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# Inclusion of lupin meal and effect of a commercial feed supplement (Synergen) in diets for carp, Cyprinus carpio

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## Copyright Statement

# Inclusion of lupin meal and effect of a commercial feed supplement (Synergen™) in diets for carp, *Cyprinus carpio*


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# Inclusion of lupin meal and effect of a commercial feed supplement (Synergen™) in diets for carp, *Cyprinus carpio*

**Ayub Younis Anwar**

**Abstract**

Plant proteins are mainly used to formulate diets for carp. Soybean meal (SBM) is one of the most nutritious of all plant protein sources in carp feeds. However, an increase in the use of soybean meal for human consumption and animal feed in both developed and developing countries has resulted in an increase market price of soybean meal globally. Therefore, using other inexpensive plant protein sources in carp feeds would be beneficial to reduce feed cost and contribute to food security as well as to sustain aquaculture production. Anti-nutritional factors (ANFs) are believed to be the most important factors limiting the use of plant protein concentrates in fish feeds. To address this limitation, one option is to use an exogenous (mixed- enzyme containing, solid state fermentation, SSF) dietary supplement. Two nutritional trials were conducted in order to assess the incorporation of extruded white lupin (*Lupinus albus*) and supplement Synergen™ in diets for juvenile carp. The first trial was designed to determine the effect of including 12.5% and 25% of white lupin as a soybean meal replacement with the addition of 0.05% of Synergen™ for common carp BSD (Basal skretting diet) based diet on growth performance, feed utilization and general health of the fish. All diets were formulated based on the summit dilution trial type. Supplementing Synergen™ to the BSD based diet and the diet including 12.5% of white lupin significantly ($P<0.05$) improved growth performance and feed utilization but this trend was very slight with the diet that contained 25% of white lupin. On the other hand, including 12.5% of white lupin significantly ($P<0.05$) improved growth performance and feed utilization. On contrary, including 12.5% of white lupin

significantly (*P<0.05*) decreased growth performance and feed utilization. No significant (*P>0.05*) differences were found in growth performance, carcass composition or liver and gut histology between the BSD based diet and diet that contained 25% of white lupin with Synergen™. The second trial was designed to determine the effects of substituting 12.5% and 25% of the soya protein concentrate (SPC) with white lupin seed meal and with the addition of 0.1% of Synergen™ for the mirror carp (*Cyprinus carpio*) plant based diet on growth performance, feed utilization and general health of the fish. All diets were formulated to be iso-nitrogenous (38% crude protein) and isolipdic (8% crude lipid). Supplementing Synergen™ to the soya protein concentrate based diet and the diets substituting 12.5% and 25% of soya protein concentrate with white lupin significantly improved growth performance, feed utilization and gut and liver histology. Additionally, substituting up to 25% of soya protein concentrate with white lupin in the complete diet for mirror carp did not have any significant negative effects on growth performance, feed utilization, carcass composition and fish health.

The results of this research program demonstrate that supplementing Synergen™ to plant based diets is beneficial to reduce the negative effect of anti-nutritional factors and to improve growth performance and nutrient utilization for carp. In addition, our findings demonstrate that lupin meal has a promising potential for use in common carp and mirror carp feeds with importance for the aquaculture industry.

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## List of Abbreviations

| | |
|---|---|
| FAO | Food agriculture organization |
| Mt | Million tonnes |
| t | Ton |
| FM | Fishmeal |
| FO | Fish oil |
| PLM | Plant protein meal |
| MF | Mucosal fold |
| L | Lupin |
| BSD | Basal skretting diet |
| SBM | Soybean meal |
| SPC | Soybean protein concentrates |
| CO | Cottonseed meal |
| CG | Corn gluten meal |
| FFSM | Full-fat soybean meal |
| RP | Rapeseed seed meal |
| GN | Groundnut |
| PN | Peanut meal |
| SF | Sunflower |
| ANFs | Anti-nutritional factors |
| S | Synergen™ |

| | |
|---|---|
| DM | Dry matter |
| MS222 | Tricane methane sulphate |
| PBS | Phosphate buffered saline |
| NFE | Nitrogen-free extracts |
| IW | Initial weight |
| FW | Final weight |
| WG | Weight gain |
| SGR | Specific growth rate (% BM day-1) |
| FCR | Feed conversion ratio |
| FCE | Feed conversion efficiency |
| PER | Protein efficiency ratio |
| LPE | Lipid efficiency ratio |
| EAA | Essential amino acid |
| EFA | Essential fatty acid |
| ER | Energy retention |
| K | Condition factor |
| ANPU | Apparent net protein utilization |
| ANOVA | Analyses of variance |
| SE | Standard error |
| AOAC | Association of Official Analytical Chemists |

| | |
|---|---|
| µl | Microliter |
| µm | Micrometer |
| L | Lymphocyte, Lumen |
| M | Monocyte, Muscularis |
| LP | Lamina properia |
| Hb | Haemoglobin |
| Hct | Haematocrit |
| H & E | Haematoxylin and eosin stain |
| LSD | Less significant difference |
| PCV | Pack cell volume |
| P | Statistical probability |
| TEM | Transmission electron microscopy |
| SEM | Scanning electron microscopy |
| AB-PAS | Alecian blue- Periodic acid Schiff |
| BSE | Bovine Spongiform Encephalopathy |
| DHA | Docosahexaenoic acid |
| EPA | Eicosapentaenoic acid |
| DDGS | Dried Distillers Grains with Solubles |

| | |
|---|---|
| ppt | part per thousand |
| Psi | Pounds per square inch |
| AU | arbitrary units |

Fish husbandry and the experiments of this thesis were in accordance with the UK Animal Scientific Procedures Act, 1986 (under the UK Home Office project license #30/2644 and personal license PIL). In addition, the study was also fully approved by the Plymouth University Ethical Review Committee.

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## Dedication

I would like to dedicate this thesis to my lovely wife, Mariya, and my son Zhanyar for all help, support and encouragements offered during the completion of a thesis. To my parents, Younis and Badiha, and my sisters (Wasiha, Sabiha, Roqia, Samira and Chnar), brothers (Edris and Mashud) deserve a huge acknowledgement and thanks for being helpful and supportive during the two years in the UK.

## Author's Declaration

At no time during the registration for the degree of research master (ResM) has the author has been registered for any other University without prior agreement of the Graduate Committee.

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# Chapter 1: General review and introduction

## 1.1 Global Fisheries and Aquaculture Status

The global population is increasing and, in order to maintain at least the current level of per-capita consumption of aquatic foods, the world will need an additional 23 million tonnes thereof by 2020. This additional supply will have to come from aquaculture.

World per capita food fish supply increased from an average of 9.9 $kg^{-1}$ (live weight equivalent) in the 1960 to 18.4 $kg^{-1}$ in 2009, and preliminary estimates for 2010 point to a further increase in fish consumption to 18.6 $kg^{-1}$. Capture fisheries and aquaculture supplied the world about 148.5 Mt of fish in 2010 (with a total value of US$217.5 billion), of which about 128 Mt was utilised for direct human consumption, and preliminary data for 2011 indicate increased production of 154 million tonnes, of which 131 million tonnes were destined as human food (see Table 1.1). Additionally, fisheries and aquaculture provided livelihoods and income for an estimated 54.8 million people engaged in the primary sector of fish production in 2010, of which an estimated 7 million were occasional fishers and fish farmers. Capture fisheries production has remained static around 90 Mt and cannot be expected to grow significantly. The best way to supply high demand for seafood is through development aquaculture. Therefore, aquaculture production has been increased dramatically in recent years (Figure 1.1) (FAO, 2012).

Aquaculture uses freshwater, brackish water and full-strength marine water as culture media. Freshwater fishes dominate global aquaculture production because freshwater fish farming has been a relatively easy entry point for practicing aquaculture in developing countries, particularly for small-scale producers. World aquaculture production in 2010 consisted of 56.4 percent of freshwater fish (33.7 Mt), 23.6 percent of molluscs (14.2 Mt), 9.6 percent of

crustaceans (5.7 Mt), 6.0 percent of diadromous fishes (3.6 Mt), 3.1 percent of marine fishes (1.8 Mt) and 1.4 percent of other aquatic animals (814 300t) (FAO, 2012). In Iraq aquaculture depends on freshwater resources (Kitto & Tabish, 2004).

The global distribution of aquaculture production across the regions and countries of different economic development levels remains imbalanced. Asia accounted for 89 % of world aquaculture production by volume in 2010 followed by America (4.3 %), Europe (4.2%), Africa (2.2%) and Oceania (0.3%) (Figure1.2). China, India, Vietnam, Indonesia, Bangladesh, Thailand, Myanmar, Philippines and Japan are the major producers of aquaculture in Asia. The number of species recorded in FAO aquaculture production statistics increased to 541 species in 2010, including 327 finishes (5 hybrids), 102 molluscs, 62 crustaceans, 6 amphibians and reptiles, 9 aquatic invertebrates and 35 algae (FAO, 2012). The most common aquaculture products are freshwater, omnivorous fish, most of which come from the cyprinid family (Mazurkiewicz, 2009). Carp still account for about 45% of the total weight of aquaculture products (Stickney, 2009).

Table 1.1: The state of fisheries and aquaculture production in the world (Excluding aquatic plants)

| | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 |
|---|---|---|---|---|---|---|
| **PRODUCTION** | | | **(Million tonnes)** | | | |
| Capture | | | | | | |
| Inland | 9.8 | 10.0 | 10.2 | 10.4 | 11.2 | 11.5 |
| Marine | 80.2 | 80.4 | 79.5 | 79.2 | 77.4 | 78.9 |
| Total capture | 90 | 90.3 | 89.7 | 89.6 | 88.6 | 90.4 |
| Aquacultur | | | | | | |
| Inland | 31.3 | 33.4 | 36.0 | 38.1 | 41.7 | 44.3 |
| Marine | 16.0 | 16.6 | 16.9 | 17.6 | 18.1 | 19.3 |
| Total aquaculture | 47.3 | 49.9 | 52.9 | 55.7 | 59.9 | 63.6 |
| Total world fisheries | 137.3 | 140.2 | 142.6 | 145.3 | 148.5 | 154.0 |
| Utilization | | | | | | |
| Human consumption | 114.3 | 117.3 | 119.7 | 123.6 | 128.3 | 130.8 |
| Non-food uses | 23.0 | 23.0 | 22.9 | 21.8 | 20.2 | 23.2 |
| Population (billions) | 6.6 | 6.7 | 6.7 | 6.8 | 6.9 | 7.0 |
| Per capita food fish | 17.4 | 17.6 | 17.8 | 18.1 | 18.6 | 18.8 |

Data for 2011 are provisional estimates. Source: FAO (2012)

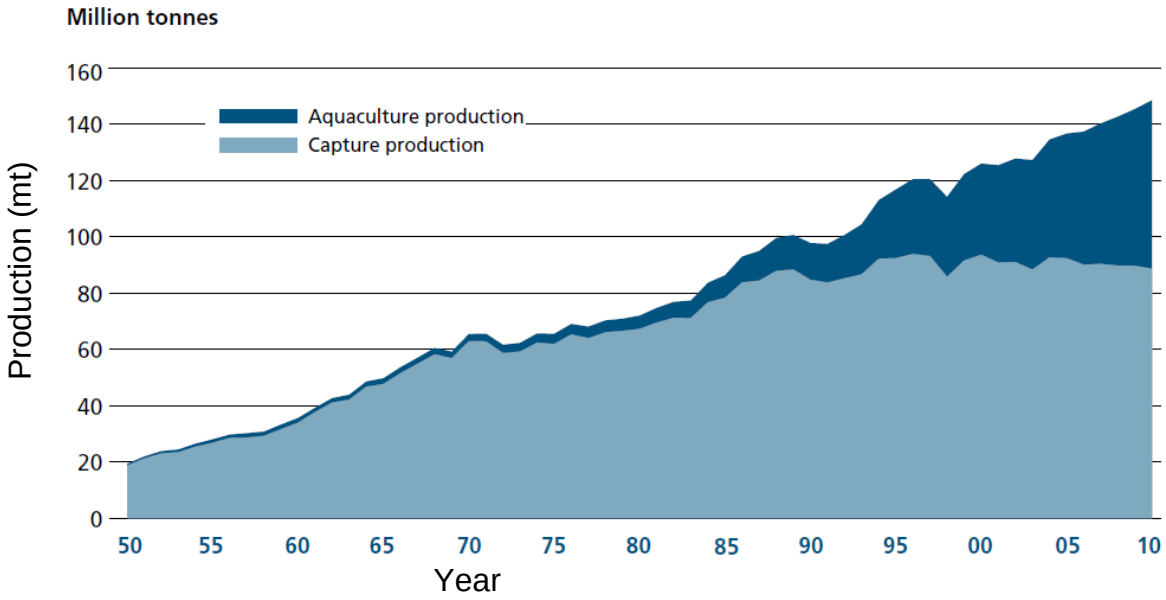



Figure 1.1: The state of capture fisheries and aquaculture production in the world.

