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The effects of selected bio-active feed additives on Nile tilapia (*Oreochromis niloticus)* production and health

By

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The effects of selected bio-active feed additives on Nile tilapia (*Oreochromis niloticus)* production and health

Ayodeji Adeoye

Three investigations were conducted to assess the effects of selected bio-active feed additives on tilapia (*Oreochromis niloticus*) growth performance, feed utilisation, haemato-immunological status, intestinal morphology and microbiology.

The first experiment was conducted to evaluate the effects of feeding tilapia semi-practical diets supplemented with exogenous enzymes (phytase, protease or carbohydrase). After six weeks of the dietary supplementation, tilapia fed diets supplemented with exogenous enzymes (phytase and carbohydrase) showed enhanced growth performance and higher microvilli density when compared to the control group. Additionally, the intestinal bacterial community profile of tilapia fed the carbohydrase supplemented diet was significantly altered in contrast to those fed the control diet.

In the second experiment, tilapia were fed with practical diets supplemented with exogenous enzymes (phytase, protease or xylanase) for eight weeks. Tilapia fed the xylanase supplemented diet demonstrated significantly higher final body weight (FBW), improved feed conversion ratio (FCR) and better protein efficiency ratio (PER) when compared to the control group. Apparent digestibility coefficients of protein, ash, energy, phosphorus, calcium and sodium were highest in tilapia fed a diet supplemented with phytase.

The third experiment was carried out to assess the combined effects of dietary exogenous enzymes (phytase, protease and xylanase) and probiotics (*Bacillus subtilis, B. licheniformis and B. pumilus*) on tilapia growth performance and health. After seven weeks of feeding, tilapia fed the diet supplemented with both exogenous enzymes and probiotics showed significantly higher FBW, specific growth rate, improved FCR and better PER. The serum lysozyme activity was observed to be significantly higher in tilapia fed the probiotic supplemented diet when compared to the control group. The dietary supplementation with combined exogenous enzymes and probiotics increased intestinal perimeter ratio, microvilli count (density), diameter and subsequently resulted in higher enterocyte absorptive area in tilapia.

This study demonstrates that feeding tilapia with dietary exogenous enzymes can enhance growth performance and modulate microbial community profile. In addition, supplementation with both exogenous enzymes and probiotics is capable of improving tilapia growth performance, immune parameters and intestinal morphology.

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List of abbreviations

ANF	Anti-nutritional factor
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AU	Arbitrary unit
BLAST	Basic local alignment search tool
CFU	Colony forming unit
CP	Charoen Pokphand
DCP	Dicalcium phosphate
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FBW	Final body weight
FCR	Feed conversion ratio
GALT	Gastro associated lymphoid tissue
H	Hour
HSI	Hepatosomatic index
IEL	Intraepithelial leucocyte
K	Condition factor
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
Min	Minute
MS222	Tricaine methane sulphonate
NRC	National Research Council

NSP	Non-starch polysaccharide
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PER	Protein efficiency ratio
SEM	Scanning electron microscopy
SGR	Specific growth rate
TAE	Tris-acetate-EDTA
TCA	Trichloroacetate
TE	Tris-EDTA
TEM	Transmission electron microscopy
VSI	Viscero-somatic index
WG	Weight gain

Dedication

To

Immortal, Invisible, God only Wise

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Author's declaration

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Conferences attended and works presented

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World Aquaculture, Adelaide - Australia (June 2014) - Presented a poster entitled "Comparative effects of supplementary exogenous enzymes on growth performance and feed utilization of Nile tilapia, *Oreochromis niloticus*"

Lupins in UK Agriculture and Aquaculture Project Meeting with Innovate UK, Shrewsbury – UK (January 2015) - Presented a poster entitled "Comparative effects of supplementary exogenous enzymes on growth performance and feed utilization of Nile tilapia, *Oreochromis niloticus*"

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Publications

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1 Chapter 1: Introduction

1.1 Importance of seafoods in human diet and influence on socio-economic impact

Seafoods (which herein refer to all major captured and farmed edible aquatic animal food products entering the human food chain) represent an important component of the world food basket, contributing to and improving nutrition, health, well-being and livelihoods of global populations. As an integral part of the human diet, aquatic foods represent one of the most healthy and nutritious food sources (Tacon and Metian, 2013), accounting for 16.6% of total supply of animal protein and 6.5% of all plant and animal protein consumed at a global level in 2009 (FAO, 2012). In comparison with terrestrial farmed food consumption, the protein content of seafoods is higher and more digestible with excellent essential amino acids profiles. Also, seafoods (mainly of marine source) have lower saturated fat content and higher concentration of long-chain omega-3 (n-3) polyunsaturated fatty acids than any other foodstuffs (Sargent and Tacon, 1999). In addition, seafoods are richer sources of most essential minerals and trace elements (e.g. iodine, selenium, zinc, etc.) as well as several important water soluble and fat soluble vitamins (e.g. vitamins A, D, E and K) than most terrestrial meats (Tacon and Metian, 2013). Apart from the importance of seafoods as sources of food and nutrient supply, seafoods also contribute to livelihoods in terms of income generation and social benefits with about 38 million people worldwide engaged directly or indirectly in fisheries and aquaculture activities (FAO, 2014).

1.2 Replacement of capture fisheries by an expanding aquaculture production

The global population reached 6 billion in 2000, 6.8 billion in 2010 and is predicted to reach 9.1 billion by 2050 (Allan, 2004). With increasing population and consumption growth, capture fisheries output continues to stagnate and will experience decline over the coming decades due to overfishing, environmental degradation and climate change. On the other hand, aquaculture (farming of aquatic food products) has continued to experience growth in production volume when compared with capture fisheries production (FAO, 2012, 2014). In the last three decades (1980–2010), world food fish production has expanded by almost twelve fold, at an annual rate of 8.8 percent. Global aquaculture production has continued to grow, with 60 million tonnes (excluding aquatic plants and non-food products) production figure in 2010 and an estimated total value of US$119 billion (FAO, 2012). In 2012, the production reached a record high of 90.4 million tonnes with an estimated value of US$144.4 billion (FAO, 2014). The global aquaculture industry contributed 42.2% to world food fish production in 2012 (Figure 1.1) and accounted for half of the world's fish for direct consumption (FAO, 2012, 2014). No doubt, aquaculture is the fastest-growing food producing industry in the world and will continue to be the engine that will drive growth in global fish production.

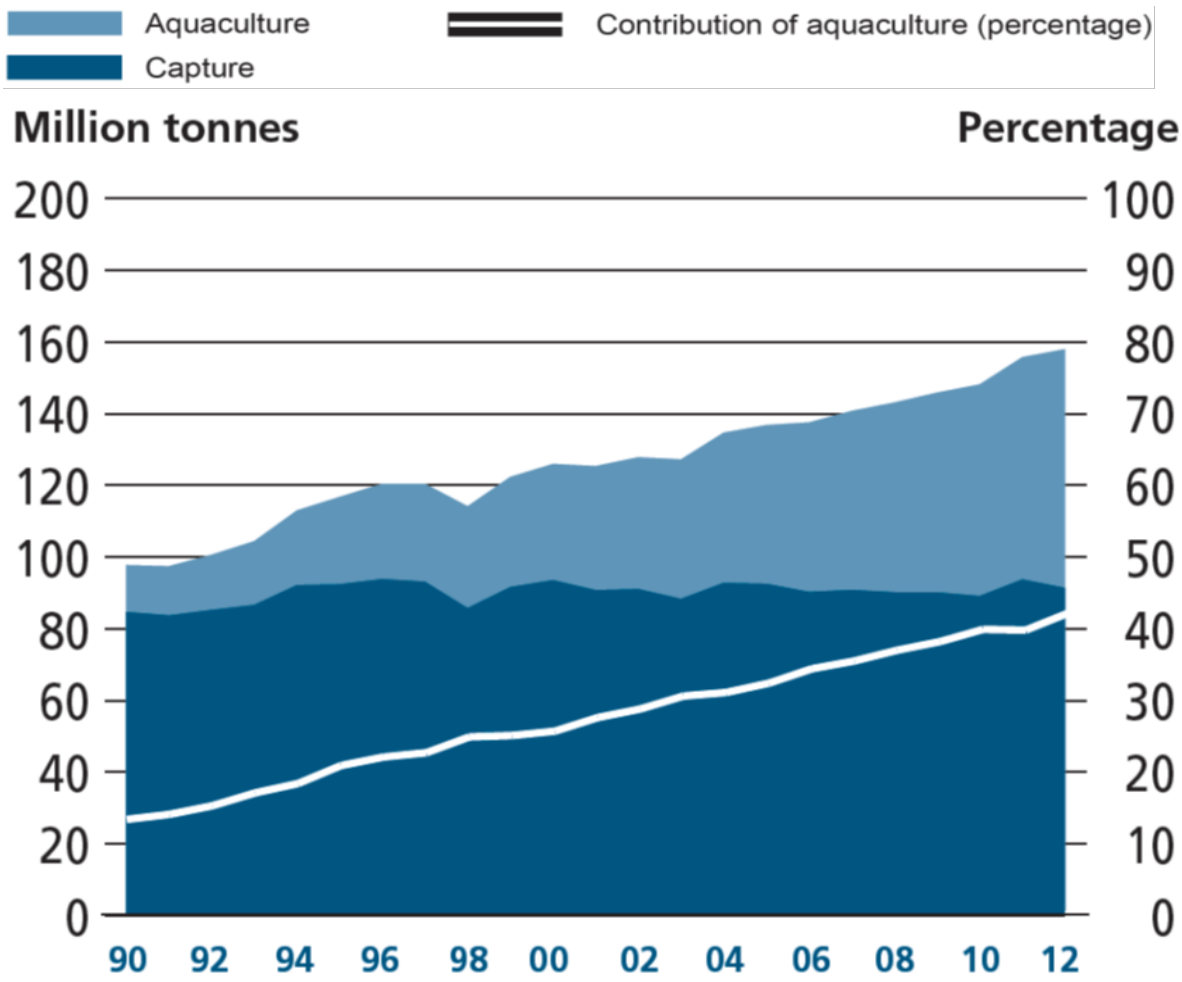

Figure 1.1: Contribution of aquaculture to global fish production (FAO, 2014)

1.3 Tilapia is a global aquaculture species

Tilapia is the common name for nearly a hundred fish species of the Cichlidae family endemic to Africa. However, as a result of aquaculture operations, recreational fishing, aquatic weed control and research purposes, tilapia are now spread world-wide (El-Sayed, 2006). Today, tilapia has become a global aquaculture species because of a number of desirable traits; fast growth, tolerance to wide range of bio-physical conditions, relative resistance to stress and disease, ability to reproduce in captivity, short generation time and ability to convert low cost feed (low trophic feeding) into high quality protein (El-Sayed, 2006). According to the FAO (2014), the farming of tilapia is the most widespread type of

aquaculture activity. In terms of production volume, tilapia are the second most important group of farmed fish after carps contributing significantly to global aquaculture production (Tacon, 2003, FAO, 2011, Fitzimmons, 2015). As shown in Figure 1.2, total tilapia production was over 4.8 million tonnes with a value of over US$8.2 billion in 2013 (FIGIS, 2013). Due to its favourable production traits and its global popularity, tilapia has been named the 'aquatic chicken'.

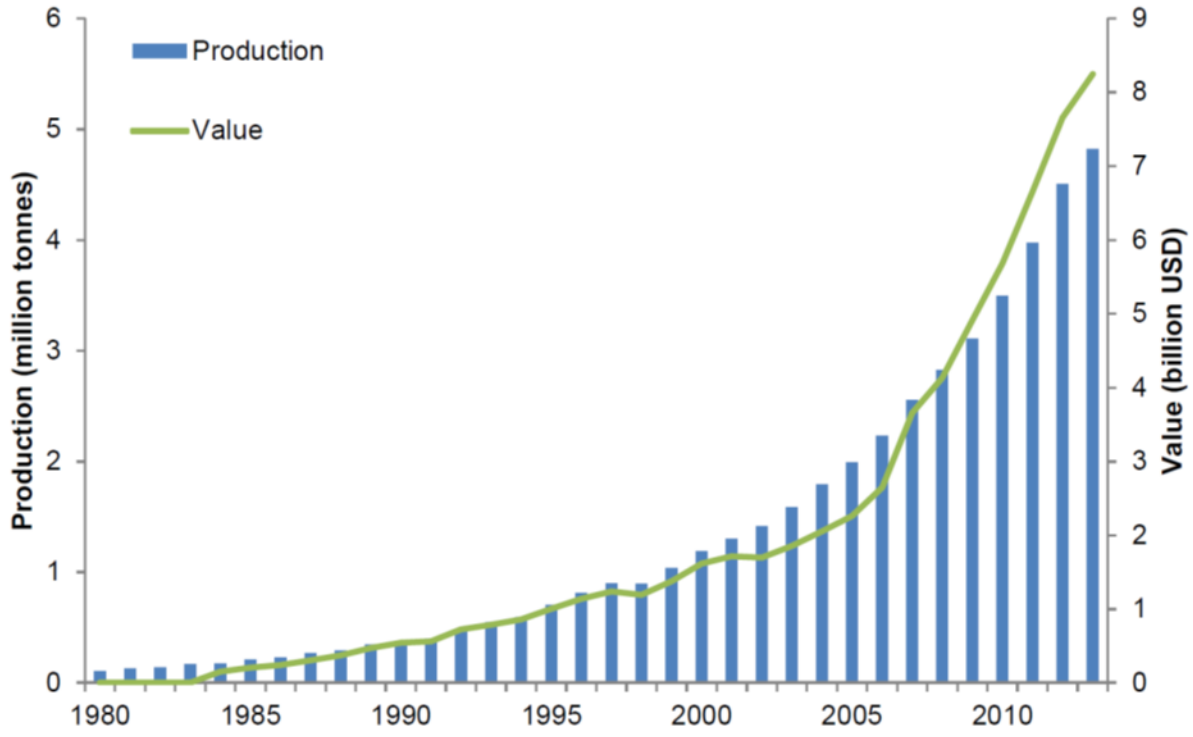

Figure 1.2: Global tilapia production and value (FIGIS, 2013)

Most of global tilapia production is attributed to Nile tilapia (*Oreochromis niloticus*; 3.4 million tonnes) distantly followed by hybrids of Nile tilapia and blue tilapia (*O. niloticus* x *Oreochromis aureus*; 414,475 tonnes) and the Mozambique tilapia (*Oreochromis mossambicus*; 34,206 tonnes) (Figure 1.3). To support the expanding Nile tilapia production, there is need to secure a sustainable supply of aquafeed.

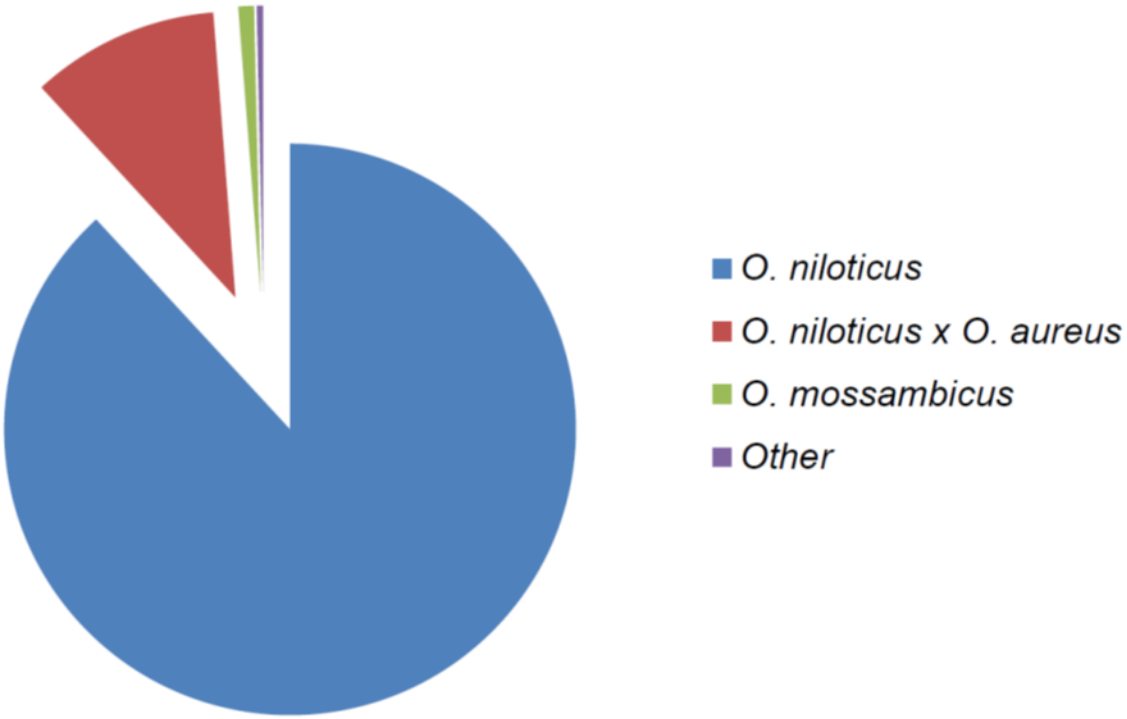

Figure 1.3: Aquaculture production by tilapia species (FIGIS, 2013)

1.4 Aquaculture production systems for tilapia

To meet the economic needs of producers, requirement of aquatic species, available resources and level of production intensity, aquaculture production systems can be extensive, semi-intensive or intensive. In extensive production system, no external input is utilised for production as neither fertilisation nor supplementary feeding is done. Fish fend for themselves and depend solely on natural productivity. This system is characterised by low stocking density and subsequently low yield. Often, this system is non-commercial and utilised for house-hold consumption. On the other hand, in semi-intensive aquaculture production system, fish grow faster and larger compared to the extensive system due to fertilisation (mainly through organic manure) and supplementary feeding (often low protein, grain-based supplementary diets). Stocking density is relatively higher compared to the extensive system and subsequently relatively higher yield. However, in intensive aquaculture

production system, the level of input is considerably much higher when compare to the semi-intensive system. More fish are produced per unit area, often with continuous water flow and aeration. In the intensive system, high quality formulated diets are supplied to meet fish nutritional requirement with little or no dependence on natural productivity; with increase in stocking rate, the contribution of natural productivity to fish yield decreases. The intensive production is often capital intensive and requires high level of technical-know-how of the targeted species and water quality parameters to ensure optimum situations that promote growth, reduce stress, control disease and reduce mortality. Additionally, production in intensive system is often market-driven.

Traditionally, tilapia are raised in extensive aquaculture production system. However, as market demand increases, aquaculture industry expands and technology develops, extensive tilapia production was replaced by semi-intensive and intensive production systems. According to El-Sayed (2008), over 90% of tilapia are produced in semi-intensive production system especially in Southeast Asia, Africa and Latin America. Though, majority of freshwater fish production (including tilapia) is based on low protein, grain-based supplementary diets and organic fertiliser in semi-intensive aquaculture production system however for aquaculture to meet future protein demand, increased outputs will require intensified aquaculture operations which depend largely on external feed (high quality formulated diets), water and energy (Bostock et al., 2010). In intensive aquaculture production systems, feed is the most expensive item (Webster and Lim, 2006, Nguyen, 2008) and major determinant for successful aquaculture growth and intensification (Agbo, 2008, Rana et al., 2009, Teves and Ragaza, 2014). Consequently, the importance of high quality formulated diets as a key factor determining future supplies of farmed tilapia.

1.5 Role of aquafeed in aquaculture production

With industrialization and intensification of aquaculture activities, new needs emerge for the production and supply of aquafeeds to enhance sustainability of aquaculture growth. This makes demand for aquafeed three times more than feed for other farm animals (IFFO, 2013). Economically viable and environmental friendly feeds must be developed to ensure successful and sustainable aquaculture production. For aquaculture growth to be sustainable, it is therefore crucial and important that resources required for such growth are secured. Aquafeeds constitute a major expense and input to intensive aquaculture production accounting for 50 - 60% of recurrent costs (Naylor et al., 2000, Rana et al., 2009). This implies that feed and feed utilization efficiency contributes significantly to the overall success of aquaculture operations and sustainability.

Principal among ingredients for producing aquafeed are fishmeal and fish oil. The finfish and crustacean aquaculture sector is still highly dependent upon marine capture fisheries for sourcing major dietary nutrient inputs, including fishmeal and fish oil. This dependency is particularly strong within compound aquafeeds for farmed carnivorous finfish species and marine shrimp (Tacon and Metian, 2008). This dependency on fishmeal and oil by aquafeed industry makes reliance and impacts of aquaculture on ocean fisheries to expand even further (Naylor et al., 2000).

1.6 Fishmeal and fish oil in aquafeeds: combating cost and sustainability

Fishmeal has traditionally been considered an important protein source for use in aquafeeds and some aquaculture formulations (especially for high trophic level fish and crustacean species) have fishmeal included at levels in excess of 65% (Glencross et al., 2007, Tacon et al., 2011). This is due to many reasons such as its high quality protein content, excellent

amino and fatty acids profile, high nutrient digestibility, general lack of anti-nutritional factors (ANFs), palatability, component concentration, wide availability and other attributes which contribute to feed intake, health and immune function in fish (Drew et al., 2007, Gatlin et al., 2007, Tacon and Metian, 2013).

According to Naylor et al. (2000) and Tacon et al. (2011), aquaculture has the fastest growing demand for fishmeal and fish oil. It has also been reported that aquaculture's shares of global fishmeal and fish oil consumption has more than doubled to 68% and 88%, respectively (Tacon and Metian, 2008). However, as the landings of reduction fish (which are reduced to fishmeal and fish oil) from the capture fisheries continue to stagnate, this adds to the uncertainty of fishmeal and fish oil production to support the growing demand of aquaculture industry. In addition, seasonal variation as well as rising cost of fishmeal and fish oil is also limiting factor that will continue to affect continuous supply of fishmeal and fish oil to support aquaculture production growth. For the past few decades, the annual global production of fishmeal and fish oil has remained relatively steady at 5 – 7 million metric tonnes of fishmeal and 0.8 – 1.5 million metric tonnes of fish oil (FAO, 2009). Thus, fishmeal has moved from a commodity to a specialized ingredient, as demand outstrips supply and the amount of whole fish converted into fishmeal decline (IFFO, 2013). The growing aquaculture industry cannot continue to rely on the finite stocks of wild-caught fish. Being too reliant on any one ingredient presents considerable risk associated with supply, price and quality fluctuations. As a strategy to reduce risk, the identification, development and use of alternative feed ingredients to fishmeal and oil in aquafeeds remains a high priority.

It is important however to note that tilapia are relatively independent from the need to use marine ingredients (fishmeal and fish oil) as feed components. Tilapia feed on a low trophic level and being omnivores are able to grow rapidly on lower protein levels and tolerate higher

carbohydrate typical of plant materials than many farmed carnivorous fish species (Watanabe et al., 2002). Subsequently plant-based diets are considered to be more suitable for tilapia (Bwanika et al., 2004) with relatively low nutritional demands compare to carnivorous farmed fish. Recent advances in tilapia nutrition have also shown that tilapia can attain optimal growth and good performance without the inclusion marine ingredients. Complete replacement of fishmeal with 63.8 % soybean (Nguyen, 2008) and 20 % full-fat soya (Abdel-Warith and Younis, 2013) in tilapia diets have been reported without detrimental effect on growth performance. Similarly, total replacement of fish oil with 8% soybean oil (Huang et al, 1998), 10% sunflower oil (Ng et al., 2001), 10 % palm oil (Ng et al., 2003) and 8% palm oil (Bahurmiz and Ng, 2007) have been shown to have no negative effects on tilapia growth performance. Recently, researchers have shown that with the use of single cell protein and algae, marine ingredients can be eliminated completely from tilapia diets without detrimental effects.

Irrespective of tilapia independence on marine ingredients, many tilapia feeds continue to contain marine ingredients (fish oil and 10% or more fishmeal). The continuous inclusion of fishmeal in tilapia feed could be due to its excellent characteristics as mentioned earlier in this section. Considering human health benefit, it is not desirable to remove fish oil entirely from formulated aquafeed and consequently reduce the n-3 highly unsaturated fatty acids (EPA and DHA) content in farmed fish species. However, with recent advances, reduction in the use of fish oil is now possible; diet rich in fish oil are used as finishing diet to 'wash out' n-6 fatty acids accumulated during the growth phase and subsequently a final product that resemble wild fish (Bostock et al., 2010).

1.7 Plant-based materials as alternatives to fishmeal and oil

The identification and use of suitable and cost-efficient ingredients as alternatives to fishmeal and oil has been the focus of continuous efforts by researchers and the aquaculture industry

(Table 1.1 and Table 1.2). Alternatives have been sought (and research is still ongoing in this regard) to reduce the level of dependency on the marine raw ingredients. Viable alternatives should possess certain characteristics, including wide availability, competitive price as well as ease of handling, shipping, storage and use in feed production. Furthermore, it must possess certain nutritional characteristics, such as low levels fibre, starch, especially non-soluble carbohydrates and ANFs, and have a relatively high protein content, favourable amino acid profile, high nutrient digestibility and reasonable palatability.

In consideration of these criteria, grain and oilseed by-products appear among the most promising alternative ingredients for aquaculture diets in the future for cultured fish species. This is in addition to their low cost, increasing abundance, potential for increased production, greater sustainability and lower health risks than other alternatives (Hardy et al., 2009).

Consequently, increasing amounts of plant-based products are being utilised in aquafeeds and this trend will continue (Allan, 2004, Tacon et al., 2011). Examples of such plant products are shown in Table 1.1 and Table 1.2, as well as examples of relevant research efforts. It therefore follows that viable utilization of plant feedstuffs formulated in aquafeeds for the production of cold, temperate and warm water aquatic species is an essential requirement for future development and expansion of aquaculture (Gatlin et al., 2007). Furthermore, the sustainability of aquaculture is likely to be linked with the use of vegetable proteins and oils especially as large amounts of aquaculture production is of non-carnivorous aquatic species (such as carps, tilapia and milkfish). This is evidenced in research efforts on the use of plant-based ingredients as fishmeal and fish oil replacements (Table 1.1 and Table 1.2).

Table 1.1: Examples of plant protein substitutes for fishmeal

Fishmeal substitute	Species	Optimum level (%)	Fishmeal level (%)	Reference
Soybean meal	Chinese sucker, *Myxocyprinus asiaticus*	22.5	33.6	Yu et al. (2013)
Soybean meal	Red drum, *Sciaenops ocellatus*	40	6	Rossi et al. (2015)
Soybean meal	Tilapia, *Oreochromis niloticus*	34.74	15.9	Kumar et al. (2012a)
Soybean meal	Tilapia, *O. niloticus*	34-46		Hasan et al. (2007)
Soybean meal	Milkfish, *Chanos chanos*	30.8		Hasan et al. (2007)
Soybean meal	Grouper, *Epinephelus sp.* Asian seabass, *Lates calcarifer*	6		Hasan et al. (2007)
Soybean meal	Gilthead seabream, *Sparus aurata*	23.74	59.21	Nengas et al. (1996)
Soybean meal	Gilthead seabream, *S. aurata*	20.5	28.6	Martínez-Llorens et al. (2007a)
Soybean meal	Gilthead seabream, *S. aurata*	20	37	Martínez-Llorens et al. (2007a)
Soybean meal	African catfish, *Clarias gariepinus*	41	0	Goda et al. (2007)

Soybean meal	African catfish, *C. gariepinus*	31	6	Goda et al. (2007)
Soybean meal	African catfish, *C. gariepinus*	40.8	29	Fagbenro and Davies (2001)
Soybean meal	Atlantic salmon, *Salmo salar*	27.2	40	Carter and Hauler (2000)
Soybean meal	European seabass, *Dicentrarchus labrax*	25	49.8	Tibaldi et al. (2006)
Soybean meal	Tilapia, *Oreochromis spp.*	63.8	0	Nguyen et al. (2009)
Soybean meal	Rainbow trout, *Oncorhynchus mykiss*	11	12	Torstensen et al. (2008)
Soybean meal	Rainbow trout, *O. mykiss*	12.1	13.3	Vielma et al. (2000)
Soybean meal	European seabass, *D. labrax*	50	34.3	Tibaldi et al. (2006)
Soy protein	Gilthead seabream, *S. aurata*	20	37.8	Kokou et al. (2015)
Soy protein	Gilthead seabream, *S. aurata*	72.5	0	Kissil et al. (2000)
Soy protein	African catfish, *C. gariepinus*	55	10.4	Fagbenro and Davies (2004)
Soy protein	Rainbow trout, *O. mykiss*	30	21.72	Collins et al. (2012)
Soy protein	Rainbow trout, *O. mykiss*	30	10	Penn et al. (2011)
Soy protein	Rainbow trout, *O. mykiss*	42.6	25	Denstadli et al. (2007)
Soy protein	Red drum, *S. ocellatus*	27.8	28.64	Moxley et al. (2014)

Soybean cake	Grass carp, *Ctenopharyngodon idella*	5-14		Hasan et al. (2007)
	Common, *Cyprinus carpio* Crucian carp, *Carassius carassius*	27-32		Hasan et al. (2007)
	Shrimp, *Litopenaeus vannamei*	15		Hasan et al. (2007)
	Freshwater prawn, *Macrobrachium rosenbergii*	21-23.2		Hasan et al. (2007)
Soybean meal	Rainbow trout, *O. mykiss*	34.38	20.17	Dalsgaard et al. (2012)
Non-GM Soybean	California yellowtail, *Seriola lalandi*	41.9	18	Buentello et al. (2015)
Non-GM Soybean	Cobia, *Rachycentron canadum*	50.7	13.4	Watson et al. (2014)
Full fat soya	Tilapia, *O. niloticus*	20	0	Abdel-Warith and Younis (2013)
Full fat soya	Gilthead seabream, *S. aurata*	10	68.95	Robaina et al. (1995)
Canola meal	Rainbow trout, *O. mykiss*	30	22	Shafaeipour et al. (2008)
Pea and canola meal	Rainbow trout, *O. mykiss*	20	33.7	Thiessen et al. (2003)
Canola protein	Rainbow trout, *O. mykiss*	30	40.7	Collins et al. (2012)

Rapeseed meal	Rainbow trout, *O. mykiss*	26.35	31.91	Dalsgaard et al. (2012)
Rapeseed cake	Grass carp, *C. idellus*	41-51		Hasan et al. (2007)
Rapeseed cake	Common, *C. carpio* Crucian carp, *C. carassius*	40-41		Hasan et al. (2007)
Rapeseed cake	Freshwater prawn, *M. rosenbergii*	21.6-26		Hasan et al. (2007)
Rapeseed protein	Gilthead seabream, *S. aurata*	74.5	0	Kissil et al. (2000)
Sunflower meal	Rainbow trout, *O. mykiss*	24.6	31.12	Dalsgaard et al. (2012)
Sunflower meal	Gilthead seabream, *S. aurata*	35.2	40.6	Mérida et al. (2010)
Sunflower meal	Gilthead seabream, *S. aurata*	11.8	53.3	Lozano et al. (2007)
Rapeseed and peanut meals	Crucian carp, *C. carassius*	25	35	Cai et al. (2013)
Pea meal	Rainbow trout, *O. mykiss*	30	38.71	Collins et al. (2012)
Pea seed meal	African catfish, *C. gariepinus*	33	57	Davies and Gouveia (2008)
Dehulled pea meal	Rainbow trout, *O. mykiss*	25	37.5	Thiessen et al. (2003)
Pea protein isolate	Tilapia, *O. niloticus*	14.88	38.96	Schulz et al. (2007)

Pea protein	Atlantic salmon, *S. salar*	27.57	40	Carter and Hauler (2000)
Pea protein	Rainbow trout, *O. mykiss*	21	6.5	Moreno-Rojas et al. (2008)
Pea protein	Rainbow trout, *O. mykiss*	30	27.54	Collins et al. (2012)
Peanut leaf meal	Tilapia, *O. niloticus*	31.4	41.5	Garduño-Lugo and Olvera-Novoa (2008)
Groundnut cake	Shrimp, *L. vannamei*	16		Hasan et al. (2007)
Lupin kernel meal	Shrimp, *L. vannamei*	18.25	16.46	Molina-Poveda et al. (2013)
Lupin meal	Gilthead seabream, *S. aurata*	39.76	44.72	Pereira and Oliva-Teles (2003)
Jatropha meal	Tilapia, *O. niloticus*	24.8	15.9	Kumar et al. (2012a)
Maize gluten	Atlantic salmon, *S. salar*	50	27	Mente et al. (2003)
Maize gluten	Gilthead seabream, *S. aurata*	40.66	24.6	Pereira and Oliva-Teles (2003)

Table 1.2: Examples of plant oil substitutes for fish oil

Fish oil substitute	Species	Optimum level (%)	Fish oil level (%)	Reference
Palm oil	African catfish, *C. gariepinus*	9	0	Sotolu (2010)
Palm oil	Atlantic salmon, *S. salar*	12.05	12.05	Bell et al. (2002)
Palm oil	Rainbow trout, *O. mykiss*	10	10	Fonseca-Madrigal et al. (2005)
Palm oil	Humpback grouper, *Cromileptes altivelis*	0.5	0.5	Shapawi et al. (2008)
Palm oil	African catfish, *C. gariepinus*	10	0	Ng et al. (2003)
Palm oil	Tilapia, *O. niloticus*	10	0	Ng et al. (2003)
Palm oil	Tilapia, *O. niloticus*	8	0	Bahurmiz and Ng (2007)
Refined palm oil	African catfish, *C. gariepinus*	10	0	Ng et al. (2003)
Palm kernel oil	African catfish, *C. gariepinus*	10	0	Ng et al. (2003)
Palm kernel oil	Tilapia, *O. niloticus*	10	0	Ng et al. (2003)
Refined palm oil	Humpback grouper, *C. altivelis*	0.5	0.5	Shapawi et al. (2008)
Combination of rapeseed,	Atlantic salmon, *S. salar*	11.48	3.83	Torstensen et al. (2005)

palm and linseed oil				
Combination of linseed, palm and rapeseed oil	European seabass, *D. labrax*	13.4	8.8	Richard et al. (2006)
Combination of soybean, rapeseed and linseed oil	European seabass, *D. labrax*	11.8	7.87	Izquierdo et al. (2003)
Benniseed oil	African catfish, *C. gariepinus*	9	0	Sotolu (2010)
Groundnut oil	African catfish, *C. gariepinus*	9	0	Sotolu (2010)
Soybean oil	African catfish, *C. gariepinus*	9	0	Sotolu (2010)
Soybean oil	White seabass, *Lates calcarifer*	7.1	0	Rombenso et al. (2015)
Soybean oil	Turbot, *Scophthalmus maximus L.*	5	2.5	Peng et al. (2014)
	Milkfish, *C. chanos*	2		Hasan et al. (2007)
Soybean oil	Atlantic halibut, *Hippoglossus hippoglossus*	4.37	0	Haugen et al. (2006)
Soybean oil	European seabass, *D. labrax*	11.8	7.87	Izquierdo et al. (2003)
Soybean oil	Rainbow trout, *O. mykiss*	10	2	Figueiredo-Silva et al. (2005)

Soybean oil	European seabass, *D. labrax*	10	2	Figueiredo-Silva et al. (2005)
Soybean oil	Japanese seabass, *Lateolabrax japonicas*	5	5	Xue et al. (2006)
Soybean oil	Humpback grouper, *C. altivelis*	0.5	0.5	Shapawi et al. (2008)
Soybean oil	Barramundi, *Lates calcarifer*	10	5	Raso and Anderson (2003)
Soybean oil	Pike perch, *Sander lucioperca*	6	0	Schulz et al. (2005)
Soybean oil	Tilapia, *O. niloticus*	8	0	Huang et al. (1998)
Soybean oil	Gilthead seabream, *S. aurata*	6.8	5.6	Martínez-Llorens et al. (2007b)
Rapeseed oil	Atlantic salmon, *S. salar*	8.4	12	Karalazos et al. (2007)
Canola/ rapeseed oil	European seabass, *D. labrax*	11.8	7.87	Izquierdo et al. (2003)
Canola oil	Humpback grouper, *C. altivelis*	0.5	0.5	Shapawi et al. (2008)
Canola/ rapeseed oil	Sunshine bass, *Morone chrysops XM saxatilis*	4	4	Wonnacott et al. (2004)
Canola oil	Barramundi, *Lates calcarifer*	10	5	Raso and Anderson (2003)

Linseed oil	European seabass, *D. labrax*	11.8	7.87	Izquierdo et al. (2003)
Linseed oil	Pike perch, *Sander lucioperca*	6	0	Schulz et al. (2005)
Linseed oil	Barramundi, *L. calcarifer*	10	5	Raso and Anderson (2003)
Corn oil	Japanese seabass, *L. japonicas*	5	5	Xue et al. (2006)
Corn oil	European eel, *Anguilla anguilla*	11.9	0	Kissil et al. (1987)
Sunflower oil	African catfish, *C. gariepinus*	10	0	Ng et al. (2003)
Sunflower oil	Tilapia, *O. niloticus*	10	0	Ng et al. (2001)

However, replacement of fishmeal with plant proteins can present a number of problems such as low crude protein content (Dongmeza et al., 2006), deficiency of essential amino acids such as lysine, methionine and tryptophan (Fagbenro and Davies, 2001, Khattab and Arntfield, 2009), low digestibility (Albrektsen et al., 2006), the presence of high amounts of carbohydrate, fibre and other ANFs such as protease inhibitors, alkaloids, tannins, saponins, lectins, adverse oligossacharides (Francis et al., 2001, Skrede et al., 2002, Refstie et al., 2005), decreased palatability as a result of presence of compounds that are offensive to olfactory receptors of fish (Dongmeza et al., 2006) and reduction of pellet quality especially with respect to water stability and stability during storage (Wood, 1987, Sogbesan and Ugwumba, 2008). Nonetheless, the affordability and availability of plant proteins is superior to fishmeal and this advantage may allow processing of crops to improve their nutritive value in aquafeeds (Drew et al., 2007).

1.8 Lupins: emerging plant ingredients for aquafeed

Due to the popularity of soybean meal as an alternative to fishmeal in animal feeds, it is becoming costlier and the trend at the moment is to identify other suitable alternatives apart from soybean meal (Ng and Romano, 2013). Lupins (which have a crude protein level of 30 - 42% and more favourable amino acids profile than beans or peas) are among the suite of ingredients being considered in Europe and Australia to complement soybean meal in the replacement of fishmeal. This is because lupin kernel meal is well digested, high in digestible protein, durable and palatable for many fish species. In addition, lupins have been shown to contain some functional properties that can contribute to the quality of fish feeds. According to (Glencross et al., 2008), lupin is an effective energy and protein source comparable to fishmeal when considered on an equivalent digestible nutrient basis. This implies that fish can utilise the plant protein as effectively as they can

use fish-based proteins. The nutritional composition of lupin meal varies from whole-seed to kernel (Table 1.3). Few nutritional problems and relative lack of ANFs in lupin is one of their strong positive features (Figure 1.4) (Glencross, 2007). Among the commercial species of lupins, *Lupinus angustifolius* (Narrow-leafed/ Sweet Lupin) dominates the other two, *Lupinus albus* (White or Albus Lupin) and *Lupinus luteus* (Yellow Lupin), in terms of production (Glencross, 2007).

Table 1.3: Composition (%) of *Lupin species*

Species	*L. angustifolius*		*L. albus*		*L. luteus*		*L. mutabilis*	
	Seed	**Kernel**	**Seed**	**Kernel**	**Seed**	**Kernel**	**Seed**	**Kernel**
Seed coat	24	0	18	0	27	0	16	0
Moisture	9	12	9	11	9	12	8	10
Protein	32	41	36	44	38	52	44	52
Fat	6	7	9	11	5	7	14	17
Ash	3	3	3	4	3	4	3	4
Lignin	1	1	1	1	1	1	1	1
Polysaccharides	22	29	17	21	8	11	9	10
Oligosaccharides	4	6	7	8	9	12	5	6
Minor components	0.5	1	0.6	1	0.9	1	1	1

(Source: www.lupins.org)

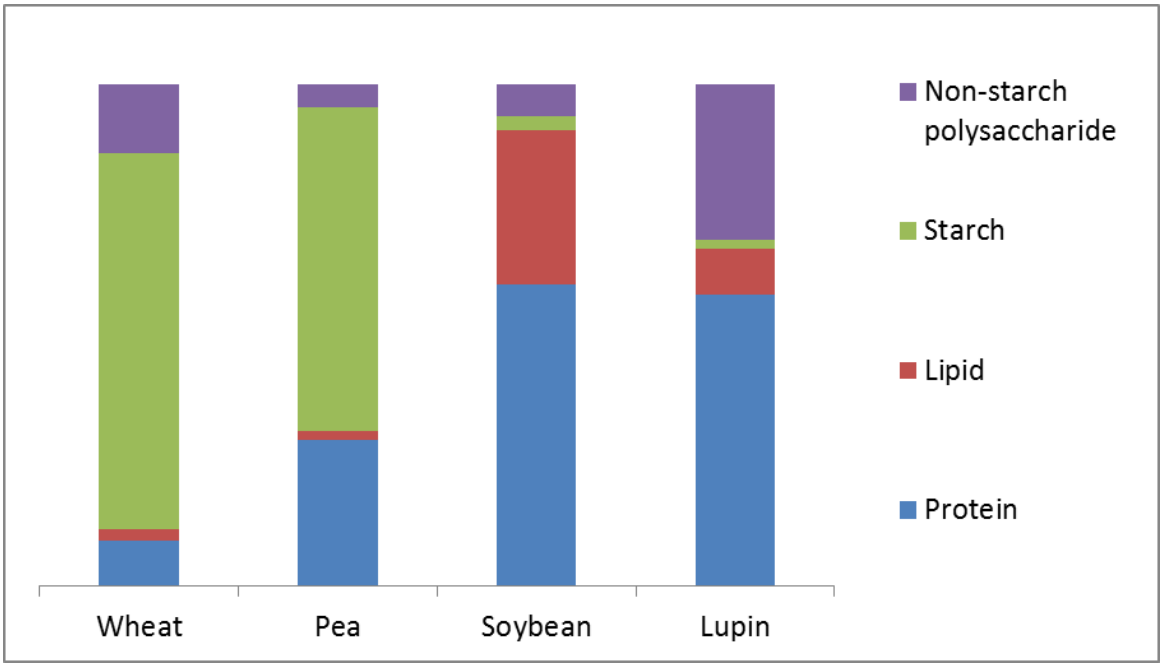

Figure 1.4: Comparative grain content of narrow-leafed lupin (source: www.lupins.org)

Several studies have been conducted to establish the viability of lupins as an effective alternative protein in fish diets. Farhangi and Carter (2001) included lupin (*L. angustifolius*) meal in rainbow trout diet as fishmeal replacement at 10 % increments up to 50 % and reported growth deterioration at each inclusion level though feed intake showed no effects of lupin level, supporting the palatability of lupin meal for salmonids. Similarly, Glencross et al. (2004) included yellow lupin (*L. luteus*) meal in a rainbow trout diet and reported a significantly reduced growth at 50 % level of inclusion when compared to a 0 % lupin reference diet. As earlier reported by Farhangi and Carter (2001), Glencross et al. (2004) also reported that feed intake was not influenced by the lupin meal inclusion level but a decline in growth was observed at each inclusion level. However, Smith et al. (2007) compare lupin meals with soybean meal in the diet of tiger shrimp (*Penaeus monodon*) and reported no negative effects with inclusion of lupin meals at 43 %.

Inherent to lupins are certain ANFs which include alkaloids and oligosaccharides with phytate, saponins, tannins, protease inhibitors and lectins. However, these ANFs tend to be present in lower concentrations compared to other plant protein sources (Glencross, 2008). Even at low concentrations, ANFs in lupin are still capable of exerting negative effects on the digestibility of protein, energy and organic matter especially the oligosaccharides (Glencross et al., 2003).

1.9 The constraints of ANFs in plant by-products

All plant-derived feedstuffs including grain and oilseed by-products have some characteristics that place them at a disadvantage to fishmeal in terms of their suitability for use in aquafeeds. Relative to fishmeal, plant feedstuffs generally have more indigestible organic matter, in the form of insoluble carbohydrates (non-starch polysaccharides) and fibre, leading to higher levels of excretion and waste production from fish. In addition, certain minerals in plant products, such as phosphorus (P), have limited uptake in fish due to other complex interaction and binding to various components such as fibre and phytate bound P. Though the palatability of many plant materials can be an issue, ANFs are the most serious concern in replacing the fishmeal in feed formulations. ANFs have an adverse impact on the digestion of feed and its efficiency.

ANFs are plant's inherent chemical defence against being eaten by herbivorous animals. The ANFs (Table 1.4), by themselves, or through their metabolic products arising in living systems, impair nutrient utilisation, interfering with performance and health (physiology) of animals (Makkar, 1993, Francis et al., 2001, NRC, 2011). In addition, ANFs can negatively affect vital physiological processes and limit the bioavailability of nutrients for utilization and retention. ANFs can broadly be categorised into four groups:

1. factors affecting protein utilisation and digestion, such as protease inhibitors, tannins, lectins, saponins;
2. factors affecting mineral utilisation, which include phytate, gossypol pigments, oxalates, glucosinolates;
3. antivitamins;
4. miscellaneous substances such as mycotoxins, mimosine, cyanogens, nitrate, alkaloids, photosensitizing agents, phytoestrogens and saponins.

ANFs can also be classified according to their ability to withstand thermal processing, the most commonly employed treatment for destroying them (Van der Poel, 1990, Rumsey et al., 1993).

Table 1.4: Important ANFs in some commonly used alternative fish feed ingredients

Plant-derived nutrient source	ANFs present
Soybean meal	Protease inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins, allergens, cellulose, galactans, arabinogalactans
Rapeseed meal	Protease inhibitors, glucosinolates, phytic acid, tannins, cellulose, arabinogalactans
Lupin seed meal	Protease inhibitors, saponins, phytoestrogens, alkaloids, cellulose, galactans, arabinogalactans
Pea seed meal	Protease inhibitors, lectins, tannins, cyanogens, phytic acid, saponins, antivitamins
Sunflower oil cake	Protease inhibitors, saponins, arginase inhibitor
Cottonseed meal	Phytic acid, phytoestrogens, gossypol, antivitamins, cyclopropenoic acid
Leucaena leaf meal	Mimosine
Alfafa leaf meal	Protease inhibitors, saponins, phytoestrogen, antivitamins
Mustard oil cake	Glucosinolates, tannins
Sesame meal	Phytic acid, protease inhibitors

Adapted from Francis et al. (2001) and Sinha et al. (2011)

1.10 Existing plants processing methods and techniques

Different processing methods have been used for many years to improve the physical characteristics and upgrade the nutritional value of aquafeed and feed ingredients. The common processing techniques include dry and wet heating, extracting with water, and addition of feed supplements. These have been widely and successfully used to reduce the concentration and remove deleterious effects of ANFs in plant-based ingredients (Francis et al., 2001). For example, cooking extrusion increases carbohydrate digestibility (Allan and Booth, 2004) and produces a more durable pellet that can be controlled to make the pellet float or sink.

Some of the common methods and techniques for upgrading plant ingredients include:

1. **Heat treatments** such as cooking, roasting (Newkirk, 2008, Davies and Gouveia, 2008), autoclaving (Ezeagu, 2006) and extrusion technology (Kraugerud et al., 2007, Davies and Gouveia, 2008) can be used to reduce the level of ANFs in plant. However this treatment also removed a major part of the minerals, nutrients and dietary fibres.
2. **Dehulling** increases crude protein concentration and digestibility through a reduction in fibre and tannins (Booth et al., 2001, Glencross et al., 2007). This method also slightly reduced the neutral non-starch polysaccharides (NSP) in seeds (Refstie et al., 2005).
3. **Soaking** in water (Olude et al., 2008, Alegbeleye and Olude, 2009) or alkali (Vadivel and Pugalenthi, 2008) reduces ANFs from plant proteins either singly or in combination with other methods. According to Kumar et al. (2012b), soaking could hydrolyse phytate at high temperature (45 - 65°C) in slightly acidic pH (5 – 6) medium. However, soaking also results in loss of water-extractable proteins and minerals (Hurrell, 2004).

