili, A. A., Ramezani, S. and Zoriezahra, S. J. (2014). Effects of two dietary exogenous multi-enzyme supplementation, Natuzyme® and beta-mannanase (Hemicell®), on growth and blood parameters of Caspian salmon (*Salmo trutta caspius*). *Comparative Clinical Pathology*, 23 (1), pp. 187-192.

Zdunczyk, Z., Jankowski, J., Jusiewicz, J. and Kwiecinski, P. (2012). The response of the gastrointestinal tract of broiler chickens to different dietary levels and sources of sodium. *Veterinarija Ir Zootechnika*, 60 (82), pp. 92-98.

Zeng, L., Lei, J., Ai, C., Hong, W. and Liu, B. (2015). Protein-sparing effect of carbohydrate in diets for juvenile turbot *Scopthalmus maximus* reared at different salinities. *Chinese Journal of Oceanology and Limnology*, 33 (1), pp. 57-69.

Zhang, w., Mullaney, E. J. and Lei, X. G. (2007). Adopting selected hydrogen bonding and ionic interactions from *Aspergillus fumigatus* phytase structure improves the thermostability of *Aspergillus niger* PhyA phytase. *Applied Environmental Microbiology*, 73 (9), pp. 3069-3076.

Zhang, G. G., Yang, Z. B., Zhang, Q. Q., Yang, W. R. And Jiang, S. Z. (2012). A multienzyme preparation enhances the utilization of nutrients and energy from pure corn and wheat diets in broilers. *Journal of Applied Poultry Research*, 21 (2), pp. 219-225.

Zhao, H., Li, C., Beck, B. H., Zhang, R., Thongda, W., Davis, D. A. and Peaman, E. (2015). Impact of feed additives on surface mucosal health and columnaris susceptibility in channel catfish fingerlings, *Ictalurus punctatus*. *Fish & Shellfish Immunology*, 46 (2), pp. 624-637.

Zhou, Y., Yuan, X., Liang, X.-F., Fang, L., Li, J., Guo, X., Bai, X. and He, S. (2013). Enhancement of growth and intestinal flora in grass carp: The effect of exogenous cellulase. *Aquaculture*, 416 (1-4), pp. 1-7.

Zhu, H. L., Hu, L. L., Hou, Y. Q., Zhang, J. and Ding, B. Y. (2014). The effects of enzyme supplementation on performance and digestive parameters of broilers fed corn-soybean diets. *Poultry Science*, 93 (7), pp. 1704-1712.

Zhu, Y. W., Xie, J. J., Li, W. X., Lu, L., Zhang, L. Y., Ji, C., Lin, X., Liu, H. C., Odle, J., Luo, X. G. (2015). Effects of environmental temperature and dietary manganese on egg production performance, egg quality, and some plasma biochemical traits of broiler breeders. *Journal of Animal Science*, 93 (7), pp. 3431-3440.

Zou, X. T., Qiao, X. J. and Xu, Z. R. (2006). Effect of beta-mannanase (Hemicell) on growth performance and immunity of broilers. *Poultry Science*, 85 (12), pp. 2176-2179.