Source (FAO, 2012)

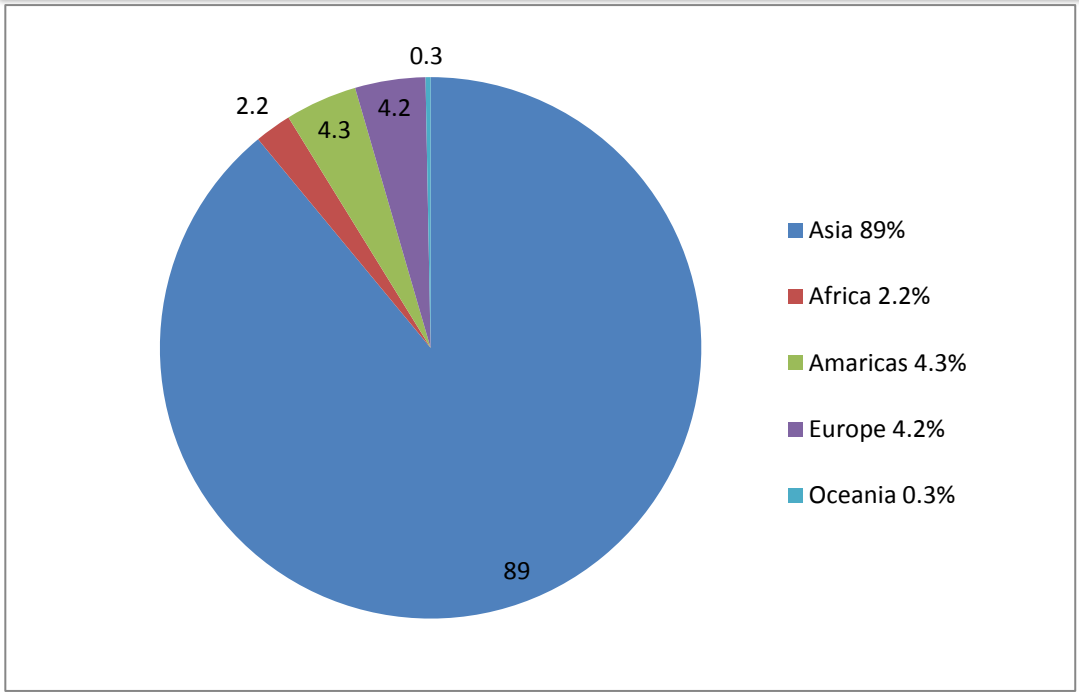



Figure 1.2 Aquaculture production by region: quantity and percentage of world total production (excluding aquatic plants and non-food products) in 2010. Data adapted from FAO (2012).

## 1.2 Aquafeed production

Aquafeeds are generally utilized for feeding carnivorous fishes (e.g. Salmon, trout, eel, sea bass, seabream and tuna), omnivorous fishes (e.g. Tilapia, catfish, common carp and milkfish) and crustacean species (marine and brackish-water shrimps, freshwater prawns, crabs and lobsters) (FAO, 2012). The rapid development of aquaculture has been accompanied by rapid growth of aquafeed production. The total industrial aquafeed production increased more than three folds, from 7.6 million tonnes in 1995 to 29.2 million tonnes in 2008, with production growing at an average rate 11 percent per year. In order to keep pace with fed aquaculture production, global aquafeed production will continue to grow, and it is expected to reach 71 Mt by 2020 (Tacon, Hasan & Metian, 2011).

Fishmeal (FM) has been used as an ideal source of protein for feed not only carnivorous and omnivorous fish, but even herbivorous fish, particularly in their early stages because of its high-protein content, excellent amino acid profile, high-nutrient digestibility with lack of anti-nutrients (Rumsey, 1993; Jackson, 2006, 2007, 2009, 2010 & 2011; Glencross, Booth & Allan, 2007; Barrows *et al*., 2008). Although there has been a gradual reduction of combined fishmeal and its use in aquaculture since 2006, the aquaculture sector has continued to remain the largest user of fishmeal (Tacon, Hasan & Metian, 2011). In 2010 aquaculture alone used 73% of total fishmeal production in the world. The major aquaculture user of fish meal are crustaceans followed by salmon and trout, marine fish, freshwater (incl. Catfish), tilapias, eels and Cyprinids (Figure 1.3) (Shepherd & Jackson 2012).

The demands for fishmeal are expected to excess in the next decade. However, the annual global production of fishmeal has steadily decreased in recent years. The price of fishmeal will increase resulting with an increase demand and decrease production. As a result, the aquaculture operation cost will increase because aquafeeds usually account for 50–70 percent of the total production costs (Barrows *et al*., 2008; Rana, Siriwardena & Hasan, 2009; Hardy, 2010; Jackson & Shepherd, 2010; Tacon, Hasan & Metian, 2011). For these reasons, finding an alternative to fishmeal is necessary to sustain aquaculture production and reduce aquaculture operation cost.

Alternatives to fishmeal are available from plant and animal protein sources as well as single–celled proteins e.g. Microalgae, bacteria and yeast. Some alternative animal protein sources which have worked well are meat and bone meal (from cattle) and poultry by- product meal (primarily from the chicken

industry), feather meal (from poultry feathers) (Stickney, 2009). They are readily available with low price, which can be used to partially replace with fishmeal.

Animal protein sources have been used to replace fishmeal in fish feeds. In the study was undertaken by El‐Saidy & Gaber (2003), the effect of the total replacing fishmeal with animal protein sources that consisted of shrimp meal, blood meal, bone meal and poultry by-product meal in the diet for Nile tilapia (*Oreochromis niloticus*) were evaluated. These workers showed that shrimp meal, meat bone meal and poultry by-product meal can totally replace with fishmeal in practical diets for this species. In spite of the promise of animal by-products in fish feeds, there is still much public concern with using animal by-products in fish feeds due to recent Bovine Spongiform Encephalopathy (BSE) commonly known as a mad-cow disease and prion risks related with such materials arising within animal and consumer food chain (Naylor *et al*., 2009; Stickney, 2009; Davies & Gouveia, 2010). Although microbial and algal species are considered innovative protein sources for aquafeeds, production costs will be an issue with some of them (FAO, 2012). Therefore, aquaculture research has mainly focused on plant protein sources in the last two decades.

## 1.3 Plant protein sources

The aquafeed industry has been using plant feedstuffs to formulate diets for cold and warm water aquatic species for many years (Gatlin III *et al*., 2007). Plant proteins are a good alternative source for fishmeal because they are readily available worldwide with low cost (Dersjant-Li, 2002 and Naylor *et al*., 2009). Plant proteins represent the major dietary protein source used within feeds for lower trophic level fish species (tilapias, carps, catfish) and the second major

source of dietary protein and lipid sources after fishmeal and fish oil for shrimps and European higher trophic level fish species (Tacon, Hasan & Metian, 2011). Among the most promising alternative protein and energy sources are varieties of grain legumes, pulses and cereals as reviewed by Gatlin III *et al*. (2007) and Hardy (2010). Furthermore, the potential of alternative dietary protein sources such as fishery by-products, terrestrial animal by-products, oil seed plants, aquatic plants in tilapia diets were widely reviewed (El-Sayed & Tacon, 1997; El-Sayed, 1999). The potential of the use soybean meal in the diets for omnivorous freshwater fish was widely reviewed (Gatlin III, 2002). The potential of using canola and rapeseed meals were widely reviewed (Enami, 2011).

Several studies have been shown that plant proteins (soybean meal, sunflower, corn gluten meal wheat, maize, yeast, maize gluten, detoxified jatropha kernel meal and wheat gluten meals) could be incorporated up to 50% for omnivores, warm water fish species diet without affecting fish performance (Pongmaneerat *et al*., 1993; Escaffre *et al*., 1997; Sahar, Ali & Naqvi, 2003; Mazurkiewicz, 2009; Kumar *et al*., 2011; Marković *et al*., 2012) with common carp (*Cyprinus carpio L*) and *(*Shiau, Chuang & Sun, 1987; Fagbenro & Davies, 2000; Gaber, 2006; Soltan, Hanafy & Wafa, 2008*)* with tilapia *(Oreochromis sp)*.

Due to its high protein content and digestibility, relatively well-balanced amino acid profile and steady supply, soybean meal is the most commonly used plant protein source in fish feeds. However, increasing use of soybean meal for both human food and animal feed demands has resulted in market increase in the global price of soybean meal as a commodity (Kumar *et al*., 2011). Therefore, utilizing other inexpensive plant protein sources in aquaculture diet would be

beneficial in reducing the feed cost and contribute to food security and to sustain aquaculture production. Lupin meal has a promising potential for use in feeds for aquaculture with importance for the aquaculture industry.

However, the inclusion of plant based proteins in aquafeeds provides a number of problems which include the occurrence of anti-nutritional factors (ANFs), reduced digestibility, issues of palatability and limitations of certain essential amino acids (Petterson, 2000; Francis, *et al*., 2001; Gatlin III *et al*., 2007; Barletta, 2010; Krogdahl *et al*., 2010). Improvements in this area continue to be made through classic breeding, transgenic manipulation and exogenous enzyme treatment (Naylor *et al*., 2009). Dietary supplemental exogenous enzymes could be one good way to reduce the negative impact of ANFs in the fish diets containing a high level of plant.

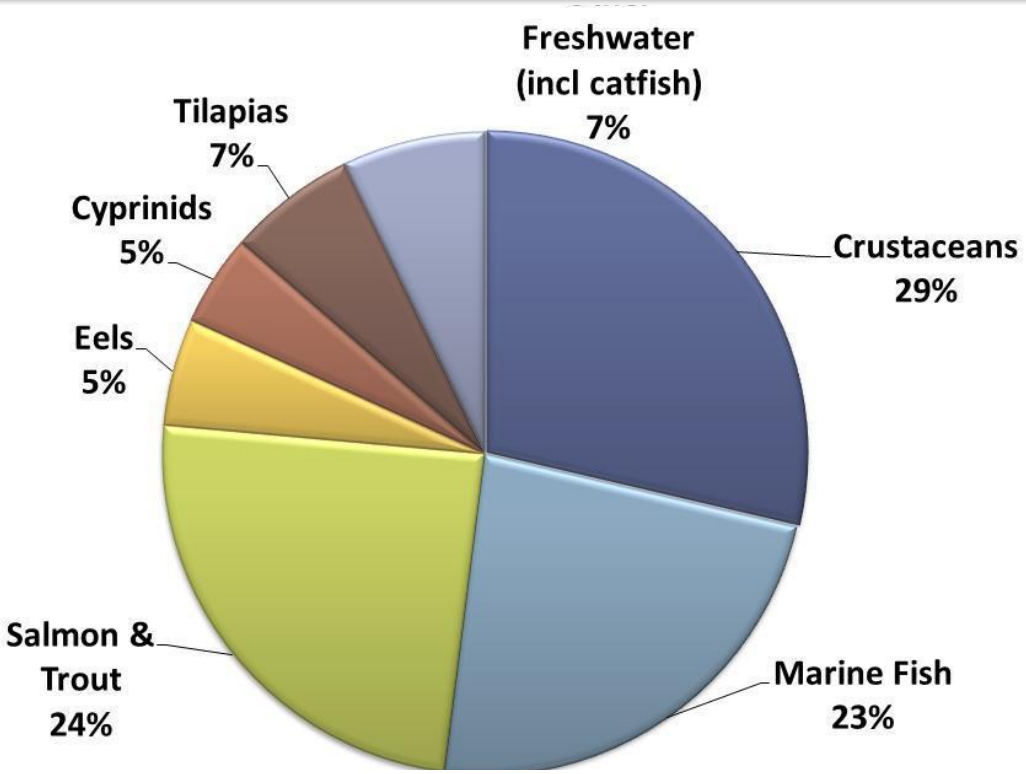



Figure 1:3: Global consumption of fish meal by major aquaculture species group in 2010.