4. **Fermentation** by either bacterial or fungal organisms (Skrede et al., 2002, Refstie et al., 2005, Alegbeleye et al., 2012) has been used by many investigators to reduce negative effects of ANFs on digestibility and growth performance in fish. Lim and Tate (1973) also reported phytate reduction through fermentation process.
5. **Sprouting and seed germination** has been demonstrated to be effective in improving the quality of many plant protein ingredients. El-Adawy (2002) and Ibrahim et al. (2002) reported that cooking pre-germinated seeds was very effective in reducing protease inhibitors, tannins, phytic acid and flatus-producing oligosaccharides (raffinose and stachyose). Urbano et al. ((2000)) reported that during seed germination, phytate is utilised as a source of inorganic phosphate for plant growth and development. However, the disappearance of ANFs during germination depends on the endogenous enzymes activity induced during germination.
6. **Plant breeding programmes** have been reported to produce improved cultivars of grains and oilseeds with reduced ANFs (Davies and Gouveia, 2008). The 'low-phytate' varieties of barley and corn have been used as component in low polluting feed for fish (Jabeen et al., 2004). However, there abounds public concern on safety of consuming fish raised with transgenic plant materials.

Caution should be exercised when using treatment methods to reduce or remove ANFs. This is because the treatment methods sometimes have unintended adverse effects on nutritional quality of feed materials. For instance, heat treatment reportedly alters the chemical nature and decreases the nutritional quality of proteins and carbohydrates (Van der Poel, 1990). Drew et al. (2007) also reported that heat-labile secondary compounds are easily destroyed by a number of heat treatments including extrusion and expander processing.

1.11 Potential of bio-active ingredient supplementation

Bio-active ingredients are essential or non-essential compounds that confer growth, health and immune-enhancing benefits beyond its basic nutritional value (Biesalski et al., 2009, Rust et al., 2012). The use of bio-active ingredients has become more relevant and crucial in plant-based diets because of requirement for specific compounds that are either unavailable or not found in plants. Bio-active ingredients are able to supply compounds that are absent or even release compounds that are bound in plants and/ or add flavour to plant-based diet. This can underpin improvements in growth performance and health benefits. Examples of bio-active ingredients include vitamins, minerals, nucleotides, solid state fermentation products, organic acids, essential amino acids, fatty acids, yeast, exogenous digestive enzymes, prebiotics, probiotics etc.

1.11.1 Potential of exogenous digestive enzymes and probiotics

1.11.1.1 Potential, modes of actions and effects of exogenous digestive enzymes

Of all the methods and techniques for improving nutritional value of plant ingredients, supplementation with exogenous digestive enzymes appears harmless, environmentally friendly and provides a natural way to transform complex feed components into absorbable nutrients. Enzyme technology is an integral tool in the brewing, baking and textile industries. Its application in poultry and pig farming has been well established and proven to have beneficial effects on plant-based diets (Adeola and Cowieson, 2011). Supplementation with exogenous enzymes allows feed producers to extend the range of raw materials used in feed and also to improve the efficiency of existing formulations.

Exogenous enzymes can be classified into:

1. Enzymes which quantitatively supplement endogenous digestive enzymes of monogastric animals (e.g. proteases, amylases, lipases). The aim of using these

enzymes is to balance the gradually occurring suboptimal synthesis of endogenous enzymes by farm animals, mainly young animals which eat large amount of plant-based feeds.

2. Enzymes which are not produced by monogastric animals (e.g. β-glucanases, pentosanases, β-galactosidases, phytases, etc).

Modes of actions of exogenous enzymes include:

1. **Breakdown of components which cannot be digested into absorbable nutrients by endogenous enzymes**. This is demonstrated with phytate phosphorus where phosphates are released by the action of phytase. Various oligosaccharides can also be broken down to their component monosaccharides (e.g. glucose and galactose by the action of β-galactosidases). In the case of complex NSPs, a number of specific enzymes are required to achieve complete breakdown due to their variable backbone and side chain configurations.
2. **Lowering of gastrointestinal viscosity in the digestive tract**. For lowering the viscosity in the digesta, the breakdown of soluble NSPs into smaller units is necessary which in turn lose their property of binding water and swelling capacity. β-glucanases, endo-xylanases, etc. are able to degrade soluble NSPs to the extent that the viscosity increasing property of these fractions is largely reduced. Due to the reduced viscosity, a better mixing of the digesta is possible thereby increasing the efficacy of the endogenous enzymes. Thus, the digestibility of nutrients as well as the utilisation of the energy contained is improved. In addition, the reduced viscosity brings about an increased passage rate of the digesta that may influence appetite and feed intake of the animal.
3. **Reduced nutrient entrapment**. This is achieved by breakdown of cell wall structures in order to release the nutrients (such as starch, protein and fats)

contained therein, and to make them accessible to the digestive enzymes. This leads to acceleration of the enzyme-substrate contact thereby enhancing the digestibility of the entrapped nutrients.

4. **Releasing other nutrients**. NSPs, proteins, phytic acid and various minerals are present as complex compounds in cell walls of plants. Nutrients bound to NSPs or phytate are released by NSP-degrading enzymes (NSPase) or phytase so that the digestibility of the protein and of various minerals (Ca, Mg, Zn) can be improved as a concomitant effect.
5. **Modulation of intestinal microbiota**. Exogenous enzymes not only influence the partitioning of nutrients to the host but also, through their action, produce nutrients which may affect the microbial communities in the gut (Bedford and Cowieson, 2012).

Exogenous digestive enzymes target specific substrates in the diet to improve digestibility and reduce/ remove the effects of anti-nutritional factors. A number of studies have been reported on the use and effects of exogenous digestive enzymes (majorly phytase, protease and carbohydrase) on feed ingredients and ANFs in plant feed materials for aquaculture production (Table 1.5)**.** Phytase enhances better phosphorus metabolism, protein utilisation and bioavailability of other minerals (e.g. calcium, magnesium, zinc, etc.) bound to phytic acid (phytate), ensuring maximum utilisation of nutrients. Several authors have reported that dietary phytase supplementation enhanced phytate-phosphorous availability, leading to improved growth performance in fish (Cain and Garling, 1995, Rodehutscord and Pfeffer, 1995, Yu and Wang, 2000). Schäfer et al. (1995) also reported enhanced weight gain, crude ash content and P content in whole body of carp fed phytase supplemented diet. Similarly, Papatryphon et al. (1999) observed significant improvements in striped bass growth, feed conversion ratios, vertebral and

scale ash concentrations when fed plant based diet supplemented with phytase. A positive effect on rainbow trout weight gain and feed efficiency was also noted when fed phytase supplemented diet (Vielma et al., 2002). Positive effects of dietary phytase were also observed in channel catfish (Jackson et al., 1996), rainbow trout (Cain and Garling, 1995), striped bass (Papatryphon et al., 1999), Atlantic salmon (Sajjadi and Carter, 2004b) and tilapia (Portz and Liebert, 2004, Liebert and Portz, 2005, 2007, Cao et al., 2008). However, Ai et al. (2007) reported that dietary phytase (200 mg kg^{-1} diet) had no significant effect on Japanese seabass specific growth rate and feed efficiency ratio. Similarly, Vielma et al. (2000) reported non-effect of dietary phytase on weight of rainbow trout. The use of phytase as a feed additive can be limited by several factors like inactivation at high temperatures required for pelleting (>80°C), loss of activity during storage, narrow optimum pH range (Debnath et al., 2005), amount of substrates in the diet, enzyme dosage and activities.

Proteases are protein-digesting enzymes capable of breaking down storage proteins (proteins generated during seed production and capable of binding to starch) in vegetable proteins, thus improving protein digestibility. Exogenous proteases may augment endogenous peptidase production, increase protease activity and subsequently improve the digestibility of dietary protein in addition to degrading protein-based ANFs (lectins or trypsin inhibitors) leading to fast absorption and increased growth (Caine et al., 1998, Hammad, 2008, Isaksen et al., 2010). Exogenous protease is also capable of increasing accessibility of nutrients by breaking down and disrupting layer of complex proteins in plant cell wall.

Carbohydrases (NSPase) are enzymes that catalyse a reduction in molecular weight of polymeric carbohydrates. Examples of NSPase include α-amylase, β-mannanase, α-galactosidase, pectinase, xylanase, cellulase, β-glucanase, etc. Mode of action of NSPase

include hydrolysis of NSPs (component of plant cell wall), reduction of NSP-induced digesta viscosity and increase in digestibility of energy-yielding nutrients (Castillo and Gatlin, 2015). NSPase are capable of reducing detrimental effects of NSPs on endogenous digestive enzymes and hydrolyse the component plant cell wall to release otherwise unavailable nutrients such as starch, protein and fat (Chesson, 1993, Dudley-Cash, 1997). In fish, the presence of NSPase that hydrolyse the β-glycosidic bonds of NSP appears to be very low or non-existent (Krogdahl et al., 2005, NRC, 2011) hence the importance of exogenous NSPase. It has been demonstrated that pre-treatment of dietary plant materials with exogenous carbohydrases (α-amylase, β-glucanases and β-xylanases) enhances energy digestibility in fish by releasing more glucose, galactose and xylose (Kumar et al., 2006b). Some studies reported improved nutrient digestibility and reduced nutrient excretion in fish when fed NSPase supplemented diets (Stone et al., 2003, Lin et al., 2007). Xylanase has been reported to improve growth performance in juvenile Jian carp (Jiang et al., 2014), Japanese sea bass (Ai et al., 2007) and African catfish (Babalola, 2006). Dietary supplementation of NSPase has also been shown to increase amylase activity in the intestine of *Labeo rohita* (Kumar et al., 2006a). However, as in the case of effects of dietary supplementation of phytase, there are also inconsistencies in the reports on the effects of NSPase to enhance feeding value of plant materials for fish. Some studies reported non-effect of NSPase on fish growth performance (Ogunkoya et al., 2006, Farhangi and Carter, 2007, Dalsgaard et al., 2012).

Among the available exogenous digestive exogenous enzymes, phytase receives the most concerted research efforts (Table 1.5). From Table 1.5, the effects of exogenous digestive enzymes have been tested in various aquaculture species including Atlantic salmon, rainbow trout, common carp, channel catfish, Nile tilapia, striped bass, rohu, African catfish, etc. The reported effects of the exogenous enzymes on growth performance, feed

efficiency, minerals bioavailability, nutrients digestibility, endogenous enzymes activities and intestinal microbiota were mainly beneficial. However, some researchers reported non-effects of dietary supplementation of exogenous digestive enzymes (Vielma et al., 2000, Yan et al., 2002, Stone et al., 2003, Ogunkoya et al., 2006, Yigit and Olmez, 2011). The non-effects is not necessary due to dosage of the dietary exogenous digestive enzymes as beneficial effects of phytase have been reported at dosage as low as 250 – 500 U kg^{-1} diet in common carp, channel catfish and Nile tilapia (Schäfer et al., 1995, Jackson et al., 1996, Li and Robinson, 1997, Furuya et al., 2001). On the other hand, non-effects of phytase on growth performance and feed efficiency have been reported in hybrid tilapia, channel catfish and rainbow trout at dosage as high as 1,000 – 8,000 U kg^{-1} diet (Vielma et al., 2000, Yan et al., 2002, Hu et al., 2016). This implies that the impact and efficiency of exogenous digestive enzymes cannot be solely based on dosage.

The likely challenge of measuring the efficiency of enzymes *in vivo* include enzymes activities at different pH conditions, proteolytic degradation and thermal inactivation (Sinha et al., 2011) in addition to differences related to aquaculture species (warm water or cold water species), diets (substrates) composition and enzymes dosage used by different researchers (Encarnação, 2015). Also, it is important to note herewith that exogenous enzymes vary greatly in activity and efficacy which is mainly determined by the producers. Irrespective of the inconsistency in research findings on the use of exogenous digestive enzymes in fish, the use of exogenous enzymes is able to reduce fishmeal inclusion in aquafeeds (Wallace et al., 2016) with potential for more as the techniques are refined (Felix and Selvaraj, 2004). The use of exogenous enzymes in diets of non-ruminants (monogastric animals including farmed fish) will continue to be promising for a variety of reasons that hinge on sustainability, economics, and the environment (Adeola and Cowieson, 2011, Kumar et al., 2012b, Castillo and Gatlin,

2015). It can therefore be stated that the utility of exogenous digestive enzymes in plant-based fish diets is an emerging area with need for further investigations to establish the benefits of exogenous enzyme supplementation for fish under a variety of conditions.

Table 1.5: Effects of exogenous digestive enzymes on aquaculture species fed plant-based diets

Species	Exogenous enzymes	Dosage	Plant-based feedstuff	Effects	Reference
Atlantic salmon, *S. salar*	Phytase	2,000 U kg^{-1} diet	Canola meal	Enhanced growth, improved feed efficiency ratio and increased P availability	Sajjadi and Carter (2004a), (2004b)
Rainbow trout, *O. mykiss*	Phytase		Soybean meal	Better growth rate and feed conversion and increased P availability	Cain and Garling (1995)
Carp, *C. carpio*	Phytase	1,000 U kg^{-1} diet	Soybean meal	Enhanced weight gain and improved P digestibility	Schäfer et al. (1995)
Rainbow trout, *O. mykiss*	Phytase	1,400 U kg^{-1} diet	Soy concentrate and sunflower meal	Increased P availability	Dalsgaard et al. (2009)
Channel catfish, *Ictalurus punctatus*	Phytase	500 - 4,000 U kg^{-1} diet		Improved weight gain, feed consumption, FCR and bioavailability of phytate P	Jackson et al. (1996)
Atlantic salmon,	Phytase	5,000 U mL^{-1}	Soy-protein	Improved protein digestibility	Storebakken et al.

S. salar			concentrate	and FCR	(1998)
Tilapia, *O. niloticus*	Phytase	700 U kg^{-1} diet	Soybean meal, Canola meal, wheat middling and corn	Improved growth performance, protein digestibility, Ca and P bioavailability	Furuya et al. (2001)
Rainbow trout, *O. mykiss*	Phytase	4,500 U kg^{-1} diet	Canola protein concentrate	Improved bioavailability of phytate P	Forster et al. (1999)
Tilapia, *O. niloticus X O. aureus*	Phytase	1 g kg^{-1} diet (2,500 U g^{-1})	Soybean meal and wheat middling	Increased activity of endogenous amylase	Li et al. (2009)
Rainbow trout, *O. mykiss*	Phytase	1,000 U kg^{-1} diet	Soybean products	Improved P digestibility and utilisation	Rodehutscord and Pfeffer (1995)
Channel catfish, *I. punctatus*	Phytase	250 – 750 U kg^{-1} diet	Soybean meal, corn screenings and wheat middling	Improved weight gain, FCR and bioavailability of phytate P	Li and Robinson (1997)
Rainbow trout, *O. mykiss*	Phytase	1,000 U kg^{-1} diet	Soybean meal	Improved digestibility of P	Lanari et al. (1998)

Rainbow trout, *O. mykiss*	Phytase	1,200 U kg^{-1} diet	Soy-derived protein	No influence on weight gain and feed efficiency	Vielma et al. (2000)
Channel catfish, *I. punctatus*	Phytase	500 - 8,000 U kg^{-1} diet	Soybean meal, corn and wheat middling	No effect on weight gain, FCR and PER	Yan et al. (2002)
Striped bass, *Morone saxatilis*	Phytase	1,000 U kg^{-1} diet	Soybean meal, corn gluten meal and wheat middling	Improved growth and FCR	Papatryphon et al. (1999)
Rainbow trout, *O. mykiss*	Phytase		Soy proteins	Improved weight gain and feed efficiency	Vielma et al. (2002)
Tilapia, *O. niloticus*	Phytase	500 - 1,250 U kg^{-1} diet	Soybean meal, wheat gluten, corn and wheat	Improved growth, FCR, SGR, PER and bioavailability of phytate P	Portz and Liebert (2004), Liebert and Portz (2005), (2007), Cao et al. (2008)
Japanese seabass, *L.*	Multi-enzyme complex (phytase,	0.2 g kg^{-1} diet (2,500 U g^{-1} phytase), 0.4 g kg^{-1}	Soybean meal, rapeseed meal	Improved growth rate, feed efficiency, increased P and	Ai et al. (2007)

japonicas	glucanase, pentosanase, cellulase and xylanase)	diet (50 U g^{-1} glucanase, pentosanase & cellulase each), 0.8 g kg^{-1} (1,000 U g^{-1} xylanase)	and peanut meal	nitrogen retention	
Labeo rohita	α-amylase	50 mg kg^{-1} diet	Corn	Enhanced energy digestibility, increased intestinal amylase and protease activities	Kumar et al. (2006a)
Silver perch, *Bidyanus bidyanus*	Natustarch® (α-amylase)	50 – 150 mg kg^{-1} diet	Wheat and dehulled lupin	Increased energy digestibility	Stone et al. (2003)
Silver perch, *B. bidyanus*	Natugrain-blend® (β-glucanase and β-xylanase)	75 – 300 µL kg^{-1} diet	Wheat starch, wheat and dehulled lupin	No effect on dry matter, energy or protein digestibility	Stone et al. (2003)
Tilapia, *O. niloticus X O. aureus*	An enzymes complex containing neutral protease, β-glucanase and xylanase	1.5 g kg^{-1} diet	Soybean meal, rapeseed meal, cottonseed meal, wheat middling, wheat bran and	Improved growth performance and feed utilisation (SGR and FER)	Lin et al. (2007)

			corn grain		
Jian Carp, *C. carpio var. Jian*	Xylanase	1,259 U kg^{-1} diet	Soybean meal, rice gluten meal, rapeseed meal, cottonseed meal and wheat middling	Improved growth performance, intestinal enzyme activities and influence the balance of intestinal microflora	Jiang et al. (2014)
African catfish, *C. gariepinus*	Xylanase	0.1g kg^{-1} diet	Maize and soybean meal	Improved growth performance	Babalola (2006)
Rainbow trout, *O. mykiss*	Superzyme® CS (a multi carbohydrase)	2.5 g kg^{-1} diet	Soybean meal, corn gluten meal and wheat	No effect on growth performance	Ogunkoya et al. (2006)
Rainbow trout, *O. mykiss*	Energex™(a multi carbohydrase), Bio-Feed™ Pro (protease) and α-galactosidase	1,800 ppm (carbohydrase), 300 ppm (protease) & 3,000 ppm (α-galactosidase)	Dehulled lupin	Increased apparent nutrient digestibility and protein efficiency ratio but no effect on growth performance	Farhangi and Carter (2007)
Rainbow trout, *O. mykiss*	β-glucanase, xylanase and	67 mg kg^{-1} diet (β-glucanase), 208 mg kg^{-1}	Soybean meal, sunflower meal	Improved apparent nutrient digestibility of soybean meal by	Dalsgaard et al. (2012)

	protease	diet (xylanase) and 228 mg kg^{-1} diet (protease)	and rapeseed meal	β-glucanase and protease but no effect on growth performance	
Atlantic salmon, *S. salar*	Enzyme mix (trypsin, alkaline protease, acid protease, amyloglucosidase, amylase and cellulase)	1 mg kg^{-1} diet	Soybean meal	Higher feed intake, final weight and growth rate. Better feed efficiency and maintenance ratio.	Carter et al. (1994)
African catfish, *C. gariepinus*	Multi-enzyme complex Farmazyme® (xylanase, β-glucanase, β-amylase, cellulase and pectinase)	0.75 g kg^{-1} diet	Commercial trout diet (48% CP)	Improved growth rate, FCR and protein efficiency ratio	Yildirim and Turan (2010)
Tilapia, *O. niloticus*	Cellulase	1 – 5 g kg^{-1} diet	Canola meal	No effect on growth performance nor nutrient	Yigit and Olmez (2011)

				digestibility	
Great sturgeon, *Huso huso*	Multi-enzyme complex Kemin® (protease, phytase, xylanase, cellulase, pectinase, β-glucanase, α-amylase and lipase)	250 mg kg^{-1} diet		Improved weight gain, growth rate, FCR, higher content of n-3 essential fatty acids and lower n-6/n-3 fatty acids ratio	Ghomi et al. (2012)
Tilapia, *O. niloticus*	Enzyme cocktail (pepsin, papain and α-amylase)	0.64 g (pepsin), 1.28 g (papain) and α-amylase per 100g^{-1} diet	Corn gluten and soybean meal	Improved growth performance, feed utilization and enhanced hematological indices	Goda et al. (2012)
Grass carp, *C. idella*	Cellulase	3 g kg^{-1} diet (1 U mg^{-1})	Duckweed and wheat flour	Improved growth performance, increased endogenous digestive enzyme activities and changes in intestinal microbiota	Zhou et al. (2013)
Caspian salmon, *Salmo*	Multi-enzyme complex	0.5 g kg^{-1} diet each	Commercial trout diet (48% CP)	Improved growth performance and feed utilization but no	ali Zamini et al. (2014)

trutta caspius	Natuzyme® (protease, xylanase, cellulase, pectinase, β-glucanase, α-amylase, lipase, phytase and phosphatase) and Hemicell® (xylanase, cellulase, galactosidase and amylase)			effects on hematological indices	
Red hybrid tilapia (*Oreochromis sp.*)	Ronozyme VP (Hemicellulases and Pectinases), Allzyme Vegpro (protease, cellulase, xylanase, α-galactosidase and amylase) and mannanase	0.05 % (Ronozyme VP), 0.1 % (Allzyme Vegpro) and 0.01 % (mannase)	Palm kernel meal	Improved dry matter and energy digestibility coefficients but no effect on growth and feed utilization efficiency	Ng and Chong (2002)

Red hybrid tilapia (*Oreochromis sp.*)	Allzyme Vegpro (protease, cellulase, xylanase, α-galactosidase and amylase)	0.1 %	Palm kernel meal	Improved dry matter, protein, lipid and energy digestibilities as well as improved growth and feed utilization efficiency	Ng et al. (2002)
Hybrid tilapia (*O niloticus* ♀ X *Oreochromis aureus* ♂)	Phytase	1,000 U kg^{-1} diet	Rapeseed meal	No effect on weight gain and FCR	Hu et al. (2016)
Red tilapia (*O. niloticus* X *Oreochromis mossambicus*)	Phytase, xylanase	0.075 g kg^{-1} diet (phytase), 0.385 g kg^{-1} diet (xylanase)	Rice bran, soybean, maize and cassava meal	Improved growth performance, increased P digestibility, minerals uptake and 2% fishmeal reduction in red tilapia diet	Wallace et al. (2016)

1.11.1.2 Potential, modes of actions and effects of probiotics

According to Merrifield et al. (2010b), probiotics are microbial cells (provided through diet or rearing water) that are capable of impacting beneficial effect on the host fish, fish farmer or fish consumer which is impart attained through the improvement of the fish intestinal microbial balance. Probiotics are able to confer these beneficial effects through varied mechanisms of actions which include:

1. Competitive exclusion of pathogenic bacteria. This is a phenomenon whereby an established microflora prevents or reduces the colonisation of competing organisms for the same intestinal site. This is done by competition for attachment sites on the mucosa (space), nutrients, oxygen and production of inhibitory substances which prevent and/or destroy the competing pathogenic bacteria and hence reduce their colonisation,
2. Source of nutrients and enzymatic contribution to digestion. Probiotics could have direct involvement in nutrient uptake or provide nutrients or vitamins and consequently improve digestibility and weight gain of host fish,
3. Production of toxic (bactericidal) or inhibitory (bacteriostatic) substances towards other pathogenic organisms,
4. Enhancement of mucosa barrier function and immune response against pathogenic microorganisms by increasing the production of innate immune molecules. The normal microbiota in gastrointestinal (GI) ecosystem influences the innate immune system and such non-specific (innate) immune system can be stimulated by probiotics (Cerezuela et al., 2011, Pandiyan et al., 2013, Pérez-Sánchez et al., 2014).

Due to the aforementioned, probiotics are becoming an integral part of aquaculture practices and are now widely used to improve fish growth and disease resistance (Nayak,

2010). Studies have been carried out on effects of probiotics (Table 1.6). Probiotics are capable of improving growth, embryo and larval development (Avella et al., 2012), stress tolerance (Rollo et al., 2006), fecundity (Gioacchini et al., 2010, Giorgini et al., 2010, Gioacchini et al., 2011, Lombardo et al., 2011, Gioacchini et al., 2012, 2013), GI morphology and microbial balance (Lara-Flores et al., 2003, Carnevali et al., 2006, El-Haroun et al., 2006, Pirarat et al., 2006, Shelby et al., 2006, Taoka et al., 2006, Aly et al., 2008b, Wang et al., 2008, Standen et al., 2013, Standen et al., 2015). It is important to note that the impacts of probiotics on host organism depend on probiotics itself, dosage, treatment duration as well as route and frequency of delivery. In addition, for probiotics to exert effect on host organism, probiotics must be viable at their site of action. This implies that probiotics have to survive stressful feed processing and storage conditions. High temperature during feed production is one of the major drawbacks causing in-feed application of probiotics in aquaculture (Castex et al., 2014).

From Table 1.6, the *Bacillus* group appears to be the most studied probiotics. This could be due to the *Bacillus* group being saprophytic Gram-positive spore forming bacteria, an attribute that allow the probiotic to be heat-stable and withstand high temperature during feed production as well as gastric conditions (Hong and Cutting, 2005) compare to other bacteria. In addition, the *Bacillus* group have been reported to secret protease (Ray et al., 2012, Liu et al., 2016) which are required for protein digestion in host organisms and naturally produce different antibiotic compounds (Moriarty, 1998) which makes the *Bacillus* group antagonistic to fish pathogen. Among the parameters investigated using probiotics (*Bacillus* group mainly) in aquaculture, growth performance, survival, immune response and disease resistance were the most reported (Table 1.6).

Table 1.6: Examples of probiotic studies in tilapia

Probiotics	Dosage	Parameters investigated	Reference
Bacillus pumilus	$10^6 – 10^{12}$ cfu g^{-1}	DR, IR, GP, SR	Aly et al. (2008c)
Commercial product containing *B. subtilis*	0.1 – 0.2 %	BC, GP, IR, SR	Salem (2010)
B. subtilis	0.1 – 0.2 g L^{-1}	DR	Mohamed and Refat (2011)
Commercial product containing *B. subtilis*	0.5 – 2.5 %	BC, GP	El-Haroun et al. (2006)
B. coagulans, B. subtilis and *Rhodopseudomonas palustris* (rearing water additive)	1 x 10^7 cfu mL^{-1}	GP, IR, SR	Zhou et al. (2010a)
B. subtilis, *Lactobacillus plantarum* and *S. cerevisae*	10^7 cfu g^{-1} (*B. subtilis* and *L. plantarum*) and 10^4 cfu g^{-1} (*S. cerevisae*)	BC, D-EA, GP, SR	Essa et al. (2010)
B. subtilis, Lb. acidophilus, Clostridium butyricum and *S. cerevisiae*	1 %	DR, IR, STR	Taoka et al. (2006)

B. subtilis and *Lb. acidophilus*	0.5 – 1 x 10^7 cfu g^{-1}	DR, GP, IR, SR	Aly et al. (2008b)
B. pumilus, B. firmus and *Citrobacter freundii*	10^7 - 10^9 cfu g^{-1}	DR, PA	Aly et al. (2008a)
B. subtilis, B. licheniformis, E. faecium, P. acidilactici and *S. cerevisiae*	10^6 – 10^8 cfu g^{-1}	DR, GM, GP, IR, SR	Shelby et al. (2006)
B. amyloliquefaciens and *Lactobacillus* sp.	10^8 cfu g^{-1}	GM, GP, IR, SR	Ridha and Azad (2012)
Bacillus sp. and presumptive LAB	5 x 10^4 cfu g^{-1}	GP, PA, SR	Apún-Molina et al. (2009)
Pediococcus acidilactici	2.81 x 10^6 cfu g^{-1}	IM, GP, GM	Standen et al. (2013)
Commercial product containing *Lactobacillus reuteri, Bacillus subtilis, Enterococcus faecium* and *Pediococcus acidilactici*	0.5 %	GM, IH, GP, IR	Standen et al. (2015)

***Key:** DR - Disease Resistance, IR - Immune Response, GP - Growth Performance, SR – Survival, BC - Body Composition, D-EA – Digestive Enzyme Activities, STR - Stress, PA – Probiotic Activity, GM – Gut Microbiota, LAB – Lactic Acid Bacteria, IH – Intestinal Histology

1.11.2 Potential of synergistic effects of exogenous digestive enzymes and probiotics

It is well established that GI microbial communities are sensitive to rearing environment, seasonality and diet changes including the supplementation of probiotics (Dimitroglou et al., 2011, Merrifield et al., 2010a, Romero et al., 2014) and exogenous digestive enzymes (Bedford and Cowieson, 2012, Geraylou et al., 2012, Zhou et al., 2013, Jiang et al., 2014, Adeoye et al., 2016). Research into the use of exogenous digestive enzymes and probiotics is on the increase as aquafeed manufacturers are interested in producing 'functional and environmentally oriented aquafeed' at almost no extra cost. The potential effects of exogenous digestive enzymes and probiotics on fish have been reviewed (Section 1.11.1). However, the combined supplementation of exogenous enzymes and probiotics could result in complimentary modes of actions:

- ability to produce fibre-degrading enzymes by probiotics (Liu et al., 2016) may complement endogenous enzyme activity,
- exogenous digestive enzymes may increase availability of suitable substrate for probiotics as well as promote the growth of other beneficial bacteria in fish gut (Bedford and Cowieson, 2012).

Given the potential complimentary modes of action of exogenous digestive enzymes and probiotics, the two products could improve the growth performance and health status of farmed fish when fed diet supplemented with both the enzymes and probiotics as a cocktail. As the pressure grows on fish farmers to reduce production cost without compromising fish health and performance, dietary supplementation of exogenous digestive enzymes and probiotics (either separately or in combination) has potential to offer healthy nutrition and performance of farmed fish.

1.12 Thesis aims and objectives

The overall aim of this project is to investigate the effects of exogenous digestive enzymes (RONOZYME® Hiphos (phytase), RONOZYME® ProAct (protease), ROXAZYME® G2 (carbohydrase), RONOZYME® WX (xylanase)) and Sanolife PRO-F (probiotics) on tilapia production and health parameters. The specific objectives of the study include:

1. Investigation of effects of dietary exogenous digestive enzymes on tilapia growth and health (Chapter 3),
2. Investigation of effects of dietary exogenous digestive enzymes on tilapia fed practical diet (Chapter 4), and
3. Investigation of combined effects of dietary digestive exogenous enzymes and probiotics on tilapia growth and health (Chapter 5).

2 Chapter 2: General materials and methods

2.1 Overview

Feeding trials were carried out at University of Plymouth (UoP), UK (Aquatic Nutrition and Health Research Aquarium) and King Mongkut's Institute of Technology Ladkrabang (KMITL), Thailand (Animal Production Technology and Fisheries Department's holding tanks). Analytical procedures described in this chapter are generic to the experimental analysis except where otherwise stated in relevant chapters. All experimental work that involved the use of fish fully conformed to the UK Animals (Scientific Procedures) Act of 1986 (with the required project license # 30/2644 and personal license # 30/10510) and University of Plymouth Animal Welfare and Ethical Review Committee.

2.2 Experimental fish and husbandry

The experimental animals used in this study were all male tilapia (*Oreochromis niloticus*). The fish were obtained from North Moore Tilapia, Goxhill UK (Chapter 3B) and Charoen Pokphand (CP) Farm, Thailand (Chapters 4 and 5). The fish were acclimatized for a period of 4 weeks before grading and random distribution into tanks prior to commencement of the feeding trials. The trials were conducted in both recirculation (Chapter 3B) and flow-through (Chapters 4 and 5) aquaculture systems.

2.3 Feeding and weighing of fish

All fish in each tank were batch weighed at the commencement of the trials and fed 2 - 5% biomass each day in three equal rations (09.00 h, 13.00 h, and 17.00 h). Total fish in individual tanks were batch weighed each week throughout the duration of the trials and feeding rate adjusted each week to the fish biomass.

2.4 Growth, feed utilisation and somatic indices

Growth performance, feed utilisation and somatic indices were assessed by weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), condition factor (K), hepatosomatic index (HSI) and viscero-somatic index (VSI). Calculations were carried out using the following formulae:

$SGR = 100 ((\ln FW - \ln IW)/T);$

$FCR = FI/WG;$

$PER = WG/PI;$

$K = (100 \times FW)/ FL^3,$

$HSI = 100 (LW/ BW)$

$VSI = 100 (VW/ BW)$

Where: FW = final weight (g), IW = initial weight (g), T = duration of feeding (days), WG = wet weight gain (g), FI = feed intake (g), PI = protein ingested (g), FL = final length (cm), LW = Liver weight (g), VW = Visceral weight (g), BW = Body weight (g),

At the beginning of each trial, 16 fish were pooled to make four samples to determine carcass composition according to AOAC (1995) protocols, as described in Section 2.5.

2.5 Proximate composition analysis

For the determination of moisture content, samples were weighed into metal dishes and left uncovered in a drying oven (Genlab Ltd, UK) set to 115°C for 72 h, or until a constant weight was achieved. Samples were transferred to a desiccator to cool and re-weighed. Percentage moisture was determined using the formula:

Moisture (%) = ((wet wt. – dry wt.)/ (wet wt.)) X 100

For ash (total mineral or inorganic content) analysis, samples were weighed into porcelain crucibles and placed in a muffle furnace (Carbolite, Sheffield, UK) at 550°C for 8 h until a light grey ash resulted. After cooling in a desiccator, samples were re-weighed and the percentage of ash determined using the understated formula.

Ash (%) = ((Sample residue wt. – crucible wt.))/ (initial sample wt.)) X 100

The Soxhlet extraction method was used for lipid analysis. Samples were weighed into extraction thimbles, plugged with cotton wool and placed into a beaker, along with anti-bumping granules. One hundred and forty millilitres of petroleum ether was added and the beakers placed on the Soxtherm unit (Tecator Systems, Hognas, Sweden; model 1043 and service unit 1046), heated to 150°C for 30 min, rinsed for 45 min and left to evaporate. Beakers were left in a fume hood until all traces of solvent had dissolved and the beaker was weighed. The percentage of total lipids was determined using the formula:

Total lipid (%) = ((final wt. of beaker – initial wt. of beaker)/ (initial sample wt.)) X 100

The Kjeldahl method was used to determine the nitrogen content of the samples (diets and fish carcass). The crude protein content was determined by multiplying the nitrogen by a factor of 6.25 for animal proteins and 5.95 for proteins of plant origin. Samples were weighed and transferred to micro Kjeldahl tubes. Catalyst tablet (3 g K_2SO_4, 105 mg $CuSO_4$ and 105 mg TiO_2; BDH Ltd. Poole, UK) was added to each tube and 10 mL of

sulphuric acid (H_2SO_4) (Sp. Gr. 1.84, BDH Ltd. Poole, UK) added. Three samples of acetanilide standard were used (theoretical nitrogen content = 10.36%) which corrected for the efficiency of nitrogen extraction. Additionally, three samples of casein were used which validated nitrogen and protein content. The tubes were transferred and digestion of samples was performed with a Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) with the following protocol; 100 ºC for 30 min, 225 ºC for 45 min (1 h if samples had high lipid content) and 380 ºC for 1 h. Once digestion was completed and following a cooling period, the samples were distilled using a Vodapest 40 automatic distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany), the distillate was neutralised with concentrated H_2SO_4 and from the titration value, the crude protein value was determined using the following formula to obtain Nitrogen (%).

$$= \frac{(mLs\ Sample\ Titrant\ -\ mLs\ Blank\ Titrant)\ X\ (Acid\ Normality)\ X\ (mW\ of\ Nitorgen)}{Sample\ Weight}$$

A Bomb Calorimeter (Parr 1356, Parr Instruments Co, IL, USA) was used to measure the gross energy content. Samples were crafted into pellets in triplicate and placed inside a stainless steel container and filled with 30 bar (435 PSI) of oxygen. The sample was electronically ignited through a wired connection inside the decomposition vessel and burned. The heat created by the combustion process was transferred to the surrounding water jacket where it was detected. This information was then converted into the energy value of the sample. Before initiating the reaction, the sample weight was keyed into the calorimeter for determination of MJ gross energy per kg as calculated by the calorimeter algorithm.

2.6 Mineral analysis

A solid nitric acid digestion procedure was utilised to completely transfer the fish tissues into solution, after which the mineral content was determined. The samples were analysed by dry weight. Prior to digestion, the samples were homogenised using a grinder. 100 - 250 mg of dried homogenised sample was weighed into a prepared boiling tube. 10 mL nitric acid (70% ANALAR grade) was added and digested in Kjeldatherm block (Gerhardt laboratory Instruments, Bonn, Germany) using 60°C (1 h), 90°C (1 h), 110°C (30 min) and 135 - 140°C (up to 4 h) temperature regime until the digest turned colourless. The digests were allowed to cool and diluted to 50 mL with ultra-pure Milli-Q water (Millipore Corp, MA, USA). Stock solutions of 100 mg L^{-1} (QC 26 from CPI International) containing 26 elements and 10 g L^{-1} (from Fisher Scientific) containing phosphorus were used to prepare standards by serial dilution. Concentration of each mineral in digests was determined using an ICP-OES instrument (iCAP 7400, Thermo Scientific) and an Inductively Coupled Plasma Mass Spectrometry, ICP-MS (Thermo Scientific, X JSeries 2, Hemel Hempstead, UK), against an external calibration and concentration in the original samples. The concentration was calculated as follows:

$$\frac{The\ ICP\ result\ (mgL^{-1}\ or\ \mu gL^{-1}) X\ Volume\ of\ diluted\ sample\ (mL)}{Weight\ of\ sample\ used\ (g)}$$

The results were expressed in mg kg^{-1} or µg kg^{-1}

2.7 Haemato – immunological analysis

2.7.1 Haematocrit

Haematocrit (packed cell volume) of whole blood was assessed in triplicate using the microhaematocrit method (Brown, 1980). Whole blood was drawn up into heparinised

capillary tubes until they were approximately two thirds full and the tubes sealed with clay. The tubes were centrifuged at 10,500 g for 5 min. Haematocrit values were read from a microhaematocrit reader and recorded as percentage packed cell volume (%PCV).

2.7.2 Haemoglobin

Haemoglobin was determined using Drabkin's cyanide-ferricyanide solution (Sigma-Aldrich Ltd, Dorset, UK). Four microliters of whole blood was mixed with 1 mL Drabkin's cyanide-ferricyanide solution (1/250 dilution factor) and measured after 5 min of incubation using a spectrophotometer set to 540nm wavelength. The original Drabkin's solution was used as blank. The haemoglobin levels (g dL^{-1}) were determined against a standard curve of haemoglobin porcine lyophilized powder (Sigma-Aldrich Ltd. Poole, UK) and calculated using the following formula:

Haemoglobin concentration (g dL^{-1}) =

$$\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard}\ X\ Dilution\ factor$$

2.7.3 Erythrocyte and leucocyte enumeration

Enumeration of erythrocytes and leucocytes was conducted as described by Dacie and Lewis (1975). Dacies solution was made up using 2 mL formaldehyde, 6.26 g tri-sodium citrate, 200 mg brilliant cresol blue (Sigma-Aldrich Ltd, Dorset, UK) and made up to 200 mL with distilled water. Twenty microliters of whole blood was mixed with 980 µL of Dacies solution (1/50 dilution factor), mixed for 60 seconds to ensure a homogenous solution. A 5 µL of the homogenous solution was aliquoted to haemacytometer and minimum of 500 cells counted for a statistically valid data.

2.7.4 Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV)

From total red blood cells count, haematocrit and haemoglobin concentrations, levels of fish whole blood mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV) were calculated using the following formula:

MCH (pg) =

$$\frac{Haemoglobin\ concentration\ (g\ dL^{-1})\ X\ 10}{Total\ red\ blood\ cell\ count\ (10^6 mm^3)}$$

MCHC (g dL^{-1}) =

$$\frac{Haemoglobin\ concentration\ (g\ dL^{-1})}{Haematocrit\ concentration\ (L\ L^{-1}\ )}$$

MCV (fL) =

$$\frac{Haematocrit\ concentration\ (L\ L^{-1}\ )\ X\ 100}{Total\ red\ blood\ cell\ count\ (10^6 mm^3)}$$

2.7.5 Leucocyte differential count

Five microliters of the whole blood was smeared unto frosted microscope slides to quantify circulatory levels of lymphocytes, granulocytes and monocytes. The smears were air-dried, fixed in methanol for 15 min and stained using May Grünwald stain (diluted 1:1 with Sorensen's buffer, pH 6.8). Slides were then rinsed in Sorensen's buffer and counter stained with Giemsa stain (diluted 1:9 with Sorensen's buffer, pH 6.8). After a final rinse in buffer, slides were left to dry. Once dried, the slides were mounted in DPX (BDH Laboratory supplies, Poole, UK). Lymphocytes, granulocytes and monocytes were identified as described by Rowley (1990), (see Figure 2.1 for examples). A minimum of

200 cells per sample were counted and the values expressed as percentage of the total leukocytes.

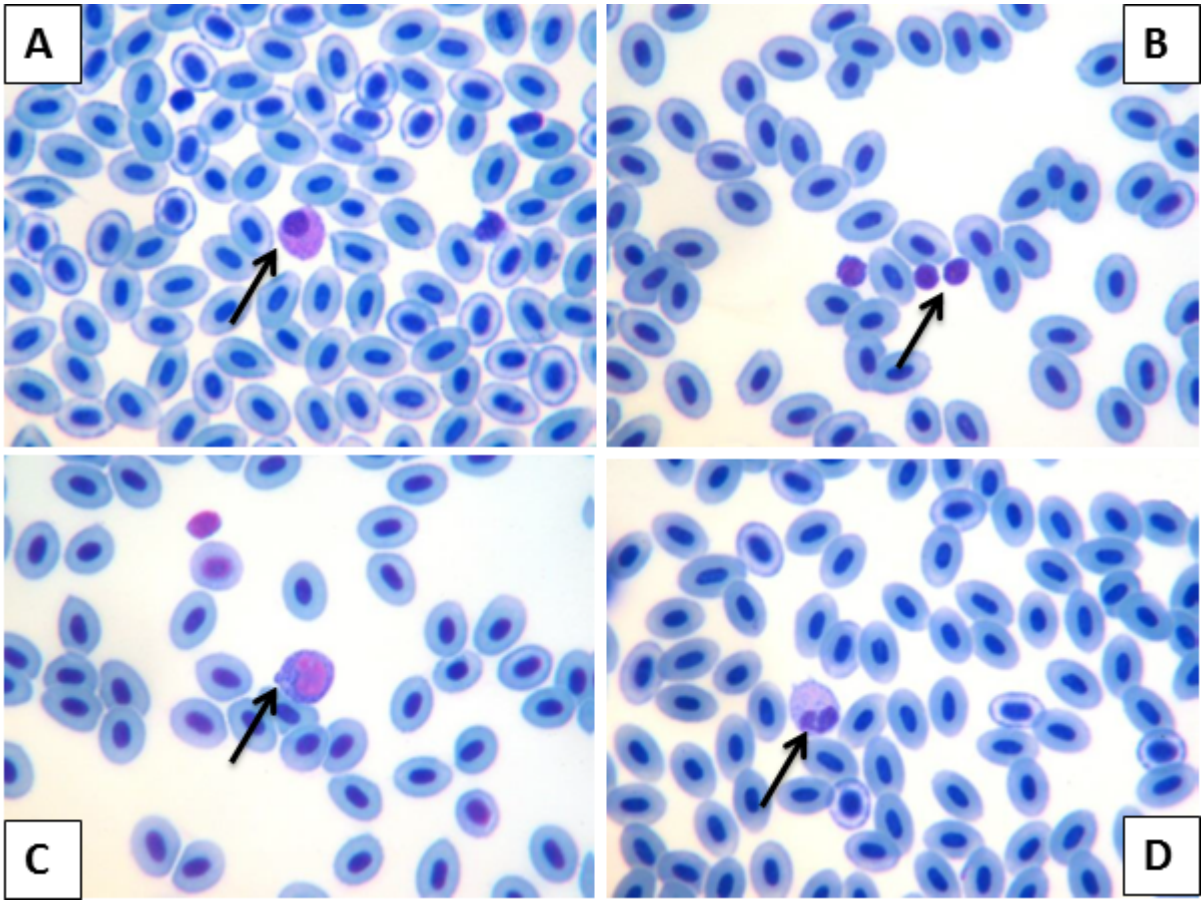

Figure 2.1: Differential leucocyte cell types (Rowley, 1990). Arrowed cells are basophilic granulocytes (A), lymphocytes (B), monocytes (C) and neutrophilic granulocytes (D).

2.7.6 Serum lysozyme activity

Serum lysozyme activity was analysed as described by Ellis (1990). Five hundred microliters of whole blood (without anticoagulant) was transferred into 1.5 mL microcentrifuge tube, placed in refrigerator overnight prior to centrifugation at 2500 g for 5 min. The serum supernatant was pipetted into new tube and stored at -20 °C until used. The lysozyme activity was determined using a turbometric assay in a 96-well microplate.

One hundred and ninety microliters of *Micrococcus lysodeikticus* (0.2 mg mL^{-1}) in 0.04 M NA_2HPO_4 buffer (pH 6.3 for tilapia) was pipetted into different 96-well microplate wells. Two columns of wells, each containing 200 µL of 0.04 M NA_2HPO_4 (without bacteria) were used as control. Ten microliters of serum was added to each of the *M. lysodeikticus*-containing wells, mixed and reduction in turbidity measured at 540 nm at 0.5 min and 4.5 min at 22 °C in a microplate reader (Optimax Tuneable Microplate Reader, Molecular Devices, CA, USA). A unit of lysozyme activity (U) was defined as the amount of serum causing a decrease in absorbance of 0.001 min^{-1}.

2.8 Digestive enzyme activities

Three fish from each tank (n = 9 per treatment) were dissected on ice 2 h after being fed to satiation. Digesta from the fish anterior intestine was obtained by gently squeezing the section with a forceps into individual 2 mL cryovials and immediately freeze in liquid nitrogen. Enzymes were extracted from the frozen samples after the samples were homogenised separately in a sonicator. The homogenates were centrifuged at 16,000 g for 15 min at 4 °C (details described in Section 7.1). The supernatant (enzyme extracts) were removed, aliquoted (30 - 50µL) into centrifuge tubes and stored at -20°C until further analysis.

2.8.1 Amylase

Amylase activity was assayed using ethylidene-*p*NP-G7 as substrate and the activity of enzyme extract (1:10 diluted with milli-Q water) determined using Amylase Activity Assay Kit (MAK009, Sigma Aldrich) according to manufacturer's instruction. The enzymatic release of ethylidene-*p*NP-G7 was measured at 405 nm in microplate reader (OPTImax microplate reader, Molecular Devices LLC), and one unit of enzyme activity

(U) was defined as the amount of enzyme that releases 1 μmol ethylidene-*p*NP-G7 per min at 25 °C.

2.8.2 Trypsin

Trypsin activity was assayed using 0.5 mM Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) (Sigma B4875) as substrate according to Erlanger et al. (1961). BAPNA substrate was prepared by dissolving 21.75 mg in 1 mL of dimethylsulphide (DMSO) to obtain 0.5 mM of the substrate solution. One hundred microliters of the BAPNA substrate was made up to 10 mL with 50 mM Tris-HCl buffer, pH 8.5 containing 20mM $CaCl_2$ to allow substrate solubilisation. Triplicate of 10 μL of enzyme extract (1:10 diluted with milli-Q water) were added to 190 μL of BAPNA substrate, pre-agitated for 20 seconds before read for a total time of 10 min at 20 seconds interval in microplate reader (OPTImax microplate reader, Molecular Devices LLC). Milli-Q water was used as blank. The enzymatic release of *p*-nitroanilide (*p*NA) was measured at 405 nm in microplate reader (OPTImax microplate reader, Molecular Devices LLC), and one unit of enzyme activity (U) was defined as the amount of enzyme that releases 1 μmol *p*NA per min, using 8,800 M cm^{-1} as extinction coefficient.

2.8.3 Chymotrypsin

Chymotrypsin activity was assayed using 0.2 mM N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (SAPNA) (Sigma S7388) as substrate according to DelMar et al. (1979). SAPNA substrate was prepared by dissolving 12.49 mg of SAPNA in 1 mL of DMSO to give a final concentration of 0.2 mM. One hundred microliters of the SAPNA substrate was made up to 10 mL with 50 mM Tris-HCl buffer (pH 8.5) containing 20mM $CaCl_2$ to allow substrate solubilisation. Triplicate of 10 μL of enzyme extract (1:10 diluted with milli-Q water) were added to 190 μL of SAPNA substrate, pre-agitated for 20 seconds before read for a total time of 10 min at 20 seconds interval in microplate reader

(OPTImax microplate reader, Molecular Devices LLC). Milli-Q water was used as blank. The enzymatic release of *p*-nitroanilide (*p*NA) was measured at 405 nm in microplate reader (OPTImax microplate reader, Molecular Devices LLC), and one unit of enzyme activity (U) was defined as the amount of enzyme that releases 1 μmol *p*NA per min, using 8800 M cm^{-1} as extinction coefficient.