Source: (Shepherd & Jackson 2012)

## 1.4 Lupins in Aquafeeds: A review

### 1.4.1 Introduction

Lupins are the harvested seed of species from the *Lupinus* genus, a group within the leguminous bean and pea family Fabaceae. Although estimates of the number of species in the genus range from about 200 to 300, only the narrow-leafed or blue lupin *(L. angustifolius),* white lupin *(L.albus)* and yellow lupin (*L.luteus*) are widely used and fully domesticated. Narrow-leafed lupin (*L. angustifolius)* dominates world lupin production (Huyghe, 1997; Gladstones, 1998; Gladstones, Atkins & Hamblin, 1998; Perry *et al*., 1998; Petterson, 2000;

Glencross, 2004; Small, 2012). Lupins are normally utilized in the diets of ruminants and terrestrial monogastric animals, primarily cattle, pigs and poultry, with considerable success (Huyghe, 1997; Edwards & Van Barneveld, 1998). Lupins were first identified in the late 1980 as having some potential as a useful feed ingredient in the diets of fish (Glencross, Curnow & Hawkins, 2003b). The total lupin production in the world was 925,412 tonnes in 2009 with the major country producer being Australia (Tacon, Hasan & Metian, 2011) (see Figure 1.4).

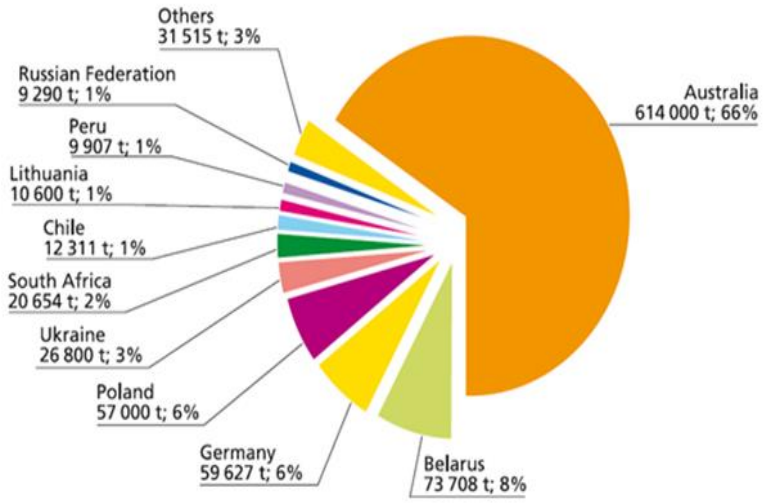



Figure 1.4 Global productions of lupins by country in 2009.

Source: (Tacon, Hasan & Metian, 2011)

## 1.4.2. Lupins composition

There is considerable variability in the composition of lupin meals depending on both lupin species and whether they are in a whole seed or kernel form (Glencross, 2007 & 2008 and Tabrett *et al*., 2012). The gross chemical composition of these three lupin species is shown in (Table 1.2).

Lupin seeds are characterized by higher protein content than most other grain legume (pulses). There is considerable variation in the protein content between

the various species and between cultivars as a result of the characteristics of the growing season and soil type. Yellow lupin is generally regarded as having the highest protein content followed by white and blue lupins (Petterson *et al*., 1997; Petterson, 2000). The amino acid profile of the protein content of lupin meals compares favourably with that soybean meal, being high in arginine, lysine, leucine and phenylalanine. The notable limitation of lupin meals is the comparative deficiency of methionine and cysteine (Glencross & Australia, 2001; Glencross, 2004) (Table 1.3).

Although white lupin kernel meal contains high level of lipid which is about 13-14%, lupins are not renowned as oilseeds. Blue lupin and yellow varieties contain low level of lipid (< 6% on dry matter basis) (Petterson *et al*., 1997; Petterson, 2000; Glencross, 2004). The general fatty acid content of the lipid in lupins is typical of that most of legumes, being high in mono-unsaturated and polyunsaturated fatty acids (Glencross & Australia, 2001). The typical lipid profiles of the three key species of lupin are shown in (Table 1.4).

The carbohydrate chemistry of lupin seed is different to most legumes. Lupins contain negligible amount of starch less than 15g $kg^{-1}$ DM in the seed of most species and low levels of lignin, while lupins contain high amounts of soluble and non- soluble non-starch polysaccharides in their seeds which have a negative effect on the nutritional value of lupins in fish diet (Petterson *et al*., 1997; Van Barneveld, 1999; Petterson, 2000; Glencross, 2004, 2007 & 2008). The key minerals in lupins are calcium, magnesium, phosphorus, potassium, sodium and sulphur. The level of mineral in lupins is different according to soil type on which

plant was grown (Petterson, 2000). The mineral profiles in major species of lupin are shown in (Table 1.5).

Lupins contain a range of vitamins, for instance (*L. angustifolius*) whole seed meal contains β_carotein (3.9mg $kg^{-1}$ DM), thiamine (5.9mg $kg^{-1}$ DM), ribovalvine (3.1), biotin (0.04), folate (0.4), choline (3.4), niacin (40), pantothenate (1.8) and tocopherol (2.4) (Petterson, 2000).

Table1.2 Chemical composition of major lupin species (%)

| | Lupin seed | | | Lupin kernel | | |
|---|---|---|---|---|---|---|
| | Blue Lupin | White Lupin | Yellow Lupin | Blue Lupin | White Lupin | Yellow Lupin |
| Seed Coat | 24 | 18 | 27 | 0 | 0 | 0 |
| Moisture | 9 | 9 | 9 | 12 | 11 | 12 |
| Protein | 32 | 36 | 38 | 41 | 44 | 52 |
| Fat | 6 | 9 | 5 | 7 | 11 | 7 |
| Ash | 3 | 3 | 3 | 3 | 4 | 4 |
| Polysaccharid | 22 | 17 | 8 | 28 | 21 | 10 |
| Oligosaccharid | 4 | 7 | 9 | 6 | 8 | 12 |
| Lignin | 1 | 1 | 1 | 1 | 1 | 1 |
| Minor Components | 0.5 | 0.6 | 0.9 | 1.0 | 1.0 | 1.0 |

Data derived from (Sipsas, 2003)

Table 1.3 Essential amino acid profile for major lupin species

| | White Lupin | Blue Lupin | Yellow Lupin | Soybean | White Lupin | Blue Lupin | Yellow Lupin |
|---|---|---|---|---|---|---|---|
| | g amino acid $kg^{-1}$ protein | | | | -- g amino acid $kg^{-1}$ seed | | |
| Arginine | 122 | 116.2 | 113 | 54.2 | 45 | 35.9 | 43.7 |
| Cysteine | 13.4 | 13.6 | 22.8 | ^ | 5.00 | 4.2 | 8.8 |
| Histidine | 18.6 | 25.7 | 33 | 24.6 | 6.8 | 7.9 | 10.5 |
| Isoleucine | 38 | 39.1 | 27 | 45.1 | 14 | 12.2 | 14.2 |
| Leucine | 69 | 66.1 | 78.9 | 68.1 | 23 | 21.2 | 30.6 |
| Lysine | 47.5 | 66.1 | 78.9 | 56.6 | 15.8 | 14.6 | 20.7 |
| Methionine | 6.6 | 7.2 | 7.00 | 12.8 | 2.4 | 2.00 | 20.7 |
| Phenylalanine | 38.5 | 36.5 | 40.4 | 36 | 12.4 | 11.8 | 15.6 |
| Threonine | 32.9 | 35.4 | 35.1 | 35.6 | 12 | 10.9 | 13.6 |
| Tryptophan | 9.7 | 10 | ^- | 13.5 | 3.6 | 3.1 | - |
| Tyrosine | 42.6 | 36.6 | 31 | 16.7 | 14.6 | 11.3 | 11.2 |

Data derived from Petterson *et al*. (1997); Glencross & Australia (2001)

^ No value reported

Table 1.4 Fatty acid profile of major lupin species (%)

| Fatty acid | Common name | White Lupin | Blue Lupin | Yellow Lupin |
|---|---|---|---|---|
| 14:0 | Myristic acid | 0.1 | 0.1 | P |
| 16:0 | Palmatic acid | 7.8 | 11.0 | 4.8 |
| 16:1 | Palmitoleic acid | 0.3 | 0.1 | P |
| 18:0 | Stearic acid | 1.6 | 3.8 | 2.5 |
| 18:1 | Oleic acid | 53.0 | 38.2 | 21.0 |
| 18:2 | Linoleic acid | 17.2 | 37.1 | 47.3 |
| 18:3 | Linolenic acid | 9.5 | 5.3 | 7.5 |
| 20:0 | Arachidonic acid | 1.2 | 0.9 | 2.7 |
| 20:1 | Hexeicosanoic acid | 4.3 | 0.3 | 1.8 |
| 20:4 | Behenic acid | 3.9 | 1.9 | 7.1 |
| 22:1 | Erucic acid | 1.9 | P | 0.8 |
| 24:0 | Lignoceric | 0.7 | 0.4 | 0.8 |
| Sterols | | P | 2.2 | P |

Data adapted from Petterson *et al*. (1997).

^ P. Present, not quantified

^ No value reported

Table 1.5 Mineral content of major species of lupin

| | White Lupin | Blue Lupin | Yellow Lupin |
|---|---|---|---|
| | ……g kg⁻¹ …… | | |
| Calcium | 2.0 | 2.2 | 2.2 |
| Magnesium | 1.3 | 1.6 | 2.1 |
| Potassium | 8.8 | 8.0 | 9.7 |
| Sodium | 0.3 | 0.4 | <0.1 |
| Sulphur | 2.5 | 2.3 | 4.6 |
| Phosphorus | 3.6 | 3.0 | 5.1 |
| | ……..mg kg⁻¹…… | | |
| Copper | 5 | 5 | 8 |
| Iron | 27 | 68 | 59 |
| Manganese | 896 | 19 | 35 |
| Molybdenum | 2 | 2 | ^ |
| Zinc | 30 | 34 | 53 |
| | …….. µ g kg⁻¹…… | | |
| Cobalt | 206 | 78 | - |
| Selenium | 85 | 89 | - |

Adapted from Petterson *et al.* (1997)

^ No value reported

P= means not quantified

### 1.4.3 Anti-nutritional Factors in Lupins (ANFs)

Lupins, like all members of the legume plant family, contain certain anti-nutritional factors (ANFs), although considerably less than other plants such as soybean meal (Francis *et al*., 2001; Glencross, Curnow & Hawkins, 2003a; Glencross, 2005). The key anti-nutritional factors present in lupins are alkaloids and oligosaccharides. Notably phytate, saponins, tannins, protease inhibitors and lectins are comparatively lower than other grain legume varieties.

The oligosaccharides in lupins are generally ∝-galactosyl homologues of sucrose. Of these oligosaccharides, lupins contain significant amounts of the raffinose, stachyose, verbascose and sucrose families. The lupin seed contains a significant proportion of oligosaccharides. The proportion of oligosaccharides in white, blue and yellow lupins is 6.6%, 4.1% and 8.9% respectively. The oligosaccharides cannot be digested and metabolized by monogastric animals, and they undergo bacterial digestion in the colon to produce carbon dioxide, methane and hydrogen. This causes abdominal discomfort, cramps, gut distension and flatulence (Petterson *et al*., 1997; Petterson, 2000; Glencross, 2005). Furthermore, Van Barneveld (1999) has reported that the non- starch polysaccharides can interfere with the digestion of major nutrients, induce osmotic effects of the oligosaccharides in the intestine and affect the fermentation of the sugars resulting in increased gas production. The effect of variations in the chemical composition of blue lupin kernel meals on their digestibility when fed to rainbow trout was assessed by Glencross, Curnow & Hawkins (2003a). Generally these later workers have demonstrated that the oligosaccharide content in lupins exerts a negative effect on digestibility of protein, energy and organic matter. In a subsequent study by Glencross, Boujard & Kaushik (2003b) these workers

showed that blue lupin oligosaccharides can reduce the protein digestibility and hence the nutritional value of lupin meals when fed to rainbow trout (*Oncorhynchus mykiss*).

It should be noted that alkaloids are not heat labile and survive the processing of pulses and plant protein concentrates with deleterious consequences in fish nutrition. Alkaloids have a bitter taste making the seed unpalatable and sometimes toxic to animals. However, lupins contain low levels of alkaloid and do not appear to present palatability problems to fish (Glencross & Australia, 2001; Gatlin III *et al*., 2007). Lupins also contain low levels of protease inhibitors. Indeed, trypsin inhibitor activity is less than 0.3mg $kg^{-1}$ and chymotrypsin inhibitor activity is less than 0.6mg $kg^{-1}$ in white, blue and yellow lupins. Petterson *et al*. (1997); Petterson (2000) and Glencross (2004) all reported that these are only a tenth of the most found other grain legume crops.

The phytate content of lupins is about 0.5%, which is similar to that of peas and soybeans, which are not likely to be of concern under any conditions within an intensive animal production (Petterson, 2000; Glencross & Australia, 2001). The concentration of the tannins in lupin is very low which is about 0.01% that is unlikely to impair protein utilization by animal species (Petterson, 2000).

Saponins are heat stable factor which have a bitter taste, which acts as a feeding deterrent due to an increase in the permeability of the small intestine mucosa cells. Only traces of saponins are present in white lupin, while concentrations in the blue range from 480 to 730 mg $kg^{-1}$ and in yellow lupin 55mg $kg^{-1}$. These concentrations are about one-tenth that in soybean meal. There is no evidence that these concentrations of saponins have any effect on feed intake or the gut absorption (Petterson, 2000).

### 1.4.4 Lupins in aquafeeds

Lupins have been investigated as having a promising potential for use in aquaculture feeds (Edwards & Van Barneveld, 1998; Van Barneveld, 1999; Glencross & Australia, 2001; Glencross, Curnow & Hawkins, 2003b; Glencross & Hawkins, 2004; Glencross, 2004; Drew *et al*., 2007; Sweetingham *et al*., 2008a; Zhang *et al*., 2012). A comprehensive review of the nutritional and biological value of lupins in aquaculture feeds has been published (Van Barneveld, 1999; Petterson, 2000; Glencross & Australia, 2001; Glencross, Curnow & Hawkins, 2003a; Glencross, 2004, 2007 and 2008). The earliest and the only study on carp was conducted by Viola, Arieli & Zohar (1988) who indicated that inclusion of 45% of whole-seed blue lupin as a fishmeal replacement in a diet for common carp did not have any significant adverse effects on growth performance and feed utilization. The possibility of replacing fishmeal by lupin meal up to 30-50% in the diets for rainbow trout without negative effects on growth performance feed utilization has been reported (Burel *et al*., 1998; Farhangi & Carter, 2001; Glencross *et al*., 2002a; Glencross *et al*. 2004a; Borquez *et al*., 2011a and Glencross, Rutherford & Hawkins, 2011). Bransden, Carter & Nowak (2001) also reported that inclusion of up 40% of de-hulled blue lupin in diets for Atlantic salmon (*Salmo salar L*.) did not have any adverse effects on growth, immune function or blood chemistry and disease resistance. However, Gouveia *et al*. (1993) reported that inclusion up to 20% of white lupin in the diet for rainbow trout did not adversely affect growth performance. Recently, Bórquez *et al*. (2011b) reported that including whole seed white lupin meal at up to 20% in extruded diets for rainbow trout did not adversely affect growth performance and feed performance. More recently, Hernández *et al*. (2012) evaluated white lupin meal

in diets for rainbow trout with favourable results in respect of growth and feed performances and concluded that up to 25% of white lupin could be included without adverse effects. Work with juvenile gilthead sea bream (*Sparus aurata L*.) (Pereira & Oliva-Teles, 2004) has also stated that incorporation of up to 30% of narrow-leafed lupin seed meal into diets for juvenile gilthead sea bream had no deleterious effects on growth performance and feed performance. Similarly, in the Burel *et al*. (2000a) study no adverse effects were encountered on growth performance, feed performance and body composition with incorporation of up to a level of 50% of extruded white lupin in diets for turbot (*Psetta maxima*).

However, there is much less information available on the substituting soybean meal with lupin meal in fish feeds. The earliest reported studies were that by Hughes (1988, 1991) who examined the nutritional value of white lupin whole-seed meal in the rainbow trout diet. It was found that full fat soybean meal can be totally replaced with lupin flour in complete diet for rainbow trout. Additionally, the economic value of using lupin meal over soybean meal was reported in this study. Similarly, Robaina *et al*. (1995) reported that soybean meal can be totally replaced by blue lupin meal in diets for juvenile gilthead seabream. Work with juvenile shrimp (*Penaeus monodon*) (Sudaryono, Tsvetnenko & Evans, 1999) also reported that 50% of soybean meal can be replaced with de-hulled sweet white lupin in practical diets for juvenile shrimp without negative effects on growth performance, feed utilization, apparent digestibility coefficient of dry matter and protein and whole body composition.

On the other hand, considerable research has been conducted to evaluate the digestible value of lupin in comparison with fishmeal and other plant protein

sources in the diets of many aquaculture species of commercial value. Morales *et al*. (1994) examined the apparent digestibility characteristics of cottonseed meal, white lupin seed meal and corn gluten meal when they substituted 40% of the fishmeal component in experimental diets for rainbow trout. The workers found that the apparent protein digestibility of the white lupin (85.2%) was higher than that of the cottonseed meal (81.2 %), but not as high as that of the corn gluten meal (88.9 %), whereas the apparent digestibility of dry matter, organic matter and energy of white lupin were (53.1%, 56.3% and 62.7%) respectively which was lower than the other plant protein sources and fishmeal. Gomes, Rema & Kaushik, (1995) also evaluated the nutritional value of a range of plant protein sources including narrow-leaf lupin whole seed meal, pea (*Pisum sativum*) seed meal, faba bean (*Vicia faba*) meal, full-fat toasted soybean meal and full-fat micronized soybean meal in test feeds for rainbow trout. Of the plant legume protein meals, full-fat micronised soybean meal had the highest apparent dry matter digestibility (86.4%) and blue lupin seed meal the lowest (63.3%). The apparent dry matter digestibility values of pea seed meal (66.6%) and faba bean meal (66.1%) were similar to that of and blue lupin seed meal. Apparent protein digestibility of the legume meals was also highest in full-fat micronized soybean meal (96.3%) and the lowest faba bean meal (80.2%). The apparent protein digestibility of blue lupin seed meal was the highest of the unprocessed whole-seed meals (85.5%). No significant differences were evident between the three whole-seed legume meals, though the soybean meals had significantly higher protein digestibility. The most comprehensive account examining the nutritive value of white lupin meal to juvenile rainbow trout was reported by Burel *et al*. (2000b) who examined the apparent digestibility of nutrients and energy of

extruded peas, extruded and de-hulled white lupin, de-hulled and solvent rapeseed and de-hulled and heat-treated rapeseed meals in juvenile rainbow trout and turbot. Extruded white lupin was found to be a promising substitute for fish meal in the diets of trout and turbot, with an acceptable digestibility of its dry matter (70% in trout and 81% in turbot) and a high digestibility of its protein (96% in trout and 98% in turbot) and its energy (77% in trout and 85% in turbot). Extruded peas had a lower digestibility of its protein in trout (88%) than in turbot (92%), and the apparent digestibility coefficient of energy, mainly supplied as starch, was relatively low (69% in trout and 78% in turbot). The digestibility of rapeseed meal was improved by a thermal treatment. Without thermal treatment, rapeseed meal had a low digestibility of its dry matter (57%) and energy (69%) in turbot. The availability of phosphorus was higher for extruded lupin (62%) in trout and 100% in turbot) compared to the other plant-ingredients. In the other digestible study was by Glencross *et al*. (2004b) it was found that the digestive value of narrow-leaf lupin and soybean meal when including at a level of 30% in the rainbow trout and Atlantic salmon diets. These researchers found that soybean or protein concentrates made from narrow-leafed lupin or field peas have excellent potential as feed ingredients for rainbow trout and Atlantic salmon. Further, Glencross & Hawkins (2004) conducted an experiment to compare the digestible value of white lupin, narrow-leaf lupin and yellow lupin kernel meal with solvent extracted, high-protein soybean meal and wheat gluten, when included at 30% in the diets for rainbow trout and red seabream. These workers found that the digestibility of protein and phosphorus of all lupin kernel meals were better than soybean meal in both species. The digestibility of dietary energy and organic matter for all lupin kernel meals was lower than soybean meal in both species.

Clearly, there is much scope to enhance the nutrition of carp as an important fish species in aquaculture. There are various options concerning the choice of feed ingredients, especially plant by-products to partially offset the use of fish meal and also soybean meal in compound feeds. An important technological development is the increasing use of exogenous enzymes that can assist in the digestion process. For these reasons this thesis explored the utilisation of a plant protein source in Europe, notably lupin meal and evaluated the potential of a commercial enzyme product Synergen™ in association with a series of experimental diets with different levels of lupin meal inclusion in experimental diets for the carp.

## 1.5 Enzyme Applications in Aquaculture

The use of exogenous enzymes is a common practice in the terrestrial animal feed industry (Sweetman, Nengas & Corneillie, 2012). Enzyme applications for monogastric feeds were widely reviewed (Campbell & Bedford, 1992; Bedford, 2000). To date the use of enzymes in aquaculture feeds has been limited, but increasing interest is growing due to the increasing inclusion of plant based protein ingredients and their by-products in fish feeds (Sweetman, Nengas & Corneillie, 2012). The positive effects of the dietary supplementation of exogenous enzyme to plant based diets for warm water and cold water freshwater fish species such as (tilapia and rainbow trout) have been investigated to a limited degree. Carter *et al*. (1994) reported that supplementing proteolytic and carbohydrase enzymes in Atlantic salmon diets that contained 34% of soybean meal enhanced growth performance and feed conversion efficiency. Ng *et al*. (2002) found a significant increase in the weight gain from (297.5% to

338.5%), specific growth rate (1.97% to 2.11%), protein efficiency ratio (2.07 to 2.25), net protein utilization (31.2% to 31.1%), apparent digestibility of dry matter (52.3% to 62.3%), protein (71.2% to 74.5%), lipid (69.6% to 75.6%) and energy ( 58.4%to 68.2%) and also significant decrease in the feed conversion ratio ( 1.56 to 1.41) with supplementing 0.1% of commercial feed enzyme (Allzyme Vegpro™) for red hybrid tilapia (*Oreochromis Sp*.) diets that contained 40% of palm kernel meal. Similarly, Lin, Mai & Tan (2007) reported that a supplement of just 0.1% commercially exogenous enzyme (neutral protease, β -glucanase and xylanase) into plant-based diets for juvenile hybrid tilapia (*Oreochromis niloticus x O. aureus*) significantly increased specific growth rate from (2.04% to 2.18%), feed efficiency ratio (56.8% to 62.9%) and apparent protein retention (28.1% to 33.1%). In the earlier study Drew *et al*. (2005) found an increase in the specific growth rate from (1.27% to 1.33%) and decrease in the feed conversion ratio (1.21 to 1.12) with the addition of 0.25g $kg^{-1}$ of protease for rainbow trout canola and peas based diet. Recently, Ai *et al*. (2007) reported that supplementing NSP enzymes (400 mg VP mainly including glucanase, pentosanase and cellulosase, each with 50 IU per gram, 800 mg WX mainly including xylanase, 1000 IU per gram, or the combination of 800 mg WX and 400 mg VP per kilogram diet) into Japanese sea bass (*Lateolabrax japonicus*) plant based diet significantly increased specific growth rate from (3.93% to 4.29%), feed efficiency ratio (0.85% to 1.20%) and nitrogein retention (27.1% to 46.1%) as well as degraded the non-starch polysaccharide (NSP).

Based on these studies, the present study was designed to use plant proteins as main protein sources, which contained anti-nutritional factors, in order to investigate the effects of dietary supplementation of exogenous enzyme

(Synergen™) to reduce the negative effects of anti-nutritional factors and increase growth performance and feed utilization in plant based diet for carp.

However, some studies could not find any significant improvement in growth performance and feed utilization by supplement exogenous enzymes. For example, Stone (2003) reported that the addition of a Natugrain-blend® [β-glucanase and β-xylanase at three nominal concentrations (0.75, 150 or 300µL $kg^{-1}$)] to a diet for silver perch (*Bidyanus bidyanus*) that contained 30% of de-hulled blue lupin did not enhance dry matter, protein and energy digestibility of the diet and ingredients. Ogunkoya *et al*. (2006) also stated that a commercial enzyme cocktail (Superzyme CS) added to soybean meal based diets containing up to 20% of soybean meal for rainbow trout was ineffective. Similarly, Farhangi & Carter (2007) showed that adding (Energex™), (Bio-Feed™ Pro), (Alpha galactosidase™) and (mixture of these products) enzymes to the diet containing 50% of de-hulled blue lupin for rainbow trout was ineffective.

## 1.6 Synergen™ (Alltech Inc, .USA)

It is a by-product of solid state fermentation of *Aspergillus niger* that contains residual enzyme activity. Synergen™ improves the profitability by maximizing nutrient release such as protein and energy and then improving growth and feed performances. Adding Synergen™ is very economical because it is not relatively expensive (£9.70 per kg; £970 per tonne). The cost of adding it is 0.0048£ per kg at an inclusion level of 0.05% or 0.0097£ at an inclusion level 0.1%. Therefore, the application of this SSF technology in this manner opens the door more flexible feed formulation and allows the incorporation of lower cost vegetable protein substitutes (Sweetman, Nengas & Corneillie, 2012).