2.8.4 Total alkaline protease

Total alkaline protease activity was measured according to procedures described by Alarcón et al. (1998) using 1% (w/v) azocasein in 50 mM Tris–HCl (pH 9.0) as substrate. One microliter of substrate solution was mixed with 100 μL of enzyme extract (1:10 diluted with milli-Q water) in 2 mL centrifuge tube and incubated for 30 min at 37 °C in a water bath. The enzymatic reaction was stopped by addition of 500 μL of tricholoroacetic acid, (TCA) and the mixture cooled for 15 min at -20 °C. Samples were centrifuged at 16,000 g for 15 min at 4 °C and supernatant absorbance measured at 366 nm in a spectrophotometer (Jenway). Blanks were prepared by adding TCA before enzyme extract. One unit of total protease activity was defined as the amount of enzyme that release 1 μg of tyrosine per min in the reaction mixture (extinction coefficient for tyrosine = 0.008 μg^{-1} mL^{-1} cm^{-1}).

2.9 Histological appraisal of mid-intestine

Samples for histological examination were taken from fish (n = 9 per treatment) deprived of feed for 24 h after the feeding trials. Fish were dissected and intestinal samples taken from mid intestine.

2.9.1 Light microscopy

Tissue samples from the fish mid-intestine (~1cm length) were fixed in 4% formalin and transferred to 70% ethanol after 24 h. Samples were then dehydrated in graded ethanol concentrations (50%, 70%, 90% and 100%) and cleared in three changes of xylene (1 h for each change) in an automated tissue processor (Leica TP1020, Germany) according to standard histological protocols prior to embedding in paraffin wax (Leica EG1150 H). From each wax block, multiple sections (5µm thick) were cut with a microtome (Leica RM2235, Germany) and placed in 50 °C for 2 min and mounted onto glass slides. Using a microsystem autostainer (Leica XL, Germany), the sections were stained with histolene and rehydrated in a series of graded ethanol concentrations. Multiple sets of sections were stained with haematoxylin and eosin (H & E) or Alcian Blue-Periodic Acid-Schiff (AB-PAS) and dehydrated once again before the sections were cleared in histolene. The stained sections were mounted with 22 mm^2 coverslips using a polystyrene resin dissolved in xylene (DPX). Slides were examined under light microscope and images captured with a digital camera (Leica DMD108). The images were analysed with ImageJ version 1.47 (National Institutes of Health, USA) to assess intestinal perimeter ratio (PR), intra epithelial leukocytes (IELs) and goblet cell abundance. PR was calculated as the ratio between the internal perimeter (IP) of the intestinal lumen (villi and mucosal folding length) and the external perimeter (EP) of the intestine (PR = IP/ EP, arbitrary units, AU), Figure 2.2. A high PR value indicates high villi length, increased mucosal folding or both (Dimitroglou et al., 2009). The IELs and goblet cell abundance were analysed across a standardized distance of 100 µm and the number of the cells were calculated by averaging the cell numbers from all replicates (Ferguson et al., 2010).

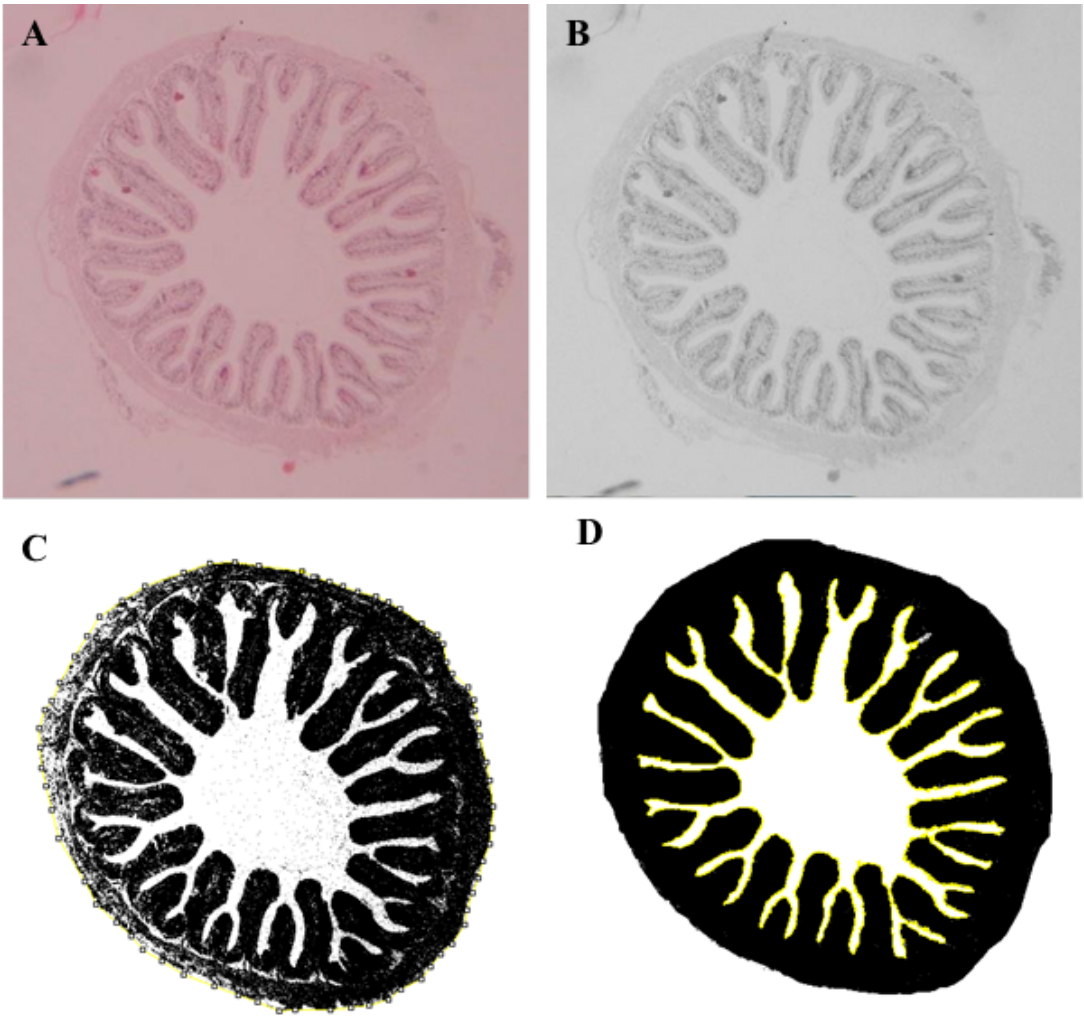

Figure 2.2: Procedure for measuring intestinal perimeter ratio. Images of transverse cross sections are loaded into Image J (A), transformed to 8-bit (B) and the threshold function applied to obtain a black and white image (C). Image was adjusted to account for sectioning artefacts (D) and both the lumen and external perimeter ratio measured (yellow)

2.9.2 Scanning electron microscope

Tissue samples from the fish mid-intestine (~2 mm length) were excised and rinsed in 1% S-carboxymethyl-L-cysteine for 30 seconds to remove epithelial mucus. Then, the samples were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (1:1 vol., pH 7.2, 3% NaCl) for 1 h before being rinsed twice in buffer for 15 min (to get rid of the fixative). Tissues were dehydrated in increasing alcohol concentrations (30%, 50%, 70%, and 90%) with each rinse lasting 15 min in each concentration and twice in 100% concentration. Samples were transferred to bombs thereafter for critical point drying (Emitech K850, UK) using ethanol as the intermediate fluid and liquid CO_2 as the transmission fluid. Samples were placed on stubs and gold sputter coated (Emitech K550) and screened with a Jeol 6610 LV electron microscope at 15kV (Jeol, Tokyo, Japan). Three images were analysed blindly with ImageJ version 1.47 (National Institutes of Health, USA) to assess microvilli density (MD), microvilli count per μm2 (MCVT) and enterocyte apical area, μm^2 (EAA). A threshold technique for images was used to differentiate the ratio between the microvilli covered area (M, foreground) to the background (B, background), MD = M/ B (arbitrary units, AU). MCVT and EAA were determined as shown in Figure 2.3.

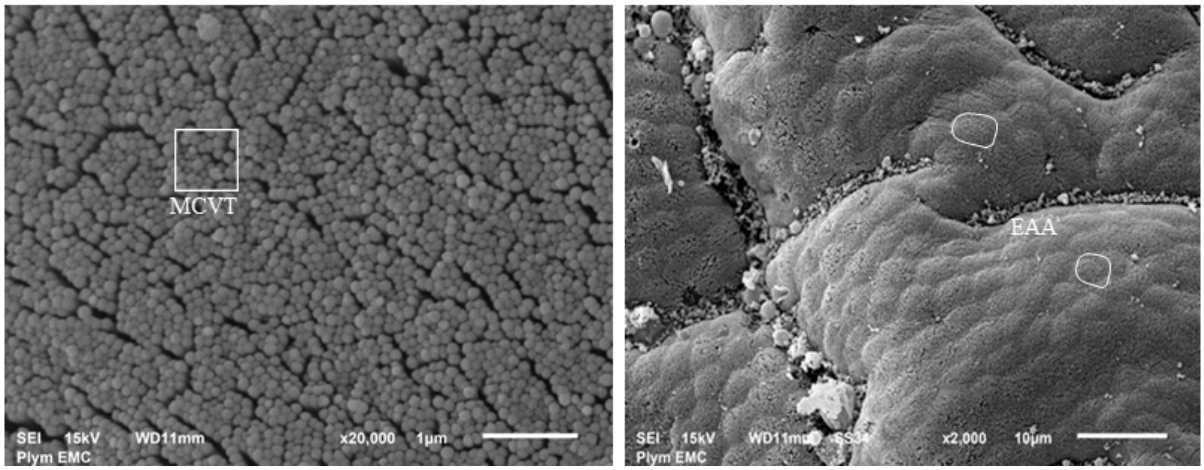

Figure 2.3: High and low magnification SEM pictures of tilapia mid-intestine assessed for microvilli count (density per µm2), MCVT (number μm^{-2}) and enterocyte apical area, EAA (μm^2)

2.9.3 Transmission electron microscopy

Tissue samples from the fish mid-intestine were excised and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (1:1 vol., pH 7.2, 3% NaCl) for 1 h before rinsed twice in buffer for 15 min (to remove the fixative). The tissue was then secondary fixed with 1% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h. After rinsing twice with buffer, the tissues were dehydrated through increasing alcohol concentrations (30%, 50%, 70%, and 90%) until 100% with each rinse lasting 15 min. The alcohol was replaced by Agar low viscosity resin by placing it in increasing concentrations of resin (30% resin: 70% ethanol, 50:50, 70:30) with 12 h for each concentration until samples were in 100% resin. The samples were then placed in Beem capsules and embedded at 60°C overnight ready for sectioning. Semi-thin sections (0.5 µm) were cut with a glass knife, placed on slide and stained with methylene blue for first examination under light microscope. From each block, ultra-thin sections (~90 nm) were cut using a diamond knife. The sections were mounted on copper grids and stained with a

saturated uranyl acetate solution for 30 min, washed thoroughly with distilled water for 15 min and post-stained with Reynolds lead citrate for 15 min. Final examination of the ultrathin sections was made on a Jeol 1200 EX II transmission electron microscope (Jeol, Tokyo, Japan). The images were analysed with ImageJ 1.47 for microvilli length and diameter. Ten well oriented individual microvilli were calculated per image, three images per sample.

2.10 Cost benefit analysis

Cost benefit analysis was estimated using incidence cost and profit index (El ‐ Dakar et al., 2007).

$$\text{Incidence cost} = \frac{Cost\ of\ feed\ consumed}{Quantity\ of\ fish\ produced}$$

$$\text{Profit index} = \frac{Local\ market\ value\ of\ fish}{Cost\ of\ feed\ consumed}$$

2.11 Statistical analysis

All statistical analysis was carried out using SPSS for Windows (SPSS Inc., 22.0, Chicago, IL, USA). Unless otherwise stated, all data were reported as mean ± standard deviation. All data were checked for normality and equality of variance using Kolmogorov-Smirnov and Bartlett's test, respectively. Where normalilty assumptions were met, data were analysed using one-way analysis of variance (ANOVA) followed by a post-hoc Duncan test to determine significant differences. Where data violated these conditions, a Kruskal- Wallis test was used on log transformed data. Differences between treatments were then determined using a Mann-Whitney U-test. All percentage data were

transformed using arcsine function prior to statistical analysis. In all cases significance was accepted at $P < 0.05$.

3 Chapter 3. *In vitro* and *in vivo* assessment of exogenous digestive enzymes

3.1 Chapter 3A. Rapid assessment of exogenous enzyme supplementation on diets using an *in vitro* digestion technique

Abstract

A study was carried out to obtain a rapid assessment of nutritional quality of diets supplemented with exogenous digestive enzymes using an *in vitro* digestion technique. The *in vitro* digestion technique consisted of 2-step digestion at 28 °C. Step 1 - a 4 h digestion of the experimental diets with pepsin at pH 2.5. Step 2 – a 14 h digestion with porcine pancreatin at pH 7.5. The undigested materials (residues) were recovered by centrifugation, oven dried at 105 °C and analysed for chemical composition. The digestion coefficients (DCs), digestible nutrients (DNs) and total digestible nutrients (TDNs) were calculated. DCs of dry matter and energy are higher ($P < 0.05$) in phytase and carbohydrase supplemented diets compared to that of a control and protease supplemented diets. DC of crude protein was highest ($P < 0.05$) in phytase supplemented diet and least ($P < 0.05$) in protease supplemented diet. However, the DC of lipid was highest ($P < 0.05$) in the protease supplemented diet. The DC of ash was significantly higher ($P < 0.05$) in the phytase supplemented diet than in the remaining three experimental diets. The DCs translated to DNs of the experimental diets in a similar trend. The TDNs of the three enzyme supplemented diets ($P < 0.05$) were higher than TDN of the control diet. Going by the DCs and TDNs, the exogenous digestive enzymes seem to

show potential for use in aquafeed and thus should be tested *in vivo* with the appropriate fish species.

3.1.1 Introduction

The search for appropriate alternate ingredients and complimentary feed additives (especially exogenous digestive enzymes) to support the growth of aquaculture production has received concerted research efforts as reviewed by Kumar et al. (2012b), Castillo and Gatlin (2015) and in Section 1.7. To determine the suitability of exogenous digestive enzymes for fish health and production, there is a need for reliable nutrient digestibility information. Information on nutrients digestibility of ingredient is an important pre-requisite in predicting the efficacy of exogenous digestive enzymes as well as the appropriateness and nutritional quality of the ingredients (as viable alternatives to finite marine resources). Plant ingredients (promising alternate ingredients to marine resources) are primarily being assessed for their appropriateness (nutrient digestibility and efficiency) through mostly *in vivo* feeding trials. However, *in vivo* feeding trials can be quite expensive in terms of elaborate construction, operation and maintenance dedicated to aquaculture systems. The trials could require large quantities of experimental diets, animals and qualified personnel for husbandry and animal welfare when running the experiments. In addition, *in vivo* feeding trials could be time-consuming and are often associated with laborious activities and associated-animal stress. These raise public concerns and are sometimes characterised by ethical difficulties. There is need for a quick and easy laboratory method capable of assessing suitability of alternate ingredients and feed additives.

In vitro digestion techniques offer an alternative approach for rapid assessment of ingredients and additives without having to use animals. It has a considerable advantage over *in vivo* feeding trials. It is simple, rapid, reproducible, economical and complies with the 3Rs principle (refinement, reduction and replacement) and supports strong ethical

justification for the use of animals in subsequent research if appropriate (Festing and Altman, 2002).

In vitro methods for estimating digestibility in monogastric animals include dialysis cell; pH-drop method and pH-stat; colorimetric; and filtration methods (Boisen and Eggum, 1991, Moyano et al., 2014). The filtration method can be further sub-divided into single or multi-enzyme filtration. Among the *in vitro* methods for estimating digestibility, only the multi-enzyme filtration technique is capable of estimating digestibility for more than one nutrient (Cronjd and Mackie, 1983, Boisen and Eggum, 1991, Moyano et al., 2014). This is because a suitable and accurate modelling of complex physiological transformations present in fish gut can be partly achieved by simulating every phase of digestion (two steps of hydrolysis at least) using suitable pH, enzyme concentrations and reaction time (Moyano and Savoie, 2001, Morales and Moyano, 2010, Guerra et al., 2012). Pascual et al. (2010) compared three different *in vitro* digestibility methods for nutritive evaluation of rabbit diets, they concluded that a multi-enzyme method was significantly better ($P < 0.05$) in terms of prediction, precision, lower variability, repeatability and reliability. Similarly, a high correlation was found between this multi-enzyme *in vitro* method and the standard *in vivo* procedures for seven diets commonly used for growing or adult pigs (Furuya et al., 1979). These findings emphasize the relevance and usefulness of this technique for obtaining rapid estimation of the digestibility of enzyme-supplemented-diets for tilapia.

The objective of the current study was to provide a rapid preliminary assessment of the potential and efficacy of exogenous digestive enzymes as feed additives in tilapia diets for improving nutrient digestibility.

3.1.2 Materials and methods

3.1.2.1 Diets preparation

Four iso-nitrogenous and iso-lipidic diets were formulated (Table 3.1) using Feedsoft® software and were used to study the *in vitro* digestion of the diets supplemented with exogenous digestive enzymes. The three exogenous digestive enzymes were RONOZYME® Hiphos (phytase), RONOZYME® ProAct (protease), and ROXAZYME® G2 (carbohydrase) from DSM Nutritional Products. Three of the formulated diets were supplemented with the exogenous enzymes (phytase, protease, and carbohydrase at 0.3 g kg^{-1}, 0.2 g kg^{-1} and 0.3 g kg^{-1}, respectively) at the expense of corn starch and the basal diet served as control diet. The feed ingredients were thoroughly mixed, moistened with warm water (400 mL kg^{-1}) and then cold press extruded to produce 2 mm pellets using a PTM extruder system (model P6, Plymouth, UK). The diets were dried to ca. 5% moisture in an air convection oven set at 45°C and their proximate composition analysed (Table 3.1) as described in Section 2.5. After drying, the diets were stored in airtight containers prior to use. Prior to the *in vitro* digestion, the diets were milled to ensure homogeneity and increase surface area to simulate the condition in fish GI tract.

Table 3.1: Formulation and composition of the experimental diets

Ingredients (g kg^{-1})	**Control**	**Phytase**	**Protease**	**Carbohydrase**
Soybean protein meal[a]	353.00	353.00	353.00	353.00
Narrow-leafed lupin meal[b]	250.00	250.00	250.00	250.00
Corn starch[c]	209.98	209.68	209.78	209.68
Herring meal LT94[d]	100.00	100.00	100.00	100.00
Corn oil	21.70	21.70	21.70	21.70
Fish oil	20.00	20.00	20.00	20.00
Lysamine pea protein concentrate[e]	20.00	20.00	20.00	20.00
Vitamin & mineral premix[f]	20.00	20.00	20.00	20.00
CMC-binder[c]	5.00	5.00	5.00	5.00
Phytase[g]	0.00	0.30	0.00	0.00
Protease[h]	0.00	0.00	0.20	0.00
Carbohydrase[i]	0.00	0.00	0.00	0.30
BHT[f]	0.075	0.075	0.075	0.075
Ethoxyquin[f]	0.0075	0.0075	0.0075	0.0075
Alpha tocopherols[f]	0.20	0.20	0.20	0.20
Composition (g kg^{-1})				
Moisture	7.04	7.43	6.49	5.98
*Crude protein	40.63	40.86	40.65	41.01
*Lipid	7.77	7.49	8.24	7.85
*Ash	6.35	6.48	6.50	6.46
Energy, MJ kg^{-1}	19.18	19.18	19.10	19.34
*NFE[j]	19.03	18.57	19.03	19.36

[a]Hamlet HP100, Hamlet Protein, Denmark.

[b]Soya UK

[c]Sigma-Aldrich Ltd., UK.

[d]Herring meal LT94 – United Fish Products Ltd., Aberdeen, UK.

[e]Roquette Frêres, France.

[f] Premier Nutrition Products vitamin/mineral premix contains: 121 g kg^{-1} calcium, Vit A 1.0 µg kg^{-1}, Vit. D3 0.1 µg kg^{-1}, Vit E (as alpha tocopherol acetate) 7.0 g kg^{-1}, Copper (as cupric sulphate) 250 mg kg^{-1}, Magnesium 15.6 g kg^{-1}, Phosphorus 5.2 g kg^{-1}

[g] RONOZYME® Hiphos (contains 10,000FYT g^{-1}) from DSM Nutritional Products

[h] RONOZYME® ProAct (contains 75,000 PROT g^{-1}) from DSM Nutritional Products

[i] ROXAZYME® G2 (contains 2700U g^{-1} xylanase, 700U g^{-1} β-glucanase and 800U g^{-1} cellulose) from DSM Nutritional Products

[j]Nitrogen - free extracts (NFE) = dry matter – (crude protein + crude lipid + ash)

*composition on dry weight basis

3.1.2.2 In vitro digestion technique

The *in vitro* digestion technique was based on the principle of enzymatic incubation of the formulated diets in a closed system followed by measurement of the indigestible material (residue) collected after filtration (Boisen and Eggum, 1991, Moyano and Savoie, 2001, Morales and Moyano, 2010). The closed system simulated the tilapia digestive process (GI tract conditions) with two successive incubation phases; short gastric digestion in an acidic environment and long intestinal digestion in an alkaline environment (Figure 3.1). In the gastric digestion phase, hydrolysis of protein was initiated by action of pepsin (P7000 from Sigma Aldrich, UK) and hydrochloric acid in the stomach (Nagase, 1964, Bowen, 1982). In the intestinal digestion phase, further hydrolysis of starch, fat and peptides was initiated by amylase, lipase and pancreatic protease (pancreatin-P1750, Sigma Aldrich, UK) in alkaline condition (Fish, 1960, Nagase, 1964). The duration of the incubation corresponds to digesta and GI tract transit time in tilapia (Riche et al., 2004, Heng et al., 2007, Uscanga et al., 2010, Hlophe and Moyo, 2011, Ray and Ringø, 2014).

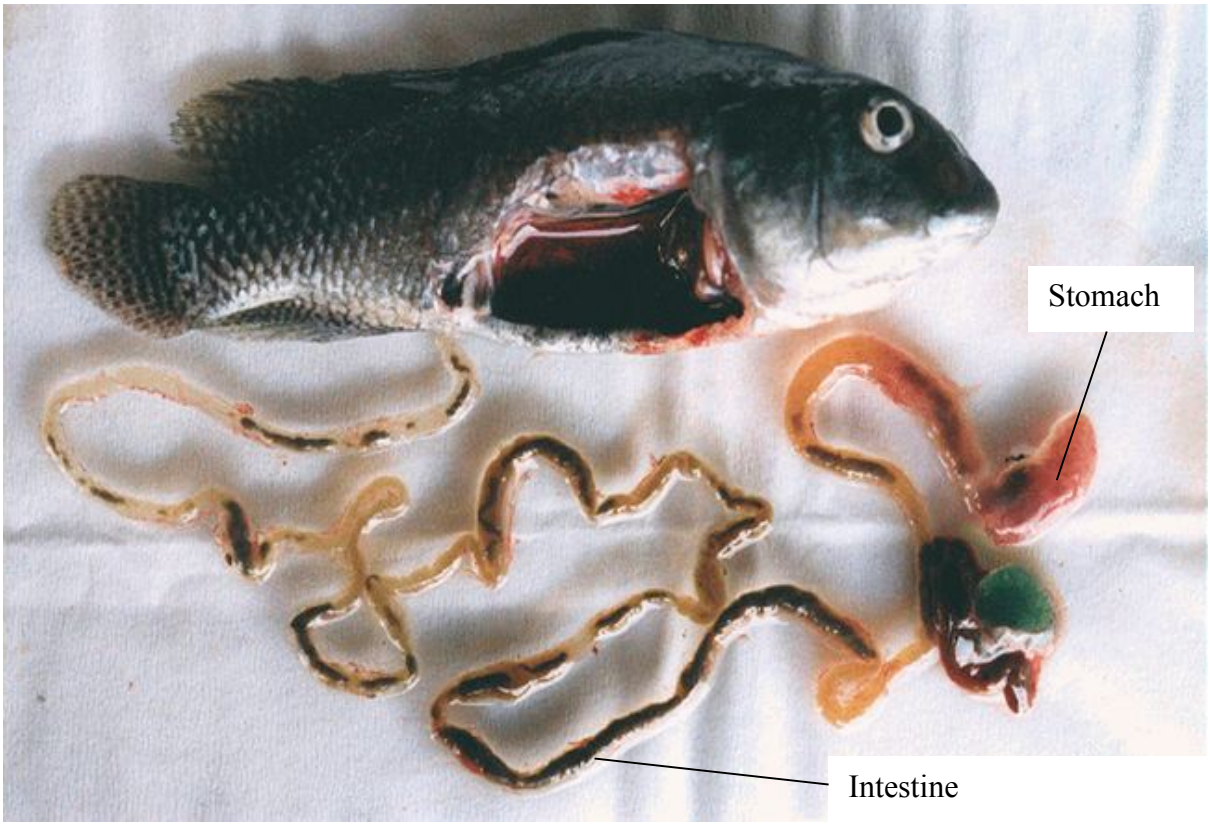

Figure 3.1: Tilapia GI tract displaying stomach (acidic pH) and long intestine (alkaline pH) (source: www.arkive.org)

A 5 g sample of each diet (Table 3.1) was placed into a digestion bottle containing 50 mL pepsin solution (1 mg pepsin mL^{-1} citrate-phosphate buffer at pH 2.5). Incubation at 28 °C was conducted in a shaking water bath for 4 h. After 4 h, the incubation bottles were centrifuged at 1200 g for 10 min and the supernatant carefully decanted. The incubation continued in 50 mL pancreatin solution (4 mg mL^{-1} citrate phosphate buffer at pH 7.5) in a shaking water bath at 28 °C for another 14 h. The incubation bottles were manually shaken occasionally during the incubation period.

After incubation, 5 mL of 40% sulphosalicylic acid was added to the incubation bottles and allowed to stand for 30 min. After 30 min, the incubation bottles were centrifuged at

1200 g and the supernatant carefully decanted. Undigested materials (residues) were collected after centrifugation and filtered through a pre-weighed 0.7µ filter paper.

The residues were dried at 105 °C for 24 h and digestibility of dry matter, ash, nitrogen and energy were measured from chemical analysis (as described in Section 2.5) of the residue (Figure 3.2).

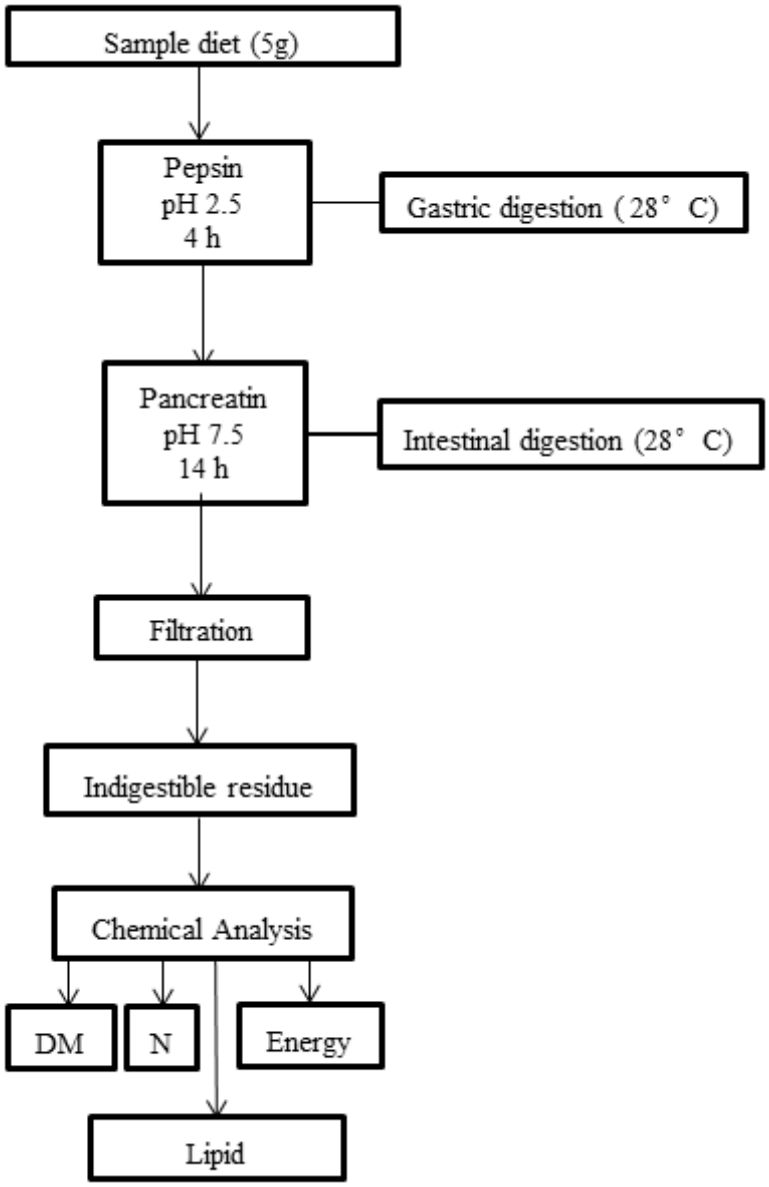

Figure 3.2: A schematic representation of *in vitro* digestion technique. DM, Dry Matter and N, Nitrogen

3.1.2.3 ***Calculations***

Digestion coefficient (DC), digestible nutrient (DN) and total digestible nutrients (TDN) were calculated as stated below.

$$\text{Digestion Coefficient, DC} = \frac{weight\ of\ sample\ diet - weight\ of\ residue}{weight\ of\ sample\ diet}\ X\ 100$$

Digestible Nutrient, DN =

$$\frac{Percentage\ nutrient\ composition\ of\ diet - Corrseponding\ DC}{100}$$

Total Digestible Nutrients, TDN = DCP + DNFE + (DL X 2.25)

Where:

DCP = Digestible crude protein

DNFE = Digestible Nitrogen-free extract

DL = Digestible lipid

3.1.3 Results

3.1.3.1 Weight and proximate composition of undigested materials after digestion

The weight and proximate composition of the undigested materials (residue) recovered after enzymatic digestion is shown in Table 3.2. The undigested material is a measure of dry matter loss and undigested component of the feed (faeces) that is passed out into the environment as faecal material. It can also be considered as measure of ileal digestibility of the diets subjected to enzymatic digestion. Undigested material (residues) recovered from phytase and carbohydrase supplemented diets were less ($P < 0.05$) than undigested materials recovered from the control and protease supplemented diets after enzymatic digestion. The crude protein content of undigested materials of protease and carbohydrase supplemented diets were higher ($P < 0.05$) than those of the control and phytase supplemented diets. The lipid content of the undigested material was highest ($P < 0.05$) in carbohydrase supplemented diet and lowest ($P < 0.05$) in protease supplemented diet. The ash content of the undigested material was highest ($P < 0.05$) in carbohydrase supplemented diet and lowest ($P < 0.05$) in the phytase supplemented diet. However, there was no significance difference ($P > 0.05$) in energy content of the undigested material in all diets subjected to enzymatic digestion.

Table 3.2: Weight and proximate composition of residues (undigested materials) after 18h of *in vitro* digestion

	Control	Phytase	Protease	Carbohydrase
Diet (g)	5.10±0.05	5.13±0.11	5.09±0.03	5.15±0.09
Residue (g)	2.11±0.10^{a}	1.80±0.03^{b}	2.06±0.03^{a}	1.82±0.02^{b}
Proximate composition				
Moisture (%)	6.00±0.34	5.19±0.28	7.05±0.06	6.38±0.14
Crude protein (%)	17.40±0.04^{a}	17.59±0.15^{a}	20.52±0.77^{b}	19.94±0.01^{b}
Lipid (%)	14.71±0.07^{c}	13.33±0.04^{b}	8.28±0.37^{a}	16.52±0.28^{d}
Ash (%)	2.40±0.01^{b}	1.87±0.10^{a}	2.54±0.11^{b}	2.78±0.04^{c}
NFE (%)	38.65±0.33^{b}	41.21±0.42^{c}	41.15±0.97^{c}	33.55±0.75^{a}
Energy (MJ Kg^{-1})	20.83±0.05	20.82±0.11	20.46±0.05	20.84±0.30

NFE represents Nitrogen-free extracts = Dry matter – (Crude protein + Crude lipid + Ash). Means in the same row with different superscripts are significantly different ($P < 0.05$).

3.1.3.2 Digestion coefficients of experimental diets subjected to enzymatic digestion

Digestion coefficient (DC) is a measure of a nutrient uptake into the digestive tract. The DC of nutrients of the experimental diets subjected to enzymatic digestion is displayed in Table 3.3. DCs of dry matter and energy are higher ($P < 0.05$) in phytase and carbohydrase supplemented diets compared to that of control and protease supplemented diets. DC of crude protein was highest ($P < 0.05$) in phytase supplemented diet and least ($P < 0.05$) in protease supplemented diet. There was no significant difference ($P > 0.05$) in lipid DC of the control and carbohydrase supplemented diets. However, the DC of lipid was highest ($P < 0.05$) in the protease supplemented diet. The DC of ash was significantly highest ($P < 0.05$) in phytase supplemented diet compare to the remaining three experimental diets.

Table 3.3: Digestion coefficients of the experimental diets (%)

	Control	Phytase	Protease	Carbohydrase
Dry matter	58.22±2.49^{a}	64.07±1.14^{b}	59.85±0.81^{a}	64.73±1.02^{b}
Crude protein	82.31±1.06^{b}	84.90±0.22^{c}	79.62±0.47^{a}	82.77±0.53^{b}
Lipid	21.58±6.28^{a}	37.52±2.07^{b}	59.37±1.46^{c}	25.29±4.25^{a}
Ash	84.36±0.81^{a}	89.88±0.70^{b}	84.21±1.00^{a}	84.75±0.31^{a}
NFE	58.21±2.45^{ab}	61.69±1.78^{b}	56.41±1.27^{a}	69.29±1.17^{c}
Energy	55.14±2.56^{a}	61.92±0.96^{b}	56.72±1.06^{a}	61.85±0.54^{b}

NFE represents Nitrogen-free extracts. Means in the same row with different superscripts are significantly different ($P < 0.05$).

3.1.3.3 Digestible nutrients of experimental diets subjected to enzymatic digestion

The DCs of the diets translated to digestible nutrients (DNs) of the experimental diets (Table 3.4). The DNs of the diets follow the same trend of significance as the DCs of the diets. However, there was no significant difference ($P > 0.05$) in the digestible lipid of phytase and carbohydrase supplemented diets.

Table 3.4: Digestible nutrients of the experimental diets (%)

	Control	Phytase	Protease	Carbohydrase
Dry matter	54.12±2.32^{a}	59.31±1.04^{b}	55.97±0.78^{a}	60.86±0.98^{b}
Crude protein	33.44±0.47^{b}	34.69±0.22^{c}	32.36±0.20^{a}	33.95±0.31^{b}
Lipid	1.69±0.55^{a}	2.81±0.16^{b}	4.89±0.35^{c}	2.00±0.44^{ab}
Ash	5.35±0.05^{a}	5.82±0.03^{b}	5.47±0.12^{a}	5.48±0.005^{a}
NFE	22.24±0.80^{ab}	23.29±0.86^{b}	21.50±0.32^{a}	26.81±0.26^{c}
Energy	10.58±0.51^{a}	11.87±0.18^{b}	10.83±0.24^{a}	11.96±0.10^{b}

Means in the same row with different superscripts are significantly different ($P < 0.05$).

3.1.3.4 Total digestible nutrients of the experimental diets

From the DNs, total digestible nutrient (TDN) was computed for each experimental diet. The TDNs of the enzymes supplemented diets (64.3±2.45%, 64.88±1.02% and 65.26±1.49% for phytase, protease and carbohydrase supplemented diets respectively) were significantly higher ($P < 0.05$) than TDN of the control diet (59.48±2.45%) (Figure 3.3).

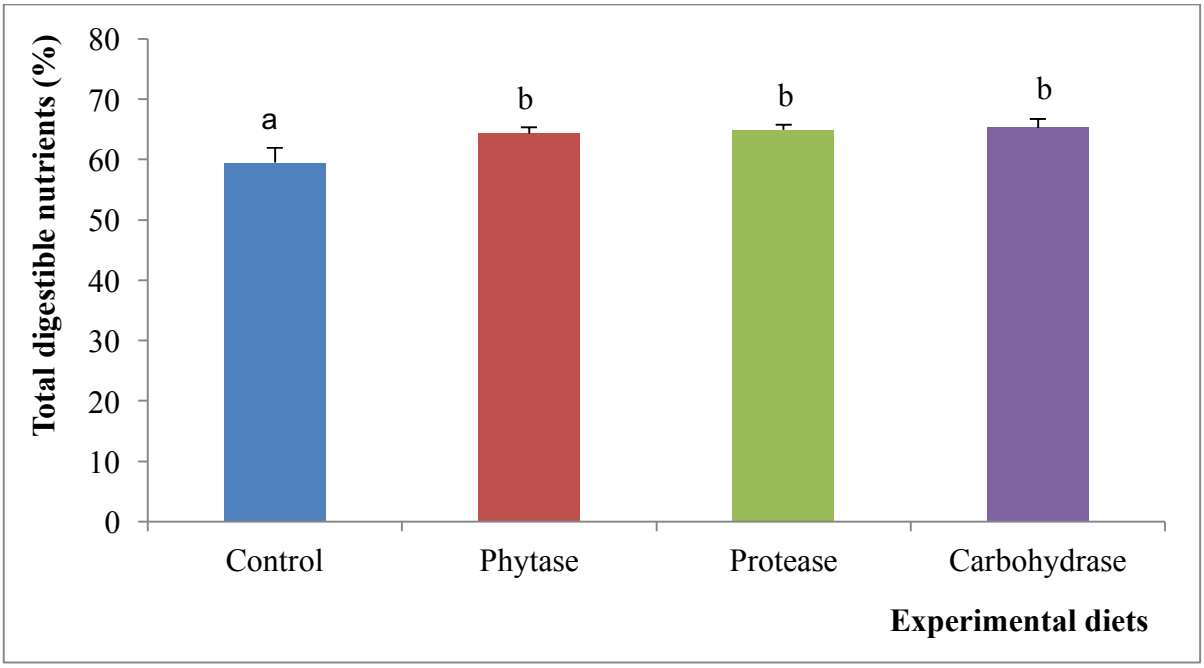

Figure 3.3: Total digestible nutrients of experimental diets. Bars with different superscript are significantly different (*P < 0.05*)

3.1.4 Discussion

Digestion is the transformation of feeds into more simple components that can be absorbed by animals' GI tract and thus digestibility (expressed in percentage) is an indicator of the efficiency of digestion process. The improved digestion coefficients and digestible nutrients which transformed to better TDNs observed in enzyme supplemented diets in the present study can be attributed to the potential effects of the exogenous digestive enzymes. There have been reports of exogenous digestive enzymes capacity to reduce the effects of ANFs and to improve utilisation of dietary nutrients resulting in improved growth performance in fish (Farhangi and Carter, 2007, Lin et al., 2007, Kumar et al., 2012b, Castillo and Gatlin, 2015). Several authors have reported that phytase supplementation enhance phytate-phosphorous availability, leading to improved growth performance in fish (Cain and Garling, 1995, Rodehutscord and Pfeffer, 1995, Yu and Wang, 2000, Liu et al., 2013). Additionally, carbohydrase is capable of hydrolysing the components of plant cell walls to release otherwise unavailable nutrients such as protein and starch (Chesson, 1993, Dudley-Cash, 1997). High digestible dry matter and energy observed in phytase and carbohydrase supplemented diets could be due to the effect of phytase on phytate bound minerals and nutrients as well as the effect of carbohydrase on NSPs. Also, high digestible crude protein and ash in phytase supplemented diets could be as a result of the effect of phytase on phytate P and its associated bound minerals and nutrients.

These findings further emphasize the relevance and usefulness of the *in vitro* digestibility technique for preliminary assessment of exogenous digestive enzymes as well as obtaining rapid digestibility values of diet. *In vitro* techniques are often validated with their correlation with *in vivo* trials because *in vitro* results may not always translate to *in vivo* system with its complex processes. Thus, the need for an *in vivo* investigation for

critical and elaborate evaluations of the full potential of the exogenous digestive enzymes will be addressed in Section 3.2.

3.2 Chapter 3B. Effects of exogenous digestive enzymes on Nile tilapia (*Oreochromis niloticus*) fed semi-purified diets

Abstract

A study was conducted to evaluate the effects of exogenous digestive enzymes on Nile tilapia growth and general health status. Tilapia (38.74 g) were fed one of four plant-based diets (40.8% protein, 7.8% lipid); one of which was a control and the remaining three were supplemented with exogenous digestive enzymes (phytase, protease and carbohydrase at 0.3 g kg^{-1}, 0.2 g kg^{-1}, and 0.3 g kg^{-1}, respectively). Tilapia fed the phytase supplemented diet displayed higher FBW (94.87±3.28 g $fish^{-1}$) and SGR (2.48 % day^{-1}) compared to tilapia fed the control (82.63±1.68 g $fish^{-1}$ FBW and 2.11 % day^{-1} SGR) and protease (85.58±0.17 g $fish^{-1}$ FBW and 2.21 % day^{-1} SGR) supplemented diets ($P < 0.05$). In terms of FCR and PER, tilapia fed diets supplemented with phytase (1.36 FCR and 1.08 PER) and carbohydrase (1.50 FCR and 0.94 PER) performed better ($P < 0.05$) than tilapia fed the control diet (1.68 FCR and 0.80 PER). However, the dietary treatments had no significant effect on tilapia somatic indices ($P > 0.05$). The body composition of tilapia fed the phytase supplemented diet contained lower lipid (70.51 g kg^{-1}) and higher ash (30.37 g kg^{-1}) than those fed the control diet (87.58 g kg^{-1} lipid and 23.23 g kg^{-1} ash). The level of circulatory red blood cells was highest ($P < 0.05$) in tilapia fed the carbohydrase supplemented diet (1.98 x 10^6 μL^{-1}). The relative proportion of monocytes of total leucocyte levels were highest in tilapia fed the phytase supplemented diet (4.54%) compared to tilapia fed the control (3.3 %) and protease (3.32 %) supplemented diets. Dietary treatments did not affect the mid-intestinal perimeter ratio, goblet cell abundance or intraepithelial leucocytes abundance. However, the microvilli density of the mid-intestine was higher ($P < 0.05$) in tilapia fed the phytase (15.55) and carbohydrase (16.01)

supplemented diets compared to those fed the control (10.40) and protease (11.47) supplemented diets. The intestinal bacterial community profile of tilapia fed the carbohydrase supplemented diet was significantly altered in contrast to those fed the control diet ($P < 0.05$). The supplementation of diets with exogenous enzymes (phytase and carbohydrase) has the potential to enhance tilapia growth without detrimental impacts on intestinal health.

3.2.1 Introduction

The use of plant ingredients in aquafeeds is gaining more attention in light of the dwindling supply of marine ingredients for this sector. Plant by-products are relatively more abundant and offer more economic advantages. Although, there are many ingredients (e.g. soybean meal, corn (gluten), sunflower meal, canola/ rapeseed meal, peas and lupins etc.) available as alternative to fishmeal, soybean is considered the most cost effective nutritive plant protein source, the most commonly used and the most abundant plant by-products (Figure 3.4). Soybean meal represents the highest proportion of plant protein in fish diets due to its high yield, relatively high crude protein content and all year round availability. Current soybean inclusion level in commercial tilapia feed ranges from 20 - 60% (Hasan et al., 2007, Rana et al., 2009, Tacon et al., 2011). For these reasons, soybeans are considered good as both food for human (especially in developing nations) and feed materials for farm animals. Therefore, the continuous use of soybean as feed ingredients could result into feed-food competition leading to market competition with human food demand.

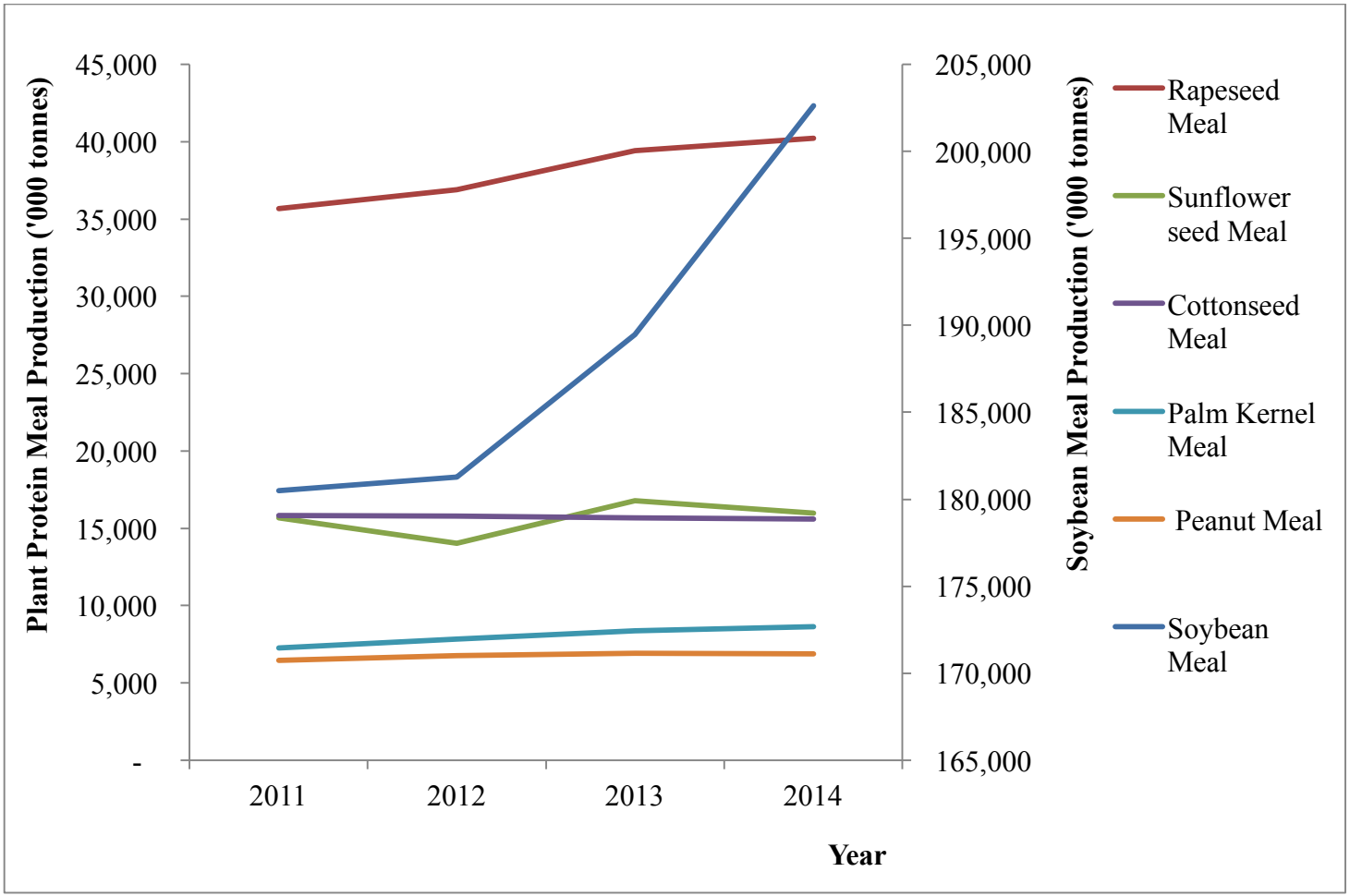

Figure 3.4: Global plant protein meal production (source: USDA, 2015)

To cope with the feed-food competition of soybean protein and ensure sustainability of aquaculture production, there is need for reduction and/ or replacement of soybean products with alternative plant products in fish diets. Lupins are among the suite of ingredients being considered in Europe and Australia to complement soybean meal in the replacement of fishmeal (see Section 1.8 for details). However, lupins being plant protein contains certain ANFs which include alkaloids and oligosaccharides with phytate, saponins, tannins, protease inhibitors and lectins. Therefore, to fully utilise lupins in aquafeed, there is a need to upgrade and increase the nutritional value of lupins and reduce the inherent ANFs. Commercially, most ANFs are extracted via elaborate processes (as described in Section 1.10) which can be quite expensive and sometimes detrimental to the nutritional value of plant proteins. For example, specific nutrients are lost in the process of upgrading plant proteins especially heat-labile nutrients (during thermal processes), water-soluble nutrients (during soaking and fermentation process), etc.