## The stages of production Synergen™

1-The solid state fermentation (SSF) process involves the careful selection of specific strains of naturally occurring fungi with the ability to modify a wide range of agricultural by-products such as DDGS, corncob, palm kernel, wheat bran, and rapeseed oil cake and soy bean.

2-The selected fungus is first propagated in liquid media to produce a large volume of inoculum, which is mixed with pre-sterilized selected solid substrate media to produce a mixture known as “Koji.” under strict aseptic conditions.

3-The Koji is then evenly distributed onto trays and introduced into environmentally controlled solid substrate fermentation culture chambers for up to five days. During this time the fungus grows rapidly, breaking down the fibrous and non-fibrous portions of the chosen substrate. Doing so dramatically changes the nutritional profile of the material and results in the generation of products that can be used to reformulate diets.

4-On day five, the Koji is extracted and the by-product is dried. It is then passed through quality control to produce the finished product, Synergen™ (www.alltech.com)

## 1.7 Carp (*Cyprinids*)

The Cyprinids are the dominant species of culture fish worldwide accounting for over 20 million metric tonnes, or 77% of the total aquaculture finfish production (Ahmed & Davies, 2010). Several species of carp are farmed in South-east Asia, on the Indian subcontinent, and in Europe, but there is little interest in carp culture in North America (Sweetman, Nengas & Corneillie, 2012). Carp have many attribute that make them an ideal candidate for aquaculture and are known as aquatic chicken such as fast growth, ability to grow in the wide range of environmental condition (tolerate a vary of temperature 1-35 ℃), disease resistance, high- quality flesh, ability to grow and reproduce in captivity and feed relatively on low trophic levels (Davis *et al*., 2009). Carp live in rich weedy ponds, lakes, canals and slow-flowing rivers (Maitland, 2000; Ahmed & Davies, 2010).

The common carp is farmed in many countries and dominates the cyprinid culture in Europe, making up about 64% of the approximately 225000 tonnes of farmed Cyprinids produced in that continent (Huntingford, Jobling & Kadri, 2012). The common carp is usually found in freshwater systems, but they can tolerate salinities of 14ppt. It is an omnivorous fish which can obtain feeds from both bottom sediments and surface waters. The typical diet for carp includes a wide variety of aquatic plants, algae, plankton, insects and their larva benthic invertebrate and small fish (Williams, 2008; Ahmed & Davies, 2010). Furthermore, in Iraq common carp is the main cultured fish species (Kitto & Tabish, 2004).

There are three recognized species of common carp: the orange – coloured scale carp (*C. Carpio var. Flavipinnis*), the partially- scaled mirror carp (*C. Carpio var.*

*specularis*) and the virtually scaleless leather carp (*C. Carpio var. nudus*) (Pillay & Kutty, 2005).

### 1.7.1 Nutrient requirements

Determining the nutritional requirements (quantity and quality) for cultured fish is essential to obtain optimum growth, health and reproduction. The nutrient requirements of fish vary according to fish species, strain, stage of development and health as well as temperature and environmental conditions of the culture system (De Silva & Anderson, 1995; Hasan, 2000). Fish, like all animals require a well-balanced diet containing protein, energy, lipids, vitamins and minerals (Davis *et al*., 2009). Numerous books and review papers have referred the nutrient requirement for carp such as (NRC, 1993; De Silva & Anderson, 1995; Hasan, 2000; Craig & Helfrich, 2002; Halver, 2002; Halver & Hardy, 2002; Lall, 2002; Sargent *et al*., 2002; Wilson, 2002; Davis *et al*., 2009; Stickney, 2009; NRC, 2011).

#### 1.7.1.1 Protein Requirement

Protein is a very important constituent of the diet, both qualitatively and quantitatively, as it is the building material for the growing animal organism as well as it is important for the production of enzymes (Robaina & Izquierdo, 2000; Davis *et al*., 2009; Stickney, 2009). Determining accurate protein requirements for each species is very significant economically because it is the most expensive part of fish feed (Craig & Helfrich, 2002; Davis *et al*., 2009).

Fish, like other animals, do not have a true protein requirement but have a requirement for a well-balanced mixture of essential (indispensable) and

nonessential (dispensable) amino acids (Wilson, 2002). Compared with other vertebrate animals, fish are characterized by high protein requirement (Steffens, 1989). The optimal dietary protein level for fish is affected by fish species, size, age, water temperature, and amino acid composition, digestibility of the dietary protein, optimal protein to energy balance and the nature of the non-protein energy sources in the diet. Protein requirements for herbivorous and omnivorous fish are lower than for carnivorous fish (Wilson, 1989; De Silva & Anderson, 1995; Craig & Helfrich, 2002; Wilson, 2002; Davis *et al*., 2009; Stickney, 2009). Inadequate protein levels in the diet results in a reduction of growth and loss of weight. However when an excess of protein is supplied in the diet, only part of it is used for protein synthesis and the remaining is transformed into energy (Robaina & Izquierdo, 2000). It should be noted that grow-out diets for this species are often formulated with lower levels of protein as compared to other species. This is due to several reasons, including the ability to consume relatively large quantities of feed as well as a reasonable use of carbohydrates as an energy source to spare protein. With suitable feed intake and protein sparing, the daily requirement of protein intake can be met from a variety of dietary protein levels (Davis *et al*., 2009). Common carp requires 38.5 % of the protein in the diet (De Silva & Anderson, 1995; NRC, 2011).

### 1.7.1.2 Amino acid requirement

It is better to consider that fish has a requirement for a well-balanced mixture of essential and non-essential amino acids rather than having a requirement for protein. All studies on finish to date have shown that fish need the same essential amino acids as most other animals. The requirement for individual amino acids

varies between species even between studies on the same species (De Silva & Anderson, 1995). Essential amino acid requirements for common carp are shown in (Table1.6).

Table 1.6 Essential amino acid requirement for common carp as percentage

| Essential amino acids | % dry diet | % of the dietary protein (38.5%) |
|---|---|---|
| Arginine (Arg) | 1.6- 1.7 | 4.3 |
| Histidine (His) | 0.8 | 2.1 |
| Isoleucine (IIe) | 0.9-1.0 | 2.5 |
| Leucine (Leu) | 1.3 | 3.3 |
| Lysine (Lys) | 2.2 | 5.7 |
| Methionine (Met) | 1.2 Cys=0% of the diet | 3.1 |
| | 0.8 Cys= 2% of the diet | 2.1 |
| Phenylalanine | 2.5 Tyr= 0% of the diet | 6.5 |
| (Phe) | 1.3Tyr= 1% of the diet | 3.4 |
| Threonine (Thr) | 1.5[a] | 3.9[a] |
| Tryptophan (Trp) | 0.3 | 0.8 |
| Valine (Val) | 1.4 | 3.6 |

Source: Data were adapted from (De Silva & Anderson, 1995; Wilson, 2002 and NRC, 2011)

[a]In the absence of cysteine

### 1.7.1.3 Energy

Energy is the one of the most important components of the diet (Steffens, 1989; Davis *et al*., 2009). Fish, like all other animals, require energy to sustain life. The energy available to the fish is dependent on how well they digest the variety of ingredients present to them (Smith, 1989). The energy requirements of fish are influenced by many factors such as fish size and age, water temperature, life cycle stage, tank water current and water supply, photoperiod, water quality and stress as reported by Robaina & Izquierdo, (2000). Furthermore, excesses of energy can reduce feed intake (limiting the intake of other nutrients) and produce fish that have higher levels of fat that may not be desired by the processor and /or consumer (Davis *et al*., 2009). Parker (2002) indicated that energy requirements for common carp (expressed as the concentration of the diet) at 13.4 MJ $kg^{-1}$ DM consistent with other research findings by Takeuchi, Satoh & Kiron (2002) which indicated that the dietary energy requirement values for the common carp of 12.97-15.06 MJ $kg^{-1}$.

### 1.7.1.4 Lipid requirements

Lipids and their constituent fatty acids play essential and dynamic roles in the maintenance of optimum growth, feed efficiency, health (immunocompetence and cardiovascular function), kidney and gill function, neural and visual development, reproduction, and flesh quality (market size) of finfish species (Lim & Webster, 2001).

Lipids typically comprise about 10- 20% of fish diets and provides optimal growth rates without producing an excessively fatty carcass (Craig & Helfrich, 2002; Stickney, 2009). Of course both the quantity and quality of the fat must be

considered, in view of the need to meet the demand for essential fatty acids (Steffens, 1989).

### 1.7.1.5 Fatty acid requirements

Fish, like all animals, cannot synthesis essential fatty acid but require for the maintenance of cellular function. When considering the essential fatty acid requirement of fish, it is useful to consider the origin of these compounds in natural systems (Hasan, 2000). Fish typically require fatty acids of the omega 3 and 6 (n-3 and n-6) families. Carp requires a combination of n-3 and n-6 fatty acids as stated by Craig & Helfrich, (2002). Previously, Takeuchi and Watanabe (1977) found that optimum growth and feed parameters were obtained in carp fed diets containing 1% 18:2n-6 and 1% 18:3n-3 in combination.

### 1.7.1.6 Carbohydrate requirements

Carbohydrates (starches and sugars) are the most economical and inexpensive sources of energy for fish diets. Although not essential, carbohydrates are included in aquaculture diets to reduce feed costs and for their binding activity during feed manufacturing (Craig & Helfrich, 2002). Carbohydrates as such are not an essential ingredient of fish diets. Owing to their relatively low digestibility of native high–molecular carbohydrate compounds they are not the primary source of energy for most fish species of high commercial value (Steffens, 1989). A study undertaken by Takeuchi *et al*. (2002) showed that carbohydrates can be easily utilized in common carp and used as dietary energy sources. Furthermore, the workers found that amylase activity in the digestive tract and the digestibility of starch in fish are generally lower than those of terrestrial animals, however, the intestinal amylase activity is greater in common carp than compared with

carnivorous fish. On the other hand, it has been reported that the optimum range of dietary carbohydrate for common carp is between 30-40% (as fed).

### 1.7.1.7 Vitamin requirements

Vitamins are organic compounds necessary in the diet for normal fish growth and health. They often are not synthesized by fish, and must be supplied in the diet (Craig & Helfrich, 2002). Fish performance and final production cost are easily affected by the dietary vitamin supplementation, so information about the vitamin requirement in fish is important to optimize the production system (Robaina & Izquierdo, 2000).

Table 1.7 Summary of the vitamin requirement of common carp (mg $kg^{-1}$ dry diet).

| Vitamin | Requirement | References |
|---|---|---|
| Thiamin | 0.5 | (Satoh, 1991; NRC, 1993; NRC, 2011) |
| Riboflavin | 6 - 7 | (Satoh, 1991; NRC, 1993; NRC, 2011) |
| Pyridoxine | 5 - 11.4 | (Satoh, 1991; NRC, 1993; NRC, 2011) |
| Pantothenate | 23 - 50 | (Satoh, 1991; De Silva & Anderson, 1995; |
| Niacin | 20 - 28 | (Satoh, 1991; NRC, 1993; NRC, 2011) |
| Biotin | 1-1.5 | (Satoh, 1991; De Silva & Anderson, 1995; |
| Vitamin B12 | 0 - 0.09 | (Satoh, 1991; NRC, 1993; NRC, 2011) |
| Folate | 0 - 4.3 | (Satoh, 1991; NRC, 1993; NRC, 2011) |
| Choline | 1000-2000 | (Steffens, 1989;Halver, 2002) |
| Inositol | 166 - 440 | (Satoh, 1991; NRC, 1993; NRC, 2011) |
| Vitamin A | 0.12 - 6 | (Satoh, 1991; NRC, 1993; NRC, 2011) |
| Vitamin E | 100 - 200 | (Satoh, 1991; NRC, 1993; NRC, 2011) |
| Vitamin K | 1.9 | (Satoh, 1991; NRC, 1993; NRC, 2011) |
| E | 80-100 | (Halver, 2002) |

### 1.7.1.8 Minerals

Minerals are required by all animals for various life processes, including the formulation of skeletal tissue, respiration, digestion and osmoregulation as well as cofactors when they are a component of protein molecules (Lall, 2002; Stickney, 2009). Determination of dietary mineral requirements is difficult in aquatic animals because they have an ability to absorb some inorganic elements not only from their diets but also from their external environment in both freshwater and seawater (De Silva & Anderson, 1995; Craig & Helfrich, 2002; Lall, 2002; NRC, 2011). Marine animals live in a medium that contains minerals in concentrations at or above those necessary to meet their requirements, while freshwater fish live in a mineral deficient medium and obtain most of their required minerals from the diet (Stickney, 2009). The recommended levels of minerals in the carp diet have been investigated by many workers (see Table 1.8).

Table 1.8 Recommended macro and micro mineral requirements for carp**.**

| Mineral | Requirement | Reference |
|---|---|---|
| Macro-mineral | g $kg^{-1}$ | |
| Phosphorus[a] | 6-7 | (Lall, 1989; De Silva & Anderson, 1995; Takeuchi, Satoh & Kiron, 2002; NRC, 2011) |
| Manganese | 0.4 - 0.6 | (Lall, 1989; De Silva & Anderson, 1995; Takeuchi, Satoh & Kiron, 2002; NRC, 2011) |
| Calcium | 3 | (Lall, 1989) |
| Sodium | ND | |
| Potassium | ND* | |
| Micro-mineral | mg $kg^{-1}$ | |
| Copper | 3 | (Lall, 1989; De Silva & Anderson, 1995;NRC, 1993; NRC, 2011) |
| Iron | 150-199 | (Satoh, 1991; NRC, 1993; De Silva & Anderson, 1995; Takeuchi, Satoh & Kiron, 2002) |
| Manganese | 12 - 13 | ( Lall, 1989; Satoh, 1991;NRC, 1993; De Silva & Anderson, 1995; Takeuchi, Satoh & Kiron, 2002) |
| Zinc | 15 -30 | (Lall, 1989; Satoh, 1991; NRC, 1993; De Silva & Anderson, 1995; Takeuchi, Satoh & Kiron, 2002) |

*ND = Not determined.

[a]Inorganic phosphorus

## 1.8 Aims and objectives of research program

The general objectives of this program were to:

1- Reduce the use of fishmeal and other high value expensive components of the diet with lupins as reliable substitutes.
2- Evaluate the benefits of the dietary supplementation of Synergen™ to reduce the negative effect of anti-nutritional factors and increase the nutritive value of the lupins in aquaculture diets.
3- Evaluate the optimum inclusion level of lupins in the diets for juvenile carp under control laboratory conditions.

The specific objectives of this research program were to:

1- Evaluate the effect of inclusion of 12.5% and 25% of white lupin as a soybean meal replacement with dietary supplementation of exogenous enzyme (Synergen™) on growth performance, feed utilization, carcass composition and general health with juvenile common carp diet.
2- Evaluate the effect of substitution of 12.5% and 25% of soya protein concentrate with white lupin with the addition of Synergen™ on growth performance, feed utilization, carcass composition and general health with juvenile mirror carp diet.

# Chapter 2

## General Materials and Methods

## 2.1 Feed formulation and Diet preparation

Experimental diets were prepared in 5 kg batches. The dry milled ingredients were weighed in plastic containers and placed into a food bench mixer. The dry milled ingredients were mixed uniformly to ensure homogeneous distribution of the diet components and then mixed for approximately 30 min using a Hobart food mixer (Hobart Food Equipment, Australia). After the initial mixing, fish oil and corn oil were gradually added in a continuous flow. After further mixing, water (~ 2.5, 3.5$L^{-1}$) was added to form light dough of each diet for first and second experimental diets respectively. The pastes were passed through an extruder (PTM Extruder System, Plymouth, Devon, UK: Model= P6, year 2006) and an appropriate aperture dies (7) was used to achieve the desired pellet size (2-mm pellets). The resulting strands were carefully broken up and spread onto trays lined with aluminium foil. These trays were subsequently transferred into a warm air oven (Genlab, MINO/ 200/ SS/F, Cheshire, UK) where they were left for 24 h at 40 ºC. Diets were crushed very well and put in a plastic vessel then labelled and kept stored in a dry dark place until used.

## 2.2 Fish and husbandry

The experimental animals used in this program of research were juvenile common carp for experiment one and juvenile mirror carp for experiment two. Fish were obtained from Hampshire Carp Farm Fisheries (Hampshire, UK). In both cases, the fish were transported from the fish farm directly to the aquarium facility in a 1000 $L^{-1}$ tank supplied with pure oxygen (BOC, UK). Total transportation time did not exceed 24h at any stage. Common carp and mirror carp were subsequently acclimatized in fiberglass tanks (each measuring 40x45x55 $cm^3$) for a period of 88 days and 75 days respectively prior to the start of the trials. During that time, fish were fed EWOS Sigma 50, (1-2% of the body weight) twice daily as a maintenance diet. The fish were subsequently re-graded and distributed randomly into experimental tanks to proceed with experimental trials. Fish were fed a control diet for two days. An automated 12h dark/light system was maintained throughout the two experimental trials (10 weeks).

## 2.3 Feed and Weighing

All fish in each trial were fed 3% of the tank biomass per day in three equal discrete rations at ~ 9:00, 13:00 and 17.00 h; for each of the experimental diets for six consecutive days per week. Fish were deprived of feed one day prior to weighing (7th day). Feeding rates were adjusted accordingly on the basis of the new total biomass in each tank.

## 2.4 Water quality

All experimental trials were conducted within a freshwater recirculation system (RS). Both experiments were undertaken at the Aquaculture and Fish Nutrition

Research Aquarium, University of Plymouth. Trials were conducted in experimental system D (see Plate 2.4); the facility used to be a freshwater recirculation system with a total water capacity of 2223 $L^{-1}$ respectively. Fish were randomly distributed into 80 $L^{-1}$ fiberglass tanks, each provided with 99% re-circulated aerated freshwater at a rate of 600 L $h^{-1}$. Water samples were collected three times a week from the aquarium system. Dissolved oxygen, temperature and pH were measured daily by HQ 40d multi-parameter meter (HACH Company, Loveland, USA). The water temperature was measured daily and held at an average value of 25 ± 0.02 ºC throughout the common carp and mirror carp experiments with an immersed heater. Dissolved oxygen (D.O) was recorded and averaged at (7.21±0.24, 6.82±0.19mg $L^{-1}$) in the common carp and mirror carp experiments respectively. The pH was maintained at an average at (6.54±0.24, 6.62±0.24) in the common carp and mirror carp experiments respectively with sodium bicarbonate ( $NaHCO_3$) used to adjust the pH level within the desired range. Partial change of water was performed daily except the feed deprivation day to reduce the ammonia concentration. Each filter was also cleaned daily. Total ammonia nitrogen (TAN) (0.089 ± 0.051, 0.11±0.06mg $L^{-1}$), nitrite (0.046 ± 0.023, 0.09±0.05mg $L^{-1}$) and nitrate (29.42 ± 11.57, 26.84±9.64mg $L^{-1}$) in the common carp and mirror carp experiments respectively were measured by the discrete automatic analyser (HACH LANGE, DR 2800 Germany) and the values were always within the acceptable ranges for intensive fish culture. Water parameter ranges were in accordance for the limits for carp.

## 2.5 Growth and feed utilization calculations

The growth performance of the fish and feed utilization were measured according to the following formulae.

Specific growth rate (SGR %) $= \frac{InFBW - In\ IBW}{\mathrm{T}} \times 100$

(Davies & Gouveia, 2010)

Feed Conversion Ratio (FCR) $= \frac{\mathrm{FI\ (g)}}{\mathrm{WG\ (g)}}$

Feed intake (g) per fish $= \frac{\mathrm{FI\ (g)}}{FN}$

Feed Conversion Efficiency (FCE %) = $\frac{WG}{FI} \times 100$

Protein efficiency ratio (PER) $= \frac{WG}{PI}$

Apparent Net Protein Utilisation (ANPU %) $= \frac{(FBP*FBW)-(IBP*IBW)}{PI} \times 100$

Protein intake (PI) $= \frac{Protein\ intake\ (G)}{\mathrm{Number\ of\ fish}}$

Condition Factor (K %) $= \frac{\mathrm{FBW}}{FL^3}$x100

Lipid efficiency ratio (LER) $= \frac{\mathrm{WG\ (g)}}{\mathrm{LI(g)}}$

Mortality (%) $= \frac{\mathrm{Intial\ Nb - Final\ Nb}}{\mathrm{Intial\ Nb}} \times 100$

$\mathrm{Energy\ retention\ ER\ (\%)} = \frac{\mathrm{final\ body\ energy - initial\ body\ energy}}{\mathrm{energy\ intake}} \times 100$

Survival (%) = 100 –Mortality (%) $= \frac{Final\ Nb}{Intial\ Nb} \times 100$

Where InFBW: is logrithim of the final body weight, InIFW: logarithm of the initial body weight, T: times (number of days), IFW: initial fish weight, FBW: final body weight, IBP: Initial body protein (g), FBP = Final body protein (g), WG: Weight gain (g), FI: Feed intake (g), PI: Protein intake (g), FL: final fork length (cm), LI: lipid intake (g), Initial Nb: initial number of fish, Final Nb: final number of fish.

## 2.6 Chemical and proximate analysis

Raw materials (ingredients), diets and fish carcass were subjected to analysis for the determination of moisture, ash, protein, lipid and gross energy content. Fish were sampled at the beginning and the end of the trial to determine carcass composition. Typically, samples were analysed in triplicate according to AOAC (2003) protocols.

### 2.6.1 Moisture

All samples were weighed and dried (in triplicate) at 105 ºC with a fan assisted oven (Gallenkamp Oven BS, Model; OV-160, England) until a constant weight was achieved. Percentage moisture was calculated by:

$$\text{Moisture (\%)} = \frac{W2-W3}{W2-W1} \times 100$$

Where

W1= initial weight of empty crucible
W2=weight of crucible+ food
W3= final weight of crucible+ food
Total solids (%) = 100 - % moisture

### 2.6.2 Ash

Ash (total mineral or inorganic) content was determined in triplicate by adding a known weight of sample (~500 mg) to a pre-weighed crucible. The crucibles were then incinerated in a muffle furnace (Carbolite, Sheffield, England) at 550C˚ for 8h until light gray ash results or to constant weight. Percentage ash was determined from the sample residue by:

$$\text{Ash (\%)} = \frac{(\text{weight of crucible} + \text{residyue}) - \text{weight of crucible (g)}}{\text{Sample weight}} \times 100$$

## 2.6.3 Lipid

Lipid content was determined in triplicate using the Rapid Soxhlet extraction method. Approximately 3g of sample material was weighted on a 3 decimal balance, placed into extraction thimble, plugged with on the top with cotton wool, placed thimble into a wire support, inserted into beaker, placed into beaker rack, added 140ml of petroleum ether to each beaker using dispenser in a fume cupboard and then placed the beakers on the SoxTecTM extraction system (Tecator Systems, Högnäs, Sweden; model Soxtec 1043 and service unit 1046). Pre-weighed cups containing 140 ml of ether extract are clamped into the condenser and the extraction settings are moved to the boiling position for 30 min, after which extraction was set to the rinsing position for a further 45 min. The cups containing extracted lipid were then transferred to a fume cupboard for 30 min before final weighing. Lipid content was determined as:

$$\text{Total lipid (\%)} = \frac{\text{final weight of beaker} - \text{intial weight of beaker}}{\text{Initial weight of sample}} \times 100$$

Plate 2.1 Soxhlet system operated in the nutrition laboratory of the University of Plymouth.