Supplementing aquafeeds with exogenous digestive enzymes (e.g. phytase, NSPase and protease) offers potential for better utilisation of nutrients from plant products based on the rapid *in vitro* digestion assessment (Section 3.1) and reported findings of enzyme applications in swine and poultry diets (Adeola and Cowieson, 2011). The application of phytase has been successful in breaking down phytate to increase mineral (especially phosphorus) and nutrient digestibility in fish (Cao et al., 2007, 2008, Kiarie et al., 2010, 2013). NSP-degrading enzymes (e.g. α-amylase, cellulase, xylanase and β-glucanase) are capable of disrupting plant cell wall integrity thereby reducing molecular size characteristics of NSP and consequently promoting rapid digestion in animals by reducing viscosity in the gut (Bedford and Cowieson, 2012, Zijlstra et al., 2010). Dietary protease supplementation has the potential to increase the utilisation of crude proteins from plant ingredients by increasing digestible crude protein and essential amino acids

available in the diets. Overall, the application of exogenous enzymes can allow better utilisation of lupins in diet formulation allowing increased inclusion rate. Apart from the potential of exogenous digestive enzymes to promote growth and nutrient utilisation, the enzymes may alter the carbohydrate composition and availability for microbial populations in the gut thus potentially altering bacterial community composition or activities as a consequence of a prebiotic effect (Bedford and Cowieson, 2012, Kiarie et al., 2013).

Although exogenous digestive enzymes have been applied to enhance the utilisation of plant nutrients in aquafeeds, the reported findings have been inconsistent (Table 1.5). Consequently, there is a need for further investigations to establish the benefits of supplementing exogenous digestive enzymes in fish diet. To the author's knowledge, previous studies on exogenous digestive enzymes have not investigated its effects on the intestinal microbiota and health of tilapia. Therefore, the objective of the present study was to investigate the effects of different exogenous digestive enzymes (phytase, protease and carbohydrase) on Nile tilapia growth performance, haematoimmunology and intestinal health when fed diets containing narrow-leafed lupin.

3.2.2 Materials and methods

3.2.2.1 Diets preparation

Four iso-nitrogenous and iso-lipidic diets were formulated (Table 3.5) according to known nutritional requirements of tilapia (NRC, 2011). The diets were prepared as previously described in Section 3.1.2.1.

3.2.2.2 Experimental design

The trial was conducted in a freshwater recirculation system (RS), system D, at the Aquatic Nutrition and Health Research Aquarium (Figure 3.5). The system contains 12 closed fibreglass tanks each with 72 L capacity. All male Nile tilapia (*Oreochromis niloticus*) were obtained from North Moore Tilapia, Goxhill, UK and stocked in the fibreglass tanks (72 L capacity each) for a period of 4 weeks to acclimatize. Thereafter, three hundred and sixty fish were randomly distributed into 12 tanks with three replicate tanks per dietary treatment (30 fish per tank; average weight = 38.74 ± 0.51 g) containing aerated recirculated freshwater. The tilapia were fed the experimental diets at 2-5% biomass per day in equal rations at 09.00, 13.00 and 17.00 h for six weeks. Daily feed was adjusted on a weekly basis by batch weighing following a 24 h starvation period.

The water temperature was maintained at 26.3±0.76 °C by aid of an inline heater. Other water parameters were monitored daily. The pH (6.20±0.7) and dissolved oxygen levels (>5 mg L^{-1}) in water system were monitored using an HQ40d pH and dissolved oxygen multi-parameter meter (HACH Company, Loveland, USA). NH_3, NO_2 and NO_3 were also monitored on a weekly basis using a nutrient analyser (SEAL AQ2 Analyser, Hampshire, UK). The water quality was maintained by changing the mechanical filters and partially (~20% of system volume) changing the water weekly. Sodium bicarbonate ($NaHCO_3$)

was used to adjust the pH level within the desired range when appropriate. A photoperiod of 12 h light and 12 h dark was used throughout the experiment.

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Figure 3.5: Sideview of recirculation system of Aquatic Animal Nutrition and Health Research Group Aquarium, University of Plymouth

3.2.2.3 Growth, feed utilisation and somatic indices

Final body weight (FBW), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), condition factor (K), hepatosomatic index (HSI) and viscero-somatic index (VSI) were assessed and calculated as described in Section 2.4.

3.2.2.4 Haemato-immunological parameters

Haematocrit, haemoglobin, blood cells count, MCH, MCHC, MCV, leucocyte differential count and serume lysozyme activity were asessed as described in Section 2.7.

Table 3.5: Formulation and composition of the experimental diets

Ingredients (g kg^{-1})	**Control**	**Phytase**	**Protease**	**Carbohydrase**
Soybean protein meal[a]	353.00	353.00	353.00	353.00
Narrow-leafed lupin meal[b]	250.00	250.00	250.00	250.00
Corn starch[c]	209.98	209.68	209.78	209.68
Herring meal LT94[d]	100.00	100.00	100.00	100.00
Corn oil	21.70	21.70	21.70	21.70
Fish oil	20.00	20.00	20.00	20.00
Lysamine pea protein concentrate[e]	20.00	20.00	20.00	20.00
Vitamin & mineral premix[f]	20.00	20.00	20.00	20.00
CMC-binder[c]	5.00	5.00	5.00	5.00
Phytase[g]	0.00	0.30	0.00	0.00
Protease[h]	0.00	0.00	0.20	0.00
Carbohydrase[i]	0.00	0.00	0.00	0.30
BHT[f]	0.075	0.075	0.075	0.075
Ethoxyquin[f]	0.0075	0.0075	0.0075	0.0075
Alpha tocopherols[f]	0.20	0.20	0.20	0.20
Composition				
Moisture	7.04	7.43	6.49	5.98
*Crude protein	40.63	40.86	40.65	41.01
*Lipid	7.77	7.49	8.24	7.85
*Ash	6.35	6.48	6.50	6.46
Energy, MJ kg^{-1}	19.18	19.18	19.10	19.34
*NFE[j]	19.03	18.57	19.03	19.36

[a]Hamlet HP100, Hamlet Protein, Denmark.

[b]Soya UK

[c]Sigma- Aldrich Ltd., UK.

[d]Herring meal LT94 – United Fish Products Ltd., Aberdeen, UK.

[e]Roquette Frêres, France.

[f] Premier Nutrition Products vitamin/mineral premix contains: 121 g kg^{-1} calcium, Vit A 1.0 µg kg^{-1}, Vit D3 0.1 µg kg^{-1}, Vit E (as alpha tocopherol acetate) 7.0 g kg^{-1}, Copper (as cupric sulphate) 250 mg kg^{-1}, Magnesium 15.6 g kg^{-1}, Phosphorus 5.2 g kg^{-1}

[g] RONOZYME® Hiphos (contains 10,000FYT g^{-1}) from DSM Nutritional Products

[h] RONOZYME® ProAct (contains 75,000 PROT g^{-1}) from DSM Nutritional Products

[i] ROXAZYME® G2 (contains 2700U g^{-1} xylanase, 700U g^{-1} β-glucanase and 800U g^{-1} cellulose) from DSM Nutritional Products

[j]Nitrogen - free extracts (NFE) = dry matter – (crude protein + crude lipid + ash)

*composition on dry weight basis

3.2.2.5 Histological appraisal of mid-intestine

At the end of the trial, three fish per tank were sampled for histological appraisal (light and scanning electron microscopy) of the mid-intestine (n = 9) as described in Section 2.9.

3.2.2.6 Intestinal microbiology

3.2.2.6.1 Fish dissection

Three fish per tank (n = 9 per treatment) were euthanized using tricaine methane sulfonate (MS222; Pharmaq, Fordingbridge, UK) (200 mg L^{-1} water) solution buffered with sodium bicarbonate (to prevent pH shock) for 15 min. Following the euthanasia, the fish brain was destroyed. The surface of the fish was wiped with 70 % industrial methylated spirit (IMS) to avoid external contamination, the peritoneal cavity of the fish opened aseptically and the intestine was entirely excised. Digesta from the intestine was obtained by gently squeezing the section with a sterile forceps into individual sterile 1.5 mL microcentrifuge tubes and stored at -20 °C.

3.2.2.6.2 DNA extraction

DNA was extracted from 100 mg digesta samples using QIAamp® Stool Mini Kit (Qiagen, Crawley, UK) with slight modifications to the manufacturer's instructions (see details in Section 7.2).

3.2.2.6.3 Polymerase chain reaction (PCR)

PCR was conducted to amplify the V3 region of the 16S rRNA gene using forward primer P3 with a GC clamp on its 5'-end (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and reverse primer P2 (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993). PCR reactions (50 µL) contained 25 µL BioMix™ Red Taq (Bioline, UK), 1 µL of each primer (50 pmol/µL each MWG-Biotech AG, Germany), 1 µL DNA template and 23 µL sterile Milli-Q water.

Positive and negative control templates were included in each assay; negative control (sterile, molecular grade water as template) and positive control (DNA extract from *Pediococcus acidilactici*, Microbiology Laboratory culture collection, Plymouth University, UK).

Touchdown thermal cycling was conducted using a GeneAmp® PCR System 9700 (Perkin-Elmer, CA, USA), under the following conditions: 94 °C for 10 min, then 30 cycles starting at 94 °C for 1 min, 65 °C for 2 min, 72 °C for 3 min (Muyzer et al., 1993). The annealing temperature decreased by 1 °C every second cycle until 55 °C and then remained at 55 °C for the remaining 10 cycles.

3.2.2.6.4 Agarose gel electrophoresis

A 1.5 % agarose gel was made with 1x Tris-acetate-EDTA (TAE) buffer pre-stained with 1 µL of SYBR® Safe™ DNA Gel Stain (Life TechnologiesTM UK) per 10 mL of agarose (Fisher Scientific) and run with 1x TAE buffer in a Pharmacia electrophoresis tank at 90 volts for 60 min. To check the purity and molecular weight characteristics of PCR product, 10 µL of the PCR products were loaded onto the agarose gel. Five microliters of Hyper Ladder IV (Bioline, UK) was run alongside the PCR products to aid assessment of DNA product sizes. The agarose gel was viewed under UV light using a Bio-Rad universal hood 11 (Bio-Rad laboratories, Italy). The PCR products were stored at 4 °C until use.

3.2.2.6.5 Denaturing gradient gel electrophoresis (DGGE)

The PCR products were used to obtain DNA fingerprints of the bacterial community present in the fish gut section by DGGE using a BioRad DGGE system (DCode™ System, Italy).

Fifteen microliters of the PCR products were loaded into 10 % acrylamide gel with a denaturing gradient of 40 - 60%. The acrylamide gel was made from stock solutions; an 80 % denaturant polyacrylamide solution consisted of 25 mL of 40 % acrylamide mix (high purity acrylamide), 2 mL of 50x TAE buffer (pH 8.3), 32 mL of molecular grade formamide (Sigma, UK), 34 g of 5.6M ultrapure urea (Sigma, UK) and volume of Milli-Q water yielding a total volume of 100 mL. Stock 0 % denaturant polyacrylamide solution consisted of 25 mL of 40 % acryμamide mix (high purity acrylamide), 2 mL of 50x TAE buffer (pH 8.3) and 73 mL of Milli-Q water. One hundred and fifty microliters of 10 % ammonium persulphate (APS, electrophoresis grade, Sigma, UK) and 17.5 mL of Tetramethylethylindiamine (TEMED) were added to the high and low denaturant solutions. Twenty one microliters of each acrylamide solution was added to separate 30 mL syringes and these were mounted onto a Bio-Rad gradient delivery system (model 475, Bio-Rad laboratories). The gel was delivered between gel plates and the gel was left to polymerize for 2 h. The gel was run at 65 V for 17 h at 60 °C in 1X TAE buffer.

The DGGE gel was stained by incubating for 30 min at room temperature in a 200 mL buffer containing 20 μL of 10000x SYBR® gold nucleic acid gel stain (Invitrogen™, UK) on a shaking platform (IKAO VIBRAX VXR) at 0.02 g. The gel was scanned in a Bio-Rad universal hood 11 (Bio-Rad Laboratories, Italy).

3.2.2.6.6 Excision of DGGE bands for sequence analysis

Bands (or 'operational taxonomic units', OTU) of interest (those showing clear and consistent specialization) were excised from the DGGE gel using sterile pipette tips and DNA was eluted overnight at 4 °C in 1.5 mL microcentrifuge tubes containing 20 μL Milli-Q water. From the eluate, 5 μL was used as template for re-amplification using the forward primers P1 (5-CCTACGGGAGGCAGCAG-3, 50pmol uL^{-1}; essentially P3 without the GC clamp at its 5'end) and the reverse primer P2 under the same conditions

as previously described (Section 3.2.2.6.3). Ten microliters was loaded onto a pre-stained agarose gel (1.5 %) to check the PCR product size. The PCR products were cleaned using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The purified products were sequenced by GATC laboratories (GATC-biotech laboratories, Germany). In order to obtain the taxonomic classification from the partial 16S rRNA sequences a blast search in GenBank database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed. The highest similarities were used to assign the taxonomic description of each sequence.

3.2.2.7 Statistical analysis

Statistical analyses of growth performance, carcass composition, histology and haemato-immunology data was carried out as described in Section 2.11.

DGGE band patterns were analysed using Quantity one® V4.6.3 software (Bio-Rad laboratories, CA, USA). A Bray Curtis similarity matrix was used to represent the relative similarities between treatments and replicates using Primer V6 previous standardization of matrices to determine relative abundance of bacterial species per sample. Standardization was calculated dividing the band intensity for each OTU by total intensity for that sample. The following diversity indexes were calculated: SIMPER similarity (%), Margalef's species richness: $d = (S - 1)/ \log(N)$, Pielou's evenness: $J' = H'/ \log(S)$ and Shannon's diversity index: $H' = -\Sigma(pi(\ln pi))$. Where N = total number of individuals (total intensity units), S = number of OTUs and pi = the proportion of the total number of individuals in the species.

3.2.3 Results

3.2.3.1 Growth, feed utilisation and somatic indices

Growth, feed utilisation and somatic indices are presented in Table 3.6. Tilapia fed the phytase supplemented diet performed significantly better ($P < 0.05$) than those fed control and protease supplemented diets in terms of FBW and SGR. Tilapia fed the phytase supplemented diet also displayed better feed utilisation in terms of improved FCR and highest PER when compared to all other treatments. The fish fed phytase and carbohydrase supplemented diets have similar growth performance; there was no significant difference ($P < 0.05$) in their FBW and SGR. Good survival was recorded in all the treatments (i.e. ≥ 90%) but higher in tilapia fed phytase and protease supplemented diets when compared to the control group. However, the dietary treatments did not affect ($P > 0.05$) the fish somatic indices assessed.

3.2.3.2 Whole body composition of tilapia fed enzymes supplemented diets

The whole body composition of tilapia fed the experimental diets is displayed in Table 3.7. The body moisture content of tilapia fed the protease supplemented diet was significantly higher ($P < 0.05$) than those fed the control diet. However, there was no significant difference ($P > 0.05$) in the body moisture contents of tilapia fed the enzymes supplemented diets. The dietary treatment did not have effect on whole body protein contents of the fish. The fish fed with phytase supplemented diet exhibited lowest lipid and highest ash body contents. While tilapia fed the control diet had the highest lipid and lowest ash body contents.

Table 3.6: Growth, feed utilisation and somatic indices of tilapia fed enzyme supplemented diets

	Control	Phytase	Protease	Carbohydrase
IBW (g fish^{-1})	38.64±0.84	38.89±0.34	38.56±0.60	38.87±0.52
FBW (g fish^{-1})	82.63±1.68^{a}	94.87±3.28^{b}	85.58±0.17^{a}	89.36±5.72^{ab}
SGR (% day^{-1})	2.11±0.1^{a}	2.48±0.08^{b}	2.21±0.05^{a}	2.31±0.18^{ab}
FCR	1.68±0.09^{a}	1.36±0.05^{c}	1.55±0.03^{ab}	1.50±0.1^{b}
PER	0.80±0.06^{a}	1.08±0.06^{c}	0.88±0.03^{ab}	0.94±0.11^{b}
HSI	1.65±0.09	1.50±0.04	1.68±0.28	1.73±0.19
K-factor	1.97±0.09	1.93±0.03	2.02±0.18	1.94±0.17
VSI	11.47±0.28	10.07±0.96	10.54±0.75	10.24±0.98
Survival (%)	90±8.82^{a}	100^{b}	100^{b}	97.78±1.92^{ab}

Means in the same row with different superscripts are significantly different ($P < 0.05$). IBW, initial body weight; FI, daily feed intake; FBW, final body weight; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficient ratio; HSI, hepatosomatic index and VSI, viscera-somatic index.

Table 3.7: Whole body composition of tilapia fed enzyme supplemented diets (g kg^{-1})

	Control	Phytase	Protease	Carbohydrase
Moisture	730.40±2.88^{a}	740.03±5.77^{ab}	747.03±3.06^{b}	738.30±7.39^{ab}
Protein	152.78±14.66	155.61±2.14	145.47±17.29	153.96±6.86
Lipid	87.58±6.68^{a}	70.51±4.63^{b}	74.14±12.68^{ab}	81.55±6.74^{ab}
Ash	23.23±1.37^{a}	30.37±1.38^{b}	25.66±5.24^{ab}	27.29±1.45^{ab}

Means in the same row with different superscripts are significantly different ($P < 0.05$).

3.2.3.3 Haemato-immunological parameters

Haematological and immunological parameters are displayed in Table 3.8. Haematocrit, haemoglobin, leucocyte levels, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and serum lysozyme activity were unaffected by the dietary treatments. However, red blood cell levels were highest ($P < 0.05$) in the blood of tilapia fed the carbohydrase diet compare to tilapia fed other diets. Consequently, mean corpuscular volume was significantly lower ($P < 0.05$) in tilapia fed the carbohydrase supplemented diet. Even though there was no difference ($P > 0.05$) in total number of circulatory leucocytes (WBC) in the blood of tilapia fed the experimental diets, the proportion of leucocyte types was significantly different ($P < 0.05$); monocyte abundance was significantly higher in tilapia fed the phytase supplemented diet compared to those fed the control and protease supplemented diets.

Table 3.8: Haemato-immunological parameters of tilapia fed enzyme supplemented diets

	Control	Phytase	Protease	Carbohydrase
Haematocrit (% PCV)	40.5±6.65	41.11±1.38	41.38±9.98	40.88±6.66
Haemoglobin (g dL^{-1})	6.6±0.21	6.94±0.42	7.80±1.31	6.93±1.18
RBC (10^6 μL^{-1})	1.5±0.17^{a}	1.66±0.02^{a}	1.64±0.13^{a}	1.98±0.14^{b}
WBC (10^3 μL^{-1})	24.3±7.95	24.05±0.32	21.19±4.02	28.37±8.16
MCV (fL)	260.9±36^{a}	250.72±12^{ab}	232.52±43^{ab}	183.77±44^{b}
MCH (pg)	46.3±8.48	42.30±1.89	48.09±9.95	35.48±6.82
MCHC (g dL^{-1})	16.4±0.75	16.86±1.69	17.24±1.23	16.94±3.36
Serum lysozyme (U)	73.6±14	74.74±19	100.89±25	80.24±20
Lymphocytes (%)	92.9±0.64	90.90±1.66	92.10±0.86	91.33±0.57
Monocytes (%)	3.3±0.18^{a}	4.54±0.78^{b}	3.32±0.67^{a}	3.97±0.59^{ab}
Granulocytes (%)	3.8±0.79	4.56±1.11	4.57±1.05	4.70±0.48
Serum lysozyme (U)	73.6±14	74.74±19	100.89±25	80.24±20

Figures in each row with different superscript are significantly different ($P < 0.05$). RBC, red blood cells; WBC, leucocytes; MCV, mean corpuscular volume (haematocrit (%PCV) x 10)/RBC 106 μL^{-1}); MCH, mean corpuscular haemoglobin (haemoglobin (g dL^{-1}) x 10)/ RBC (106 μL^{-1}); MCHC, mean corpuscular haemoglobin concentration (haemoglobin (g dL^{-1}) x 100)/ haematocrit (% PCV); U, lysozyme activity unit (activity mL^{-1} min^{-1}); and %, mean percentage of total leucocytes

3.2.3.4 Intestinal histology

Light and scanning electron microscopy revealed a normal and healthy morphology of the mid-intestines of tilapia fed the experimental diets. The intestines of the fish showed intact epithelial barrier with well organised villi-like mucosal folds, abundant IELs and goblet cells (Figure 3.6a – h). The dietary treatments had no significant effects ($P > 0.05$) on the intestinal perimeter ratio, number of goblet cells or IELs (per 100 μm) of the fish fed the experimental diets (Table 3.9). The fish intestines displayed healthy brush border with well organised and tightly packed microvilli revealing no signs of damage (Figure 3.6i – l). However, microvilli of the brush border of tilapia fed control and protease supplemented diets appeared to be less tightly packed (Figure 3.6i & k) compared to those fed phytase and carbohydrase supplemented diets (Figure 3.6j & l). Consequently, the microvilli density of the fish intestines was significantly different among tilapia fed the experimental diets; the microvilli density of tilapia fed the phytase and carbohydrase supplemented diets were significantly higher ($P < 0.05$) than that of tilapia fed the control and protease supplemented diets (Table 3.9).

Table 3.9: Intestinal histology of tilapia fed enzyme supplemented diets

	Control	**Phytase**	**Protease**	**Carbohydrase**
Perimeter ratio (AU)	4.86±0.85	6.22±0.72	5.84±1.94	5.84±1.85
Goblet cells (per 100μm)	7.35±0.33	6.87±0.94	7.26±0.41	7.75±0.25
IELs (per 100μm)	47.73±2.85	49.12±6.03	53.45±15.70	46.28±7.87
Microvilli density (AU)	10.40±1.27[a]	15.55±0.70[b]	11.47±0.49[a]	16.01±0.98[b]

Figures in each row with different superscripts indicate significant differences ($P < 0.05$).

AU, arbitrary units and IELs, Intraepithelial leucocytes.

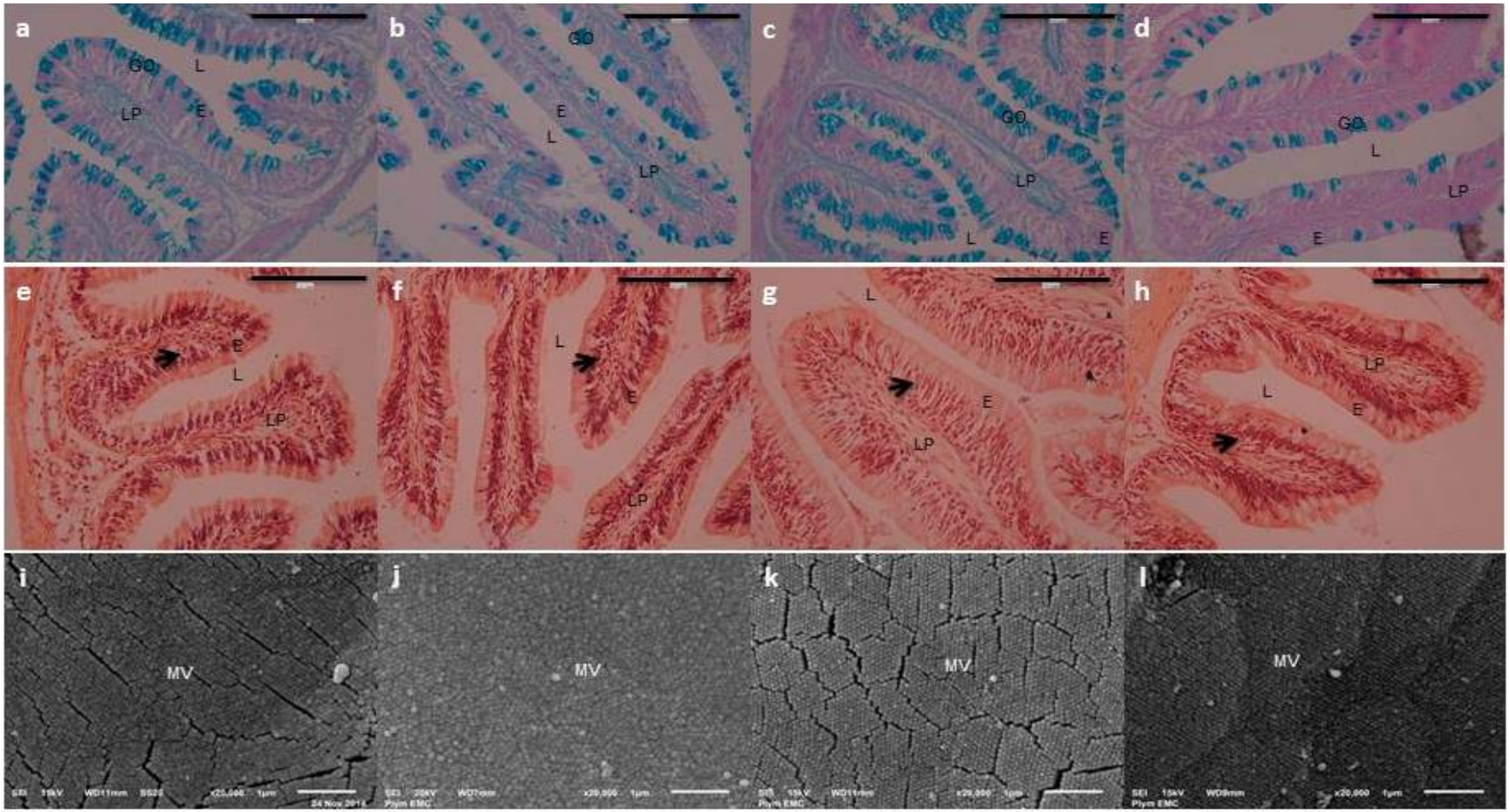

Figure 3.6: Light (a - h) and scanning electron (i - l) micrographs of the mid-intestine of tilapia fed the control (a,e & i), phytase (b, f & j), protease (c,g & k) and carbohydrase (d, h & l) diets. Goblet cells are filled with abundant acidic mucins (blue; a – d) in all treatments and abundant IELs (arrows) are present in the epithelia. A Abbreviations are E enterocytes, LP lamina propria, L lumen, GO goblet cell, and, MV microvilli. Light microscopy staining: [a-d] Alcian Blue-PAS; [e-h] H & E. Scale bars = 100 µm (a-h) or 1 µm (i-l)

3.2.3.5 Intestinal microbiota

The bacterial community of tilapia fed the experimental diets were analysed by PCR-DGGE. The DGGE banding patterns of the 16S rRNA V3 region from the fish intestinal content is displayed in Figure 3.7 and the taxonomic affiliation of the DGGE bands is displayed in **Table 3.10**. A total of eleven OTUs were obtained from the DGGE for sequencing. Some OTUs were common to all treatments, for example, OTU (#9) was detected in all tilapia fed all of the experimental diets and had 100% sequence alignment to *Clostridium ghonii*. In contrast, OTU (#5) was uniquely detected in tilapia fed the carbohydrase supplemented diet and was closely aligned (97%) to *Acinetobacter schindleri*. OTU (#7) was common in tilapia fed phytase and protease supplemented diets and had 99% sequence alignment to *Arthrobacter russicus*. OTU (#8) was common in tilapia fed protease and carbohydrase supplemented diets and had 99% sequence alignment to *Sporosarcina aquimarina*. OTUs (#10 and #11) were common in tilapia fed control and phytase supplemented diets and both had 99% sequence alignment to *Austwickia chelonae* and *Intrasporangium calvum*, respectively. OTUs (#1 and #3) were present in all tilapia fed the exogenous supplemented diets and had 96% and 83% sequence alignment to *Aquisphaera giovannonii* and uncultured bacterium clone AMD-A65, respectively. OTUs (#2, #4 and #6) were common in tilapia fed the control diet and had 93%, 81% and 99% sequence alignment to *Marinobacter hydrocarbonoclasticus*, *Desulforegula conservatrix*, and *Arthrobacter russicus*, respectively. Firmicutes was the most frequently identified phylum across all the treatments and high frequency of the OTUs from the *Clostridium* genus was also evident.

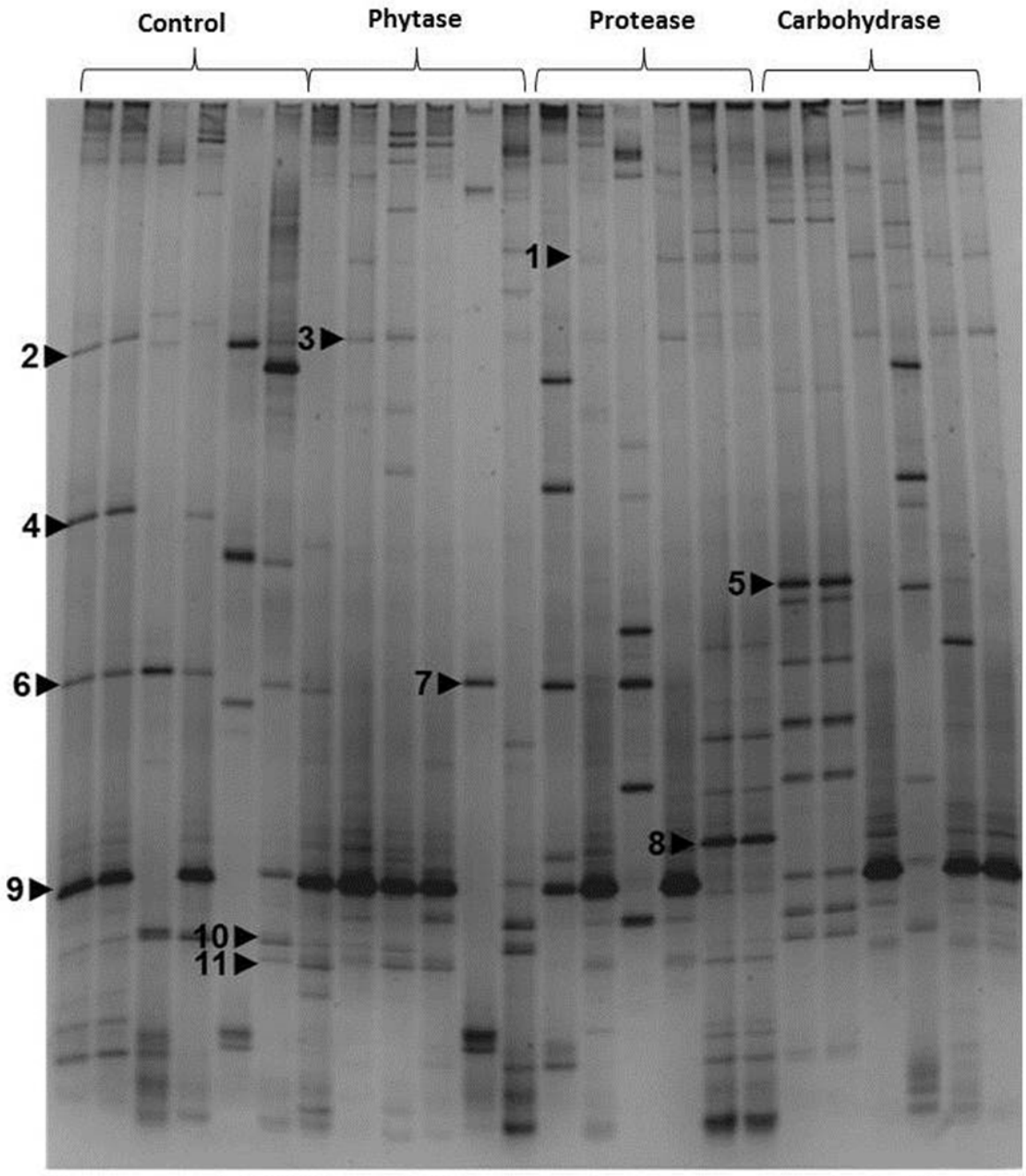

Figure 3.7: 40 - 60% DGGE banding pattern of V3 region of 16S rRNA fragments from tilapia distal intestinal contents

Table 3.10: Taxonomic affiliation of DGGE bands sequenced from intestinal content of Nile tilapia fed enzyme supplemented diets

Band #	Phylum	Nearest neighbour identified by BLASTn	Similarity (%)	Treatment
1	Planctomycetes	*Aquisphaera giovannonii*	96	Protease (4) Carbohydrase (3)
5	Proteobacteria	*Acinetobacter schindleri*	97	Carbohydrase (4)
6	Actinobacteria	*Arthrobacter russicus*	99	Control (4)
7	Actinobacteria	*Arthrobacter russicus*	99	Phytase (1) Protease (2)
8	Firmicutes	*Sporosarcina aquimarina*	99	Protease (2) Carbohydrase (3)
9	Firmicutes	*Clostridium ghonii*	100	Control (4) Phytase (5) Protease (3) Carbohydrase (6)
10	Actinobacteria	*Austwickia chelonae*	99	Control (5) Phytase (3) Protease (3)
11	Actinobacteria	*Intrasporangium calvum*	99	Phytase (4)

Numbers in parenthesis represents number of replicates, out of 6, where the respective OTU was detected

In terms of the number of OTUs, species richness and diversity, no significant differences were observed between the treatments (Table 3.11). However, high variability in the bacterial community structure was observed between individuals in the same group in all the treatments; the control group showed the greatest differences (Figure 3.8). The dietary treatments did not significantly affect ($P > 0.05$) the ecological parameters of PCR-DGGE fingerprints. However, Permanova analysis revealed a significant difference in bacterial community composition of fish fed the control and carbohydrase supplemented diets (Table 3.11).

Table 3.11: Ecological parameters of PCR - DGGE fingerprints of the intestinal bacterial of tilapia (n = 6)

Treatment	OTUs[1]	Richness[2]	Evenness[3]	Diversity[4]	SIMPER (%)	Permanova P (perm)	Similarity (%)
Control	16.3 ± 2.36	3.3 ± 0.51	0.95 ± 0.00	2.6 ± 0.16	25.7		
Phytase	16.0 ± 2.21	3.2 ± 0.48	0.94 ± 0.01	2.5 ± 0.19	36.3		
Protease	20.2 ± 1.85	4.1 ± 0.40	0.94 ± 0.00	2.8 ± 0.10	39.4		
Carbohydrase	19.2 ± 1.65	4.0 ± 0.36	0.95 ± 0.01	2.8 ± 0.09	43.7		
Control vs phytase						0.260	28.5 ± 17.5
Control vs protease						0.187	27.6 ± 13.0
Control vs carbohydrase						0.029	24.8 ± 12.8
Phytase vs protease						0.464	34.8 ± 16.4
Phytase vs carbohydrase						0.086	34.7 ± 17.6
Protease vs carbohydrase						0.085	36.5 ± 17.1

Results are presented as mean ±SD in each group (n=6). There were no significant differences between the treatments.

[1] Operational taxonomic unit.

[2] Margalef species richness: $d = (S - 1) / \log (N)$.

[3] Pielou's evenness: $J' = H' / \log(S)$.

[4] Shannons diversity index: $H' = -\Sigma(pi(\ln pi))$.

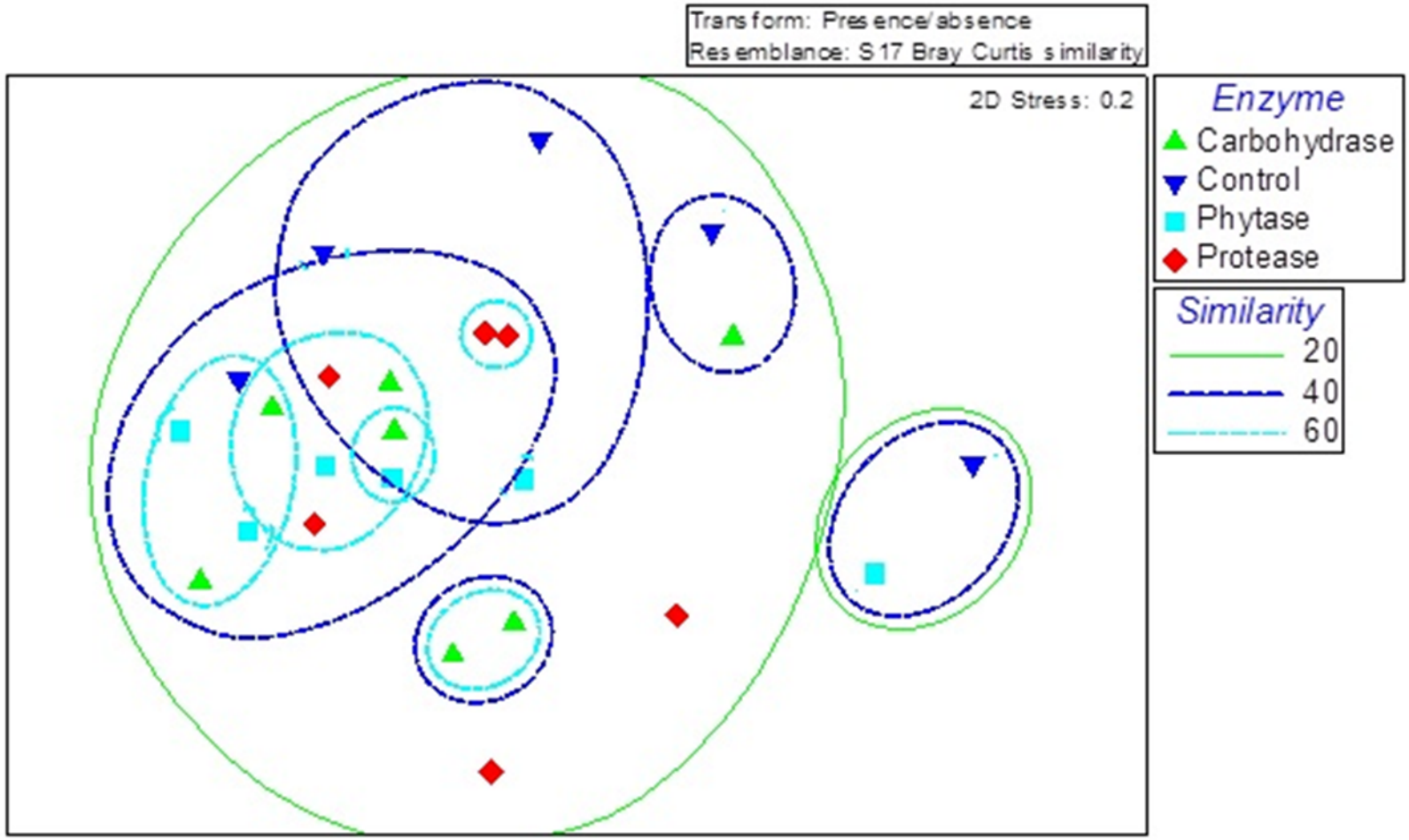

Figure 3.8: Cluster analysis based on DGGE profile of V3 region fragments of 16S rRNA from distal intestinal content of tilapia fed enzyme supplemented diets

3.2.4 Discussion

The potential of exogenous enzymes to enhance aquaculture production by liberating potentially unavailable plant nutrients within specific plant feed ingredients warrants more study to validate their effectiveness in fish feeds. To this end, the present trial was conducted to establish and document the effects of dietary phytase, protease and carbohydrase on Nile tilapia production and health when supplemented to diets containing narrow-leafed lupin and soybean meal proteins. Diet supplementation with exogenous enzymes, especially phytase and carbohydrase, may neutralise some of the negative effects of anti-nutritional factors, increase nutrient bioavailability and consequently improve diet nutritional quality. In the present study, improved growth (FBW and SGR) of tilapia fed the phytase supplemented diet infers improved nutrient bioavailability. Confirming this, tilapia fed the phytase or carbohydrase supplemented diets displayed FCR and PER significantly better than tilapia fed the control diet. In the case of phytase, this could be attributed to better utilisation of previously sequestered nutrients released by the effect of phytase on phytate-bound nutrients especially bound phosphorus in the diet. Cao et al. (2008) reported the same effect when Nile tilapia were fed with a phytase supplemented diet; the phytase supplemented diet gave better growth performance, FCR and PER compared with the control group. This is also in agreement with previous findings from Portz and Liebert (2004), Liebert and Portz (2005), (2007) and Nwanna and Schwarz (2007) on improved digestibility and growth performance effects of phytase on plant-based diets fed to Nile tilapia. However, there are some reports of non-effects of dietary phytase provision on growth performance and nutrient utilisation in fish (Cao et al., 2007). This could possibly be due to the fact that removal of phytate could enhance the influence of other ANFs and shield amino acids from degradation or reduce leaching of water soluble components (Cao et al., 2007). This could also be

attributed to enzyme dosage (activity) and substrates available for enzymatic reaction. Tilapia fed the carbohydrase supplemented diet had similar growth performance (FBW & SGR) with tilapia fed phytase supplemented diet. The similarity in performance could be attributed to the ability of the carbohydrase to reduce the molecular size of NSPs present in the basal diet and consequently promote better digestion and absorption of nutrients (Castillo and Gatlin, 2015). However, the positive effect of carbohydrase on tilapia growth performance and nutrient utilisation was contrary to findings by Yigit and Olmez (2011) who reported no benefits on growth when tilapia were fed a carbohydrase supplemented diet. It was hypothesised by the authors that protease supplementation could degrade complex proteins in the diet into usable amino acids and peptides thereby resulting in improved protein digestibility and growth performance. In the current study however, growth performance and nutrient utilisation of tilapia fed the protease supplemented diet were not significantly different from those fed the control diet. Contrary to this, Dias et al. (2014) reported a positive effect of protease on tilapia growth performance fed a lower crude protein diet compared to the higher crude protein diet in the present study. It could be inferred from this report that the protease effect is likely to be more pronounced in a lower crude protein and fishmeal diet. The non-effect of protease in a relatively higher crude protein diet could be as a result of non-beneficial effects of digestible protein when the level exceeds the requirement for fish maintenance and growth.

The ash content in whole tilapia body fed the phytase supplemented diet was significantly higher than those fed the control diet at the end of the feeding trial. This would indicate that phytase supplementation increased mineral uptake and possibly elevate nutrient, mineral and phosphorus retention. Lovell (1998) stated that the percentage of body lipid reduced linearly as dietary phosphorus increase above the requirement for normal fish

growth. This perhaps explains the reason for higher ash and lower lipid contents in whole fish body fed the phytase supplemented diet compare to lower ash and higher lipid contents in whole fish body fed the control diet. This finding is supported by the investigation of Cao et al. (2008) on increased ash and low lipid contents in whole body of tilapia fed phytase supplemented diet compared to whole body of tilapia fed control diet. Contrary to findings in this study, Nwanna (2007) reported non-effect on carcass composition of tilapia fed phytase supplemented diets.

Haematological parameters are useful for monitoring fish general health and physiological responses to stress. In this study, an elevated red blood cell count was observed in fish fed the carbohydrase supplemented diet. Monocytes are one of the main immune cells of the innate immune system and are precursor cells to macrophages and dendritic cells (which are phagocytic). The proportional abundance of these cells (monocytes) was significantly higher in the blood of tilapia fed a phytase supplemented diet compared to levels in the blood of tilapia fed the control and protease supplemented diets. This may confer better immune response of the tilapia fed phytase supplemented diets but further studies on the immune response are required to test the speculative hypothesis.

In terms of GI morphology, there was no significant difference in mid-intestine with respect to perimeter ratios, goblet cells levels and IELs levels, but significantly higher microvilli density (a measure of absorptive intestinal surface area) was observed in tilapia fed the phytase and carbohydrase supplemented diets. This is in line with improved growth performance and nutrient utilisation mentioned earlier and may have been a contributory factor to the observed growth parameters.

To the authors' knowledge, this is the first study investigating the effect of exogenous enzymes (phytase, protease and carbohydrase) on the intestinal microbiota of tilapia in a feeding trial. Previous studies have demonstrated that different feed additives such as antibiotics (He et al., 2010), probiotics (He et al., 2013) and prebiotics (Qin et al., 2014) can modulate the gut microbiota in tilapia. Zhou et al. (2013) reported significant changes in bacteria species and density of the intestinal microbiota of grass carp (*Ctenopharyngodon idella*) fed cellulase supplemented duckweed-based diets. In the present study, the predominant allochthonous bacterial species in the intestine of tilapia was *Clostridium ghonii*, which was the only phylotype found in all the individual tilapia regardless of the dietary treatment. Other authors have also found members of Family *Clostridiaceae* in tilapia's intestine suggesting that members of this family may be adapted to play an important role in the tilapia gut system (Zhou et al., 2011). There were some phylotypes that were only present in the intestine of fish fed with certain exogenous enzymes. For instance, OTU #1 which had 96% similarity with *Aquisphaera giovannonii* was present in intestines of tilapia fed phytase, protease and carbohydrase supplemented diets but absent in the intestine of tilapia fed control treatment. The occurrence of specific bacterial members of *Proteobacteria* and *Actinobacteria* were selectively associated to particular dietary treatment. The Proteobacteria was present in the control and carbohydrase treatments while the Actinobacteria was detected in control, phytase and protease treatments. The presence of *Proteobcateria* in the intestine of tilapia is in agreement with previous research that used molecular techniques to assess gut microbiota in tilapia. Even though, *Actinobacteria* is not frequently reported in intestine of tilapia; this group has been found to be among the most abundant groups in freshwater habitats (Ghai et al., 2014) and reported to be part of gut microbial community in other freshwater fish species (Standen et al., 2013, Ye et al., 2014, Etyemez and Balcázar, 2015, Standen

et al., 2015). Permanova analysis revealed that inclusion of exogenous carbohydrase in diet of tilapia altered significantly the bacterial community composition in the intestine of fish in contrast to that of tilapia fed the control diet. This finding suggests that this specific enzyme may have a modulating effect on the diet substrate profile thereby promoting or decreasing certain bacterial groups in the intestine.

In conclusion, tilapia fed diets supplemented with phytase and carbohydrase exhibited superior growth performance in contrast to fish fed the control and protease supplemented diets. A significant difference was noted in the intestinal microbiota of tilapia fed carbohydrase supplemented diet when compared to those fed the control diet. Although the microbiota ecological parameters were not affected by dietary treatment, Permanova analysis revealed differences in the community profiles. Further quantitative studies are necessary to confirm how exogenous digestive enzymes (especially carbohydrase) modulate intestinal microbiota and if these modulations contribute towards the improved growth performance of the host. It will also be more beneficial for the aquaculture industry and tilapia farmers to test the effect of exogenous digestive enzymes in practical diet so as to establish if the effects of the enzymes will be same in practical conditions.

4 Chapter 4. Effects of exogenous digestive enzymes on Nile tilapia (*Oreochromis niloticus*) fed a practical diet in tropical conditions

Abstract

A study was carried out to evaluate the effects of exogenous digestive enzymes on Nile tilapia (*Oreochromis niloticus*) fed practical diet. Tilapia (29.52±0.3g) were fed one of diets supplemented with phytase (75 mg kg^{-1}), protease (300 mg kg^{-1}), xylanase (250 mg kg^{-1}) and control diet without any exogenous digestive enzyme. Tilapia were fed at 3 % biomass per day in triplicate tanks per treatment for 8 weeks. The FBW (14.43±0.31 g $fish^{-1}$) of tilapia fed xylanase supplemented diet was significantly higher than that of tilapia fed the remaining three diets (136.42±0.41, 137.09±1.5 and 135.26 g $fish^{-1}$ for control, phytase and protease supplemented diets respectively). FCR of tilapia fed phytase and xylanase supplemented diets were significantly better ($P < 0.05$) than tilapia fed the control diet. The PER was highest in tilapia fed xylanase supplemented diet. However, the dietary treatments did not have significant effects on tilapia survival and somatic indices. Whole body lipid content of tilapia fed phytase supplemented diet was not different from those fed control and xylanase supplemented diets but higher ($P < 0.05$) in tilapia fed protease supplemented diet. The highest ($P < 0.05$) apparent digestibility coefficient of protein, ash, energy, phosphorus, calcium and sodium was observed in tilapia fed the phytase supplemented diet. The level of circulatory leucocytes was higher in tilapia fed phytase and protease supplemented diets when compared to the control group. The dietary treatments did not affect the endogenous enzyme activities of tilapia. It could therefore be concluded that supplementation of a commercial diet with phytase is capable of reducing nutrient loads in intensive aquaculture operations.