## 2.6.4 Protein

Determination of crude protein (CP) of feed ingredients, diets and whole fish carcass was performed by the Kjeldahl method to gain the total nitrogen (N) content. This value is then multiplied by a factor 6.25 (5.72 for proteins originating from plant sources) to calculate the crude protein content. Briefly, 100 mg of sample (the raw ingredient, dried feed or whole body carcass) was weighed directly into a Kjeldahl digestion tube along with a catalyst tablet (3g $K_2SO_4$ ,105mg $CuSo_4$ , $5H_2O$ and 105 mg $TiO_2$; BDH Ltd. Poole, UK) and 10 ml of concentrated sulphuric acid ($H_2SO_4$) (Sp. Gr. BDH Ltd. Poole, UK). Digestion was performed with a Gerhardt Kejldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) at 100 ºC for 30 min, 225℃ for 45 min and at 380℃ for 60 min. The tube rack was removed from the heating block and allowed to cool down during the additional 30 min. After this digestion stage the samples are distilled using Vodapest 40 automatic distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany). The distillate was neutralized with concentrated ($H_2SO_4$) and from the titration value crude nitrogen determined as a:

$$\text{Nitrogen (\%)} = \frac{(\text{mls sample Titrant} - \text{mls Blank Titrant}) \times Acid\ Normality \times MW\ Nitrogen}{\text{Sample weight}}$$

Where: Acid normality = 0.2, MW of Nitrogen = 14.01 if sample weight.
To determine Protein Content:

$$\text{Protein (\%)} = \%\ \text{Nitrogen} \times \text{Conversion Factor}$$

Where Conversion Factor = 6.25

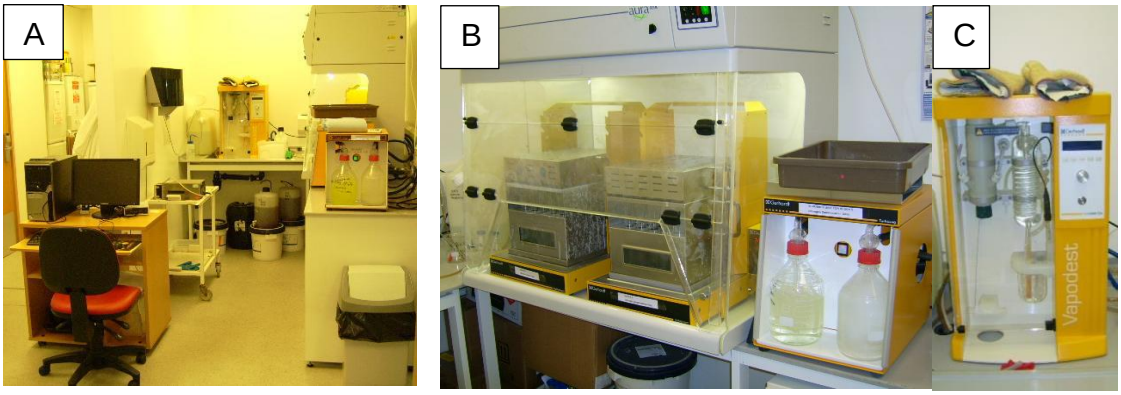



Plate 2.2 A, B and C are computerized digestion block and distillation unit of the Kjeldahl system utilized (Gerhardt Laboratory instruments) at the University of Plymouth.

## 2.6.6 Gross Energy

Gross energy was determined on samples of material in duplicate by means of an Adiabatic Bomb Calorimeter model 1356 (Parr Instrument Company, IL, and USA). The ground and dried sample was first compressed into a 1±0.1 $g^{-1}$ pellet and weighed. The pellet was then loaded into a nickel crucible with a 10cm length of fuse wire, which was formed into a “U” shape to touch the pellet. After having added 1 ml of distilled water to the bomb, this one reconstituted and filled with oxygen to a pressure of 300 psi (20 bars). Precisely two litters of water were used with the instrument to determine released heat energy. This was weighed at two kg prior to loading the bomb. The crucible was then loaded and sample weigh keyed into the calorimeter for calculation of MJ Gross Energy per kg (see Plate 2.3).

Plate 2.3 Bomb calorimeter in the nutrition laboratory at the University of Plymouth


Plate 2.4 Recirculation system _D located at Plymouth University. White arrow shows the direction of water overflow to experimental tanks from the drum filter through the biological filter (not visible).

## 2.7 Histology

Fish were anesthetized with buffered tricane methane sulphate (MS222) at 200mg $L^{-1}$ (Sodium bicarbonate 400 mg $L^{-1}$) followed by the destruction of the brain. The intestinal and liver samples were retained for histological examination by both light and electron microscopy. Intestinal sections of the mid and posterior regions (1cm) were excised for both light and electron microscopy. Liver samples were analysed using light microscopy (LM).

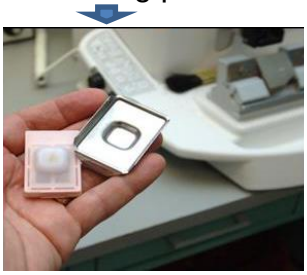
### 2.7.1 Light microscopy

Sample for LM were fixed at 4% formal saline buffered preparation for histological examination. All samples were dehydrated in graded ethanol and equilibrated in xylene using a Leica TP1020 automatic tissue processor for 23 h. The samples were embedded in paraffin wax to create blocks for sectioning, samples were then sectioned at a 5µm thickness with a RM2235 microtome and stained using either haematoxylin and eosin staining of liver samples with a Lica Autostainer XL or alecian blue periodic acid-Schiff staining (AB-PAS) for intestine samples. Slides were mounted with a cover slip and DPX. A Photograph of slides at an appropriate magnification was taken with an Olympus e-620 digital camera mounted Vanox Olympus research microscope model AHBT.

Fixation of tissue 10% of formaldehyde solution 48h

Tissue processing

Embedding paraffin



Tissue blocking

Trimming and sectioning 5um

Embedding



Staining

Plate 2.5 Major steps for the sectioning of tissues for histological studies using different instruments.

## 2.7.2 Electron microscopy

### 2.7.2.1 Scanning electron microscopy (SEM)

Fish were dissected as described in section 2.7. SEM samples were taken from six fish per treatment unless otherwise stated. Typically, intestinal samples from posterior region (ca. 2 mm) were excised and washed thoroughly in 1% Scarboxymethyl- L-cysteine for 30 Sec in order to remove epithelial mucus. Samples were then fixed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer (1: 1 vol., pH 7.2, 3% NaCI). Fixative removal of samples was carried out by rinsing two times with distilled water for 15 min. Dehydration was achieved by placing samples in graded ethanol solutions (30%, 50%, 70%, 90%) for at least 15 min each and then twice in 100%. After the dehydration process samples were critically point dried with ethanol as the intermediate fluid and C02 as the transition fluid (Emitech K850; Kent, UK) for one hour. Dried samples are then mounted on aluminium stubs and gold coated using an Emitech K550 sputter coater (Kent, UK). Samples were then screened with a Jeol JSM 6610 LV electron microscope at 15 kV (Jeol, Tokyo, Japan) (see plat 2.6). SEM images were taken with high magnification (x20, 000) and analysed using image J 1.43 in order to calculate the density of the microvilli (MD). A thresholding technique for Images was used to differentiate the ratio between the microvilli covered area (*M*, foreground) to the background (*B*, background), MD=*M/B*, and was measured in arbitrary units (AU). Images were analysed blind to prevent bias and typically three images per sample were analysed.

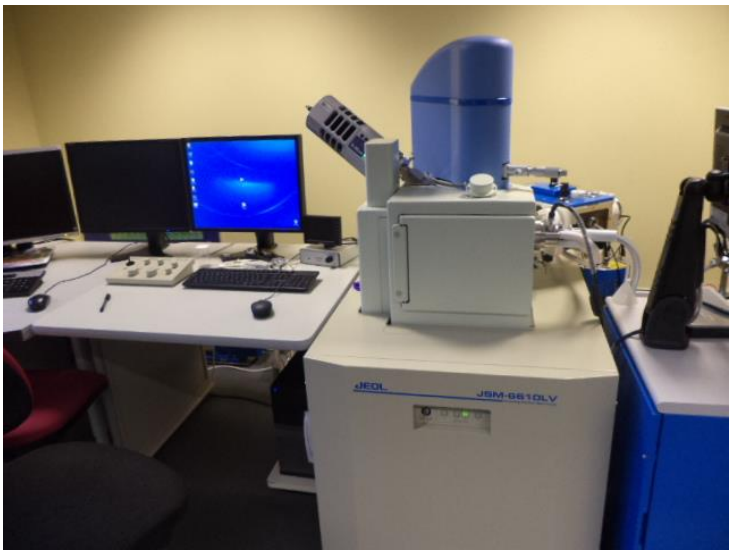



Plate 2.6 JEOL JSM 6610 LV electron microscope at 15 kV (Jeol, Tokyo, Japan) used for SEM analysis.

### 2.7.2.2 Transmission electron microscopy (TEM)

Fish were dissected as described in section 2.7. Samples for TEM were taken from three fish per tank unless otherwise indicated. Typically, intestinal samples from the posterior regions were excised and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (1: 1 vol., pH 7.2, 3% NaCl). Samples were rinsed again twice 10 min in order to remove fixative. The samples were then rinsed and post-fixed in osmium tetroxide for 1h $OSO_4$. Afterwards, they were rinsed again twice with 1% M cacodylate sodium buffer PH 7.2 in order to remove the residual osmium. Samples were then dehydrated with graded ethanol solutions 30%, 50%, 70%, and 90% respectively (for 15 min) and twice the 100 % (for 15 min). Alcohol was then removed with resin using an ethanol/resin at several ratio graded concentrations: 30% resin: 70% ethanol for at least 24 h, 50% resin: 50% ethanol for 24 h, 70% resin: 30% ethanol for 24h, and finally in 100% resin for 24 h. Resin blocks were trimmed; semi-thin sections (0.5 µm) were cut with a glass knife

placed onto a slide and stained with methylene blue for a first examination under the light microscope. From each blocks ultrathin sections (~90 nm) were cut using a diamond knife. The resulting sections were mounted on the 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 JSM 1200 EX transmission electron microscope at 120 KV (Joel, Tokyo, Japan). Transmission electron microscopy (TEM) images were analysed using Image J 1.43 (Magnification x 20, 000) to calculate the length of microvilli. Ten well orientated individual microvilli were calculated per image, with typically three images per sample.

## 2.8 Haematological parameters

Blood samples were collected from six fish per treatment (three from each tank) at the end of the growth trial. Fish were anesthetized with buffered tricane methane sulphate (MS222) at 200mg $L^{-1}$ (Sodium bicarbonate 400 mg $L^{-1}$) followed by the destruction of the brain. Blood was sampled from the caudal vein using a 25-gauge needle and 1-ml syringe and placed into eppendrof tubes.

### 2.8.1 Haematocrit (Hct)

Haematocrit is used as an indicator of animal health and is the percentage of packed blood cells to plasma volume (Rao & Deshpande, 2005). In order to measure haematocrit fresh blood was drawn into heparinised haematocrit tubes by capillary rise and sealed with Cristaseal. Capillaries were centrifuged at 12500 rpm for five min. Haematocrit values were measured as the total percentage packed cell volume (PCV) using a Hawksley reader.

## 2.8.2 Haemoglobin (Hb)

Haemoglobin (Hb) concentration was calculated based on Drabkin's cyanide-ferricyanide solution as described by Rao & Deshpande (2005). Briefly, the Drabkin's reagent consists of dissolved 50mg of potassium cyanide, 20mg of potassium ferricyanide and 1g of sodium bicarbonate made the volume to 1l in a conical flask using distilled water and stored in a borosilicate glass bottle for later use.

The assay was performed by adding 20 µL of whole blood to 5 ml of Drabkin"s reagent, and vortexes immediately. The haemoglobin was measured at 540 nm using a spectrophotometer (Thermo spectronic, Helious Epsilon, USA) against a blank containing 5 ml Drabkin"s reagent and 20 µL distilled water. Haemoglobin absorbance was measured from a curve prepared from reference standards (cyanmethaemoglobin; Sigma diagnostic kit Nº 525 A). The values obtained are expressed in g $dL^{-1}$.

**Calculation:**

Content of haemoglobin in the test sample is calculated as follows:

$$\text{Blood Haemoglobin (g/dl)} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of standard}} \times \frac{61\ (\text{Conc.of Hb}) \times 251\ (\text{dilution factor})}{1000}$$

## 2.8.3 Determination of differential leukocyte counts

Blood smears were made by dropping 5µl of fresh whole blood onto a glass slide; the end of the second slide ("spreader slide") was placed against the surface of the slide with the blood drop, at an angle of 45°. By drawing the "spreader slide" up against the drop of blood, it spread across the end of the slide by capillary attraction and filled the angle between the two slides. The "spreader slide" was then pushed back along the other slide (Dacie & Lewis, 1995). The prepared smears were left to dry at room temperature and kept prior to staining. Slides were stained using May Grunwald Giemsa stain. Slides were fixed in Methanol for 5 minutes after that slides were put in May Grunwald Sorensens Buffer solution 1:1 for 5 minutes after that put slides were rinsed in Sorensens Buffer (PH 6.8) three times then slides were put in Sorensens Buffer and Giemsa stain solution slides were rinsed in Sorensens Buffer for minutes min. Slides were allowed to dry at room temperature. When thoroughly dried, slides were mounted with coverslips (glasses) using DPX mountant. Counting was accomplished by observing the slides under the light microscope (Olympus Vanox-T microscope) using oil immersion at a final magnification of x1000. To prevent potential errors arising from uneven distribution of leukocytes, the slide was divided into four segments and 50 leukocytes per segments were counted. Leukocytes were counted in a parallel row beginning from the outside edge of the slide to the inside. The 200 leukocytes counted per slide were classified according to their general form, identified and recorded in a table as a specific cell type (for example a lymphocyte or monocyte) by dividing the sum of each type of leukocyte by two, the percentage of each cell was obtained. Photographs of selected slides were

also taken using a digital camera (Olympus camedia C-2020 Z) at a total magnification of x 1000 (zoom on the camera was x2.5)

## 2.9 Microbiological investigations

### 2.9.1 Fish dissection

Under strict aseptic conditions, nitrile gloves regularly wiped down with 99% ethanol were used for dissecting carp. Such aseptic techniques were maintained throughout the entire dissection process. Fish were examined externally to ensure good health and fish were sampled as follows: the underside of the fish was washed with 70% ethanol and the peritoneal cavity was opened with a sterile scalpel blade. Four fish were sampled from each treatment at the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth, UK. Fish were euthanized with sulphate (MS222) at 200 mg $L^{-1}$ followed by destruction of the brain. The time between termination and dissection did not exceed 2h30 minutes. The whole intestine was removed and the digesta and mucosa from two fish per tank were pooled together to avoid interfaces variation (Merrifield, 2009). The samples were stored at -20 ˚C for further use.