4.1 Introduction

With high commodity prices, fish farmers are pressured to increase productivity without compromising food safety, fish performance and welfare. High commodity prices could lead to inclusion of low quality and cheap raw materials (mainly plant materials) in aquafeeds and subsequently increase feed complexity resulting in more ANFs which could impair the performance and welfare of farmed fish (Francis et al., 2001). In addition to this is the environmental pressure of waste output from aquaculture operations. With the evolvement of aquafeed over the last half a century, the aquafeed industry is constantly seeking to optimise product quality in a cost effective manner. Hence, the importance of functional ingredients as supplements to optimise the aquafeed at little or no extra cost.

The potential of exogenous digestive enzymes (as reviewed in Section 1.11.1.1 and observed in Section 3.2) has been demonstrated to mitigate against the effects of ANFs common in cost effective feed ingredients, enhance the ingredients' nutritional value thereby improve nutrient utilisation and subsequently improve fish growth performance. Unlike other feed additives and supplements (such as antibiotic growth promoters) which may have adverse impact on human health and the environment, exogenous digestive enzymes are perceived to be harmless, environment friendly and natural (Liu and Baidoo, 1997). The exogenous digestive enzymes have the potential to reduce environmental pollution arising from aquaculture operations (Kumar et al., 2012b).

Research and development are useful to establish efficacy of feed supplements especially exogenous digestive enzymes. Usually, the research is conducted in ideal conditions with large variations to what is obtainable in practical conditions where the end users (i.e. fish farmers and aquafeed producers) operate. Practical conditions (e.g. commercial availability, dosage, etc.) are more suitable to account for the wide range of factors

affecting overall fish performance. This further emphasises the importance of validating research findings using practical conditions.

Therefore, the objective of the study was to evaluate the efficacy of exogenous digestive enzymes in practical conditions using commercially relevant dosage and diet within a tropical location.

4.2 Materials and methods

4.2.1 Experimental design and diets preparation

The trial was conducted in a freshwater flow-through aquaculture system (Figure 4.1) at the Division of Animal Production Technology and Fisheries of King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok – Thailand. The flow-through system (Figure 4.1) contains 12 circular concrete tanks (580 L capacity each) and were supplied with freshwater sourced from a local river system. Four hundred and eighty all male Nile tilapia (*Oreochromis niloticus*) of mean weight 29.52±0.15 g obtained from Charoen Pokphand (CP) farm in Thailand were randomly distributed (40 fish per tank) into the 12 tanks after four weeks of acclimatization. During acclimatization, the tilapia were fed ad libitum same commercial diet. The photoperiod and water temperature (30.83±0.29 °C) was maintained at ambient condition of 30.83±0.29 °C. The pH (6.48±0.34) and dissolved oxygen levels (>5.0 mg L^{-1}) in water system were monitored daily using an HQ40d pH meter and dissolved oxygen multi-parameter meter (HACH Company, Loveland, USA). NH_3 (0.34±0.1 mg L^{-1}), NO_2 (0.008±0.005 mg L^{-1}) and NO_3 (1.28±0.12 mg L^{-1}) were also monitored on a weekly basis using a nutrient analyser (SEAL AQ2 Analyser, Hampshire, UK). The flow (3.98 L min^{-1}) of water through the system maintained the water quality by washing off metabolic wastes without causing marked alteration in the water quality.

Figure 4.1: Flow-through system (FS) at the Division of Animal Production Technology and Fisheries, KMITL where tilapia were held during the trial. The experimental system consisted of circular concrete tanks with a capacity of 580 L. Water was sourced from the local river system and was monitored daily (pH, DO and temperature) and weekly (nitrogen wastes – ammonia, nitrite, nitrate) to ensure appropriate conditions for tilapia.

A commercial diet (No. 461; 35% protein, 5% lipid) containing fishmeal, soybean meal, full-fat soybean meal, yeast, corn meal, broken rice, rice bran, wheat bran, DCP, salt, vitamins, minerals, amino acids, fish oil and preservatives was obtained from INTEQC Feed Co. Ltd., Thailand and was used as the basal formulation. The diet was ground in a blender to powder and sieved to remove large particles. Exogenous digestive enzymes obtained from DSM Nutritional Products were added to the ground diets separately; RONOZYME® HiPhos (phytase) at 75 mg kg^{-1}, RONOZYME® ProAct (protease) at 300 mg kg^{-1} and RONOZYME® WX (xylanase) at 250 mg kg^{-1} (Table 4.1). The diets were mixed thoroughly to ensure homogeneity. Warm water was added to form consistency

suitable for cold press extrusion (2mm pellet size). After pelleting, the diets were placed in trays and dried in an air convection oven set at 45 °C for 24 h. The basal diet served as the control diet and was prepared in the same way to those supplemented with exogenous digestive enzymes except that it did not receive the enzymes. The diets were analysed for proximate composition as described in Section 2.5 (Table 4.1). Tilapia were fed the experimental diets for eight weeks at a rate of 3 % biomass per day in three equal rations. Total fish in individual tanks were batch weighed each week after 24 h starvation and feeding rate was adjusted weekly to the fish biomass.

Table 4.1: Dietary formulation and proximate composition (%) of experimental diets

	Control	Phytase	Protease	Xylanase
Commercial feed[a]	100	99.9925	99.97	99.975
Phytase[b]	0	0.0075	0	0
Protease[c]	0	0	0.03	0
Xylanase[d]	0	0	0	0.025
Proximate composition (% as fed basis)				
Moisture	8.03±0.04	6.87±0.14	8.06±0.06	6.63±0.09
Protein	34.32±0.28	34.78±0.09	34.43±0.13	34.56±0.08
Lipid	5.49±0.04	5.33±0.10	6.38±0.70	5.22±0.08
Ash	13.13±0.11	13.13±0.17	13.16±0.04	13.4±0.04
Energy (MJ kg^{-1})	17.06	17.56±0.01	17.31±0.04	17.66±0.21
Fibre	3.65±0.06	3.15±0.12	3.15±0.07	3.21±0.05

[a]No. 461, INTEQC Feed Co Ltd., Thailand

[b]RONOZYME® Hiphos (contains 10,000 FYT g^{-1}) from DSM Nutritional Products

[c]RONOZYME® ProAct (contains 75,000 PROT g^{-1}) from DSM Nutritional Products

[d]RONOZYME® WX (contains 1000 FXU g^{-1}) from DSM Nutritional Products

4.2.2 Growth performance, feed utilisation and somatic indices

Growth performance, feed utilisation and somatic indices were assessed by FBW, WG gain, feed intake (FI), SGR, PER, K–factor, HSI and VSI as described in Section 2.4.

4.2.3 Whole body composition of tilapia

Three tilapia per tank (n = 9) were sampled and the whole body composition were analysed as described in Sections 2.5 and 2.6.

4.2.4 Digestibility protocol

A 10-day digestibility study was carried out after the termination of the feeding trial to determine apparent digestibility coefficient (ADC) of nutrients and minerals. On termination of the feeding trial, the tilapia were allowed one week of rest during which the tilapia were fed a maintenance ration of the basal diet before the commencement of digestibility study. Chromic oxide was used as inert diet marker at 10 g kg^{-1} inclusion level in the diet. The tilapia were fed twice (10.00 h and 1.00 h) a day at 4 % biomass per day. Faecal collection started four days after feeding with the experimental diets to allow evacuation of all previously ingested material. Velocity of water flow was adjusted to minimize collection of faeces in drain pipe and to maximize faeces recovery in fish tanks. The tanks were cleaned of faeces prior to feeding each day. The uneaten feed was siphoned from tanks after 1 h of feeding and dried to constant weight at 45 °C in an oven to determine feed consumption. Faecal collection was done twice daily after first and second feedings. Expelled faecal material (less than 1 h in water) was carefully siphoned and collected using a fine mesh net. Only intact strands of faecal material were collected. Faecal samples collected from each tank of same treatment were pooled accordingly. Pooled faeces were dried at 60 °C in an oven for 24 h, labelled and frozen at -20 °C until ready for analysis. The analysis of nutrients, chromic oxide and minerals were done as described in Sections 2.5 and 2.6. ADC was determined using the formula below:

$$ADC = \left(1 - \frac{\%\ marker\ in\ diet}{\%\ marker\ in\ feces}\ X\ \frac{\%\ nutreint\ in\ feces}{\%\ nutrient\ in\ diet}\right) X\ 100$$

4.2.5 Haemato-immunoligical analysis

Blood samples were taken at the end of the trial to assess haematocrit, haemoglobin, blood cell counts, MCV, MCH and MCHC (n = 9 per treatment). Serum was collected to assess serum lysozyme activity (n = 15 per treatment). All sampling and analyses were carried out as described in Section 2.7.

4.2.6 Endogenous enzymes activities

Digesta from anterior intestine of three tilapia per tank (n = 9 per treatment) were sampled for endogenous enzyme activities. The sampling and analyses were carried out as described in Section 2.8.

4.2.7 Histological appraisal of mid-intestine

Three tilapia per tank were sampled (n = 9 per treatment) to assess perimeter ratio, IELs and goblet cell abundance as described in Section 2.9.1.

4.2.8 Cost benefit analysis

Cost benefit analysis of feeding tilapia exogenous digestive enzymes was estimated as described in Section 2.10.

4.2.9 Statistical analysis

Statistical analyses were carried out as described in Section 2.11.

4.3 Results

4.3.1 Growth performance, feed utilisation and somatic indices

Growth performance was assessed by means of FBW, WG, SGR, FCR and PER (Table 4.2). Tilapia in all treatments showed excellent growth performance with survival in each treatment exceeding 99%. The highest ($P < 0.05$) FBW was observed in tilapia fed xylanase supplemented diet. Tilapia fed protease supplemented diet displayed lower performance ($P > 0.05$) in terms of WG and SGR when compared with those fed xylanase supplemented diet. However, tilapia fed the control and protease supplemented diets performed similarly ($P > 0.05$) in in terms of FBW, WG, SGR, FCR and PER. On the other hand, tilapia fed phytase and xylanase supplemented diets had similar performance in terms of WG, SGR, FCR and PER. In addition, tilapia fed phytase and xylanase supplemented diets showed improved FCR when compared to the control group. None of the dietary treatments affected the somatic indices and survival of the tilapia.

Table 4.2: Growth, feed utilisation and somatic indices of tilapia fed experimental diets

	Control	Phytase	Protease	Xylanase
IBW (g fish^{-1})	29.57±0.76	29.31±0.55	29.73±0.58	29.33±0.17
FBW (g fish^{-1})	136.42±0.72^{a}	137.09±2.59^{a}	135.26±1.74^{a}	140.43±0.54^{b}
WG (%)	461.62±13.9^{ab}	467.74±3.99^{ab}	455.19±12.56^{a}	478.89±1.54^{b}
SGR (% day^{-1})	3.18±0.06^{ab}	3.21±0.02^{ab}	3.16±0.06^{a}	3.26±0.01^{b}
FI (g fish^{-1})	100.95±0.38	100.53±2.25	99.52±1.46	101.28±0.59
FCR	0.99±0.01^{a}	0.97±0.00^{b}	0.98±0.01^{ab}	0.96±0.01^{b}
PER	2.47±0.04^{a}	2.53±0.01^{ab}	2.48±0.04^{a}	2.57±0.02^{b}
HSI	2.01±0.27	2.30±0.53	2.04±0.35	2.69±0.32
K-factor	1.97±0.08	2.02±0.14	1.98±0.02	2.14±0.17
VSI	20.57±2.55	18.70±1.03	19.61±0.32	20.05±1.77
Survival (%)	100	99.17±1.44	100	100

Means in the same row with different superscripts are significantly different ($P < 0.05$). IBW, initial mean body weight; FI, daily feed intake; FBW, final mean body weight; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficient ratio; HSI, hepatosomatic index and VSI, viscero-somatic index.

4.3.2 Whole body composition

Whole body composition of tilapia fed the experimental diets is shown in Table 4.3. No differences were observed in the tilapia body composition except for the lipid content. The lipid content of tilapia fed the protease supplemented diet was significantly lower ($P < 0.05$) compare to that of tilapia fed the phytase supplemented diet. However, there was no significant difference ($P > 0.05$) in the lipid content of tilapia fed the control, xylanase or phytase supplemented diets.

Table 4.3: Whole body composition of tilapia fed enzyme supplemented diets (wet weight per 100 g)

	Control	**Phytase**	**Protease**	**Xylanase**
Moisture (g)	71.86±1.62	72.92±1.96	73.1±0.96	73.79±1.32
Protein (g)	16.15±0.83	14.95±0.98	15.32±0.86	15.41±0.40
Lipid (g)	$6.08±0.32^{ab}$	$6.24±0.53^{b}$	$5.38±0.20^{a}$	$5.58±0.41^{ab}$
Ash (g)	4.39±0.46	4.05±0.65	4.31±0.15	4.15±0.22
Energy (MJ kg^{-1})	6.03±0.33	5.88±0.40	5.52±0.34	5.67±0.30
Phosphorus (mg kg^{-1})	54.73±3.02	57.59±13.17	61.86±3.93	66.22±26.85

Means in the same row with different superscripts are significantly different ($P < 0.05$).

4.3.3 Apparent digestibility coefficients (ADCs) of nutrients and minerals

Apparent Digestibility Coefficient (ADC) is a measure of amount of nutrients and minerals available for utilisation from the experimental diets. Table 4.4 displays the ADCs for dry matter, crude protein, lipid, ash, energy, as well as the mineral bioavailability for phosphorus (P), calcium (Ca), magnesium (Mg), potassium (K), sodium (Na) selenium (Se) and cobalt (Co) from the digestibility study. The phytase supplemented diet is shown to have the most available ($P < 0.05$) crude protein, ash, energy, P, Ca and Na than the remaining experimental diets. There was no significant difference ($P > 0.05$) in the availability of lipid in the experimental diets. The xylanase supplemented diet has the least available ($P < 0.05$) K among the experimental diets. However, the availability of Se and Co was lowest ($P < 0.05$) in the control diet when compared to the enzyme supplemented diets.

Table 4.4: Apparent digestibility coefficients (ADC) of nutrients and minerals (%)

	Control	Phytase	Protease	Xylanase
Dry matter	72	77.57	73.25	66.68
Protein	88.43±0.41^{b}	91.15±0.05^{d}	89.15±0.17^{c}	86.30±0.21^{a}
Lipid	93.11±1.74	93.23±2.87	95.57±1.88	92.37±2.54
Ash	21.92±0.75^{c}	37.75±0.25^{d}	17.58±0.07^{b}	6.39±0.82^{a}
Energy	80.84±0.05^{b}	84.32±0.23^{d}	82.10±0.15^{c}	77.89±0.51^{a}
Phosphorus	37.16±5.06^{b}	48.83±1.23^{c}	36.63±0.82^{b}	35.32±1.87^{a}
Calcium	18.95±4.97^{c}	35.08±1.14^{d}	11.93±3.05^{b}	-3.82±1.32^{a}
Magnesium	63.08±5.48^{a}	66.64±0.87^{a}	53.96±1.12^{b}	50.02±0.29^{b}
Potassium	97.08±0.49^{a}	97.46±0.06^{a}	97.04±0.06^{a}	95.97±0.10^{b}
Sodium	93.09±0.98^{b}	94.56±0.08^{c}	93.54±0.14^{b}	91.62±0.02^{a}
Selenium	38.79±14.63^{a}	72.91±1.49^{b}	69.66±7.04^{b}	62.45±8.89^{b}
Cobalt	-25.48±37.84^{a}	67.18±0.79^{c}	58.06±2.28^{bc}	26.14±5.59^{b}

Means in the same row with different superscripts are significantly different ($P < 0.05$).

4.3.4 Haemato-immunological parameters

The haemato-immunological status of tilapia fed the enzyme supplemented diets was assessed by the measurement of haematocrit, haemoglobin, blood cells count, MCV, MCH, MCHC and serum lysozyme (Table 4.5). Tilapia in all treatments displayed good haemato-immunological status compatible with profiles for this species. No differences were observed between treatments in any parameter measured as a result of dietary treatments.

Table 4.5: Haemato-immunological parameters of tilapia fed enzyme supplemented diets

	Control	Phytase	Protease	Xylanase
Haematocrit, (%PCV)	48.00±2.84	44.56±0.84	45.44±3.15	47.33±3.84
Haemoglobin, (g dL^{-1})	13.03±1.11	13.33±1.03	13.49±1.39	15.16±1.15
RBC (10^6 μL^{-1})	1.43±0.16	1.69±0.33	1.54±0.26	1.58±0.28
WBC (10^3 μL^{-1})	20.49±1.55^{a}	22.81±0.87^{b}	23.14±0.97^{b}	22.32±0.72^{ab}
MCV (fL)	349.9±65.86	284.3±68.10	301.9±45.79	312.7±67.70
MCH (pg)	93.97±7.40	84.95±23.77	89.49±15.49	99.59±27.29
MCHC (g dL^{-1})	27.28±3.88	29.89±1.78	29.66±1.05	32.08±2.89
Lymphocytes (%)	92.64±2.05	94.11±0.72	93.21±0.71	93.84±2.56
Monocytes (%)	4.34±1.47	2.69±0.39	3.99±0.53	3.78±2.36
Granulocytes (%)	3.02±0.60	3.20±0.40	2.81±0.18	2.37±0.39
Serum lysozyme (U)	77.81±6.93	94.31±54	94.09±48.66	145.79±46.85

Figures in each row with similar superscript are not significantly different ($P > 0.05$). RBC, red blood cells; MCV, mean corpuscular volume (haematocrit (%PCV) x 10)/RBC 10^6 μL^{-1}); MCH, mean corpuscular haemoglobin (haemoglobin (g dL^{-1}) x 10)/RBC (10^6 μL^{-1}); MCHC, mean corpuscular haemoglobin concentration (haemoglobin (g dL^{-1}) x 100)/haematocrit (%PCV); U is lysozyme activity unit (activity mL^{-1} min^{-1}); %, mean percentage of total leucocytes

4.3.5 Endogenous enzymes activities

Endogenous digestive enzymes produced in the GI tract are essential part of digestive process, breaking nutrients (macro-molecules) down to smaller forms to enhance absorption and assimilation by the intestinal tract. A measure of quantity of active enzyme present in GI tract is referred to as enzyme activity. Activities of the endogenous enzymes of tilapia fed diets supplemented with exogenous digestive enzymes are displayed in Figure 4.2 -Figure 4.5. Amylase, chymotrypsin, trypsin and total alkaline protease were assessed but there were no significant differences ($P > 0.05$) observed in their activities as a result of dietary treatment.

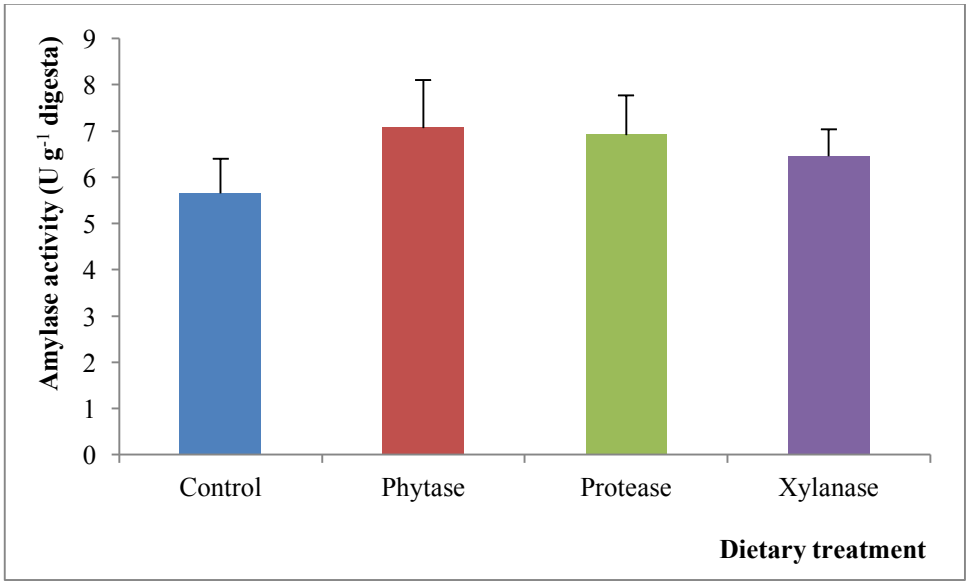

Figure 4.2: Amylase activities (U g^{-1} digesta) of tilapia fed enzyme supplemented diets

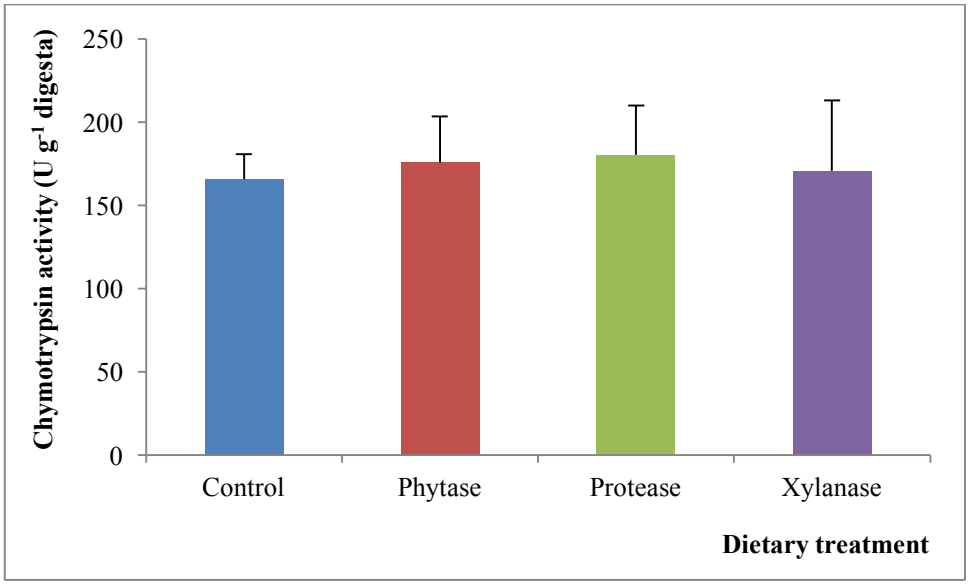

Figure 4.3: Chymotrypsin activities (U g^{-1} digesta) of tilapia fed enzyme supplemented diets

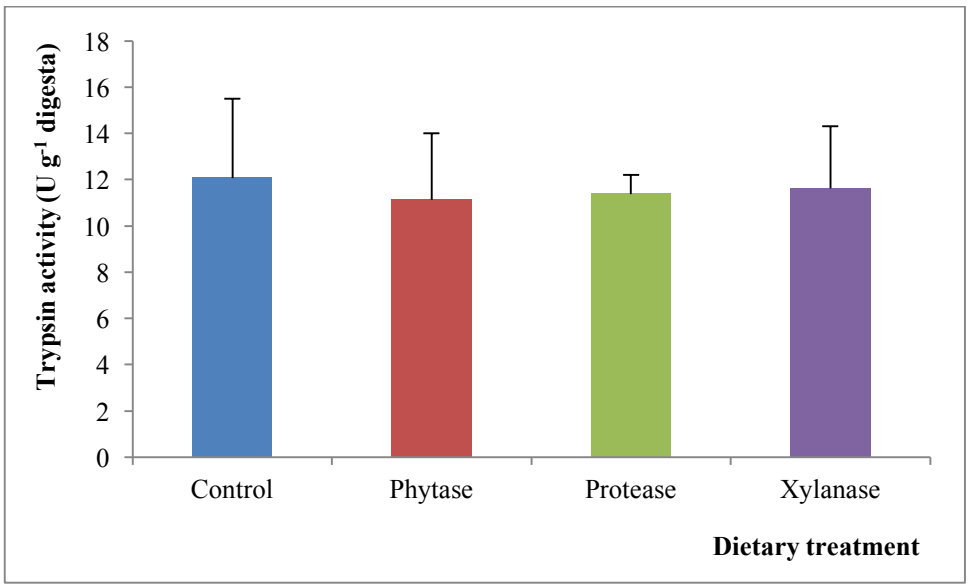

Figure 4.4: Trypsin activities (U g^{-1} digesta) of tilapia fed enzyme supplemented diets

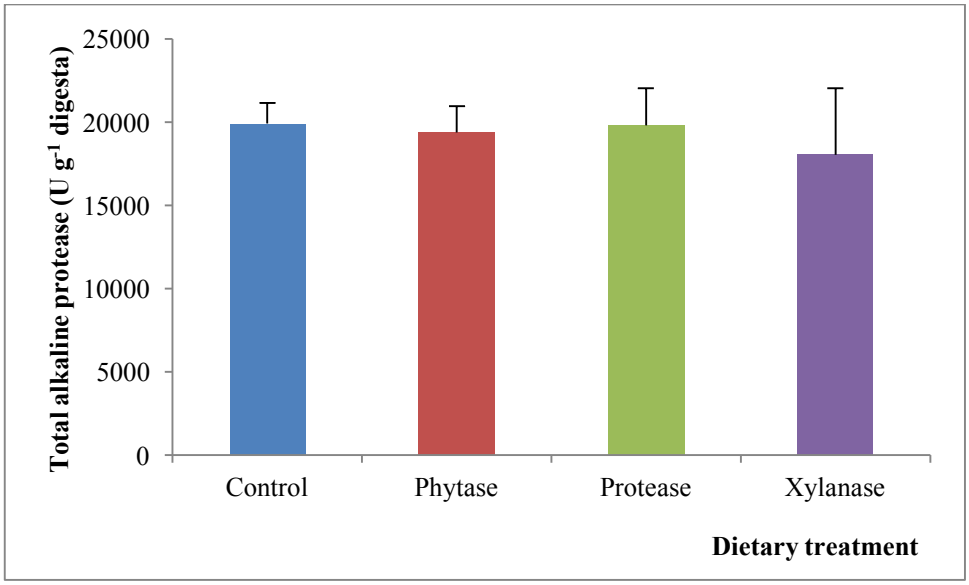

Figure 4.5: Total alkaline protease activities (U g^{-1} digesta) of tilapia fed enzyme supplemented diets

4.3.6 Intestinal histology

The mid-intestine of the tilapia was examined under light microscope after eight weeks of feeding experimental diets. Tilapia from all treatments displayed intact epithelial barrier with extensive mucosal folds which extend into the lumen. Each fold consists of simple lamina propria that house abundant IELs and goblet cells (Figure 4.6). No differences were observed in mid-intestine perimeter ratio and number of goblet cells in the epithelial of tilapia fed the experimental diets (Table 4.6). Tilapia fed the control diet had the highest ($P < 0.05$) IELs abundance when compared to tilapia fed the protease and xylanase supplemented diets. However, there was no significant difference ($P > 0.05$) in the abundance of IELs of tilapia fed the control diet and phytase supplemented diet.

Table 4.6: Intestinal histology of tilapia fed enzyme supplemented diets

	Control	Phytase	Protease	Xylanase
Perimeter ratio	4.14±1.55	3.85±0.20	4.00±0.61	4.55±0.74
Goblet cells (per 100µm)	5.42±1.38	4.61±1.26	3.82±1.07	4.01±0.83
IELs (per 100µm)	36.45±0.90^{a}	31.45±5.01^{ab}	26.96±3.29^{b}	26.83±1.71^{b}

Values with different superscripts indicate significant differences ($P < 0.05$). IELs, Intraepithelial leucocytes

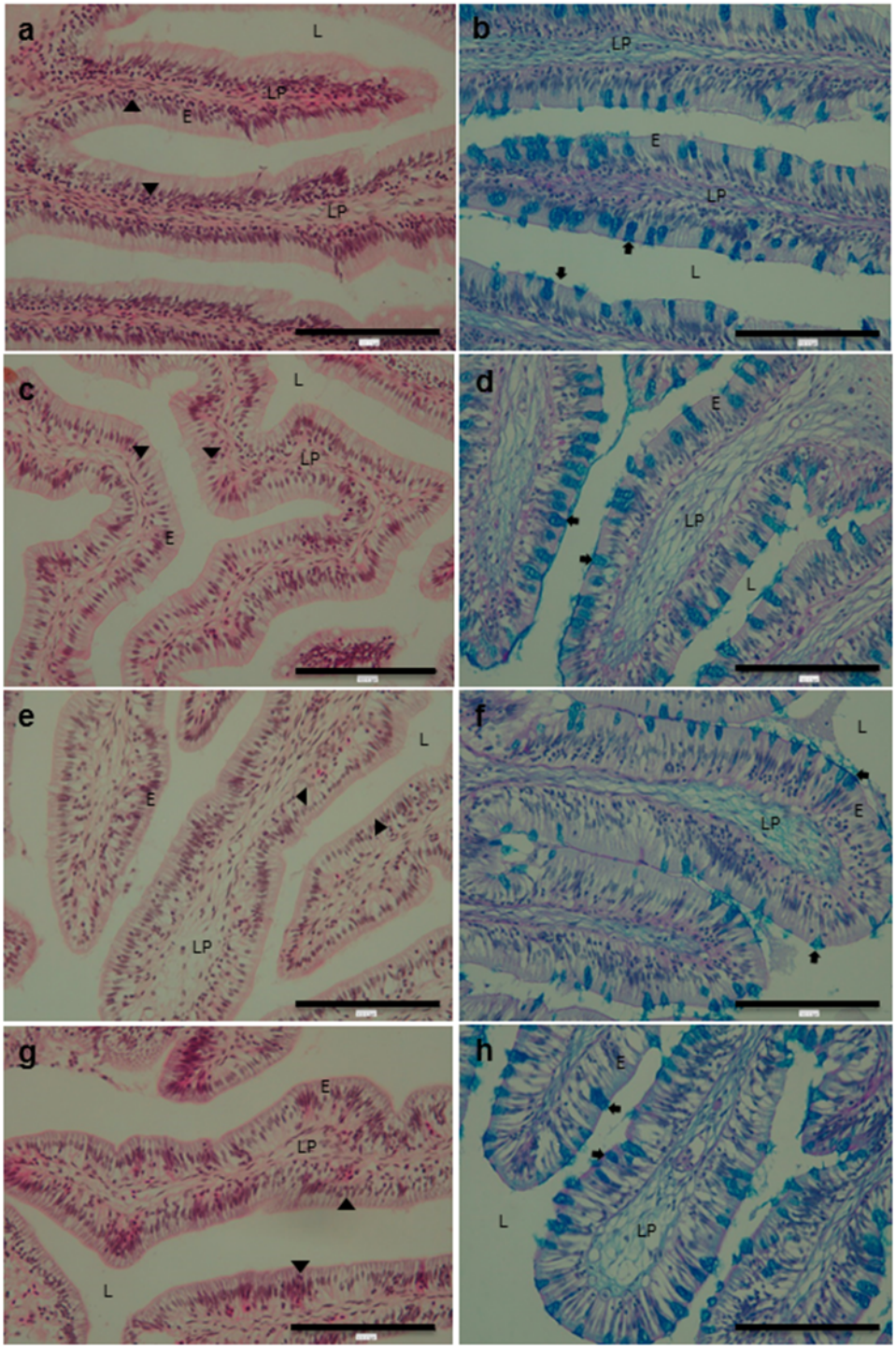

Figure 4.6: Light micrograph of the mid-intestine of tilapia fed control (a & b), phytase (c & d), protease (e & f) and xylanase (g & h) diets. Goblet cells (arrows) are in all treatments and abundant IELs (arrowheads) are present in the epithelia. Abbreviations are E enterocytes, LP lamina propria and L lumen. Light microscopy staining: [a, c, e & g] H & E; [b, d, f & h] Alcian Blue-PAS. Scale bars = 100 µm.

4.3.7 Cost benefit analysis

The incidence costs and profit indices of feeding tilapia exogenous enzymes supplemented diets are displayed in Table 4.7. The incidence cost of feeding tilapia xylanase supplemented diet is lower ($P < 0.05$) compared to the control and protease supplemented diets. However, there was no difference in the incidence cost of feeding tilapia either xylanase or phytase supplemented diet. The profit index was highest ($P < 0.05$) when tilapia were fed the diet supplemented with xylanase.

Table 4.7: Cost benefit analysis

	Control	Phytase	Protease	Xylanase
Cost per kg diet (US $)	0.85	0.85	0.86	0.85
Incidence cost (US $)	0.68^{a}	0.67^{bc}	0.68^{ab}	0.66^{c}
Profit index	2.74 ± 0.02^{a}	2.76 ± 0.01^{a}	2.73 ± 0.01^{a}	2.80 ± 0.01^{b}

Values with different superscripts indicate significant differences ($P < 0.05$). Incidence cost is cost of feed consumed to produce 1 kg weight fish

4.4 Discussion

For cost and sustainability reasons, commercial aquafeeds are increasingly being modified to contain more plant based materials. However the presence of ANFs in plant materials could impair full optimisation of nutrients and consequently result in reduced production and growth performance. Supplementation with bio-active (functional) feed additives may enhance optimisation of nutrient value in the plant materials. Exogenous digestive enzymes as additives offer the potential to deactivate ANFs, optimise nutrient utilisation and also lower P and N excretion into the environment. Exogenous enzymes are also perceived to be harmless, environmentally friendly and natural (Liu and Baidoo, 1997, Dalsgaard et al., 2012, Kumar et al., 2012b, Castillo and Gatlin, 2015). The activity of endogenous digestive enzymes constitutes a considerable factor in the process of digestion and absorption, in particular those located in the brush border section of the intestine. Exogenous enzymes (not naturally produced by fish) complement endogenous digestive enzyme and enhance the degradation of ANFs to release bound nutrients and minerals.

In this study, a commercial aquafeed was supplemented with three exogenous digestive enzymes (phytase, protease and xylanase) separately to assess the most effective enzyme(s) in terms of nutrient utilisation, bioavailability, haemato-immunology, intestinal histology and endogenous enzymes activities in tilapia. The final body weight of tilapia fed xylanase supplemented diet was the highest compared to tilapia fed other experimental diets. Although not significant, the same trend was also observed in FCR and PER performance; tilapia fed a xylanase supplemented diet slightly outperformed tilapia fed the control diet. This could be attributed to the presence of a relatively larger amount of plant substrates (arabinoxylans found in plant materials - soybean, full-fat soya, corn meal, broken rice, rice bran and wheat bran) in the experimental diets. The

availability of the substrate for enzyme activation is one of the factors influencing the efficacy of an enzyme *in vivo*. Xylanase, a NSP-degrading enzyme is able to hydrolyse cell wall components in the plant material, efficiently reducing NSP content of the plant materials and consequently releasing the bound nutrients. Similarly, Jiang et al. (2014) reported improved growth performance in Jian carp (*Cyprinus carpio* var. Jian) fed xylanase (800 mg kg^{-1}) supplemented plant-based diet. Improved growth performance was also reported in Japanese sea bass when fed plant-based diet supplemented with xylanase (Ai et al., 2007). However at lower level of inclusion (67 mg kg^{-1}) in plant based diet fed to rainbow trout, no significant effect was observed in growth (Dalsgaard et al., 2012). It is important to note however that the high quality diet used in this study could have possibly disguised the potential impacts of the supplemented exogenous enzymes that might have occurred on lower quality diet as reported by Wallace et al. (2016) given that the fish appears to attain optimal performance (good FCR and survival) irrespective of dietary treatment.

ADCs, a measure of a nutrient digestibility and mineral bioavailability were also measured in this study. The ADC for protein ranged from 86.30±0.12% (lowest) in the xylanase supplemented diet to 91.15±0.03% (highest) in the phytase supplemented diet for tilapia. Similarly, ADCs of ash, energy, P, Ca, Na and Co were highest in the phytase supplemented diet. The high ADCs in the phytase supplemented diet could be attributed to the degrading effect of phytase on phytate and phytic acid bound minerals and nutrients thereby causing the liberation of the bound minerals and nutrients. P is particularly of environmental concern among the minerals assessed. High bioavailability of P in aquafeed is an important factor for controlling pollution arising from level of nutrients in aquaculture operation wastes. Similarly, a significant improvement in digestibility of P was reported when Nile tilapia were fed phytase supplemented diets (Portz and Liebert,

2004, Liebert and Portz, 2007, Cao et al., 2008). Liu et al. (2013) also reported improvement in digestibilities of crude protein, ash, P and Ca in phytase supplemented diets fed to grass carp.

The haemato-immunological status of tilapia was good and within the healthy range for this species. No differences were observed in the parameters measured possibly due to unestablished interaction between the exogenous digestive enzymes and the tilapia haematology and immune system. Exogenous digestive enzymes mode of action is mainly in the digestive process to enhance better digestibility and utilisation of nutrients rather than modulation of immune factors. However, the level of abundance of IELs (components of gut associated lymphoid tissue – GALT) was significantly lower in tilapia fed protease and xylanase supplemented diets compare to tilapia fed control diet. To the author's knowledge, this is the first time this parameter was measured in tilapia fed exogenous supplemented diets. This requires further study to establish the effect of exogenous digestive enzymes in tilapia immune-stimulation or immune-depression.

One of the factors that improve efficacy of digestive process could be attributed to increase in activity of endogenous digestive enzymes. In this study, the dietary treatments did not affect the activity of the tilapia endogenous digestive enzymes (amylase, chymotrypsin, trypsin and total alkaline protease). This could be the reason why growth performance and feed efficiency (FBW, WG, SGR, and PER) of tilapia fed the phytase and protease supplemented diets were not different from tilapia fed the control diet. Li et al. (2009) reported that neither NSP-degrading enzyme nor phytase affected the activities of endogenous protease but influence the activities of endogenous amylase in digestive tract of tilapia. Also, Hlophe-Ginindza et al. (2015) reported a significant increase in the activities of endogenous enzymes of *Oreochromis mossambicus* fed exogenous digestive enzymes. This difference could be because enzymes used in this study are commercial

single enzymes (phytase, protease or xylanase) unlike the commercial multi-enzyme used by Hlophe-Ginindza et al. (2015).

This trial provides further information on the efficacy of exogenous digestive enzymes (xylanase and phytase) in practical diet to improve nutrients digestibility and minerals bioavailability (especially P that is of environmental concern). Future work could consider combined supplementation of aquafeed with exogenous digestive enzymes and additive (such as probiotics) to enhance fish health and immunity in addition to improved growth and nutrients utilisation. More pronounced effects would more likely be obtained when inferior diets formulated with poorer quality materials are supplemented with exogenous digestive enzymes. Such plant by-products having higher fibre and NSPs may be more sensitive to the effects of exogenous digestive enzymes supplementation in tilapia feed.

5 Chapter 5. Combined effects of exogenous digestive enzymes and probiotics on Nile tilapia (*Oreochromis niloticus*) fed a practical diet

Abstract

The combined effect of exogenous digestive enzymes and probiotics was investigated on the growth, health status, intestinal morphology and microbiota parameters of Nile tilapia (*O. niloticus*). Tilapia (34.56±0.05 g) were fed one of four diets (35% protein, 5% lipid); one of which was a control and the remaining three were supplemented with enzymes (containing 75 mg kg^{-1} phytase, 300 mg kg^{-1} protease and 250 mg kg^{-1} xylanase), 200 mg kg^{-1} probiotics (containing 1 x 10^{10} cfu g^{-1} *Bacillus subtilis, B. licheniformis* and *B. pumilus*) and a combination of both the exogenous digestive enzymes and probiotics. Tilapia fed diet supplemented with a combination of enzymes and probiotics performed significantly better ($P < 0.05$) than tilapia fed the control and probiotics supplemented diets in term of FBW, WG, SGR, FCR and PER. However, there was no significant difference ($P > 0.05$) in the performance of tilapia fed diet supplemented with enzymes and those fed a combination of enzymes and probiotics in terms of FBW, WG, SGR and FCR. The dietary treatment did not affect the tilapia somatic indices. Whole body protein content was highest ($P < 0.05$) in tilapia fed the control diet and lowest in tilapia fed diet supplemented with enzymes. The highest ($P < 0.05$) lipid content was observed in the body of tilapia fed the diet supplemented with a combination of enzymes and probiotics compared to tilapia fed the remaining experimental diets. The serum lysozyme activity was observed to be highest ($P < 0.05$) in tilapia fed probiotics supplemented diet compared to those fed the control diet and diet supplemented with a combination of both enzymes and probiotics. The dietary treatments did not affect endogenous digestive

enzyme activities in the tilapia intestine. The intestinal perimeter ratio was observed to be higher ($P < 0.05$) in tilapia fed diet supplemented with a combination of enzymes and probiotics when compared to those fed with the control or probiotics supplemented diets. Goblet cells abundance, microvilli diameter and enterocyte absorptive area was significantly higher ($P < 0.05$) in tilapia fed diet supplemented with a combination of enzymes and probiotics than those fed the control diet. High-throughput sequencing revealed that majority of reads derived from the tilapia digesta belonged to members of *Fusobacteria* (*Cetobacterium*) distantly followed by *Proteobacteria* and *Firmicutes*. The alpha and beta diversities did not differ among dietary treatments indicating that the overall microbial community was not modified to a large extent by dietary treatment. Conclusively, supplementation of diet with a combination of enzymes and probiotics as a cocktail is capable of improving tilapia growth and intestinal histology without deleterious effect on the fish microbial composition.

5.1 Introduction

The growth of aquaculture as the world's fastest food production sector is linked to population increases and consequently the intensification of the aquaculture operations to meet the arising demand (Msangi et al., 2013). The rearing technologies for intensive operations in aquaculture are often accompanied by sub-optimum environmental conditions (oxygen levels, pH, temperature, nitrogen wastes, etc.) as a result of overcrowding and overfeeding. These conditions may be stressful for fish, leading to decreased performance and subsequently compromise immune response leaving fish prone to infection and disease by opportunistic pathogens. However, with the need to meet global animal protein demand and the growing pressure on fish farmers to reduce production cost without necessarily transferring the cost to the consumers, the stressful conditions associated with intensive aquaculture production is likely to continue. However, the growing concept of immunonutrition (production of high quality feed with optimal growth and immune boosting effects) could be of benefit to intensive aquaculture operation (Nakagawa et al., 2007, Kiron, 2012).

The GI microbiota of fish has been reported to play a role in nutrition and immunity. According to Nayak (2010), GI microbiota is involved in a number of nutritional functions which include digestion, nutrient utilisation and the production of amino acids, enzymes, short-chain fatty acids, vitamins and minerals. The nutritional role of GI microbiota is possibly due to the fact that microbes within the fish digestive tract are capable of secreting digestive enzymes (protease from *Bacillus* sp., cellulase from *Clostridium,* etc.) that could promote nutrient digestion as well as synthesise nutrients (vitamin B12 from *Cetobacterium*) required by fish (Okutani et al., 1967, Saha et al., 2006, Li et al., 2010, Liu et al., 2016). In addition, GI microbiota is capable of influencing immune status, disease resistance, survival, feed utilisation and may have a role in

preventing pathogens from colonising the host (Denev et al., 2009, Ringø et al., 2015). Apart from the nutrition and immunity effects, GI microbiotas of fish have important functions in host metabolism, mucosal development and promote gut maturation (Bates et al., 2006, Rawls et al., 2004, Round and Mazmanian, 2009). The effects of probiotics and exogenous digestive enzymes on fish GI microbiotas (1.11.2) can be further harnessed to improve production of farmed fish. Given the potential complimentary modes of action of exogenous digestive enzymes and probiotics, the two products could improve the growth performance and health status of farmed fish when fed diet supplemented with both the enzymes and probiotics as a cocktail.

Therefore, the objective of the study was to evaluate the combined effects of dietary exogenous digestive enzymes and probiotics on Nile tilapia (*Oreochromis niloticus*) growth, intestinal morphology and microbiome composition.

5.2 Materials and methods

5.2.1 Experimental design and diets preparation

The trial was conducted in a freshwater flow-through aquaculture system (Figure 5.1**)** at the Division of Animal Production Technology of KMITL, Bangkok – Thailand. The flow-through system contains 12 square concrete tanks (508 L capacity each) and were supplied with freshwater sourced from a local river system. Three hundred and sixty all male Nile tilapia (*Oreochromis niloticus*) of mean weight 34.56±0.05 g obtained from CP farm in Thailand were randomly distributed (30 fish per tank) into the 12 tanks after four weeks of acclimatization. During acclimatization, the tilapia were fed ad libitum same commercial diet. The photoperiod and water temperature (30.34±0.15 °C) was maintained at ambient condition. The pH (6.20±0.22) and dissolved oxygen levels (>5.0 mg L^{-1}) in water system were monitored daily using an HQ40d pH meter and dissolved oxygen multi-parameter meter (HACH Company, Loveland, USA). NH_3 (0.304±0.08 mg L^{-1}), NO_2 (0.016±0.002 mg L^{-1}) and NO_3 (1.46±0.19 mg L^{-1}) were also monitored on a weekly basis using a nutrient analyser (SEAL AQ2 Analyser, Hampshire, UK). The flow (4.9 L min^{-1}) of water through the system maintained the water quality by washing off metabolic wastes without causing marked alteration in the water quality.

Figure 5.1: Flow-through system (FS) at the Division of Animal Production Technology and Fisheries, KMITL where tilapia were held during the trial. The experimental system consisted of square concrete tanks with a capacity of 508 L. Water was sourced from the local river system and was monitored daily (pH, DO and temperature) and weekly (nitrogen wastes – ammonia, nitrite, nitrate) to ensure appropriate conditions for tilapia.

A commercial diet (No. 461; 35% protein, 5% lipid) was obtained from INTEQC Feed Co. Ltd., Thailand and was used as the basal formulation. The commercial diet was ground in a blender to powder and sieved to remove large particles. An enzyme cocktail (containing phytase, protease and xylanase), Sanolife PRO-F (a mixture of *Bacillus subtilis*, *B. licheniformis* and *B. pumilus*) and a combination of the enzyme cocktail and Sanolife PRO-F were added to the respective diets (Table 5.1). The diets were coded as control (zero supplementation), enzymes (phytase, protease and xylanase supplementation), probiotics (probiotics supplementation) and enzyprob (enzymes and probiotics supplementation). The diets were mixed thoroughly to ensure homogeneity. Warm water was added to form a consistency suitable for subsequent cold press extrusion

in a pelleting machine (2mm pellet size). After pelleting, the diets were dried in an air convection oven set at 45°C for 24 h. The basal diet served as the control and was prepared in the same way as those supplemented with the enzymes and probiotics except that it did not receive any supplements. The diets were analysed for proximate composition as described in Section 2.5 (Table 5.1). Tilapia were fed the experimental diets for seven weeks at 3% biomass per day in three equal rations. Daily feed was adjusted on a weekly basis by batch weighing following a 24 h starvation.

Table 5.1: Dietary formulation and proximate composition (%) of experiemental diets

	Control	Enzymes	Probiotics	EnzProb
Commercial feed[a]	100	99.9125	99.98	99.8925
Phytase[b]	0	0.0075	0	0.0075
Protease[c]	0	0.03	0	0.03
Xylanase[d]	0	0.025	0	0.025
Probiotics[e]	0	0	0.02	0.02 ($1.9x10^7$)
Proximate composition (% as fed basis)				
Moisture	8.03±0.04	6.87±0.14	8.06±0.06	6.63±0.09
Protein	34.32±0.28	34.78±0.09	34.43±0.13	34.56±0.08
Lipid	5.49±0.04	5.33±0.10	6.38±0.70	5.22±0.08
Ash	13.13±0.11	13.13±0.17	13.16±0.04	13.4±0.04
Energy (MJ kg^{-1})	17.06	17.56±0.01	17.31±0.04	17.66±0.21
Fibre	3.65±0.06	3.15±0.12	3.15±0.07	3.21±0.05

[a]No. 461, INTEQC Feed Co Ltd., Thailand

[b]RONOZYME® Hiphos (contains 10,000FYT g^{-1}) from DSM Nutritional Products

[c]RONOZYME® ProAct (contains 75,000 PROT g^{-1}) from DSM Nutritional Products

[d]RONOZYME® WX (contains 1000 FXU g^{-1}) from DSM Nutritional Products

[e]Sanolife PRO-F (contains 1 x 10^{10} cfu g^{-1} *B. subtilis, B. licheniformis* and *B. pumilus*) from INVE Aquaculture

5.2.2 Growth performance, feed utilisation and somatic indices

Growth performance, feed utilisation and somatic indices were assessed by FBW, WG, FI, SGR, PER, K-factor, HSI and VSI as described in Section 2.4.

5.2.3 Whole body composition of tilapia

Three tilapia per tank (n = 9 per treatment) were sampled and analysed for whole body composition as described in Sections 2.5 and 2.6.

5.2.4 Haemato-immunological analysis

Blood samples were taken at the end of the trial from anaesthetised tilapia to assess haematocrit, haemoglobin, blood cell counts, MCV, MCH and MCHC (n = 9 per treatment). Serum was collected to assess lysozyme activity (n = 15 per treatment). All sampling and analyses were carried out as described in Section 2.7.

5.2.5 Endogenous enzymes activities

Digesta from anterior intestine of three tilapia per tank (n = 9 per treatment) were sampled as described in Section 2.8. Activities of chymotrypsin, trypsin and total alkaline protease were measured.

5.2.6 Histological appraisal of mid-intestine

Samples were obtained from mid-intestine of three tilapia per tank (n = 9 per treatment), processed and screened as described in Section 2.9. Perimeter ratio (AU), goblet cells abundance (per 100 μm), IELs (per 100μm), microvilli count (per μm^2), enterocyte apical area (μm^2), microvilli length (μm) and microvilli diameter (μm) were assessed. Enterocyte total absorptive surface, (μm^2) was calculated as stated below.

ETAS = ((2 x π x ½ MVD x MVL) + (π x ½ MVD2)) x MVCT x EAA

Where ETAS = enterocyte total absorptive surface (μm^2), MVD = microvilli diameter (μm), MVL = microvilli length (μm), MVCT = microvilli count (per μm^2), and EAA = enterocyte apical area (μm^2).

5.2.7 Intestinal microbiology

5.2.7.1 Fish dissection

Five fish per treatment were euthanized in (10 mL L^{-1} water) benzocaine (ethyl aminobenzoate) solution (100 g L^{-1} ethanol) for 10 min. Following the euthanasia, the fish brain was destroyed. The surface of the fish was wiped with 70 % industrial methylated spirit (IMS) to avoid external contamination, the peritoneal cavity of the fish opened aseptically and the intestine was entirely excised. Digesta from the intestine was obtained by gently squeezing the section with a sterile forceps into individual sterile 1.5 mL microcentrifuge tubes. All microbiology samples were stored and transported to Plymouth University in 100 % molecular grade ethanol (Sigma, UK).

5.2.7.2 DNA extraction

Prior to DNA extraction, samples were centrifuged and the ethanol was removed. DNA was extracted from 100 mg digesta samples after lysozyme incubation (50 mg mL^{-1} in TE buffer) for 30 min at 37 °C using PowerFecal® DNA Isolation Kit according to the manufacturer's instructions. The integrity of the extracted DNA was checked using a 1.5% agarose gel (described in Section 3.2.2.6.4) and DNA concentrations were analysed using a Nanodrop™ 1000 (Thermo Scientific Ltd., DE, USA). Samples were stored at -20 °C and subsequently used for downstream procedures.

5.2.7.3 High-throughput sequencing analysis

The DNA samples were prepared for high-throughput sequence analysis as described by (Standen et al., 2015). PCR amplification of the 16S rRNA V1-V2 region was conducted

using primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 338R (5′-GCW GCC WCC CGT AGG WGT-3′). Each PCR contained 0.5 µL primer 27F and 338R (50 pmol μL^{-1}; Eurofins MWG, Germany), 25 µL MyTaq™ Red Mix (Bioline), 22 µL sterile molecular grade water and 2 µL DNA template (diluted 1/10 in molecular grade water). Thermal cycling was conducted using a TC-512 thermal cycler (Techne, Staffordshire, UK) under the following conditions: initial denaturation at 94 °C for 7 min, then 10 cycles at 94 °C for 30 seconds, touchdown of 1 °C per cycle from 62-53 °C for 30 seconds and 72 °C for 30 seconds. Furthermore, 20 cycles were performed at 94 °C for 30 seconds, 53 °C for 30 seconds and 72 °C for 30 seconds before a final extension for 7 min at 72 °C. The quality of the PCR products was checked using agarose gel electrophoresis (as described in Section 3.2.2.6.4). Afterwards, the PCR products were purified (QIAquick PCR Purification Kit; Qiagen) and quantified using a Qubit® 2.0 Fluorometer (Invitrogen). Before sequencing, the amplicons were assessed for fragment concentration using an Ion Library Quantitation Kit (Life Technologies TM, USA), the concentrations were then adjusted to 26 pM. Amplicons were attached to Ion Sphere Particles using Ion PGM Template OT2 400 kit (Life Technologies™, USA) according to the manufacturer's instructions. Multiplexed sequencing was conducted using Ion Xpress Barcode Adapters (Life Technologies™) and a 318™ chip (Life Technologies™) on an Ion Torrent Personal Genome Machine (Life Technologies™). The sequences were binned by sample and filtered within the PGM software to remove low quality reads. Then, data were exported as FastQ files.

Phylogenetic analyses were performed after the removal of low quality scores ($Q < 20$) with FASTX-Toolkit (Hannon Laboratory, USA). Sequences were concatenated and sorted by sequence similarity into a single fasta file, denoised and analysed using the QIIME 1.8.0 pipeline (Caporaso et al., 2010b). The USEARCH quality filter pipeline

(Edgar, 2010) was used to filter out putative chimeras and noisy sequences and carry out OTU picking on the remaining sequences. The taxonomic affiliation of each OTU was determined based on the Greengenes database (DeSantis et al., 2006) using the RDP classifier (Wang et al., 2007) clustering the sequences at 95 % similarity with a 0.80 confidence threshold and a minimum sequence length of 150 base pairs. Non-chimeric OTUs were identified with a minimum pairwise identity of 95 %, and representative sequences from the OTUs were aligned using PyNAST (Caporaso et al., 2010a). To estimate bacterial diversity, the number of OTUs present in the samples was determined and a rarefaction analysis was performed by plotting the number of observed OTUs against the number of sequences. Good's coverage, Shannon-Wiener (diversity) and Chao1 (richness) indices were calculated in addition. The similarities between the microbiota compositions of the intestinal samples were compared using weighted principal coordinate analysis (PCoA).

5.2.8 Cost benefit analysis

Incidence cost and profit index were calculated as described in Section 2.10.

5.2.9 Statistical analysis

Statistical analyses for all data, except high-throughput sequencing, were carried out as described in Section 2.11.

For high-throughput sequence data, a Kruskal-Wallis was performed followed by pairwise comparison to compare alpha diversity metrics, Vegan and ape packages of R were used to analyse the beta diversity of the groups. STAMP v2.1.3 and PRIMER V7 software (PRIMER-E Ltd., Ivybridge, UK) were used to distinguish differences at each taxonomic level for high-throughput sequence data.

5.3 Results

5.3.1 Growth performance, feed utilisation and somatic indices

Growth performance and feed utilisation was assessed using tilapia FBW, WG, SGR, FCR and PER (Table 5.2). Tilapia in all treatments had excellent growth performance with 100% survival in all treatments. Tilapia fed the diet supplemented with a combination of enzymes and probiotics performed significantly better ($P < 0.05$) than tilapia fed the control and probiotics supplemented diets in term of FBW, WG, SGR, FCR and PER. However, there was no significant difference ($P > 0.05$) in the performance of tilapia fed the diet supplemented with enzymes and those fed a combination of enzymes and probiotics in terms of FBW, WG, SGR and FCR. The dietary treatment did not have any noticeable effect on the tilapia somatic indices.

Table 5.2: Growth, feed utilisation and somatic indices of tilapia fed the experimental diets

	Control	Enzymes	Probiotics	EnzProb
IBW (g fish^{-1})	34.5±0.18	34.54±0.05	34.6±0.13	34.61±0.29
FBW (g fish^{-1})	138.04±2.44^{a}	139.49±2.83^{ab}	136.61±1.34^{a}	143.42±3.06^{b}
WG (%)	400.12±7.77^{a}	403.80±7.70^{ab}	394.80±2.39^{a}	414.37±7.46^{b}
SGR (% day^{-1})	3.30±0.05^{a}	3.32±0.04^{ab}	3.27±0.02^{a}	3.38±0.04^{b}
FI (g fish^{-1})	92.24±0.92	92.83±1.22	92.35±0.27	93.00±1.39
FCR	0.94±0.02^{a}	0.93±0.02^{ab}	0.96±0.02^{a}	0.9±0.01^{b}
PER	2.49±0.06^{ab}	2.53±0.06^{b}	2.42±0.05^{a}	2.63±0.02^{c}
HSI	3.19±0.23	3.18±0.26	2.86±0.46	3.10±0.02
VSI	21.72±0.66	21.44±2.96	23.40±1.31	21.83±1.61
K-factor	2.11±0.08	2.06±0.05	2.10±0.07	2.06±0.04
Survival (%)	100	100	100	100

Means in the same row with different superscripts are significantly different ($P < 0.05$). IBW, initial mean body weight; FI, daily feed intake; FBW, final mean body weight; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficient ratio; HSI, hepatosomatic index and VSI, viscero-somatic index.

5.3.2 Whole body composition of tilapia fed the experimental diets

The whole body compositions of tilapia fed the experimental diets are displayed in Table 5.3. Whole body protein content was lowest ($P < 0.05$) in tilapia fed a diet supplemented with the enzymes and highest among tilapia fed the control diet. In the whole body lipid content, tilapia fed the diet supplemented with a combination of enzymes and probiotics had the highest ($P < 0.05$) lipid content compared to tilapia fed the remaining experimental diets. On the other hand, whole body ash and energy content of tilapia fed the control diet were higher ($P > 0.05$) when compared to that of tilapia fed diets supplemented with the enzymes and probiotics. However, no difference ($P > 0.05$) was observed in the whole body ash content of tilapia fed control diet and diet supplemented with a combination of enzymes and probiotics.

Table 5.3: Whole body composition of tilapia fed the experimental diets (per 100g wet weight)

	Control	**Enzymes**	**Probiotics**	**EnzProb**
Protein (g)	15.58 ± 0.24^{a}	13.42 ± 0.05^{c}	14.43 ± 0.31^{b}	14.51 ± 0.33^{b}
Lipid (g)	5.86 ± 0.15^{c}	5.31 ± 0.02^{b}	4.87 ± 0.04^{a}	6.23 ± 0.05^{d}
Ash (g)	4.23 ± 0.28^{c}	2.84 ± 0.32^{a}	3.43 ± 0.52^{ab}	3.93 ± 0.34^{bc}
Energy (MJ)	5.82 ± 0.02^{c}	5.12 ± 0.02^{a}	5.09 ± 0.05^{a}	5.63 ± 0.02^{b}

Means in the same row with different superscripts are significantly different ($P < 0.05$)

5.3.3 Haemato-immunological parameters of tilapia fed experimental diets

The haemato-immunological status of tilapia fed the experimental diets was assessed by the measurement of haematocrit, haemoglobin, blood cells count, MCV, MCH, MCHC and serum lysozyme (Table 5.4). The serum lysozyme activity was observed to be higher ($P < 0.05$) in tilapia fed the probiotics supplemented diet than serum lysozyme activity in tilapia fed the control diet and diet supplemented with a combination of enzymes and probiotics. However, there was no significant difference ($P > 0.05$) in the serum lysozyme activity of tilapia fed diets supplemented with enzymes and diet supplemented with probiotics. No differences were observed between treatments in any other haematological parameters measured.

Table 5.4: Haemato-immunological parameters of tilapia fed the experimental diets

	Control	Enzymes	Probiotics	EnzProb
Haematocrit, (%PCV)	40.11±3.34	39.11±1.35	41.67±3.48	39.66±1.53
Haemoglobin, (g dL^{-1})	11.35±1.21	10.66±0.91	11.93±2.50	11.33±0.22
RBC (10^6 μL^{-1})	1.74±0.10	2.02±0.47	1.92±0.32	1.87±0.09
WBC (10^3 μL^{-1})	20.28±1.34	20.37±4.00	20.59±0.08	20.64±2.82
MCV (fL)	232.53±12.95	207.97±36.80	223.30±34.69	213.04±12.66
MCH (pg)	66.10±4.60	56.25±6.66	62.76±7.18	61.00±4.19
MCHC (g dL^{-1})	28.29±1.59	27.25±1.59	28.75±3.98	28.62±0.97
Lymphocytes (%)	90.43±2.57	91.40±2.38	91.77±1.30	89.43±3.54
Monocytes (%)	5.14±1.87	4.26±2.06	3.94±0.54	5.74±1.97
Granulocytes (%)	4.42±0.70	4.34±0.33	4.29±0.76	4.83±1.62
Serum lysozyme (U)	115.31 ± 22.87^{a}	154.21 ± 24.93^{ab}	170.39 ± 22.98^{b}	127.97 ± 6.43^{a}

Figures in each row with different superscript are significantly different ($P < 0.05$). RBC, red blood cells; WBC, leucocytes; MCV, mean corpuscular volume (haematocrit (%PCV) x 10)/RBC 106 μL^{-1}); MCH, mean corpuscular haemoglobin (haemoglobin (g dl-1) x 10)/RBC (106 μL^{-1}); MCHC, mean corpuscular haemoglobin concentration (haemoglobin (g dL^{-1}) x 100)/haematocrit (%PCV); U, lysozyme activity (activity mL^{-1} min^{-1}) ; %, mean percentage of total leucocytes.

5.3.4 Endogenous enzymes activities

The activities of chymotrypsin, trypsin and total alkaline protease of tilapia fed the experimental diets were assessed (Table 5.5) as a measure of quantity of active enzymes present in the tilapia GI tract. The dietary treatments did not significantly affect the endogenous enzyme activities in the intestine of tilapia fed the respective experimental diets.

Table 5.5: Endogenous enzymes activities (U g^{-1} digesta)

	Control	Enzymes	Probiotics	EnzProb
Chymotrypsin	190.77±21	226.68±70	179.42±27	174.49±37
Trypsin	17.00±2.5	23.46±11.9	18.45±2	18.41±3.8
Total alkaline protease (x10^3)	16.98±2	25.67±10	21.26±1.6	20.84±0.4

Values with different superscripts indicate significant differences ($P < 0.05$)

5.3.5 Intestinal histology of tilapia fed the experimental diets

The mid-intestine of tilapia was examined after seven weeks of feeding the experimental diets by means of light microscopy (Figure 5.2), scanning and transmission electron microscopy (Figure 5.3). Tilapia from all treatments showed intact epithelial barrier with extensive mucosal folds extending into the lumen. Each fold consisted of simple lamina propria that housed abundant IELs and goblet cells (Figure 5.2). Tilapia fed a diet supplemented with a combination of enzymes and probiotics produced significantly higher perimeter ratio and microvilli count (density) compared to tilapia fed probiotic supplemented and control diets (Table 5.6). Goblet cells abundance was significantly higher ($P < 0.05$) in tilapia fed the diet supplemented with a combination of enzymes and probiotics than fish fed the control diet. However, there was no significant difference ($P > 0.05$) observed in IELs abundance, microvilli length and enterocyte apical area of tilapia in all the treatments evaluated. However, microvilli diameter of tilapia fed diet supplemented with a combination of enzymes and probiotics was significantly larger ($P < 0.05$) than that of tilapia fed the control diet. This translated to higher ($P < 0.05$) enterocyte absorptive area observed in tilapia fed the diet supplemented with a combination of enzymes and probiotics than tilapia fed with the control diet.

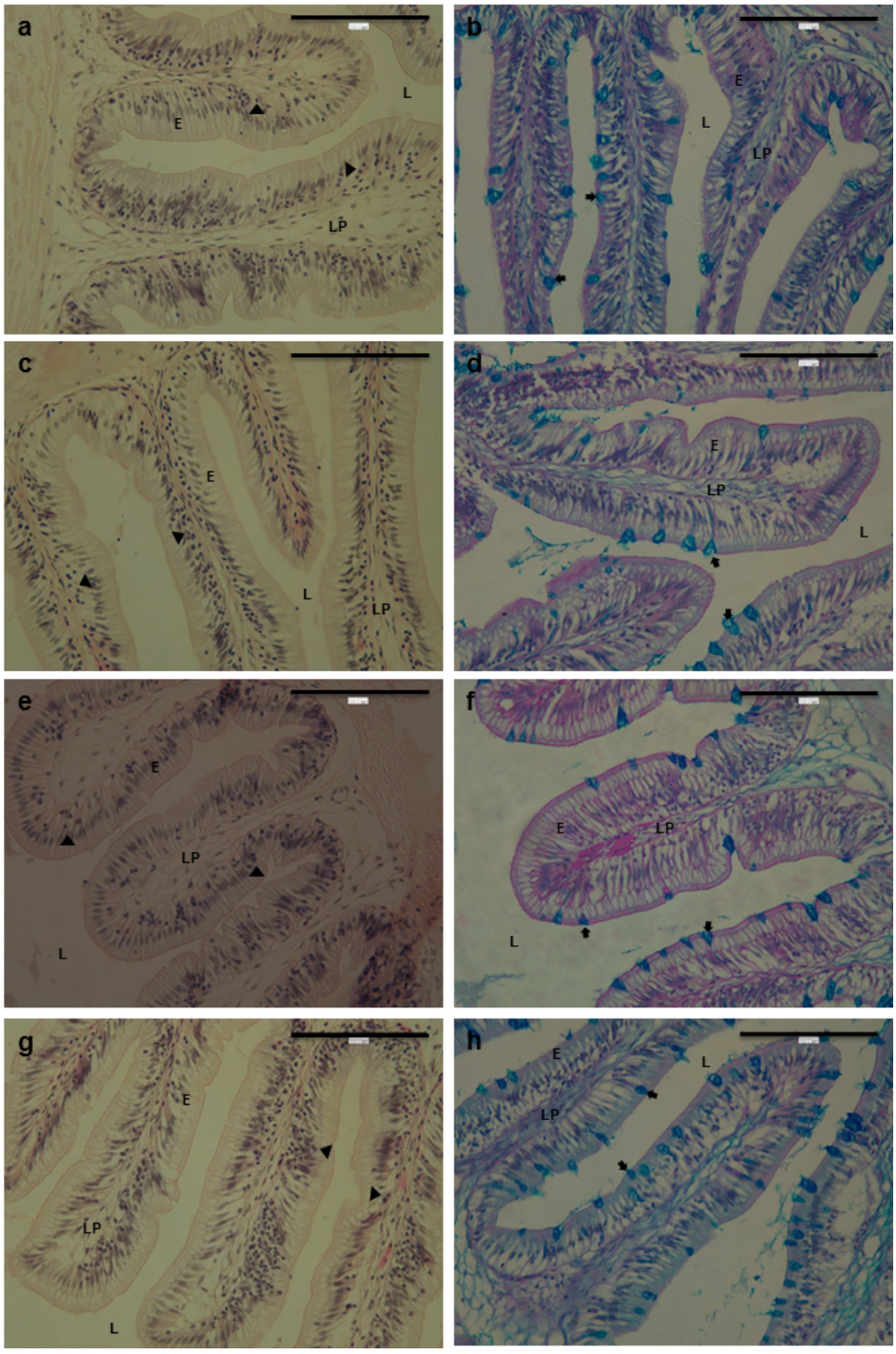

Figure 5.2: Light micrograph of the mid-intestine of tilapia fed control (a & b), enzyme cocktail (c & d), probiotics (e & f) and a combination of enzyme cocktail and probiotics (g & h) diets. Goblet cells (arrows) and abundant IELs (arrowheads) are present in the epithelia. Abbreviations are E enterocytes, LP lamina propria, L lumen and IELs intraepithelial leucocytes. Light microscopy staining: [a, c, e & g] H & E; [b, d, f & h] Alcian Blue-PAS. Scale bars = 100 µm.

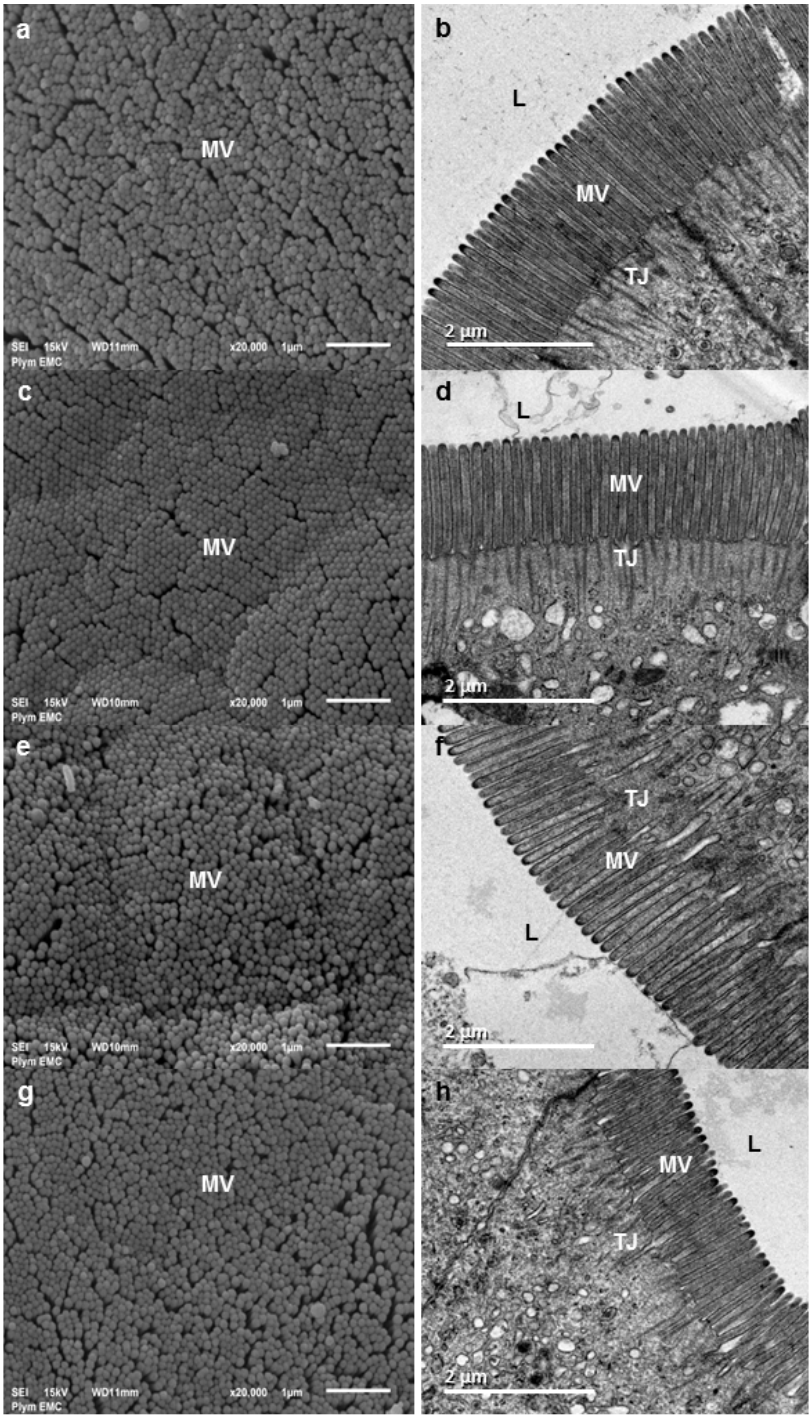

Figure 5.3: Scanning electron (a, c, e & g) and transmission electron (b, d, f & h) micrographs of the mid-intestine of tilapia fed control (a & b), enzyme cocktail (c & d), probiotics (e & f) and a combination of enzyme cocktail and probiotics (g & h) diets after seven weeks. Abbreviations are L lumen, TJ tight junction, MV microvilli. Scale bars = 1 µm (a, c, e & g), 2 µm (b, d, f & h).

Table 5.6: Intestinal histology of tilapia fed the experimental diets

	Control	Enzymes	Probiotics	EnzProb
Perimeter ratio	5.30±0.7^{a}	5.84±0.4^{ab}	5.22±0.5^{a}	6.72±0.8^{b}
Goblet cells (per 100µm)	3.85±0.6^{a}	4.66±0.6^{ab}	4.55±0.6^{ab}	5.11±0.2^{b}
IELs (per 100µm)	29.16±5	29.48±2	29.85±5	28.68±4
Microvilli count (per µm^2)	91.82±4^{a}	110.30±2.2^{bc}	103.75±5.9^{b}	115.17±6.5^{c}
Enterocyte apical area (µm^2)	11.30±1.3	12.39±1.4	12.06±1	12.47±2.1
Microvilli length (µm)	1.24±0.04	1.35±0.03	1.32±0.2	1.27±0.04
Microvilli diameter (µm)	0.117±0.01^{a}	0.123±0.01^{ab}	0.123±0.01^{ab}	0.130±0.00^{b}
ETAS (µm^2)	499.9±82^{a}	762.17±85^{b}	674.55±145^{ab}	773.7±151^{b}

Values with different superscripts indicate significant differences ($P < 0.05$). IELs, Intraepithelial leucocytes; ETAS = enterocyte total absorptive surface.

5.3.6 Intestinal microbiology

A total of 536,602 reads from the tilapia digesta were retained after trimming by quality; after removing low quality reads, 24,521±14,451, 25,588±12,901, 32,708±10,388 and 24,503±12,255 sequences for control, enzymes, probiotics and enzyprob treatments, respectively were used for downstream analyses. Good's coverage rarefaction curves for the treatments reached a plateau close to 1 (0.9994 – 0.9996) (Figure 5.4 and Table 5.7), an indication that sufficient coverage was achieved and that the OTUs detected in the samples are representative of the sampled population.

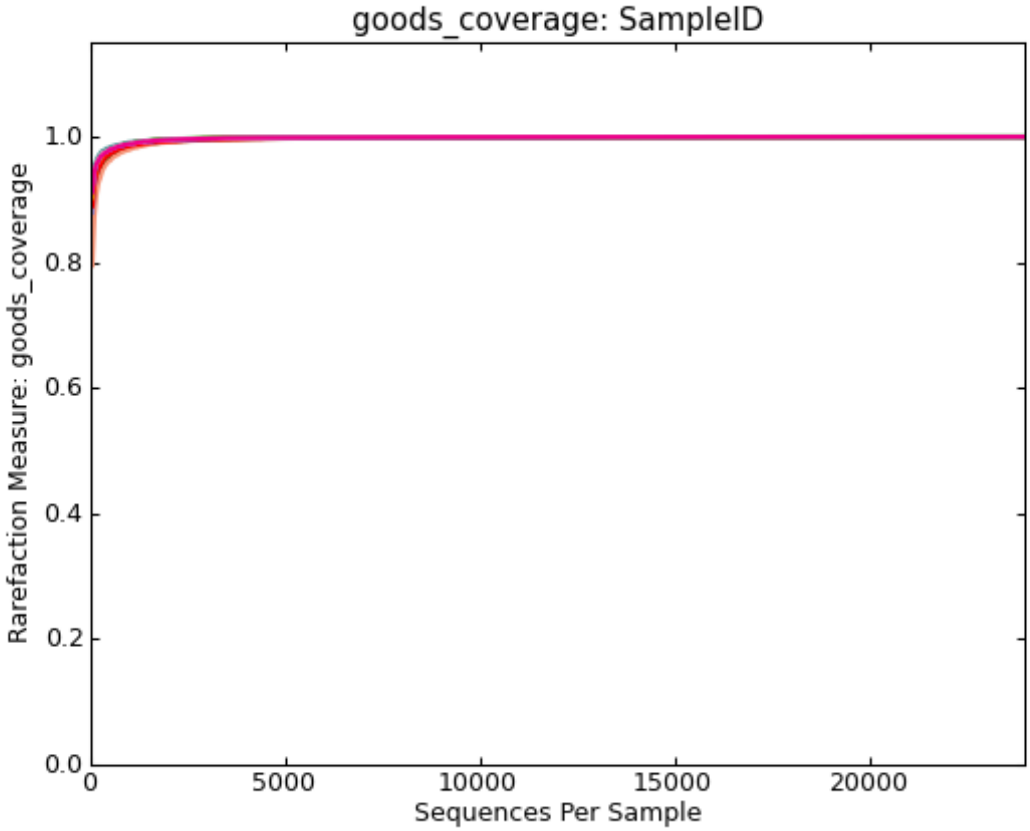

Figure 5.4: Good's coverage rarefaction curves of tilapia digesta

Table 5.7: Number of reads, reads assigned to OTU's, Good's coverage and alpha diversity indices of allochthonous intestinal microbiota composition between control, enzymes, probiotics and enzyprob treatments after 7 weeks of experimental feeding

	Reads (pre-trimming)	Reads assigned (post trimming)	Good's coverage	Observed species	Shanon's diversity index	Chao1 Index
Control	41,748±22,108	24,521±14,451	0.9994±0.0001	75.90±9.54	2.82±0.10	92.00±11.19
Enzymes	42,898±20,096	25,588±12,901	0.9995±0.00007	75.18±14.54	2.78±0.14	88.77±12.04
Probiotics	57,638±15,492	32,708±10,388	0.9996±0.0002	76.95±17.94	3.20±0.60	87.28±16.15
EnzyProb	40,244±18,342	24,503±12,255	0.9994±0.0001	72.12±7.10	2.94±0.25	88.04±8.18

There were no significant differences between the treatments.

The majority of reads derived from the tilapia digesta belonged to members of *Fusobacteria* (> 89%) distantly followed by *Proteobacteria* (> 7%) and *Firmicutes* (≥ 0.4%) (Figure 5.5). Table 5.8 shows the most abundant genera in tilapia digesta. *Cetobacterium, Aquaspirillum, Edwardsiella* and *Plesiomonas* as well as unknown genera from the order *Clostridiales,* family *Clostridiaceae,* class *Gammaproteobacteria* and order *Aeromonadales* were present in all treatments with *Cetobacterium* being dominant in all treatments. *Cetobacterium* accounted for 92.1%, 89.3%, 84.2% and 91% of the 16S reads in tilapia fed the control, enzymes, probiotics and enzyprob diets respectively. Unknown genera from the families *Leuconostocaceae* and *Methylocystaceae* were present in the control, enzymes and probiotics treatments only. *Weissella* and unknown genus from the family *Methylocystaceae* were present in enzymes and probiotics treatments. *Balneimonas* was detected in enzymes and enzyprob treatments. Unknown genus from the class *Betaproteobacteria* was also present in the control, probiotics and enzyprob treatments. However, *Corynebacterium*, *Bacillus, Staphylococcus* and *Rhodobacter* were detected in probiotics treatment only.

The alpha diversity parameters are presented in Table 5.7. There was nominally a higher number of species richness (Chao1) in the control group when compared to tilapia fed the remaining three experimental diets but the difference was not statistically significant. Figure 5.6 shows the beta diversity of the digesta through PCoA plots (based on Bray-Curtis dissimilarity matrix). The PCoA plot shows a spatial differentiation among the treatments.

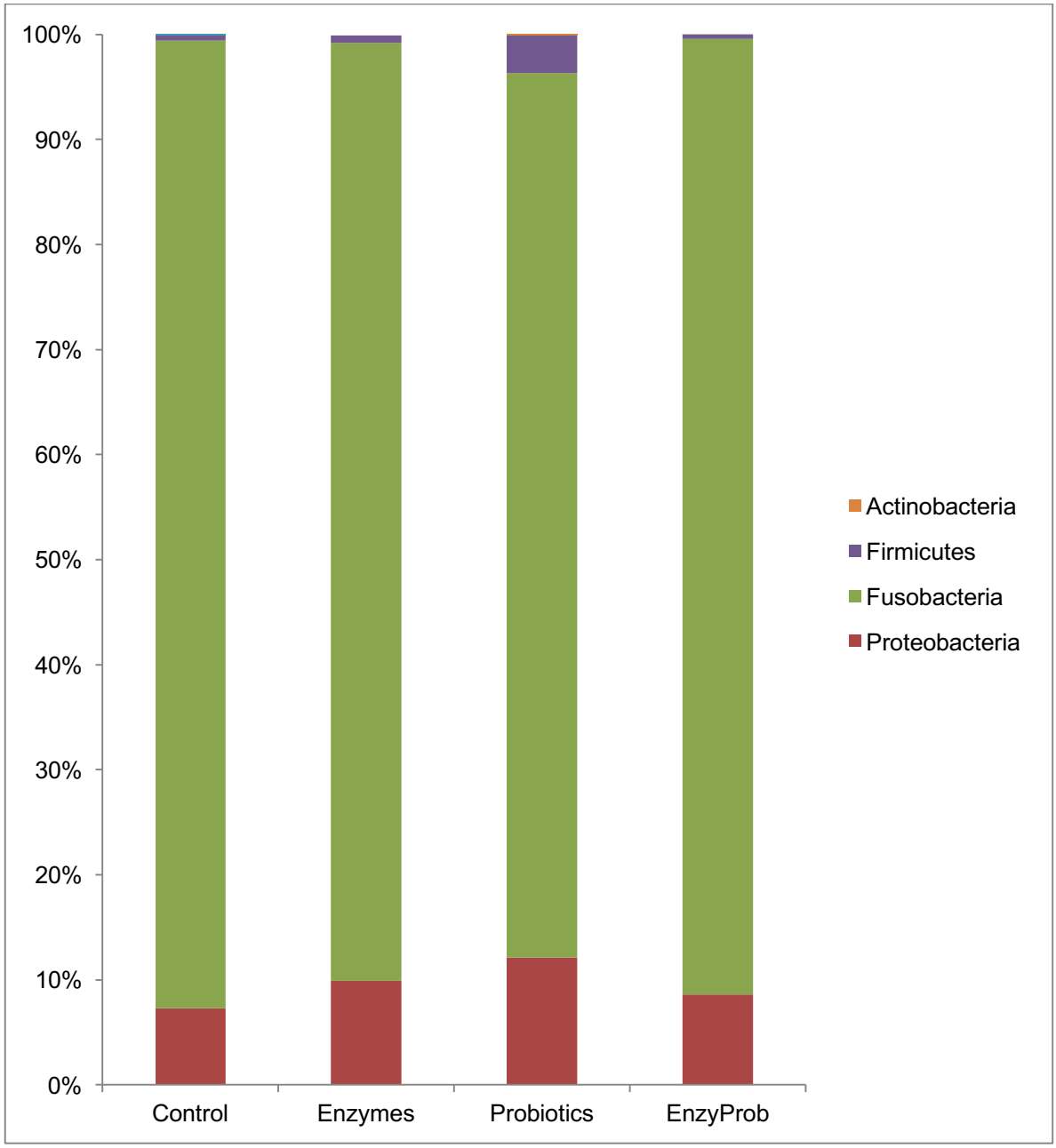

Figure 5.5: Proportion of reads from the digesta samples by dietary treatment assigned at the phylum level. There was no significant difference in the phylum across the treatment.

Table 5.8: Abundance of the OTUs present in digesta samples (expressed as %). General level identification is presented where possible

OTU	Control	Enzymes	Probiotics	EnzyProb
Cetobacterium	92.1±3.8	89.3±4.8	84.21±4.3	91.0±3.4
Plesiomonas	4.0±2.5	7.7±4.4	5.6±1.9	4.0±2.2
Unknown genus from order *Aeromonadales*	2.4±2.4	1.0±0.5	3.1±2.4	2.7±2.4
Aquaspirillum	0.9±0.4	0.4±0.3	1.2±1.3	0.7±0.7
Unknown genus from family *Leuconostocaceae*	0.1±0.1	0.2±0.3	1.5±2.9	0.0±0.0
Unknown genus from family *Leuconostocaceae*	0.1±0.2	0.2±0.3	2.0±3.9	0.0±0.0
Edwardsiella	0.2±0.1	0.6±0.7	1.2±1.4	0.3±0.1
Unknown genus from order *Clostridiales*	0.1±0.1	0.2±0.1	0.1±0.0	0.1±0.1
Unknown genus from family *Clostridiaceae*	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1
Unknown genus from class *Gammaproteobacteria*	0.1±0.0	0.1±0.1	0.3±0.2	0.1±0.1
Unknown genus from class *Betaproteobacteria*	0.2±0.3	0.0±0.0	0.6±1.2	0.1±0.0
Weissella	0.0±0.0	0.1±0.2	0.7±1.4	0.0±0.0
Unknown genus from family *Methylocystaceae*	0.1±0.1	0.2±0.4	0.3±0.6	0.0±0.0
Balneimonas	0.0±0.0	0.1±0.1	0.0±0.0	0.6±1.2
Unknown genus from family *Methylocystaceae*	0.0±0.0	0.1±0.1	0.2±0.3	0.0±0.0
Rhodobacter	0.0±0.0	0.0±0.0	0.4±0.9	0.0±0.0
Leuconostoc	0.0±0.0	0.1±0.1	0.1±0.2	0.0±0.0
Staphylococcus	0.0±0.0	0.0±0.0	0.2±0.4	0.0±0.0
Corynebacterium	0.0±0.0	0.0±0.0	0.1±0.2	0.0±0.0
Bacillus	0.0±0.0	0.0±0.0	0.1±0.2	0.0±0.0

There was no significant difference in genus composition across the treatments

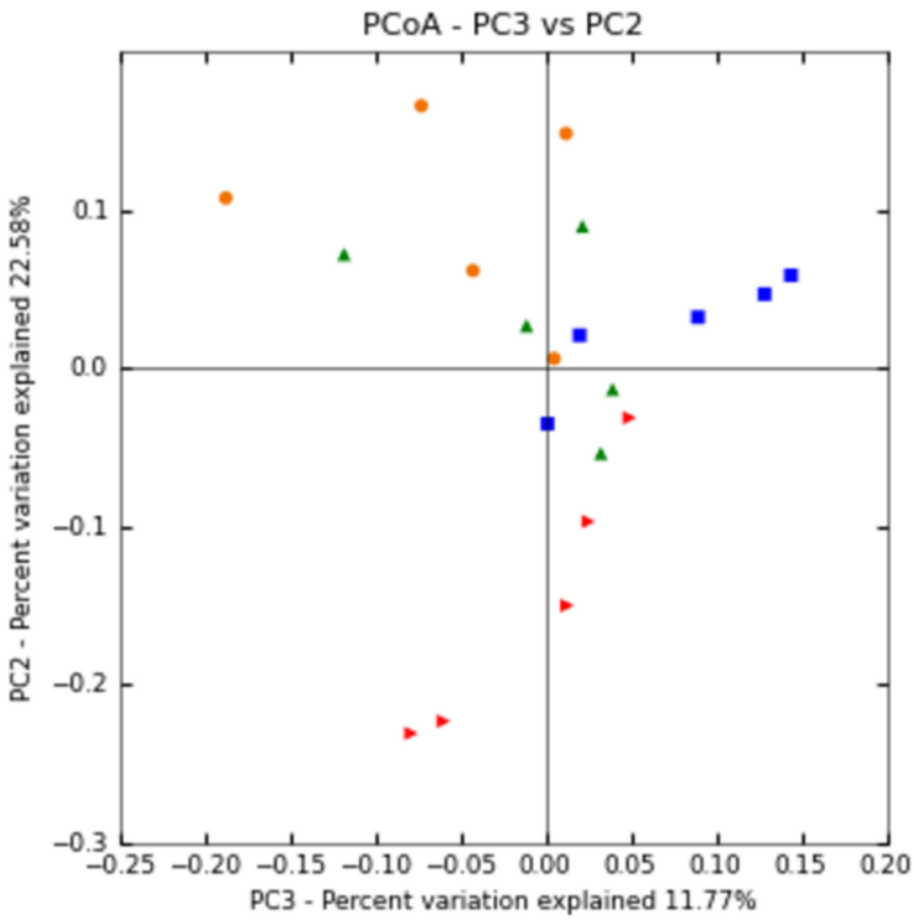

Figure 5.6: Principal coordinates analysis (PCoA) of digesta samples using Bray-Curtis dissimilarity matrix where data points represent samples from tilapia fed a control diet (red triangles), enzymes supplemented diet (blue squares), probiotic (green triangles) and enzyprob diet (orange circles)

5.3.7 Cost benefit analysis

The incidence costs and profit indices of feeding tilapia the experimental diets are displayed in Table 5.9. The incident costs of feeding tilapia either of diet supplemented with enzymes or probiotics is higher ($P < 0.05$) than feeding tilapia with diet supplemented with a combination of enzymes and probiotics. However, there was no significant difference in the incidence cost of the control diet and diet supplemented with a combination of exogenous enzymes and probiotics. Consequently, the profit index was significantly more in tilapia production with diet supplemented with a combination of exogenous enzymes and probiotics compared to tilapia production with diets supplemented with the enzymes or probiotics.

Table 5.9: Cost benefit analysis

	Control	**Enzymes**	**Probiotics**	**EnzyProb**
Cost per kg diet (US $)	0.85	0.86	0.85	0.86
Incidence cost (US $)	0.63 ± 0.00^{ab}	0.63 ± 0.00^{b}	0.64 ± 0.01^{b}	0.62 ± 0.00^{a}
Profit index	2.95 ± 0.02^{ab}	2.93 ± 0.02^{a}	2.90 ± 0.03^{a}	2.99 ± 0.02^{b}

Values with different superscripts indicate significant differences ($P < 0.05$). Incidence cost is feed cost consumed to produce 1 kg of weight gain of fish

5.4 Discussion

The effects of exogenous digestive enzymes and probiotics to enhance fish growth performance as individual supplement have been reported (as reviewed in Section 1.11.1). However, to the authors' knowledge no research has been conducted previously on the combined effects of exogenous digestive enzymes and probiotics on growth, intestinal morphology and microbiome of Nile tilapia. In this study, Nile tilapia were fed diets supplemented with enzymes, probiotics and a combination of both the enzymes and probiotics. Given the potential complimentary modes of actions of exogenous digestive enzymes and probiotics, the two products (when used in combination) could offer more benefits than when used alone. This is confirmed in this study with improved growth performance in terms of FBW, SGR, FCR and PER observed in tilapia fed diet supplemented with the enzyprob (a combination of enzymes and probiotics). The enhanced growth performance could be attributed to the ability of probiotics to produce fibre-degrading enzymes that may complement endogenous enzyme activity for digestion in fish (Roy et al., 2009, Ray et al., 2010, Ray et al., 2012) as well as the external exogenous enzyme capacity to increase the availability of suitable substrates for probiotic action (Bedford and Cowieson, 2012). In addition, enzymes could positively affect the gut microbiota through improved digestibility and enhanced nutrient absorption and assimilation. The indigestible NSPs and trypsin inhibitors that appear to induce necrotic enteritis in certain fish species are well known substrates for xylanase and protease enzymes respectively. Furthermore, xylanase may increase the digestion of NSPs (e.g. arabinoxylans) which could provide substrates for utilisation by beneficial bacteria (Bedford, 2000).

The use of enzymes and probiotics as single supplement in this study do not have significant effect on the growth performance of tilapia. The growth of tilapia fed the

enzymes supplemented diet in current study is contrary to Hlophe‐Ginindza et al. (2015) observation of high growth performance when an exogenous enzyme cocktail, Natuzyme® (containing protease, lipase, α-amylase, cellulase, amyloglucosidase, β-glucanase, pentosonase, hemicellulose, xylanase, pectinase, acid phosphatase and acid phytase) were added to a plant-based diet and fed to *Oreochromis mossambicus*. The inconsistency in the findings may be due to lower application dosage of enzymes (75 mg kg^{-1} phytase, 300 mg kg^{-1} protease and 250 mg kg^{-1} xylanase) used in the current study compared to 500 mg kg^{-1} used by Hlophe‐Ginindza et al. (2015). On the other hand, the insignificance difference in growth of tilapia fed probiotics supplemented diet when compared to the control group in the current study is similar to findings of Ng et al. (2014) who reported that dietary probiotics (*B. subtilis, B. licheniformis* or *Pediococcus* sp.) had no effect on growth or feeding efficiencies of tilapia. Shelby et al. (2006) also reported the non-effect of dietary *Enterococcus faecium* or *Pediococcus acidilactici* or mixtures of *B. subtilis* and *B. licheniformis* on growth of tilapia. However, *B. subtilis* when used solely as a dietary supplement was reported to be an effective growth promoter in tilapia (Aly et al., 2008a), yellow croaker, *Larimichthys crocea* (Ai et al., 2011) and rohu, *Labeo rohita* (Nayak and Mukherjee, 2011).

The improvement in intestinal morphology in the current study could be the result of complimentary changes to meet the increased rates of digestion and absorption after exposure to the diets. In this study, tilapia fed enzyprob diet yielded higher perimeter ratio, microvilli count (density) and larger diameter which translated to increased enterocyte absorptive area and subsequently resulted in the improved growth performance when compared with tilapia fed the control diet. This could be attributed to the combined effect of enzymes and probiotics to confer a superior beneficial effect than when used alone. However, there was no significant difference between intestinal histology of tilapia fed

the control and probiotics supplemented diets. This is contrary to Standen et al. (2015) who reported increased population of IELs, a higher absorptive surface area index and higher microvilli density in the intestine of tilapia fed a diet supplemented with AquaStar® Growout, a multi-species probiotics containing *Lactobacillus reuteri, Bacillus subtilis, Enterococcus faecium and Pediococcus acidilactici*. This difference could be attributed to different probiotic composition as well as application dosage which is 20 mg kg^{-1} in the present study compared to 5 g kg^{-1} used by Standen et al. (2015).

In this study, the dietary treatment did not have significant effect on the tilapia haematological parameters. Emadinia et al. (2014) reported that dietary supplementation of poultry diets with an enzyme cocktail (xylanase, β-glucanase, cellulase, pectinase, phytase, protease, lipase, and α-amylase) had no obvious effect on haemato-immunological parameters. However, the serum lysozyme activity was significantly higher in tilapia fed the probiotic supplemented diet compared to those fed the control and enzyprob supplemented diets. This is similar to the findings of Mandiki et al. (2011) who reported that dietary *Bacillus* probiotics have potential stimulating impact on lysozyme activity in Eurasian perch, *Perca fluviatilis*. The increased lysozyme activity could be due to the effects of probiotics alone as this effect was not observed in tilapia fed the remaining experimental diets. Standen et al. (2013) also suggested that dietary probiotics are able to stimulate innate immune response in tilapia.

Dietary supplementation of exogenous enzymes has the potential to complement endogenous enzymes produced by fish to enhance digestibility of plant nutrients. In addition, dietary supplementation of exogenous enzymes could lead to increase in the activities of endogenous enzymes and consequently improves the efficacy of digestive process (Hlophe-Ginindza et al., 2015). The activity of digestive enzymes in fish is directly related to fish digestive ability (Wen et al., 2009) and generally correlates with

growth rate of fish (Hidalgo et al., 1999). This is confirmed in findings on effect of exogenous enzymes and probiotics on endogenous digestive activities. Hlophe - Ginindza et al. (2015) reported high levels of digestive enzyme activities in fish fed diet supplemented with 0.5 g kg^{-1} Natuzyme50®. Similarly, dietary supplementation of exogenous enzyme (containing protease, β-glucanase and xylanase) to a plant based diet (at 1.0 and 1.5 g kg^{-1} dosage) fed to Nile tilapia resulted in increased secretion of the tilapia endogenous enzymes (amylase and protease) (Lin et al., 2007). Li et al. (2009) also reported that NSP-degrading enzyme (1g kg^{-1}) and phytase (1g kg^{-1}) increased the activities of endogenous amylase but did not have effect on activities of protease and lipase in tilapia digestive organs. In addition an increase in the activities of trypsin, chymotrypsin, lipase and amylase were reported in Jian carp when fed xylanase supplemented diets (Jiang et al., 2014). Essa et al. (2010) also reported that probiotics (*Bacillus subtilis*) improved the activities of Nile tilapia endogenous enzymes (amylase, protease and lipase). However, in the present study there were no significant differences recorded in the activities of the endogenous enzymes measured. The non-effect may be due to low application dosage of exogenous enzymes (75 mg kg^{-1} phytase, 300 mg kg^{-1} protease and 250 mg kg^{-1} xylanase) used in the current study compared to enzymes application dosage in the positive findings obtained from other researchers. In addition, the difference in findings could also be as a result of different production methods employed by products (probiotics and exogenous enzymes) manufacturers.

Gut microbiota may function to prevent pathogens from colonization of the intestinal tract. The importance of commensal gut microbiota is highly significant for normal functioning of the immune apparatus of GI tract in fish (Rawls et al., 2004, Pérez et al., 2010, Ringø et al., 2015). The population size and composition of intestinal microbiota could influence the extent of nutrient digestion and absorption in their host environment (Merrifield et al.,

2010a, Dimitroglou et al., 2011, Bedford and Cowieson, 2012, Ray et al., 2012). In addition, GI microbiota are understood to influence immune status, disease resistance, survival and feed utilisation (Denev et al., 2009). For instance, Jiang et al. (2014) reported that dietary supplementation of xylanase affected the amount of *Lactobacillus*, *Escherichia coli* and *Aeromonas* in the intestine of juvenile Jian carp thus influencing the intestinal microbial population of juvenile Jian carp. The intestinal microbiota of grass carp fed supplemental cellulase changed in respect to bacteria species and density (Zhou et al., 2013). Adeoye et al. (2016) also reported alteration in the intestinal bacterial community profile of tilapia fed carbohydrase supplemented diet. Similarly, several studies have reported the modulating effect of probiotics on fish GI microbiota (as reviewed in Section 1.11.1). However, in the present study exogenous enzymes and probiotics did not modify to a large extent microbial community of tilapia fed the experimental diets. Regardless of the dietary treatments, certain OTUs such as *Clostridiales, Cetobacterium, Aquaspirillum, Gammaproteobacteria, Aeromonadales, Edwardsiella* and *Plesiomonas* were found in the intestinal tract of tilapia. This is similar to findings by Larsen et al. (2014) who reported dominance of genus *Cetobacterium* in warm water fish species. Shared core gut microbiota was observed in zebrafish irrespective of geographical locations (Roeselers et al., 2011). Wong et al. (2013) also reported core intestinal microbiota in rainbow trout being resistant to variation in diet and rearing density. Similarly, the tilapia microbiome was quite stable and resistant to potential changes in community abundance and diversity in response to the dietary supplements used in this study. However, the functionality of the microbiome may have been altered and this may have contributed towards the improved performance of the tilapia fed the enzymes and probiotics (enzyprob) supplemented diet. Future studies

should include metagenomics and transcriptomics of the gut microbiome to investigate this hypothesis.

In conclusion, supplementation of tilapia diets with combination of enzymes and probiotics is capable of improving tilapia growth and intestinal histology without deleterious effect on the fish health or intestinal microbiota. It is pertinent therefore to consider these findings for the future development of diets specific for tilapia under a variety of culture conditions and stages of growth from fry to fingerlings and on-growing to production (harvest) size.

6 Chapter 6: General discussion

Plant materials (which are often of low quality and cheaper than marine ingredients) have economic and production stability (Gatlin et al., 2007, Hardy, 2010) and can support the sustainability and growth of aquaculture. Studies have shown that plant materials contain ANFs as a part of their inherent defence mechanisms which can impair fish physiology and interfere with digestive processes as well as intestinal tissue in fish (Francis et al., 2001, Krogdahl et al., 2010, Sinha et al., 2011, Chikwati et al., 2013). Due to the increasing dependence of aquaculture on plant materials, the use of bio-active feed additives has become important to reduce the effects of ANFs and optimise the low quality cheaper alternative ingredients (plant materials). The focus of this thesis is to assess the potential benefit(s) of selected bioactive feed additives (i.e. exogenous digestive enzymes and probiotics) in intensive tilapia aquaculture. The investigation compared the efficacy of exogenous digestive enzymes (phytase, protease and carbohydrase) in tilapia fed semi-purified diet (Chapter 3) as a proof of concept. Afterwards, the efficacy of the exogenous digestive enzymes (phytase, protease and xylanase) was investigated in practical conditions when tilapia were fed a commercial diet for this species (Chapter 4). Finally the study investigated combined effects of exogenous digestive enzymes (containing phytase, protease and xylanase) and probiotics (containing *B. subtilis, B. licheniformis* and *B. pumilus*) on tilapia fed a practical diet (Chapter 5). Each of the three experiments conducted in this research programme investigated aspects which have not (or only marginally) been in the focus of research related to the dietary administration of exogenous digestive enzymes and probiotics in tilapia aquaculture.

An *in vitro* digestion technique was useful for a preliminary and rapid screening of the exogenous digestive enzymes (Chapter 3A) before embarking on expensive and comprehensive *in vivo* feeding trial (Chapters 3B and 4) for elaborate evaluations of the full potential of the exogenous digestive enzymes. Data obtained from the *in vitro* digestion technique could have been validated with an *in vivo* digestibility data. Unfortunately, digestibility study was not carry out due to technical difficulties associated with obtaining faecal material from tilapia for more comprehensive nutrients digestibility data. Further work is required to refine the *in vitro* technique. Nonetheless, the potential benefits elucidated by the *in vitro* study indicated that *in vivo* assessment of the enzymes was warranted.

The results of Chapter 3B revealed that supplementation of a semi-purified diet with phytase resulted in better growth performance of tilapia under defined conditions. However, when a practical diet was supplemented with phytase, the observed growth performance (FBW, WG and SGR) was not significantly different from that of tilapia fed the control diet (Chapter 4). This could be attributed to a number of factors including differences in enzyme dosage and diets. The application dosage of phytase in the practical diet (75 mg kg^{-1}) was lower (a more practical and cost-effective dosage rate) compare to testing dosage in semi-purified diet (300 mg kg^{-1}). In addition, the practical diet could be of higher quality (0.97 FCR) compare to the semi-purified diet (1.36 FCR) going by series of refinement by the feed manufacturer to gain market competitive edge as well as meeting customers' demand.

Works conducted in Chapters 3B and 4 showed that there was no significant difference in growth performance (SGR) of tilapia fed diets supplemented with NSP-degrading enzymes (ROXAZYME® G2 in Chapter 3B and RONOZYME® WX in Chapter 4) when compared with tilapia fed a diet supplemented with phytase. Work conducted in Chapter

3B suggested that the improved performance (FCR and PER) in carbohydrase treatment could be as a result of the presence of xylanase rather than β-glucanase nor cellulase components of ROXAZYME® G2. When xylanase (RONOZYME® WX) was used alone in Chapter 4, the effect on growth performance (3.26 SGR) was more pronounced than when used in combination with β-glucanase and cellulase as components of ROXAZYME® G2 (2.31 SGR). The combination of three exogenous digestive enzymes (phytase, protease and xylanase) as a cocktail in Chapter 5 revealed a marginal difference in growth performance (3.32 SGR) compared to work carried out in Chapter 4 when the enzymes were used as individual supplement (3.21, 3.16 and 3.26 SGR for phytase, protease and xylanase treatments respectively). This suggests that all enzymes may not be active when used as a cocktail. The non-effect of protease supplemented diets on the growth performance of tilapia (Chapters 3B and 4) could suggest that the marginal improvement observed in the growth of tilapia fed an enzyme cocktail diet (Chapter 5) could be attributed to the presence of phytase and NSP-degrading enzyme in the enzyme cocktail. Further study is required to establish this speculation.

Though, many studies on dietary probiotics have reported improved growth performance in tilapia (Abdel-Tawwab et al., 2008, Aly et al., 2008b, Aly et al., 2008c, Wang et al., 2008, El-Rhman et al., 2009, Zhou et al., 2010a, Zhou et al., 2010b, Gonçalves et al., 2011, Jatobá et al., 2011, Ayyat et al., 2014, Eissa and Abou-ElGheit, 2014, Ridha and Azad, 2015) but in work carried out in Chapter 5, growth performance of tilapia fed diet supplemented with probiotics was not significantly different from those fed the control diet. The difference in finding in this study and past findings could be attributed to different composition of probiotics as well as different application dosage rate. However, a combination of probiotics and enzymes resulted in a better growth performance than

when probiotics alone was used. Again, this requires further research to establish the modes of operation between exogenous digestive enzymes and probiotics.

It is important to note that growth performance similar to control treatment can be considered as a positive effect if the additive (the exogenous enzymes and probiotics) manifested other benefits (such as reduction of nutrient load to the environment, immune modulation or improvement in intestinal morphology) which would have required the utilisation of energy and resources. As observed in Chapter 4, tilapia receiving the phytase treatment (75 mg kg^{-1}) had the same growth performance with the control group but the phytase treatment manifested additional benefit of positive environmental effects (i.e. high ADCs of ash, phosphorus and calcium, an indication of reduction of nutrient waste from an intensive aquaculture operation). Similarly, though tilapia fed with a protease supplemented diet yielded the same growth performance with those fed the control diet yet the protease treatment produced a large proportion of IELs (Chapter 3B) which could stimulate or enhance localised GI immune response. Also, in Chapter 5, tilapia fed probiotics supplemented diet had the same growth performance with those fed the control diet but serum lysozyme activity in the probiotics treatment was significant higher than the control group. It is important however to note that the high quality of the diets used in this study probably disguised the potential impacts of exogenous digestive enzymes that might have occurred in lower quality diets as the fish appears to be performing close their optimal.

Haemato-immunological analysis is an important diagnostic tool in the assessment of dietary treatment on fish health and immune status. Both the exogenous digestive enzymes and the probiotics did not appear to have marked effect on haemato-immunological parameters in the present study except for carbohydrase effect on red blood cells, phytase effect on monocytes (Chapter 3) and probiotics effect on serum

lysozyme activity (Chapter 5). The increased red blood cells count could infer better immune response (Jiang et al., 2007). Monocytes are one of the main immune cells of the innate immune system in fish and are precursor cells to macrophages and dendritic cells (which are phagocytic). The proportionally high abundance of these cells (monocytes) in tilapia fed phytase diet (Chapter 3) could also result in better immune response and improved health status. Lysozyme, an important index of fish innate immunity, is one of the humoral components of innate immune mechanism in fish (Pohlenz and Gatlin, 2014), possess lytic activity against Gram positive bacteria and plays a role in fish defence processes. An increase in the serum lysozyme activity of tilapia fed the probiotic supplemented diet (Chapter 5) is a boost in innate immunity of tilapia fed this diet. The elevated proportion of mucus-producing goblet cells residing in the intestine of tilapia fed the diet supplemented with a combined enzymes and probiotics (Chapter 5) is likely to improve the intestinal barrier function, ultimately retarding pathogen attachment and subsequent infection.

The histological appraisals (either through light or electron microscopy) of fish intestine assist in determining fish GI integrity and intestinal health as well as level of nutrient absorption and utilisation efficiency. The improved growth performance of tilapia in this study could be the consequence of an increased enterocyte absorptive area as observed in tilapia fed the diet supplemented with a combination of enzymes and probiotics (Chapter 5). In the present studies, the trend of intestinal perimeter ratio is directly proportional to fish growth performance (In Chapter 3, 6.22 for phytase treatment and 6.77 for enzyprob treatment in Chapter 5). Increased perimeter ratio is synonymous to enhanced mucosal fold depth and microvilli length and subsequently enhances the absorptive area of the lumen intestinal interface. This consequently increases nutrient digestibility as well as improvement in growth performance and feed utilisation.

To the author's knowledge, no research has been conducted previously on the effects of exogenous digestive enzymes on intestinal microbial communities of tilapia. In this study, intestinal microbiota of tilapia was examined using two methods. First the DGGE (Chapter 3), a semi-quantitative approach that separate dominant OTU amplicons based on nucleotide denaturing properties (Ferguson et al., 2010, He et al., 2013) and the second used high-throughput sequencing (Chapter 5) which offers the ability to obtain large number of sequence reads in short period of time (Ghanbari et al., 2015) to obtain a higher resolution (i.e. including the rare biosphere) and better understanding of the entirety of microbiome. High-throughput sequencing was incorporated into this study as DGGE technique has various limitations. With DGGE, different organisms with same denaturing properties could migrate to same place and thus mistaken for one another. In addition to DGGE being a semi-quantitative technique, OUT amplicons here cannot be longer than 500 nucleotides. However, DGGE technique is better than using cultured-based technique to assess fish GI microbiota. Work carried out in Chapter 3 indicated that the intestinal bacterial community profile of tilapia fed carbohydrase (ROXAZYME® G2) supplemented diet was significantly altered compared to those fed the control diet. This finding is in line with the findings of Zhou et al. (2013) and Jiang et al. (2014) who reported a change in intestinal microbiota of carps when fed exogenous digestive enzymes (cellulase and xylanase respectively). However, the use of high-throughput (Ion Torrent PGM) sequencing to assess tilapia gut microbiota (Chapter 5) did not indicate a change in diversity of the microbiota. To the author's knowledge, this is the first time an investigation has been carried out on the combined effect of exogenous enzymes and probiotics on tilapia gut microbiota. According to Ghanbari et al. (2015), diversity of gut microbiota may be affected by biotic and abiotic factors including diet and environmental factors. The non-effect of diet supplemented with enzymes (containing xylanase) on

tilapia gut microbiota could be as a result of different diet composition and water temperature. Tilapia were fed semi-purified diets and kept at 26.3±0.76 °C in Chapter 3 but in Chapter 5, tilapia were fed a practical diet and kept at 30.34±0.15 °C. However, regardless of dietary treatment, geographical locations and rearing conditions, the presence of *Actinobacteria, Firmicutes and Proteobacteria* in the intestinal tract of tilapia (in both Chapters 3 and 5) suggests that these bacterial phyla may be of significance to host functions and may contribute towards a core gut microbiome assemblage. Wong et al. (2013) also reported consistency and stability in intestinal microbiota composition in rainbow trout irrespective of different dietary treatments and holding density over a period of 10 months.

Fish farming and aquafeed production, like other business enterprises, are profit-driven; every producer seeks to reduce production cost in order to maximise profit. This emphasises the importance of the economic consideration and merit of supplementing aquafeeds with exogenous digestive enzymes and probiotics. Cost benefit analysis of supplementation with additives was assessed using incidence cost and profit index to present a viable economic possibility of their use. Going by incidence costs, supplementation of diet with phytase ($ 0.67, Chapter 4), xylanase ($ 0.66, Chapter 4) and a combination of enzymes and probiotics ($0.62, Chapter 5) are cheaper than using the control diet ($ 0.68 and $0.63 in Chapters 4 and 5 respectively). The economic viability of producing premium diets at low cost may make these products (exogenous digestive enzymes and probiotics) more acceptable to feed manufacturers. The cheaper options translated to higher profit indexes of using the xylanase (2.80 in Chapter 4.2.1) and a combination of enzymes and probiotics (2.99 in Chapter 5).

On a final note, the current study focuses on selected additives (exogenous enzymes and probiotics) in juvenile tilapia over a short period of time (8 weeks maximum). It would be

pertinent to carry out further research on the selected bio-active feed additives on tilapia in different life stages (e.g. in fry from first feeding onwards or in adult tilapia) for a longer duration to establish the efficacy and effects of the exogenous enzymes and probiotics observed herewith. The further study could also investigate the use of the selected bio-active feed additives in other aquatic species relevant to aquaculture production.

In conclusion, the supplementation of diets with phytase (RONOZYME® Hiphos), carbohydrase (ROXAZYME® G2), xylanase (RONOZYME® WX) and a combination of enzymes (containing phytase, protease and xylanase) and probiotics (containing *B. subtilis, B. licheniformis* and *B. pumilus*) resulted in improved growth performance in tilapia. The profile of the tilapia gut microbiota was altered by carbohydrase inclusion. This work adds to the growing body of knowledge surrounding the usage of feed additives (exogenous digestive enzymes and probiotics) in key fish species including tilapia. These findings are both novel and highly relevant for the aquafeed industry as well as in the development of cost effective diets (supplemented with additives capable of enhancing both growth performance and immunological status of fish) for a warmwater fish species of important value especially intensive tilapia cage culture given its challenge of open status and risk of pathogen transfer. In addition, the finding provides opportunities to enhance the nutritional value of wide variety of plant products. This will present much more scope for fish feed formulation and raise the nutritive value of key commodities for use in balanced diets for different fish species throughout the world minimising wastage, environmental impact and upholding sustainability. There is a widespread interest in a myriad of natural bioactive feed supplements and additives for animal production and increasingly with application for farmed fish. This programme of

work has demonstrated the feasibility of a selected group only, but there is clearly much scope for the potential benefits of many such agents for different fish species globally.

7 Appendices

7.1 Appendix 1: Enzyme extraction protocol

1. Switch on centrifuge (set at 4 °C)
2. Weigh samples (g) into Eppendorf centrifuge tubes (4 decimal places)
3. Add twice weight of each sample of distilled water (iced) to individual sample
    - First add equivalence sample weight (in g) of distilled water (iced)
    - Crush and mix with pestle over ice
    - Add second half of the distilled water (iced)
    - Crush as homogenised as possible
4. Samples were kept in a beaker of ice all through the extraction process

Sonicator

1. Samples were exposed to short pulses (3 times)
2. Samples were not allowed to heat up

Centrifuge

1. Centrifuge samples at 16,000 g for 15 min at 4°C

After Centrifugation

1. The top layer (fat), middle layer (enzyme extract) and bottom layer (digesta sample residue)
2. Pipette out carefully the enzyme extract avoiding fat and/or digesta residue
3. Transfer enzyme extract to new eppendorfs centrifuge tubes
4. Check for fat in the new sample
5. If contain fat (lipid contamination), centrifuge again
6. It is safe to centrifuge again to be sure there is no lipid contamination

7.2 Appendix 2: DNA extraction protocol

Before Start

- Wear gloves, use new/ filter tips, sterilized/DNA free tubes.
- Ensure awareness of relevant COSHH/ Risk regulations.
- Work on ice where possible. Labelling all tubes in advance will save time!
- Ensure isopropanol is in the -20 °C freezer.
- Use the same weight of sample and process them together where possible.

Lysis

1. Use up to 350 mg of sample and add 500 µL of lysozyme (fresh, 50mg/mL in TE). Incubate minimum 30 min at 37 °C. (Samples may be frozen at this point).
2. Add 800 µL of Buffer ASL and vortex until thoroughly mixed.
3. Heat the suspension for 10 min at 90 °C.
4. Vortex for 15 seconds and centrifuge for 2 min/ max speed (14K).

Inhibitor removal

5. Place 800 µL of the supernatant into an Eppendorf and add half an Inhibitex tablet. Vortex **immediately** until suspended. Stand for 1 min. (Process tubes in pairs).
6. Centrifuge for 3 - 6 min (sample dependent) and pipette all of the supernatant into a new tube. Retain the remaining sample for future extraction if required.
7. Centrifuge for 3 - 4 min (sample dependent).

Protein removal

8. Place 20 µL of Proteinase K into a fresh tube and place 230/400 µL of the supernatant into this tube. (230 µL to do the next phenol-chloroform method in 1.5 mL tubes/ 400 if using 15 mL Falcon tubes).
9. Add 230/400 µL of Buffer AL and vortex.

10. Incubate at 70 °C for minimum 60 min (incubation time dependent on sample type).

Phenol Chloroform Clean-up

Wear goggles. Perform 11-12, 14 in fume hood

11. Carefully pour the entire sample into a 1.5 mL Eppendorf tube/15 mL falcon tube and add an equal volume of ice-cold Tris-buffered phenol solution (460 or 800 depending on the size of tube you are using). Mix by hand and leave on ice for 10 min.
12. Add an equal volume of chloroform/ isoamyl alcohol (24:1) and mix.
1. (The latter is optional- it stabilises the chloroform).
13. Repeat step 12 if the sample is not clean.
14. Centrifuge 6000 rcf/ 5 min in D301 refrigerated centrifuge, or 5 min in a bench top microcentrifuge (max speed).
15. Carefully pipette off the aqueous layer and place in new 1.5 mL Eppendorf tube. Discard the organic layer into appropriate waste container.

Precipitation

16. Add 230/400 µL ice-cold isopropanol. Vortex and place in -20 °C freezer for 10 min overnight. (Recovery may be enhanced by addition of 3M Na Acetate to final conc. of 0.3M (96 µL).
17. Centrifuge in a bench top microcentrifuge (max speed) for 30 min (at 4 °C)
18. Carefully pipette off supernatant and discard.
19. Slowly add 100 - 500 µL 70 % molecular grade ethanol

    Pipette up and down carefully and discard.

Alternatively, slowly vortex then centrifuge in a bench top microcentrifuge (max speed) for 10 min. Discard the supernatant

20. Repeat (19)
21. Dry pellet for 5 min maximum, ideally under vacuum, or leave the samples open above clean side of a blue roll next to a blue flame for 5 min
22. Re-suspend overnight at 4 °C using 30 µL of either molecular grade water or 1/10 TE (in molecular grade water)
23. Check yield on Nanodrop/ agarose gel before progressing or go directly for PCR and check in the agarose gel

7.3 Appendix 3: Denaturing gradient gel electrophoresis protocol

Store refrigerated or keeps on ice in darkness the reagents when outside of the fridge.

Stock 0 % Denaturant Polyacrylamide solution for a 10 % gel

25 mL 40 % Acrylamide (high purity acrylamide, molecular grade)

2 mL 50x TAE

Fill up to 100 mL with Milli-Q Water

Stock 80 % Denaturant Polyacrylamide solution for a 10 % gel

25 mL 40 % Acrylamide

32 mL Formamide (molecular grade)

34 g Ultrapure Urea (5.6M, Sigma)

2 mL 50x TAE

Fill up to 100 mL with Milli-Q Water

50x TAE (Tris-acetate-EDTA) Buffer (Maniatis et al., 1982)

242.3 g Tris Base (2 M)

18.61 g EDTA di-Sodium salt (50 mM)

Approximately 57.1 mL Glacial Acetic acid

Adjust pH to 7.8 with additional Glacial Acetic acid

Fill up to 1 L with Milli-Q Water

Preparing to pour the gel

- Clean gel plates with Acetone.
- Assemble plates absolutely straight and apply grease in the spacers' bottom side.
- Make the running buffer (140 mL 50x TAE in 7 L of Milli-Q water if possible).
- Pre-warm the buffer in the running tank to 60 ^{o}C.

Making the gel

Depending on the total volume of the gel, calculate the volume required for each solution. Afterwards, calculate the percentage desired for high and low denaturant solution. In this case, the total volume is 40 mL, 60 and 40 % for high and low denaturant solution.

	Stock 0%	Stock 80%	Volume
60%	5.0 mL	15.0 mL	20.0 mL
40%	11 mL	11 mL	22 mL
Total volume			42 mL

To these two mixes, add 143 µL of FRESH 10% Ammonium persulphate (APS) (Electrophoresis grade, Sigma). When ready to pour, add 11 µL of TEMED (N,N,N Tetramethylethylenediamine) to each of the above mixes, load into syringes (avoid bubbles) and attach to the gradient maker.

The gel get polymerize in about 20 - 30 min, then use a needle and syringe to clean 4 times each well with the pre-warm running buffer, before fixing the gel inside the running tank.

Loading and running

- 20 wells comb: 15 µL of sample + 4 µL of loading buffer
- 32 wells comb: 10 µL of sample + 2.7 µL of loading buffer.
- Load with extreme care, avoiding contamination between wells
- Run the gel for 16 – 17 h at 65 Volts and 60 ^{o}C (minimum 50 ^{o}C)

Staining with SYBR® Gold Nucleic Acid Gel Stain

Incubate the gel in 200 mL 1x TAE with 20 uL of SYBR Gold for 30 min on a shaking platform at 30 ^{o}C, then visualize the gel in a UV scanner

8 References

ABDEL-TAWWAB, M., ABDEL-RAHMAN, A. M. & ISMAEL, N. E. 2008. Evaluation of commercial live bakers' yeast, *Saccharomyces cerevisiae* as a growth and immunity promoter for Fry Nile tilapia, *Oreochromis niloticus* (L.) challenged in situ with Aeromonas hydrophila. *Aquaculture,* 280**,** 185-189.

ABDEL-WARITH, A.-W. A.-M. & YOUNIS, E. M. 2013. Influence of dietary inclusion of full-fat soybean meal and amino acids supplementation on growth and digestive enzymes activity of Nile tilapia, *Oreochromis niloticus*. *Turkish Journal of Fisheries and Aquatic Sciences,* 13.

ADEOLA, O. & COWIESON, A. 2011. Board-invited review: opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. *Journal of animal science,* 89**,** 3189-3218.

ADEOYE, A., JARAMILLO-TORRES, A., FOX, S., MERRIFIELD, D. & DAVIES, S. 2016. Supplementation of formulated diets for tilapia (*Oreochromis niloticus*) with selected exogenous enzymes: overall performance and effects on intestinal histology and microbiota. *Animal Feed Science and Technology*.

AGBO, N. W. 2008. Oilseed meals as dietary protein sources for juvenile Nile tilapia (*Oreochromis niloticus* L.).

AI, Q., MAI, K., ZHANG, W., XU, W., TAN, B., ZHANG, C. & 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 Part A: Molecular & Integrative Physiology,* 147**,** 502-508.

AI, Q., XU, H., MAI, K., XU, W., WANG, J. & ZHANG, W. 2011. Effects of dietary supplementation of *Bacillus subtilis* and fructooligosaccharide on growth performance, survival, non-specific immune response and disease resistance of juvenile large yellow croaker, *Larimichthys crocea*. *Aquaculture,* 317**,** 155-161.

ALARCÓN, F., DÍAZ, M., MOYANO, F. & ABELL, 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**,** 257-267.

ALBREKTSEN, S., MUNDHEIM, H. & AKSNES, A. 2006. Growth, feed efficiency, digestibility and nutrient distribution in Atlantic cod (*Gadus morhua*) fed two different fish meal qualities at three dietary levels of vegetable protein sources. *Aquaculture,* 261**,** 626-640.

ALEGBELEYE, W., OBASA, S., OLUDE, O., MORONKEJI, T. & ABDULRAHEEM, I. 2012. Growth Performance and Nutrient Utilization of African Mud Catfish (*Clarias gariepinus*) Fingerlings fed Different Levels of Fermented Pigeon Pea (*Cajanus cajan*) meal. *The Israeli Journal of Aquaculture - Bamigdeh,* 64**,** 8.

ALEGBELEYE, W. & OLUDE, O. 2009. An evaluation of diets containing various levels of fermented cottonseed hull on digestibility and growth of Nile tilapia (*Oreochromis niloticus*) fingerlings. *Asian Fisheries Science,* 22**,** 857-866.

ALI ZAMINI, A., KANANI, H. G., AZAM ESMAEILI, A., RAMEZANI, S. & 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**,** 187-192.

ALLAN, G. Fish for feed vs fish for food. Fish, Aquaculture and Food Security: Sustaining Fish as a Food Supply. Record of a conference conducted by ATSE Crawford Fund, Parliament House, Cambera, 2004. Citeseer, 20-26.

ALLAN, G. L. & BOOTH, M. A. 2004. Effects of extrusion processing on digestibility of peas, lupins, canola meal and soybean meal in silver perch *Bidyanus bidyanus* (Mitchell) diets. *Aquaculture Research,* 35**,** 981-991.

ALY, S. M., ABD-EL-RAHMAN, A. M., JOHN, G. & MOHAMED, M. F. 2008a. Characterization of some bacteria isolated from *Oreochromis niloticus* and their potential use as probiotics. *Aquaculture,* 277**,** 1-6.

ALY, S. M., AHMED, Y. A.-G., GHAREEB, A. A.-A. & MOHAMED, M. F. 2008b. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. *Fish & Shellfish Immunology,* 25**,** 128-136.

ALY, S. M., MOHAMED, M. F. & JOHN, G. 2008c. Effect of probiotics on the survival, growth and challenge infection in Tilapia nilotica (*Oreochromis niloticus*). *Aquaculture research,* 39**,** 647-656.

AOAC 1995. Official Methods of Analysis. *Association of Official Analytical Chemists.* Arlington, VA.

APPER, E., WEISSMAN, D., RESPONDEK, F., GUYONVARCH, A., BARON, F., BOISOT, P., RODILES, A. & MERRIFIELD, D. 2016. Hydrolysed wheat gluten as part of a diet based on animal and plant proteins supports good growth performance of Asian seabass (*Lates calcarifer*), without impairing intestinal morphology or microbiota. *Aquaculture,* 453**,** 40-48.

APÚN-MOLINA, J. P., SANTAMARÍA-MIRANDA, A., LUNA-GONZÁLEZ, A., MARTÍNEZ-DÍAZ, S. F. & ROJAS-CONTRERAS, M. 2009. Effect of potential probiotic bacteria on growth and survival of tilapia *Oreochromis niloticus* L., cultured in the laboratory under high density and suboptimum temperature. *Aquaculture Research,* 40**,** 887-894.

AVELLA, M. A., PLACE, A., DU, S.-J., WILLIAMS, E., SILVI, S., ZOHAR, Y. & CARNEVALI, O. 2012. *Lactobacillus rhamnosus* accelerates zebrafish backbone calcification and gonadal differentiation through effects on the GnRH and IGF systems. *PLoS ONE,* 7.

AYYAT, M., LABIB, H. M. & MAHMOUD, H. K. 2014. A Probiotic Cocktail as a Growth Promoter in Nile Tilapia (*Oreochromis niloticus*). *Journal of Applied Aquaculture,* 26**,** 208-215.

BABALOLA, T. O. O. 2006. THE EFFECTS OF FEEDING MOINA, MICRODIET AND XYLANASE SUPPLEMENTED MICRODIET ON GROWTH AND SURVIVAL OF *Clarias gariepinus* (BURCHELL) LARVAE. *Nigerian Journal of Fisheries,* 2**,** 205 - 217.

BAHURMIZ, O. M. & NG, W.-K. 2007. Effects of dietary palm oil source on growth, tissue fatty acid composition and nutrient digestibility of red hybrid tilapia, *Oreochromis* sp., raised from stocking to marketable size. *Aquaculture,* 262**,** 382-392.

BATES, J. M., MITTGE, E., KUHLMAN, J., BADEN, K. N., CHEESMAN, S. E. & GUILLEMIN, K. 2006. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. *Developmental biology,* 297**,** 374-386.

BEDFORD, M. 2000. Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimise subsequent problems. *World's Poultry Science Journal,* 56**,** 347-365.

BEDFORD, M. & COWIESON, A. 2012. Exogenous enzymes and their effects on intestinal microbiology. *Animal Feed Science and Technology,* 173**,** 76-85.

BELL, J. G., HENDERSON, R. J., TOCHER, D. R., MCGHEE, F., DICK, J. R., PORTER, A., SMULLEN, R. P. & SARGENT, J. R. 2002. Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects muscle fatty acid composition and hepatic fatty acid metabolism. *The Journal of nutrition,* 132**,** 222-230.

BIESALSKI, H.-K., DRAGSTED, L. O., ELMADFA, I., GROSSKLAUS, R., MÜLLER, M., SCHRENK, D., WALTER, P. & WEBER, P. 2009. Bioactive compounds: Definition and assessment of activity. *Nutrition,* 25**,** 1202-1205.

BOISEN, S. & EGGUM, B. 1991. Critical evaluation of in vitro methods for estimating digestibility in simple-stomach animals. *Nutrition research reviews,* 4**,** 141-162.

BOOTH, M. A., ALLAN, G. L., FRANCES, J. & PARKINSON, S. 2001. Replacement of fish meal in diets for Australian silver perch, *Bidyanus bidyanus*: IV. Effects of dehulling and protein concentration on digestibility of grain legumes. *Aquaculture,* 196**,** 67-85.

BOSTOCK, J., MCANDREW, B., RICHARDS, R., JAUNCEY, K., TELFER, T., LORENZEN, K., LITTLE, D., ROSS, L., HANDISYDE, N. & GATWARD, I. 2010. Aquaculture: global status and trends. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 365**,** 2897-2912.

BOWEN, S. 1982. Feeding, digestion and growth-qualitative considerations. *The biology and culture of tilapias,* 7**,** 141-156.

BROWN, B. 1980. Routine hematology procedures. *Hematology: Principles and Procedures***,** 71-112.

BUENTELLO, A., JIRSA, D., BARROWS, F. T. & DRAWBRIDGE, M. 2015. Minimizing fishmeal use in juvenile California yellowtail, *Seriola lalandi*, diets using non-GM soybeans selectively bred for aquafeeds. *Aquaculture,* 435**,** 403-411.

BWANIKA, G., MAKANGA, B., KIZITO, Y., CHAPMAN, L. & BALIRWA, J. 2004. Observations on the biology of Nile tilapia, *Oreochromis niloticus* L., in two Ugandan crater lakes. *African Journal of Ecology,* 42**,** 93-101.

CAI, C., SONG, L., WANG, Y., WU, P., YE, Y., ZHANG, Z. & YANG, C. 2013. Assessment of the feasibility of including high levels of rapeseed meal and peanut meal in diets of juvenile crucian carp (*Carassius auratus gibelio*♀× *Cyprinus carpio*♂): Growth, immunity, intestinal morphology, and microflora. *Aquaculture,* 410**,** 203-215.

CAIN, K. D. & GARLING, D. L. 1995. Pretreatment of soybean meal with phytase for salmonid diets to reduce phosphorus concentrations in hatchery effluents. *The Progressive Fish-Culturist,* 57**,** 114-119.

CAINE, W., VERSTEGEN, M., SAUER, W., TAMMINGA, S. & 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**,** 177-183.

CAO, L., WANG, W., YANG, C., YANG, Y., DIANA, J., YAKUPITIYAGE, A., LUO, Z. & LI, D. 2007. Application of microbial phytase in fish feed. *Enzyme and Microbial Technology,* 40**,** 497-507.

CAO, L., YANG, Y., WANG, W., YAKUPITIYAGE, A., YUAN, D. & DIANA, J. 2008. Effects of pretreatment with microbial phytase on phosphorous utilization and growth performance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture Nutrition,* 14**,** 99-109.

CAPORASO, J. G., BITTINGER, K., BUSHMAN, F. D., DESANTIS, T. Z., ANDERSEN, G. L. & KNIGHT, R. 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics,* 26**,** 266-267.

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. 2010b. QIIME allows analysis of high-throughput community sequencing data. *Nature methods,* 7**,** 335-336.

CARNEVALI, O., DE VIVO, L., SULPIZIO, R., GIOACCHINI, G., OLIVOTTO, I., SILVI, S. & CRESCI, A. 2006. Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.), with particular attention to IGF-1, myostatin and cortisol gene expression. *Aquaculture,* 258**,** 430-438.

CARTER, C. & HAULER, R. 2000. Fish meal replacement by plant meals in extruded feeds for Atlantic salmon, *Salmo salar* L. *Aquaculture,* 185**,** 299-311.

CARTER, C., HOULIHAN, D., BUCHANAN, B. & MITCHELL, A. 1994. Growth and feed utilization efficiencies of seawater Atlantic salmon, *Salmo salar* L., fed a diet containing supplementary enzymes. *Aquaculture Research,* 25**,** 37-46.

CASTEX, M., DURAND, H. & OKEKE, B. 2014. Issues with Industrial Probiotic Scale-up. *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics***,** 347-359.

CASTILLO, S. & GATLIN, D. M. 2015. Dietary supplementation of exogenous carbohydrase enzymes in fish nutrition: a review. *Aquaculture,* 435**,** 286-292.

CEREZUELA, R., MESEGUER, J. & ESTEBAN, M. 2011. Current knowledge in synbiotic use for fish aquaculture: a review. *J Aquac Res Development S,* 1**,** 2.

CHESSON, A. 1993. Feed enzymes. *Animal feed science and technology,* 45**,** 65-79.

CHIKWATI, E. M., GU, J., PENN, M. H., BAKKE, A. M. & KROGDAHL, Å. 2013. Intestinal epithelial cell proliferation and migration in Atlantic salmon, *Salmo salar* L.: effects of temperature and inflammation. *Cell and tissue research,* 353**,** 123-137.

COLLINS, S. A., DESAI, A. R., MANSFIELD, G. S., HILL, J. E., VAN KESSEL, A. G. & DREW, M. D. 2012. The effect of increasing inclusion rates of soybean, pea and canola meals and their protein concentrates on the growth of rainbow trout: concepts in diet formulation and experimental design for ingredient evaluation. *Aquaculture,* 344**,** 90-99.

CRONJD, P. & MACKIE, R. 1983. Colorimetric determination of in vitro feed protein degradation. *South African journal of animal science= Suid-Afrikaanse tydskrif vir veekunde*.

DACIE, J. & LEWIS, S. 1975. Basic hematology techniques. *Dacie JU, Lewis SM: Practical haematology, London, Churchill Livingston***,** 21-96.

DALSGAARD, J., EKMANN, K. S., PEDERSEN, P. B. & 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**,** 105-112.

DALSGAARD, J., VERLHAC, V., HJERMITSLEV, N., EKMANN, K. S., FISCHER, M., KLAUSEN, M. & 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**,** 181-191.

DAVIES, S. & GOUVEIA, A. 2008. Enhancing the nutritional value of pea seed meals (*Pisum sativum*) by thermal treatment or specific isogenic selection with comparison to soybean meal for African catfish, *Clarias gariepinus*. *Aquaculture,* 283**,** 116-122.

DEBNATH, D., SAHU, N., PAL, A., BARUAH, K., YENGKOKPAM, S. & MUKHERJEE, S. 2005. Present scenario and future prospects of phytase in aquafeed. *Asian-australasian journal of animal sciences,* 18**,** 1800-12.

DELMAR, E., LARGMAN, C., BRODRICK, J. & GEOKAS, M. 1979. A sensitive new substrate for chymotrypsin. *Analytical biochemistry,* 99**,** 316-320.

DENEV, S., STAYKOV, Y., MOUTAFCHIEVA, R. & BEEV, G. 2009. Microbial ecology of the gastrointestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. *International aquatic research,* 1**,** 1-29.

DENSTADLI, V., STOREBAKKEN, T., SVIHUS, B. & 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**,** 414-426.

DESANTIS, T. Z., HUGENHOLTZ, P., LARSEN, N., ROJAS, M., BRODIE, E. L., KELLER, K., HUBER, T., DALEVI, D., HU, P. & ANDERSEN, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology,* 72**,** 5069-5072.

DIAS, J., REMA, P., NUNES, C., VOGEL, K. & VERLHAC, V. 2014. Efficacy of dietary protease supplementation on growth performance and nutrient digestibility and utilization of juvenile Nile tilapia fed low fishmeal diets with variable crude protein level. *FeedInfo News.*

DIMITROGLOU, A., MERRIFIELD, D., MOATE, R., DAVIES, S., SPRING, P., SWEETMAN, J. & BRADLEY, G. 2009. Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout,(Walbaum). *Journal of animal science,* 87**,** 3226-3234.

DIMITROGLOU, A., MERRIFIELD, D. L., CARNEVALI, O., PICCHIETTI, S., AVELLA, M., DANIELS, C., GÜROY, D. & DAVIES, S. J. 2011. Microbial manipulations to improve fish health and production–a Mediterranean perspective. *Fish & Shellfish Immunology,* 30**,** 1-16.

DONGMEZA, E., SIDDHURAJU, P., FRANCIS, G. & BECKER, K. 2006. Effects of dehydrated methanol extracts of moringa (*Moringa oleifera* Lam.) leaves and three of its fractions on growth performance and feed nutrient assimilation in Nile tilapia (*Oreochromis niloticus* (L.)). *Aquaculture,* 261**,** 407-422.

DREW, M., BORGESON, T. & THIESSEN, D. 2007. A review of processing of feed ingredients to enhance diet digestibility in finfish. *Animal Feed Science and Technology,* 138**,** 118-136.

DUDLEY-CASH, W. 1997. NSP: a simple classification for a complex group of chemicals. *Feed Stuffs,* 69.

EDGAR, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics,* 26**,** 2460-2461.

EISSA, N. & ABOU-ELGHEIT, E. 2014. Dietary Supplementation Impacts of Potential Non-Pathogenic Isolates on Growth Performance, Hematological Parameters and Disease Resistance in Nile Tilapia (*Oreochromis Niloticus*). *Journal of Veterinary Advances,* 4**,** 712-719.

EL-ADAWY, T. A. 2002. Nutritional composition and antinutritional factors of chickpeas (Cicer arietinum L.) undergoing different cooking methods and germination. *Plant Foods for Human Nutrition,* 57**,** 83-97.

EL-RHMAN, A. M. A., KHATTAB, Y. A. & SHALABY, A. M. 2009. *Micrococcus luteus* and *Pseudomonas species* as probiotics for promoting the growth performance and health of Nile tilapia, *Oreochromis niloticus*. *Fish & Shellfish Immunology,* 27**,** 175-180.

EL-SAYED, A.-F. M. 2006. *Tilapia culture*, CABI.

EL-SAYED, A. Tilapia feed and feeding in semi-intensive culture systems. 8th International Symposium on Tilapia in Aquaculture (ISTA8) Cairo, Egypt, 2008. 717-723.

EL-DAKAR, A., SHALABY, S. & SAOUD, I. 2007. Assessing the use of a dietary probiotic/prebiotic as an enhancer of spinefoot rabbitfish *Siganus rivulatus* survival and growth. *Aquaculture Nutrition,* 13**,** 407-412.

EL-HAROUN, E., GODA, A. S. & CHOWDHURY, K. 2006. Effect of dietary probiotic Biogen® supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *Oreochromis niloticus* (L.). *Aquaculture Research,* 37**,** 1473-1480.

ELLIS, A. E. 1990. Lysozyme assays. *Techniques in fish immunology,* 1**,** 101-103.

EMADINIA, A., TOGHYANI, M., GHEISARI, A., TABEIDIAN, S. A., ALE SAHEB FOSOUL, S. S. & MOHAMMADREZAEI, M. 2014. Effect of wet feeding and enzyme supplementation on performance and immune responses of broiler chicks. *Journal of Applied Animal Research,* 42**,** 32-37.

ENCARNAÇÃO, P. 2015. Functional feed additives in aquaculture feeds. *Aquafeed Formulation***,** 217.

ERLANGER, B. F., KOKOWSKY, N. & COHEN, W. 1961. The preparation and properties of two new chromogenic substrates of trypsin. *Archives of biochemistry and biophysics,* 95**,** 271-278.

ESSA, M. A., EL-SERAFY, S., EL-EZABI, M. M., DABOOR, S. M., ESMAEL, N. A. & LALL, S. P. 2010. Effect of different dietary probiotics on growth, feed utilization and digestive enzymes activities of Nile tilapia, *Oreochromis niloticus*. *Journal of the Arabian Aquaculture Society,* 5**,** 143-161.

ETYEMEZ, M. & BALCÁZAR, J. L. 2015. Bacterial community structure in the intestinal ecosystem of rainbow trout (*Oncorhynchus mykiss*) as revealed by pyrosequencing-based analysis of 16S rRNA genes. *Research in veterinary science*.

EZEAGU, I. E. 2006. Efficiency of inactivation of trypsin inhibitory activity in some selected tropical plant seeds by autoclaving. *Biokemistri,* 18.

FAGBENRO, O. & DAVIES, S. 2001. Use of soybean flour (dehulled solvent-extracted soybean) as fish meal substitute in practical diets for African catfish, *Clarias gariepinus* (Burchell 1822): growth, feed utilization and digestibility. *Journal of Applied Ichthyology,* 17**,** 64-69.

FAGBENRO, O. A. & DAVIES, S. J. 2004. Use of High Percentages of Soy Protein Concentrate as Fish Meal Substitute in Practical Diets for African Catfish, *Clarias gariepinus* (Burchell 1822) Growth, Diet Utilization, and Digestibility. *Journal of Applied Aquaculture,* 16**,** 113-124.

FAO 2009. The State of world Fisheries and aquaculture. Food and Agriculture Organization of United Nations, Rome.

FAO 2011. Tilapia market report. Globefish. Food and Agriculture Organization of the United Nations, Rome.

FAO 2012. Year Book. Fishery and aquaculture statistics. Rome.

FAO 2014. The State of World Fisheries and Aquaculture. Opportunities and challenges. Food and Agriculture Organization of the United Nations, Rome.

FARHANGI, M. & CARTER, C. 2001. Growth, physiological and immunological responses of rainbow trout (Oncorhynchus mykiss) to different dietary inclusion levels of dehulled lupin (*Lupinus angustifolius*). *Aquaculture Research,* 32**,** 329-340.

FARHANGI, M. & CARTER, C. G. 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**,** 1274-1282.

FELIX, N. & SELVARAJ, S. 2004. Enzymes for sustainable aquaculture. *Aquaculture Asia,* 9**,** 5-6.

FERGUSON, R., MERRIFIELD, D. L., HARPER, G. M., RAWLING, M. D., MUSTAFA, S., PICCHIETTI, S., BALCÀZAR, J. L. & DAVIES, S. J. 2010. The effect of Pediococcus acidilactici on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*). *Journal of Applied Microbiology,* 109**,** 851-862.

FESTING, M. F. & ALTMAN, D. G. 2002. Guidelines for the design and statistical analysis of experiments using laboratory animals. *ILAR journal,* 43**,** 244-258.

FIGIS 2013. The Fisheries Global Information System. *Food and Agriculture Organization of the United Nations.* online.

FIGUEIREDO-SILVA, A., ROCHA, E., DIAS, J., SILVA, P., REMA, P., GOMES, E. & VALENTE, L. 2005. Partial replacement of fish oil by soybean oil on lipid distribution and liver histology in European sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) juveniles. *Aquaculture Nutrition,* 11**,** 147-155.

FISH, G. 1960. The comparative activity of some digestive enzymes in the alimentary canal of tilapia and perch. *Hydrobiologia,* 15**,** 161-178.

FITZIMMONS, K. Tilapia global supply and demand in 2014. Aquaculture America 2015 New Orleans.

FONSECA-MADRIGAL, J., KARALAZOS, V., CAMPBELL, P., BELL, J. G. & TOCHER, D. R. 2005. Influence of dietary palm oil on growth, tissue fatty acid compositions, and fatty acid metabolism in liver and intestine in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Nutrition,* 11**,** 241-250.

FORSTER, I., HIGGS, D. A., DOSANJH, B. S., ROWSHANDELI, M. & 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**,** 109-125.

FRANCIS, G., MAKKAR, H. P. & BECKER, K. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture,* 199**,** 197-227.

FURUYA, S., SAKAMOTO, K. & TAKAHASHI, S. 1979. A new in vitro method for the estimation of digestibility using the intestinal fluid of the pig. *British Journal of Nutrition,* 41**,** 511-520.

FURUYA, W. M., GONÇALVES, G. S., FURUYA, V. R. B. & HAYASHI, C. 2001. Phytase as feeding for Nile tilapia (*Oreochromis niloticus*). Performance and digestibility. *Revista Brasileira de Zootecnia,* 30**,** 924-929.

GARDUÑO-LUGO, M. & OLVERA-NOVOA, M. Á. 2008. Potential of the use of peanut (*Arachis hypogaea*) leaf meal as a partial replacement for fish meal in diets for Nile tilapia (*Oreochromis niloticus L.*). *Aquaculture research,* 39**,** 1299-1306.

GATLIN, D. M., BARROWS, F. T., BROWN, P., DABROWSKI, K., GAYLORD, T. G., HARDY, R. W., HERMAN, E., HU, G., KROGDAHL, Å. & NELSON, R. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Research,* 38**,** 551-579.

GERAYLOU, Z., SOUFFREAU, C., RURANGWA, E., D'HONDT, S., CALLEWAERT, L., COURTIN, C. M., DELCOUR, J. A., BUYSE, J. & OLLEVIER, F. 2012. Effects of arabinoxylan-oligosaccharides (AXOS) on juvenile Siberian sturgeon (*Acipenser baerii*) performance, immune responses and gastrointestinal microbial community. *Fish & shellfish immunology,* 33**,** 718-724.

GHAI, R., MIZUNO, C. M., PICAZO, A., CAMACHO, A. & RODRIGUEZ-VALERA, F. 2014. Key roles for freshwater Actinobacteria revealed by deep metagenomic sequencing. *Molecular ecology,* 23**,** 6073-6090.

GHANBARI, M., KNEIFEL, W. & DOMIG, K. J. 2015. A new view of the fish gut microbiome: advances from next-generation sequencing. *Aquaculture,* 448**,** 464-475.

GHOMI, M. R., SHAHRIARI, R., LANGROUDI, H. F., NIKOO, M. & VON ELERT, E. 2012. Effects of exogenous dietary enzyme on growth, body composition, and fatty acid profiles of cultured great sturgeon *Huso huso* fingerlings. *Aquaculture International,* 20**,** 249-254.

GIOACCHINI, G., DALLA VALLE, L., BENATO, F., FIMIA, G. M., NARDACCI, R., CICCOSANTI, F., PIACENTINI, M., BORINI, A. & CARNEVALI, O. 2013. Interplay between autophagy and apoptosis in the development of Danio rerio follicles and the effects of a probiotic. *Reproduction, Fertility and Development,* 25**,** 1115-1125.

GIOACCHINI, G., GIORGINI, E., MERRIFIELD, D. L., HARDIMAN, G., BORINI, A., VACCARI, L. & CARNEVALI, O. 2012. Probiotics Can Induce Follicle Maturational Competence: The Danio rerioCase. *Biology of reproduction,* 86**,** 65.

GIOACCHINI, G., LOMBARDO, F., MERRIFIELD, D., SILVI, S., CRESCI, A., AVELLA, M. & CARNEVALI, O. 2011. Effects of probiotics on Zebrafish reproduction. *J Aquac Res Development S,* 1**,** 2.

GIOACCHINI, G., MARADONNA, F., LOMBARDO, F., BIZZARO, D., OLIVOTTO, I. & CARNEVALI, O. 2010. Increase of fecundity by probiotic administration in zebrafish (*Danio rerio*). *Reproduction,* 140**,** 953-959.

GIORGINI, E., CONTI, C., FERRARIS, P., SABBATINI, S., TOSI, G., RUBINI, C., VACCARI, L., GIOACCHINI, G. & CARNEVALI, O. 2010. Effects of *Lactobacillus rhamnosus* on zebrafish oocyte maturation: an FTIR imaging and biochemical analysis. *Analytical and bioanalytical chemistry,* 398**,** 3063-3072.

GLENCROSS, B. Lupins in aquafeeds. *In:* GLENCROSS, B. D., ed. Harvesting the benefits of grain in aquaculture feeds, Proceedings of a workshop, 13 February 2007. Fisheries Occasional Publication No. 41, Department of Fisheries, Western Australia, 102.

GLENCROSS, B. Harvesting the benefits of lupin meals in aquaculture feeds. Lupins for health and wealth. Proceedings of the 12th International Lupin Conference, Fremantle, Western Australia, 14-18 September 2008., 2008. International Lupin Association, 496-505.

GLENCROSS, B., BOOTH, M. & ALLAN, G. 2007. A feed is only as good as its ingredients–a review of ingredient evaluation strategies for aquaculture feeds. *Aquaculture nutrition,* 13**,** 17-34.

GLENCROSS, B., EVANS, D., HAWKINS, W. & JONES, B. 2004. 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**,** 411-422.

GLENCROSS, B., HAWKINS, W., EVANS, D., RUTHERFORD, N., DODS, K., MCCAFFERTY, P. & SIPSAS, S. 2008. Evaluation of the influence of Lupinus angustifolius kernel meal on dietary nutrient and energy utilization efficiency by rainbow trout (*Oncorhynchus mykiss*). *Aquaculture nutrition,* 14**,** 129-138.

GLENCROSS, B. D., BOUJARD, T. & KAUSHIK, S. J. 2003. Influence of oligosaccharides on the digestibility of lupin meals when fed to rainbow trout, *Oncorhynchus mykiss*. *Aquaculture,* 219**,** 703-713.

GODA, A., EL-HAROUN, E. & CHOWDHURY, K. 2007. Effect of totally or partially replacing fish meal by alternative protein sources on growth of African catfish *Clarias gariepinus* (Burchell, 1822) reared in concrete tanks. *Aquaculture Research,* 38**,** 279-287.

GODA, A., MABROUK, H., WAFA, M. A. E.-H. & EL-AFIFI, 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 and Technology B,* 2**,** 15-28.

GONÇALVES, A. T., MAITA, M., FUTAMI, K., ENDO, M. & KATAGIRI, T. 2011. Effects of a probiotic bacterial *Lactobacillus rhamnosus* dietary supplement on the crowding stress response of juvenile Nile tilapia *Oreochromis niloticus*. *Fisheries Science,* 77**,** 633-642.

GUERRA, A., ETIENNE-MESMIN, L., LIVRELLI, V., DENIS, S., BLANQUET-DIOT, S. & ALRIC, M. 2012. Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in biotechnology,* 30**,** 591-600.

HAMMAD, M. S. 2008. Biomass production of saccharomyces cerevisiae (baker's yeast) using the cactus cladodes extract as a culture medium. Master Degree, Islamic University of Gaza.

HARDY, R., BURNELL, G. & ALLAN, G. 2009. Aquaculture feeds and ingredients: an overview. *New technologies in aquaculture: improving production efficiency, quality and environmental management,* 178**,** 370.

HARDY, R. W. 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Aquaculture Research,* 41**,** 770-776.

HASAN, M. R., HECHT, T., DE SILVA, S. & TACON, A. 2007. *Study and analysis of feeds and fertilizers for sustainable aquaculture development*, Food and Agriculture Organization of the United Nations.

HASSAAN, M., SOLTAN, M. & GHONEMY, M. 2014. Effect of synbiotics between Bacillus licheniformis and yeast extract on growth, hematological and biochemical indices of the Nile tilapia (*Oreochromis niloticus*). *The Egyptian Journal of Aquatic Research,* 40**,** 199-208.

HAUGEN, T., KIESSLING, A., OLSEN, R., RØRÅ, M., SLINDE, E. & NORTVEDT, R. 2006. Seasonal variations in muscle growth dynamics and selected quality attributes in Atlantic halibut (*Hippoglossus hippoglossus* L.) fed dietary lipids containing soybean and/or herring oil under different rearing regimes. *Aquaculture,* 261**,** 565-579.

HE, S., ZHANG, Y., XU, L., YANG, Y., MARUBASHI, T., ZHOU, Z. & YAO, B. 2013. Effects of dietary *Bacillus subtilis* C-3102 on the production, intestinal cytokine expression and autochthonous bacteria of hybrid tilapia *Oreochromis niloticus*♀× *Oreochromis aureus*♂. *Aquaculture,* 412**,** 125-130.

HE, S., ZHOU, Z., LIU, Y., CAO, Y., MENG, K., SHI, P., YAO, B. & RINGØ, E. 2010. Effects of the antibiotic growth promoters flavomycin and florfenicol on the autochthonous intestinal microbiota of hybrid tilapia (*Oreochromis niloticus*♀× *O. aureus*♂). *Archives of microbiology,* 192**,** 985-994.

HENG, H. G., ONG, T. & HASSAN, M. 2007. Radiographic assessment of gastric emptying and gastrointestinal transit time in hybrid tilapia (*Oreochromis niloticus*× *O. mossambicus*). *Veterinary Radiology & Ultrasound,* 48**,** 132-134.

HIDALGO, M., UREA, E. & SANZ, A. 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture,* 170**,** 267-283.

HLOPHE-GININDZA, S. N., MOYO, N. A., NGAMBI, J. W. & NCUBE, I. 2015. The effect of exogenous enzyme supplementation on growth performance and digestive enzyme activities in *Oreochromis mossambicus* fed kikuyu-based diets. *Aquaculture Research*.

HLOPHE, S. & MOYO, N. 2011. The effect of different plant diets on the growth performance, gastric evacuation rate, and carcass composition of *Tilapia rendalli*. *Asian J Anim Vet Adv,* 6**,** 1001-1009.

HONG, H. A. & CUTTING, S. M. 2005. The use of bacterial spore formers as probiotics. *FEMS microbiology reviews,* 29**,** 813-835.

HU, J., RAN, C., HE, S., CAO, Y., YAO, B., YE, Y., ZHANG, X. & ZHOU, Z. 2016. Dietary microbial phytase exerts mixed effects on the gut health of tilapia: a possible reason for the null effect on growth promotion. *The British journal of nutrition,* 115**,** 1958-1966.

HUANG, C.-H., HUANG, M.-C. & HOU, P.-C. 1998. Effect of dietary lipids on fatty acid composition and lipid peroxidation in sarcoplasmic reticulum of hybrid

tilapia, *Oreochromis niloticus× O. aureus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 120**,** 331-336.

HURRELL 2004. Phytic acid degradation as a means of improving iron absorption. *International Journal for Vitamin and Nutrition Research,* 74**,** 445-452.

IBRAHIM, S., HABIBA, R., SHATTA, A. & EMBABY, H. 2002. Effect of soaking, germination, cooking and fermentation on antinutritional factors in cowpeas. *Food/Nahrung,* 46**,** 92-95.

IFFO 2013. Present supply status of fish meal and fish oil. Global Aquaculture Advocate.

ISAKSEN, M., COWIESON, A. & KRAGH, K. 2010. Starch-and Protein-degrading Enzymes: Biochemistry, Enzymology and Characteristics Relevant to Animal Feed Use. *Enzymes in Farm Animal Nutrition***,** 85-95.

IZQUIERDO, M., OBACH, A., ARANTZAMENDI, L., MONTERO, D., ROBAINA, L. & ROSENLUND, G. 2003. Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. *Aquaculture Nutrition,* 9**,** 397-407.

JABEEN, S., SALIM, M. & AKHTAR, P. 2004. Feed conversion ratio of major carp *Cirrhinus mrigala* fingerlings fed on cotton seed meal, fish meal and barley. *Pakistan Veterinary Journal,* 24**,** 42-45.

JACKSON, L. S., LI, M. H. & ROBINSON, E. H. 1996. Use of microbial phytase in channel catfish *Ictalurus punctatus* diets to improve utilization of phytate phosphorus1. *Journal of the World Aquaculture Society,* 27**,** 309-313.

JATOBÁ, A., DO NASCIMENTO VIEIRA, F., BUGLIONE-NETO, C. C., MOURINO, J. L. P., SILVA, B. C., SEIFTTER, W. Q. & ANDREATTA, E. R. 2011. Diet supplemented with probiotic for Nile tilapia in polyculture system with marine shrimp. *Fish physiology and biochemistry,* 37**,** 725-732.

JIANG, N., TAN, N. S., HO, B. & DING, J. L. 2007. Respiratory protein–generated reactive oxygen species as an antimicrobial strategy. *Nature immunology,* 8**,** 1114-1122.

JIANG, T. T., FENG, L., LIU, Y., JIANG, W. D., JIANG, J., LI, S. H., TANG, L., KUANG, S. Y. & ZHOU, X. Q. 2014. Effects of exogenous xylanase supplementation in plant protein-enriched diets on growth performance, intestinal enzyme activities and microflora of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture Nutrition,* 20**,** 632-645.

KARALAZOS, V., BENDIKSEN, E., DICK, J. R. & BELL, J. G. 2007. Effects of dietary protein, and fat level and rapeseed oil on growth and tissue fatty acid composition and metabolism in Atlantic salmon (*Salmo salar* L.) reared at low water temperatures. *Aquaculture Nutrition,* 13**,** 256-265.

KHATTAB, R. & ARNTFIELD, S. 2009. Nutritional quality of legume seeds as affected by some physical treatments 2. Antinutritional factors. *LWT-Food Science and Technology,* 42**,** 1113-1118.

KIARIE, E., NYACHOTI, C., VITTI, D. & KEBREAB, E. 2010. Bioavailability of Calcium and Phosphorus in Feedstuffs for Farm Animals. *Phosphorus and Calcium Utilization and Requirements in Farm Animals***,** 76.

KIARIE, E., ROMERO, L. F. & NYACHOTI, C. M. 2013. The role of added feed enzymes in promoting gut health in swine and poultry. *Nutrition research reviews,* 26**,** 71-88.

KIRON, V. 2012. Fish immune system and its nutritional modulation for preventive health care. *Animal Feed Science and Technology,* 173**,** 111-133.

KISSIL, G. W., LUPATSCH, I., HIGGS, D. & HARDY, R. 2000. Dietary substitution of soy and rapeseed protein concentrates for fish meal, and their effects on growth and nutrient utilization in gilthead seabream Sparus aurata L. *Aquaculture Research,* 31**,** 595-601.

KISSIL, G. W., YOUNGSON, A. & COWEY, C. B. 1987. Capacity of the European eel (*Anguilla anguilla*) to elongate and desaturate dietary linoleic acid. *J. Nutr,* 117**,** 1379-1384.

KOKOU, F., SARROPOULOU, E., COTOU, E., RIGOS, G., HENRY, M., ALEXIS, M. & KENTOURI, M. 2015. Effects of Fish Meal Replacement by a Soybean Protein on Growth, Histology, Selected Immune and Oxidative Status Markers of Gilthead Sea Bream, *Sparus aurata*. *Journal of the World Aquaculture Society,* 46**,** 115-128.

KRAUGERUD, O. F., PENN, M., STOREBAKKEN, T., REFSTIE, S., KROGDAHL, Å. & SVIHUS, B. 2007. Nutrient digestibilities and gut function in Atlantic salmon (*Salmo salar*) fed diets with cellulose or non-starch polysaccharides from soy. *Aquaculture,* 273**,** 96-107.

KROGDAHL, Å., HEMRE, G. I. & MOMMSEN, T. 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. *Aquaculture nutrition,* 11**,** 103-122.

KROGDAHL, Å., PENN, M., THORSEN, J., REFSTIE, S. & BAKKE, A. M. 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. *Aquaculture Research,* 41**,** 333-344.

KUMAR, S., SAHU, N., PAL, A., CHOUDHURY, D. & MUKHERJEE, S. 2006a. Studies on digestibility and digestive enzyme activities in *Labeo rohita* (Hamilton) juveniles: effect of microbial α-amylase supplementation in non-gelatinized or gelatinized corn-based diet at two protein levels. *Fish physiology and Biochemistry,* 32**,** 209-220.

KUMAR, S., SAHU, N. P., PAL, A. K., CHOUDHURY, D. & MUKHERJEE, S. C. 2006b. Non-gelatinized corn supplemented with α-amylase at sub-optimum protein level enhances the growth of *Labeo rohita* (Hamilton) fingerlings. *Aquaculture Research,* 37**,** 284-292.

KUMAR, V., AKINLEYE, A., MAKKAR, H., ANGULO-ESCALANTE, M. & BECKER, K. 2012a. Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing Jatropha platyphylla kernel meal as a protein source. *Journal of animal physiology and animal nutrition,* 96**,** 37-46.

KUMAR, V., SINHA, A., MAKKAR, H., DE BOECK, G. & BECKER, K. 2012b. Phytate and phytase in fish nutrition. *Journal of animal physiology and animal nutrition,* 96**,** 335-364.

LANARI, D., D'AGARO, E. & TURRI, C. 1998. Use of nonlinear regression to evaluate the effects of phytase enzyme treatment of plant protein diets for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture,* 161**,** 345-356.

LARA-FLORES, M., OLVERA-NOVOA, M. A., GUZMÁN-MÉNDEZ, B. Z. E. & LÓPEZ-MADRID, W. 2003. Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). *Aquaculture,* 216**,** 193-201.

LARSEN, A., MOHAMMED, H. & ARIAS, C. 2014. Characterization of the gut microbiota of three commercially valuable warmwater fish species. *Journal of applied microbiology,* 116**,** 1396-1404.

LI, H., ZHENG, Z., CONG-XIN, X., BO, H., CHAO-YUAN, W. & GANG, H. 2010. Isolation of cellulose—producing microbes from the intestine of grass carp (*Ctenopharyngodon idellus*). *Chinese Fishes.* Springer.

LI, J., LI, J. & WU, T. 2009. Effects of non-starch polysaccharides enzyme, phytase and citric acid on activities of endogenous digestive enzymes of tilapia (*Oreochromis niloticus× Oreochromis aureus*). *Aquaculture Nutrition,* 15**,** 415-420.

LI, M. H. & ROBINSON, E. H. 1997. Microbial Phytase Can Replace Inorganic Phosphorus Supplements in Channel Catfish *Ictalurus punctatus* Diets1. *Journal of the World Aquaculture Society,* 28**,** 402-406.

LIEBERT, F. & 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**,** 111-119.

LIEBERT, F. & 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**,** 292-299.

LIM, P. & TATE, M. 1973. The phytases. II. Properties of phytase fractions F 1 and F 2 from wheat bran and the myo-inositol phosphates produced by fraction F 2. *Biochimica et Biophysica Acta (BBA)-Enzymology,* 302**,** 316-328.

LIN, S., MAI, K. & 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**,** 1645-1653.

LIU, C.-H., CHIU, C.-H., WANG, S.-W. & CHENG, W. 2012. Dietary administration of the probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. *Fish & shellfish immunology,* 33**,** 699-706.

LIU, H., GUO, X., GOONERATNE, R., LAI, R., ZENG, C., ZHAN, F. & WANG, W. 2016. The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. *Scientific reports,* 6.

LIU, L., SU, J., ZHANG, T., LIANG, X. F. & LUO, Y. 2013. Apparent digestibility of nutrients in grass carp (*Ctenopharyngodon idellus*) diet supplemented with graded levels of neutral phytase using pretreatment and spraying methods. *Aquaculture Nutrition,* 19**,** 91-99.

LIU, Y. & BAIDOO, S. K. 1997. Exogenous enzymes for pig diets: an overview. *Enzymes in poultry and swine nutrition*.

LOMBARDO, F., GIOACCHINI, G. & CARNEVALI, O. 2011. Probiotic-based nutritional effects on killifish reproduction. *Fish Aquacult J FAJ-33*.

LOVELL, T. 1998. Dietary requirement. *In:* LOVELL, T. (ed.) *Nutrition and feeding of fish.* Springer US.

LOZANO, N. B. S., VIDAL, A. T., MARTÍNEZ-LLORENS, S., MÉRIDA, S. N., BLANCO, J. E., LÓPEZ, A. M., TORRES, M. P. & CERDÁ, M. J. 2007. Growth and economic profit of gilthead sea bream (*Sparus aurata*, L.) fed sunflower meal. *Aquaculture,* 272**,** 528-534.

MAKKAR, H. 1993. Antinutritional factors in foods for livestock. *BSAP occasional publication: An occasional publication of the British Society of Animal Production*.

MANDIKI, S., MILLA, S., WANG, N., BLANCHARD, G., DJONKACK, T., TANASCAUX, S. & KESTEMONT, P. 2011. Effects of probiotic bacteria on growth parameters and immune defence in Eurasian perch *Perca fluviatilis* L. larvae under intensive culture conditions. *Aquaculture Research,* 42**,** 693-703.

MARTÍNEZ-LLORENS, S., MOÑINO, A. V., TOMÁS VIDAL, A., SALVADOR, V. J. M., PLA TORRES, M. & JOVER CERDÁ, M. 2007a. Soybean meal as a protein source in gilthead sea bream (*Sparus aurata* L.) diets: effects on growth and nutrient utilization. *Aquaculture Research,* 38**,** 82-90.

MARTÍNEZ-LLORENS, S., VIDAL, A. T., MOÑINO, A. V., TORRES, M. P. & CERDÁ, M. J. 2007b. Effects of dietary soybean oil concentration on growth, nutrient utilization and muscle fatty acid composition of gilthead sea bream (*Sparus aurata* L.). *Aquaculture Research,* 38**,** 76-81.

MENTE, E., DEGUARA, S., SANTOS, M. B. & HOULIHAN, D. 2003. White muscle free amino acid concentrations following feeding a maize gluten dietary protein in Atlantic salmon (*Salmo salar* L.). *Aquaculture,* 225**,** 133-147.

MÉRIDA, S. N., TOMÁS-VIDAL, A., MARTÍNEZ-LLORENS, S. & CERDÁ, M. J. 2010. Sunflower meal as a partial substitute in juvenile sharpsnout sea bream (*Diplodus puntazzo*) diets: amino acid retention, gut and liver histology. *Aquaculture,* 298**,** 275-281.

MERRIFIELD, D., BRADLEY, G., BAKER, R. & DAVIES, S. 2010a. Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) II. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria postantibiotic treatment. *Aquaculture nutrition,* 16**,** 496-503.

MERRIFIELD, D. L., DIMITROGLOU, A., FOEY, A., DAVIES, S. J., BAKER, R. T., BØGWALD, J., CASTEX, M. & RINGØ, E. 2010b. The current status and future

focus of probiotic and prebiotic applications for salmonids. *Aquaculture,* 302**,** 1-18.

MOHAMED, M. H. & REFAT, N. 2011. Pathological evaluation of probiotic, *Bacillus subtilis*, against Flavobacterium columnare in tilapia nilotica (*Oreochromis niloticus*) fish in Sharkia Governorate, Egypt. *Journal of American Science,* 7**,** 244-256.

MOLINA-POVEDA, C., LUCAS, M. & JOVER, M. 2013. Evaluation of the potential of Andean lupin meal (*Lupinus mutabilis* Sweet) as an alternative to fish meal in juvenile Litopenaeus vannamei diets. *Aquaculture,* 410**,** 148-156.

MORALES, G. A. & 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**,** 244-251.

MORENO-ROJAS, J., TULLI, F., MESSINA, M., TIBALDI, E. & GUILLOU, C. 2008. Stable isotope ratio analysis as a tool to discriminate between rainbow trout (O. mykiss) fed diets based on plant or fish-meal proteins. *Rapid Communications in Mass Spectrometry,* 22**,** 3706-3710.

MORIARTY, D. 1998. Control of luminous Vibrio species in penaeid aquaculture ponds. *Aquaculture,* 164**,** 351-358.

MOXLEY, J. D., ROSSI, W., BUENTELLO, A., POHLENZ, C., GATLIN, D. M. & TOMASSO, J. R. 2014. Replacement of fish meal with plant feedstuffs in the diet of red drum, *Sciaenops ocellatus*: effects on production characteristics and tolerance to aquaculture-related stressors. *Journal of the World Aquaculture Society,* 45**,** 192-198.

MOYANO, F. J., SAÉNZ DE RODRIGÁÑEZ, M. A., DÍAZ, M. & TACON, A. G. 2014. Application of in vitro digestibility methods in aquaculture: constraints and perspectives. *Reviews in Aquaculture*.

MOYANO, F. J. & SAVOIE, L. 2001. Comparison of in vitro systems of protein digestion using either mammal or fish proteolytic enzymes. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 128**,** 359-368.

MSANGI, S., KOBAYASHI, M., BATKA, M., VANNUCCINI, S., DEY, M. & ANDERSON, J. 2013. Fish to 2030: Prospects for fisheries and aquaculture. *World Bank Report*.

MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and environmental microbiology,* 59**,** 695-700.

NAGASE, M. G. 1964. Contribution to the physiology of digestion in *Tilapia mossambica* Peters: digestive enzymes and the effects of diets on their activity. *Zeitschrift für vergleichende Physiologie,* 49**,** 270-284.

NAKAGAWA, H., SATO, M. & GATLIN III, D. M. 2007. *Dietary supplements for the health and quality of cultured fish*, Cabi.

NAYAK, S. K. 2010. Role of gastrointestinal microbiota in fish. *Aquaculture Research,* 41**,** 1553-1573.

NAYAK, S. K. & MUKHERJEE, S. C. 2011. Screening of gastrointestinal bacteria of Indian major carps for a candidate probiotic species for aquaculture practices. *Aquaculture Research,* 42**,** 1034-1041.

NAYLOR, R. L., GOLDBURG, R. J., PRIMAVERA, J. H., KAUTSKY, N., BEVERIDGE, M. C., CLAY, J., FOLKE, C., LUBCHENCO, J., MOONEY, H. & TROELL, M. 2000. Effect of aquaculture on world fish supplies. *Nature,* 405**,** 1017-1024.

NENGAS, I., ALEXIS, M. & DAVIES, S. 1996. Partial substitution of fishmeal with soybean meal products and derivatives in diets for the gilthead sea bream *Sparus aurata* (L.). *Aquaculture Research,* 27**,** 147-156.

NEWKIRK, R. W. 2008. *The effects of processing on the nutritional value of canola meal for broiler chickens.* Doctor of Philosophy, University of Saskatchewan.

NG, W.-K. & CHONG, K.-K. 2002. The nutritive value of palm kernel and the effect of enzyme supplementation in practical diets for red hybrid tilapia (*Oreochromis* sp). *Asian Fisheries Science,* 15**,** 167-176.

NG, W.-K., KIM, Y.-C., ROMANO, N., KOH, C.-B. & YANG, S.-Y. 2014. Effects of Dietary Probiotics on the Growth and Feeding Efficiency of Red Hybrid Tilapia, *Oreochromis* sp., and Subsequent Resistance to Streptococcus agalactiae. *Journal of Applied Aquaculture,* 26**,** 22-31.

NG, W.-K., LIM, P.-K. & BOEY, P.-L. 2003. Dietary lipid and palm oil source affects growth, fatty acid composition and muscle α-tocopherol concentration of African catfish, *Clarias gariepinus*. *Aquaculture,* 215**,** 229-243.

NG, W.-K., LIM, P.-K. & SIDEK, H. 2001. The influence of a dietary lipid source on growth, muscle fatty acid composition and erythrocyte osmotic fragility of hybrid tilapia. *Fish Physiology and Biochemistry,* 25**,** 301-310.

NG, W. K., LIM, H. A., LIM, S. L. & 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**,** 1199-1207.

NG, W. K. & ROMANO, N. 2013. A review of the nutrition and feeding management of farmed tilapia throughout the culture cycle. *Reviews in Aquaculture,* 5**,** 220-254.

NGUYEN, T. N. The utilization of soybean products in tilapia feed-A review. Proceedings of the 8th International Symposium on Tilapia in Aquaculture. The Central Laboratory for Aquaculture Research, Cairo, 2008. 53-65.

NGUYEN, T. N., DAVIS, D. A. & SAOUD, I. P. 2009. Evaluation of alternative protein sources to replace fish meal in practical diets for juvenile tilapia, *Oreochromis* spp. *Journal of the World Aquaculture Society,* 40**,** 113-121.

NRC 2011. *Nutrient Requirements of Fish and Shrimp, The National Academies Press,* Washington DC.

NWANNA, L. 2007. Effect of dietary phytase on growth, enzyme activities and phosphorus load of Nile tilapia (*Oreochromis niloticus*). *Journal of Engineering and Applied Sciences,* 2**,** 972-976.

NWANNA, L. C. & SCHWARZ, F. J. 2007. Effect of supplemental phytase on growth, phosphorus digestibility and bone mineralization of common carp (*Cyprinus carpio* L). *Aquaculture Research,* 38**,** 1037-1044.

OGUNKOYA, A. E., PAGE, G. I., ADEWOLU, M. A. & 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**,** 466-475.

OKUTANI, K., KAWADA, I. & KIMATA, M. 1967. The chitinolytic enzyme present in the digestive tracts of yellow tail. *Bull. Jap. Soc. Sc. Fish,* 33**,** 848-852.

OLUDE, O., ALEGBELEYE, W. & OBASA, S. 2008. The use of soaked copra meal as a partial substitute for soybean meal in the diet of Nile tilapia (*Oreochromis niloticus*) fingerlings. *Lipids,* 8**,** 9.62.

PANDIYAN, P., BALARAMAN, D., THIRUNAVUKKARASU, R., GEORGE, E. G. J., SUBARAMANIYAN, K., MANIKKAM, S. & SADAYAPPAN, B. 2013. Probiotics in aquaculture. *Drug Invention Today,* 5**,** 55-59.

PAPATRYPHON, E., HOWELL, R. A. & SOARES, J. H. 1999. Growth and mineral absorption by striped bass *Morone saxatilis* fed a plant feedstuff based diet supplemented with phytase. *Journal of the World Aquaculture Society,* 30**,** 161-173.

PASCUAL, J., CERVERA, C. & FERNÁNDEZ-CARMONA, J. 2010. Comparison of different in vitro digestibility methods for nutritive evaluation of rabbit diets. *World Rabbit Science,* 8.

PENG, Y.-L., GUO, Y.-M. & YUAN, J.-M. 2003. Effects of xylanase and phytase on the growth and nutrient digestibility in broilers fed wheat-based diets [J]. *Acta Zoonutrimenta Sinica,* 3**,** 010.

PENG, M., XU, W., MAI, K., ZHOU, H., ZHANG, Y., LIUFU, Z., ZHANG, K. & AI, Q. 2014. Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. *Aquaculture,* 433**,** 442-449.

PENN, M. H., BENDIKSEN, E. Å., CAMPBELL, P. & KROGDAHL, Å. 2011. High level of dietary pea protein concentrate induces enteropathy in Atlantic salmon (*Salmo salar* L.). *Aquaculture,* 310**,** 267-273.

PEREIRA, T. & OLIVA-TELES, A. 2003. Evaluation of corn gluten meal as a protein source in diets for gilthead sea bream (*Sparus aurata* L.) juveniles. *Aquaculture Research,* 34**,** 1111-1117.

PÉREZ-SÁNCHEZ, T., RUIZ-ZARZUELA, I., BLAS, I. & BALCÁZAR, J. L. 2014. Probiotics in aquaculture: a current assessment. *Reviews in Aquaculture,* 6**,** 133-146.

PÉREZ, T., BALCÁZAR, J., RUIZ-ZARZUELA, I., HALAIHEL, N., VENDRELL, D., DE BLAS, I. & MÚZQUIZ, J. 2010. Host–microbiota interactions within the fish intestinal ecosystem. *Mucosal immunology*.

PIRARAT, N., KOBAYASHI, T., KATAGIRI, T., MAITA, M. & ENDO, M. 2006. Protective effects and mechanisms of a probiotic bacterium *Lactobacillus rhamnosus* against experimental Edwardsiella tarda infection in tilapia (*Oreochromis niloticus*). *Veterinary immunology and immunopathology,* 113**,** 339-347.

POHLENZ, C. & GATLIN, D. M. 2014. Interrelationships between fish nutrition and health. *Aquaculture,* 431**,** 111-117.

PORTZ, L. & LIEBERT, F. 2004. Growth, nutrient utilization and parameters of mineral metabolism in Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) fed plant-based diets with graded levels of microbial phytase. *Journal of Animal Physiology and Animal Nutrition,* 88**,** 311-320.

QIN, C., ZHANG, Y., LIU, W., XU, L., YANG, Y. & ZHOU, Z. 2014. Effects of chito-oligosaccharides supplementation on growth performance, intestinal cytokine expression, autochthonous gut bacteria and disease resistance in hybrid tilapia *Oreochromis niloticus*♀× *Oreochromis aureus*♂. *Fish & shellfish immunology,* 40**,** 267-274.

RANA, K. J., SIRIWARDENA, S. & HASAN, M. R. 2009. Impact of rising feed ingredient prices on aquafeeds and aquaculture production, Food and Agriculture Organization of the United Nations (FAO).

RASO, S. & ANDERSON, T. A. 2003. Effects of dietary fish oil replacement on growth and carcass proximate composition of juvenile barramundi (*Lates calcarifer*). *Aquaculture Research,* 34**,** 813-819.

RAWLS, J. F., SAMUEL, B. S. & GORDON, J. I. 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 4596-4601.

RAY, A., GHOSH, K. & RINGØ, E. 2012. Enzyme-producing bacteria isolated from fish gut: a review. *Aquaculture Nutrition,* 18**,** 465-492.

RAY, A. K. & RINGØ, E. 2014. The Gastrointestinal Tract of Fish. *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics***,** 1-13.

RAY, A. K., ROY, T., MONDAL, S. & RINGØ, E. 2010. Identification of gut-associated amylase, cellulase and protease-producing bacteria in three species of Indian major carps. *Aquaculture Research,* 41**,** 1462-1469.

REFSTIE, S., SAHLSTRÖM, S., BRÅTHEN, E., BAEVERFJORD, G. & KROGEDAL, P. 2005. Lactic acid fermentation eliminates indigestible carbohydrates and antinutritional factors in soybean meal for Atlantic salmon (*Salmo salar*). *Aquaculture,* 246**,** 331-345.

RICHARD, N., MOURENTE, G., KAUSHIK, S. & CORRAZE, G. 2006. Replacement of a large portion of fish oil by vegetable oils does not affect lipogenesis, lipid

transport and tissue lipid uptake in European seabass (*Dicentrarchus labrax* L.). *Aquaculture,* 261**,** 1077-1087.

RICHE, M., HALEY, D., OETKER, M., GARBRECHT, S. & GARLING, D. 2004. Effect of feeding frequency on gastric evacuation and the return of appetite in tilapia *Oreochromis niloticus* (L.). *Aquaculture,* 234**,** 657-673.

RIDHA, M. T. & AZAD, I. S. 2012. Preliminary evaluation of growth performance and immune response of Nile tilapia *Oreochromis niloticus* supplemented with two putative probiotic bacteria. *Aquaculture Research,* 43**,** 843-852.

RIDHA, M. T. & AZAD, I. S. 2015. Effect of autochthonous and commercial probiotic bacteria on growth, persistence, immunity and disease resistance in juvenile and adult Nile tilapia *Oreochromis niloticus*. *Aquaculture Research*.

RINGØ, E., ZHOU, Z., VECINO, J., WADSWORTH, S., ROMERO, J., KROGDAHL, Å., OLSEN, R., DIMITROGLOU, A., FOEY, A. & DAVIES, S. 2015. Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquaculture Nutrition*.

ROBAINA, L., IZQUIERDO, M., MOYANO, F., SOCORRO, J., VERGARA, J., MONTERO, D. & FERNANDEZ-PALACIOS, H. 1995. Soybean and lupin seed meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications. *Aquaculture,* 130**,** 219-233.

RODEHUTSCORD, M. & PFEFFER, E. 1995. Effects of supplemental microbial phytase on phosphorus digestibility and utilization in rainbow trout (*Oncorhynchus mykiss*). *Water Science and Technology,* 31**,** 143-147.

ROESELERS, G., MITTGE, E. K., STEPHENS, W. Z., PARICHY, D. M., CAVANAUGH, C. M., GUILLEMIN, K. & RAWLS, J. F. 2011. Evidence for a core gut microbiota in the zebrafish. *The ISME journal,* 5**,** 1595-1608.

ROLLO, A., SULPIZIO, R., NARDI, M., SILVI, S., ORPIANESI, C., CAGGIANO, M., CRESCI, A. & CARNEVALI, O. 2006. Live microbial feed supplement in aquaculture for improvement of stress tolerance. *Fish Physiology and Biochemistry,* 32**,** 167-177.

ROMBENSO, A. N., TRUSHENSKI, J. T., JIRSA, D. & DRAWBRIDGE, M. 2015. Successful fish oil sparing in White Seabass feeds using saturated fatty acid-rich soybean oil and 22: 6n-3 (DHA) supplementation. *Aquaculture,* 448**,** 176-185.

ROMERO, J., RINGØ, E. & MERRIFIELD, D. L. 2014. The Gut Microbiota of Fish. *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics***,** 75-100.

ROSSI, W., TOMASSO, J. R. & GATLIN, D. M. 2015. Production performance and non-specific immunity of cage-raised red drum, Sciaenops ocellatus, fed soybean-based diets. *Aquaculture,* 443**,** 84-89.

ROUND, J. L. & MAZMANIAN, S. K. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology,* 9**,** 313-323.

ROWLEY, A. F. 1990. Collection, separation and identification of fish leucocytes. *Techniques in fish immunology,* 1**,** 113-136.

ROY, T., MONDAL, S. & RAY, A. K. 2009. Phytase-producing bacteria in the digestive tracts of some freshwater fish. *Aquaculture research,* 40**,** 344-353.

RUMSEY, G. L., HUGHES, S. G. & WINFREE, R. A. 1993. Chemical and nutritional evaluation of soya protein preparations as primary nitrogen sources for rainbow trout (*Oncorhynchus mykiss*). *Animal feed science and technology,* 40**,** 135-151.

RUST, M., BARROWS, F., HARDY, R., LAZUR, A., NAUGHTEN, K. & SILVERSTEIN, J. 2012. The Future of Aquafeeds: A Report of the NOAA/USDA Alternative Feeds Initiative. *NOAA Technical Memorandum NMFS F/SPO-124*.

SAHA, S., ROY, R. N., SEN, S. K. & 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**,** 380-388.

SAJJADI, M. & CARTER, C. 2004a. Dietary phytase supplementation and the utilisation of phosphorus by Atlantic salmon (*Salmo salar* L.) fed a canola-meal-based diet. *Aquaculture,* 240**,** 417-431.

SAJJADI, M. & CARTER, C. 2004b. Effect of phytic acid and phytase on feed intake, growth, digestibility and trypsin activity in Atlantic salmon (*Salmo salar*, L.). *Aquaculture Nutrition,* 10**,** 135-142.

SALEM, M. F. 2010. Evaluation of (Bio-Nutra 200) as A Commercial Probiotic Product in Nile Tilapia (*Oreochromis niloticus*) Diets. *Evaluation,* 5.

SARGENT, J. & TACON, A. 1999. Development of farmed fish: a nutritionally necessary alternative to meat. *Proceedings of the Nutrition Society,* 58**,** 377-383.

SCHÄFER, A., KOPPE, W., MEYER-BURGDORFF, K.-H. & GÜNTHER, K. 1995. Effects of a microbial phytase on the utilization of native phosphorus by carp in a diet based on soybean meal. *Water Science and technology,* 31**,** 149-155.

SCHULZ, C., KNAUS, U., WIRTH, M. & RENNERT, B. 2005. Effects of varying dietary fatty acid profile on growth performance, fatty acid, body and tissue composition of juvenile pike perch (*Sander lucioperca*). *Aquaculture Nutrition,* 11**,** 403-413.

SCHULZ, C., WICKERT, M., KIJORA, C., OGUNJI, J. & RENNERT, B. 2007. Evaluation of pea protein isolate as alternative protein source in diets for juvenile tilapia (*Oreochromis niloticus*). *Aquaculture Research,* 38**,** 537-545.

SELLE, P., RAVINDRAN, V., RAVINDRAN, G., PITTOLO, P. & BRYDEN, W. 2003. Influence of phytase and xylanase supplementation on growth performance and nutrient utilisation of broilers offered wheat-based diets. *Asian Australasian Journal of Animal Sciences,* 16**,** 394-402.

SHAFAEIPOUR, A., YAVARI, V., FALAHATKAR, B., MAREMMAZI, J. G. & GORJIPOUR, E. 2008. Effects of canola meal on physiological and biochemical parameters in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Nutrition,* 14**,** 110-119.

SHAPAWI, R., MUSTAFA, S. & NG, W. K. 2008. Effects of dietary fish oil replacement with vegetable oils on growth and tissue fatty acid composition of humpback grouper, *Cromileptes altivelis* (Valenciennes). *Aquaculture Research,* 39**,** 315-323.

SHELBY, R. A., LIM, C., YILDIRIM-AKSOY, M. & DELANEY, M. A. 2006. Effects of probiotic diet supplements on disease resistance and immune response of young Nile tilapia, *Oreochromis niloticus*. *Journal of Applied Aquaculture,* 18**,** 23-34.

SINHA, A. K., KUMAR, V., MAKKAR, H. P., DE BOECK, G. & BECKER, K. 2011. Non-starch polysaccharides and their role in fish nutrition–A review. *Food Chemistry,* 127**,** 1409-1426.

SKREDE, G., STOREBAKKEN, T., SKREDE, A., SAHLSTRØM, S., SØRENSEN, M., SHEARER, K. & SLINDE, E. 2002. Lactic acid fermentation of wheat and barley whole meal flours improves digestibility of nutrients and energy in Atlantic salmon (*Salmo salar* L.) diets. *Aquaculture,* 210**,** 305-321.

SMITH, D., TABRETT, S. & GLENCROSS, B. 2007. Growth response of the black tiger shrimp, *Penaeus monodon* fed diets containing different lupin cultivars. *Aquaculture,* 269**,** 436-446.

SOGBESAN, A. & UGWUMBA, A. 2008. Nutritional evaluation of termite (*Macrotermes subhyalinus*) meal as animal protein supplements in the diets of

Heterobranchus longifilis (Valenciennes, 1840) fingerlings. *Turkish Journal of Fisheries and Aquatic Sciences,* 8**,** 149-157.

SOTOLU, A. O. 2010. Feed utilization and biochemical characteristics of *Clarias gariepinus* (Burchell, 1822) fingerlings fed diets containing fish oil and vegetable oils as total replacements. *World Journal of Fish and Marine Sciences,* 2**,** 93-98.

STANDEN, B., RAWLING, M., DAVIES, S., CASTEX, M., FOEY, A., GIOACCHINI, G., CARNEVALI, O. & MERRIFIELD, D. 2013. Probiotic *Pediococcus acidilactici* modulates both localised intestinal-and peripheral-immunity in tilapia (*Oreochromis niloticus*). *Fish & shellfish immunology,* 35**,** 1097-1104.

STANDEN, B., RODILES, A., PEGGS, D., DAVIES, S., SANTOS, G. & MERRIFIELD, D. 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**,** 8403-8417.

STONE, D., ALLAN, G. & ANDERSON, A. 2003. 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**,** 135-147.

STOREBAKKEN, T., SHEARER, K. & ROEM, A. 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**,** 365-379.

TACON, A. J. 2003. Aquaculture production trends analysis. *FAO Fisheries Circular,* 886**,** 5-29.

TACON, A. G., HASAN, M. R. & METIAN, M. 2011. Demand and supply of feed ingredients for farmed fish and crustaceans: trends and prospects, FAO Fisheries and Aquaculture Technical Paper No. 564. .

TACON, A. G. & METIAN, M. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture,* 285**,** 146-158.

TACON, A. G. & METIAN, M. 2013. Fish matters: Importance of aquatic foods in human nutrition and global food supply. *Reviews in Fisheries Science,* 21**,** 22-38.

TACON, A. J. 2003. Aquaculture production trends analysis. *FAO Fisheries Circular,* 886**,** 5-29.

TAOKA, Y., MAEDA, H., JO, J. Y., KIM, S. M., PARK, S. I., YOSHIKAWA, T. & SAKATA, T. 2006. Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*. *Fisheries Science,* 72**,** 755-766.

TEVES, J. F. C. & RAGAZA, J. A. 2014. The quest for indigenous aquafeed ingredients: a review. *Reviews in Aquaculture*.

THIESSEN, D., CAMPBELL, G. & ADELIZI, P. 2003. Digestibility and growth performance of juvenile rainbow trout (*Oncorhynchus mykiss*) fed with pea and canola products. *Aquaculture Nutrition,* 9**,** 67-75.

TIBALDI, E., HAKIM, Y., UNI, Z., TULLI, F., DE FRANCESCO, M., LUZZANA, U. & HARPAZ, S. 2006. Effects of the partial substitution of dietary fish meal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (*Dicentrarchus labrax*). *Aquaculture,* 261**,** 182-193.

TORSTENSEN, B., ESPE, M., SANDEN, M., STUBHAUG, I., WAAGBØ, R., HEMRE, G.-I., FONTANILLAS, R., NORDGARDEN, U., HEVRØY, E. & OLSVIK, P. 2008. Novel production of Atlantic salmon (*Salmo salar*) protein based on combined replacement of fish meal and fish oil with plant meal and vegetable oil blends. *Aquaculture,* 285**,** 193-200.

TORSTENSEN, B. E., BELL, J. G., ROSENLUND, G., HENDERSON, R. J., GRAFF, I. E., TOCHER, D. R., LIE, Ø. & SARGENT, J. R. 2005. Tailoring of Atlantic salmon (*Salmo salar* L.) flesh lipid composition and sensory quality by replacing fish oil with a vegetable oil blend. *Journal of Agricultural and Food Chemistry,* 53**,** 10166-10178.

URBANO, G., LOPEZ-JURADO, M., ARANDA, P., VIDAL-VALVERDE, C., TENORIO, E. & PORRES, J. 2000. The role of phytic acid in legumes: antinutrient or beneficial function? *Journal of physiology and biochemistry,* 56**,** 283-294.

USCANGA, A., MOYANO, F. & ALVAREZ, C. 2010. Assessment of enzymatic efficiency on protein digestion in the tilapia *Oreochromis niloticus*. *Fish physiology and biochemistry,* 36**,** 1079-1085.

VADIVEL, V. & PUGALENTHI, M. 2008. Removal of antinutritional/toxic substances and improvement in the protein digestibility of velvet bean (*Mucuna pruriens*) seeds during processing. *JOURNAL OF FOOD SCIENCE AND TECHNOLOGY-MYSORE,* 45**,** 242-246.

VAN DER POEL, A. 1990. Effect of processing on antinutritional factors and protein nutritional value of dry beans (*Phaseolus vulgaris* L.). A review. *Animal Feed Science and Technology,* 29**,** 179-208.

VIELMA, J., MÄKINEN, T., EKHOLM, P. & KOSKELA, J. 2000. Influence of dietary soy and phytase levels on performance and body composition of large rainbow trout (*Oncorhynchus mykiss*) and algal availability of phosphorus load. *Aquaculture,* 183**,** 349-362.

VIELMA, J., RUOHONEN, K. & PEISKER, M. 2002. Dephytinization of two soy proteins increases phosphorus and protein utilization by rainbow trout, *Oncorhynchus mykiss*. *Aquaculture,* 204**,** 145-156.

WALLACE, J., MURRAY, F. & LITTLE, D. 2016. Effects of β-xylanase and 6-phytase on digestibility, trace mineral utilisation and growth in juvenile red tilapia, *Oreochromis niloticus* (Linnaeus, 1758) x *O. mossambicus* (Peters, 1852), fed declining fishmeal diets. *Journal of Applied Ichthyology*.

WANG, Q., GARRITY, G. M., TIEDJE, J. M. & COLE, J. R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology,* 73**,** 5261-5267.

WANG, Y.-B., TIAN, Z.-Q., YAO, J.-T. & LI, W.-F. 2008. Effect of probiotics, Enteroccus faecium, on tilapia (*Oreochromis niloticus*) growth performance and immune response. *Aquaculture,* 277**,** 203-207.

WATANABE, W. O., LOSORDO, T. M., FITZSIMMONS, K. & HANLEY, F. 2002. Tilapia production systems in the Americas: technological advances, trends, and challenges. *Reviews in fisheries science,* 10**,** 465-498.

WATSON, A. M., BUENTELLO, A. & PLACE, A. R. 2014. Partial replacement of fishmeal, poultry by-product meal and soy protein concentrate with two non-genetically modified soybean cultivars in diets for juvenile cobia, Rachycentron canadum. *Aquaculture,* 434**,** 129-136.

WEBSTER, C. D. & LIM, C. 2006. *Tilapia: biology, culture, and nutrition*, CRC Press.

WEN, Z. P., ZHOU, X. Q., FENG, L., JIANG, J. & LIU, Y. 2009. Effect of dietary pantothenic acid supplement on growth, body composition and intestinal enzyme activities of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture Nutrition,* 15**,** 470-476.

WONG, S., WALDROP, T., SUMMERFELT, S., DAVIDSON, J., BARROWS, F., KENNEY, P. B., WELCH, T., WIENS, G. D., SNEKVIK, K. & RAWLS, J. F.

2013. Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. *Applied and environmental microbiology,* 79**,** 4974-4984.

WONNACOTT, E. J., LANE, R. L. & KOHLER, C. C. 2004. Influence of dietary replacement of menhaden oil with canola oil on fatty acid composition of sunshine bass. *North American Journal of Aquaculture,* 66**,** 243-250.

WOOD, J. F. 1987. The functional properties of feed raw materials and their effect on the production and quality of feed pellets. *Animal Feed Science and Technology,* 18**,** 1-17.

XUE, M., LUO, L., WU, X., REN, Z., GAO, P., YU, Y. & PEARL, G. 2006. Effects of six alternative lipid sources on growth and tissue fatty acid composition in Japanese sea bass (*Lateolabrax japonicus*). *Aquaculture,* 260**,** 206-214.

YAN, W., REIGH, R. C. & XU, Z. 2002. Effects of Fungal Phytase on Utilization of Dietary Protein and Minerals, and Dephosphorylation of Phytic Acid in the Alimentary Tract of Channel Catfish *Ictalurus punctatus* Fed an All-Plant-Protein Diet. *Journal of the World Aquaculture Society,* 33**,** 10-22.

YE, L., AMBERG, J., CHAPMAN, D., GAIKOWSKI, M. & LIU, W.-T. 2014. Fish gut microbiota analysis differentiates physiology and behavior of invasive Asian carp and indigenous American fish. *The ISME journal,* 8**,** 541-551.

YIGIT, N. & OLMEZ, M. 2011. Effects of cellulase addition to canola meal in tilapia (*Oreochromis niloticus* L.) diets. *Aquaculture Nutrition,* 17**,** e494-e500.

YILDIRIM, Y. B. & TURAN, F. 2010. Effects of exogenous enzyme supplementation in diets on growth and feed utilization in African catfish, *Clarias gariepinus*. *Journal of Animal and Veterinary Advances,* 9**,** 327-331.

YU, D., GONG, S., YUAN, Y. & LIN, Y. 2013. Effects of replacing fish meal with soybean meal on growth, body composition and digestive enzyme activities of juvenile Chinese sucker, *Myxocyprinus asiaticus*. *Aquaculture Nutrition,* 19**,** 84-90.

YU, F. & WANG, D. 2000. The effects of supplemental phytase on growth and the utilization of phosphorus by crucian carp *Carassius carassius*. *J. Fish Sci. Chin,* 7**,** 106-109.

ZHOU, X., TIAN, Z., WANG, Y. & LI, W. 2010a. Effect of treatment with probiotics as water additives on tilapia (*Oreochromis niloticus*) growth performance and immune response. *Fish physiology and biochemistry,* 36**,** 501-509.

ZHOU, X., WANG, Y., YAO, J. & LI, W. 2010b. Inhibition ability of probiotic, Lactococcus lactis, against A. hydrophila and study of its immunostimulatory effect in tilapia (*Oreochromis niloticus*). *International Journal of Engineering, Science and Technology,* 2.

ZHOU, Y., YUAN, X., LIANG, X.-F., FANG, L., LI, J., GUO, X., BAI, X. & HE, S. 2013. Enhancement of growth and intestinal flora in grass carp: the effect of exogenous cellulase. *Aquaculture,* 416**,** 1-7.

ZHOU, Z., HE, S., LIU, Y., CAO, Y., MENG, K., YAO, B., RINGØ, E. & YOON, I. 2011. Gut microbial status induced by antibiotic growth promoter alters the prebiotic effects of dietary DVAQUA® on Aeromonas hydrophila-infected tilapia: Production, intestinal bacterial community and non-specific immunity. *Veterinary microbiology,* 149**,** 399-405.

ZIJLSTRA, R., OWUSU-ASIEDU, A. & SIMMINS, P. 2010. Future of NSP-degrading enzymes to improve nutrient utilization of co-products and gut health in pigs. *Livestock Science,* 134**,** 255-257.