### 2.9.2 Plating and colony counts

Typically, the resulting material from 2 fish per tank was pooled into one sample. The feed was suspended from $10^{-1}$ to $10^{-10}$ dilution of PBS and homogenized in a stomacher (Seward laboratory, London, UK). Intestinal, feed and water samples were then serially diluted to $10^{-7}$ with PBS and 100 µL was spread onto appropriate duplicate TSA and MRS agar plates and incubated. Viable counts were then performed using a Gallenkamp colony counter (Weiss Gallenkamp, 46

Loughborough, UK). Aerobic heterotrophic counts were performed after 7 days aerobic incubation at 30℃. TSA is a general purpose medium which supports a wide range of bacterial species and has shown a high correlation between viable counts and the number of bacteria enumerated by direct counts. Colony forming units (CFU) $mL^{-1}$ or $g^{-1}$ was determined for viable bacterial populations

## 2.10 Statistical analysis

All data statistics were carried out using Minitab v.16 statistical software (Minitab, Coventry, UK). Significance was accepted at level of $P< 0.05$. Results are presented as mean ± standard error (SE) unless otherwise indicated. Typically a two way ANOVA and Fisher LSD were used for normally distributed data.

# Chapter 3

## Evaluate the effect of white lupin inclusion and dietary supplementation of Synergen™ on growth performance and feed utilization in diet for common carp (*Cyprinus carpio*)

### 3.2 Introduction

Fish meal has been widely used as an ideal protein source for aquaculture feeds, particularly carnivorous and omnivorous species, because of it contains high level of protein with an excellent amino acid profile, it also has a high level of unsaturated lipid with EPA and DHA as well as having good palatability with no anti-nutritional factors. Additionally, fishmeal is very digestible compared to plant ingredients (Rumsey, 1993; Dersjant-Li, 2002; Francis-Floyd, 2002; Jackson, 2006; Gatlin III *et al*., 2007; Glencross, Booth & Allan, 2007; Barrows *et al*., 2008; Jackson & Shepherd, 2010; Tan, 2010). However, increasing demand, uncertain availability, and high price of fishmeal with the expansion of aquaculture has made it necessary to search for alternative protein sources (Sweetman, Nengas & Corneillie, 2012). Alternatives to fishmeal are available from plant and animal protein sources as well as single–celled proteins (e.g. Microalgae, bacteria and yeast) which are now considered as attractive alternatives to fish meal for many fish species (Filer, 2010). Animal by-product proteins include poultry by-product, meat, bone, blood and feather meals are viable commodities to replace fish meal in fish feeds (Owen, 2011). In spite of the promise of animal by-product in fish feeds, there is still much public concern with using animal by-products in fish feeds due to the recent Bovine Spongiform Encephalopathy (BSE) commonly known as a Mad-Cow Disease and prion risks related with such materials arising

within animal and consumer food chain (Davies & Gouveia, 2010). Therefore, in the last two decades aquaculture research has mainly focused on the plant protein sources such as grain, pulses and oilseeds as they are widely available with cheaper price owing to the greater world production of grains and oilseeds as a result of higher yields and increased plantings (Davies & Gouveia, 2010; Hardy, 2010).

Plant proteins are mainly used to formulate diets for carp (Tacon, Hasan & Metian, 2011). Numerous studies have been undertaken to evaluate the potential of several plant protein sources in carp feeds (Van den Ingh *et al*., 1991; Pongmaneerat *et al*., 1993; Escaffre *et al*., 1997; Mazurkiewicz, 2009) evaluated soybean, (Hasan, Macintosh & Jauncey, 1997; Hossain *et al.*, 2001; El‐Saidy & Gaber, 2003; Kumar *et al*., 2011) evaluated mustard, linseed, groundnut, sesame, copra and leucaena, Sesbania aculeata Pers, hazelnut meal, cluster bean seed and meal and detoxified Jatropha curcas kernel meal. Of these plant protein sources soybean meal is one of the most promising in feeds for carp due to its high protein content with the favourable amino acid profile. However, an increase of the use of soybean meal for human consumption and animal feed in both developed and developing countries has resulted in an increase market price of soybean meal globally. Furthermore, soybean meal is limited in lysine and methionine concentration and contains a wide variety of anti-nutritional factors (Kumar *et al*., 2011). Therefore, utilization of other inexpensive plant protein source in carp feeds would be beneficial in reducing the feed cost and contribute to food security and also to sustain aquaculture production.

Recently, lupin meals have attracted considerable attention due to high protein content, highly digestible protein and energy with a low market price (Van Barneveld, 1999; Edwards & Van Barneveld, 1998; Glencross & Australia, 2001; Glencross, Curnow & Hawkins, 2003a; Glencross & Hawkins, 2004; Glencross, 2004; Drew, Borgeson & Thiessen, 2007; Sweetingham *et al*., 2008). The possibility of replacing fishmeal by lupin meal up to 30-50% in the rainbow trout diet without negative effects on growth performance feed utilization has been reported (Burel *et al*., 1998; Farhangi & Carter, 2001; Glencross *et al*., 2002a; Glencross *et al*., 2004a; Borquez *et al*., 2011a; Glencross, Rutherford & Hawkins, 2011). Similarly, Bransden, Carter & Nowak (2001) reported that inclusion of up 40% of de-hulled blue lupin for rainbow trout diet did not adversely affect growth, immune function or blood chemistry and disease resistance. Gouveia *et al*. (1993) also reported that inclusion up to 20 % of white lupin in the diet for rainbow trout did not adversely affect growth performance. Recently, Bórquez *et al*. (2011b) reported that including whole seed white lupin meal at up to 20% in extruded diets for rainbow trout did not have any deleterious effects on growth performance and feed performance. More recently, Hernández *et al*. (2012) reported that including whole seed white lupin meal up to 25% in extruded diets for rainbow trout did not have any negative effects on growth performance and feed performance. In another study was performed by Pereira & Oliva-Teles (2004) these workers also reported that incorporation of up to 30% of narrow-leafed lupin seed meal into diet for juvenile gilthead sea bream did not have any negative effects on growth performance and whole-body composition. Similarly, Burel *et al*. (2000a) also reported that extruded white lupin can be incorporated into the diets of turbot up to a level of 50% without compromising growth performance and body

composition in this species. Only, Viola *et al.* (1988) examined the use of whole seed in the common carp diet.

Given the varieties of lupin throughout the world, the majority of work has focused on the white lupin strain in aquafeeds. However, there is no information concerning the application of white lupin meal in formulated diets for the common carp given the importance of this feed ingredient and this fish species in Central Europe and Asia.

The main drawbacks for the use of lupins in fish feeds is largely due to inadequacies in the protein composition (essential amino acid deficiencies such as methionine and cysteine), relatively high levels of some deleterious carbohydrate fraction such as soluble and non-soluble non-starch polysaccharides with the presence of several anti-nutritional factors (ANFs) such as oligosaccharides, alkaloids, phytate, saponins and tannins can contribute to reduce the nutritional value of lupin in fish feeds (Peterson, 2000; Francis *et al*,. 2001). The positive effects of dietary supplementation of exogenous enzyme to reduce the negative effect of ANFs with improve growth performance and feed utilization in the fish feeds containing high level of plant ingredients have been investigated for many aquaculture species. For example, Carter *et al*. (1994) reported the benefits of dietary supplementation of proteolytic and carbohydrases enzymes for Atlantic salmon diets that contained 34% of soybean meal to improve growth and food conversion efficiency. Furthermore, the aadvantages of treating of palm kernel meal with commercial feed enzyme (Allzyme Vegpro™) in diets for red hybrid tilapia with respect to growth, feed utilization and nutrient and energy digestibility were also reported by Ng *et al*. (2002). Subsequently, Drew *et*

*al*. (2005) reported that application of a supplementary protease enzyme product to a rainbow trout canola and peas based diet significantly improved feed efficiency and overall performance in this species. Similarly, Lin, Mai & Tan (2007) reported that a supplement of just 0.1% commercially exogenous enzyme (neutral protease, b-glucanase and xylanase) into plant-based diets for juvenile hybrid tilapia can significantly enhance growth performance and feed utilization as well as promote the secretion of the endogenous enzymes. Recently, Ai *et al*. (2007) investigated that supplementing of NSP enzymes (400 mg VP mainly includes glucanase, pentosanase and cellulosase, each with 50 IU per gram, 800 mg WX mainly includes xylanase, 1000 IU per gram, or the combination of 800 mg WX and 400 mg VP per kilogram diet) for Japanese sea bass plant based diet degrade the anti-nutritional effects of non-starch polysaccharide (NSP) and enhance feed utilization and growth performance.

Therefore, dietary supplementation of Synergen™ to the common carp diet containing white lupin and soybean meal could be reduced the negative effects of anti-nutritional factors and enhance the nutritional value of lupin and soybean meals.

For these reasons, the prime purpose of the present study was to evaluate the effect of inclusion of 12.5% and 25% of white lupin as a soybean replacement and together with the addition of Synergen™ on growth performance, feed utilization and general health in the diet for juvenile common carp.

## 3.3 Materials and methods

### 3.3.1 Experimental dietary preparation

The summit dilution trial type was used to formulate diets for the current experimental protocol. According to this type of trial a reference diet provides optimum protein and energy levels for use with a respective aquaculture species, but protein and energy specifications are not maintained with the progressive inclusion of the test ingredient (Glencross, Booth & Allan, 2007). Two reference diets were formulated to contain 55.87±0.17% of protein, 8.9 % of lipid and approximately (20 MJ $kg^{-1}$). A separate group of (BSD) based diet and diets that included 12.5% and 25% of de-hulled white lupin with the addition of 0.05% of Synergen™ (SSF, Alltech, Ireland) were formulated. The dry milled ingredients were weighed in plastic containers and placed into a food bench mixer. The dry milled ingredients were mixed uniformly to ensure homogeneous distribution of the diet components and then mixed for approximately 30 min using a Cater-Bake food mixer (Cater –Bake UK). After the initial mixing, fish oil and corn oil were gradually added in a continuous flow. After further mixing, water (~ 2.5 $L^{-1}$) was added to form light dough of each diet. The resulting was passed through an extruder (PTM Extruder System, Plymouth, Devon, UK: Model= P6, year 2006) and an appropriate aperture die (7) was used to achieve the desired pellet size (2-mm pellets). The resulting strands were carefully broken up and spread onto trays lined with aluminium foil. These trays were subsequently transferred into a warm air oven (Genlab, MINO/ 200/ SS/F, Cheshire, UK) where they were left for 24h at 40 ºC. Diets were crushed very well and put in a plastic vessel then labelled and kept stored in a dry dark place until used. The composition of the

main ingredients used in the current study is shown in (Table 3.1). The diet composition and chemical analyses of the experimental diet are shown in (Table 3.2).

Table 3.1 Proximate analysis of the diet components.

| Chemical composition (%) | BSD | SBM | White lupin |
|---|---|---|---|
| Moisture | 9.56±0.01 | 7.6±0.07 | 9.56±0.01 |
| Protein | 54.48±0.47 | 59.35±0.48 | 42.85±0.43 |
| Lipid | 8.73±0.22 | 2.36±0.02 | 10.11±0.04 |
| Ash | 9.99±0.25 | 5.32±0.84 | 2.55±0.07 |
| NFE | 17.24 | 25.37 | 34.93 |
| Gross Energy (MJ $kg^{-1}$) | 18.88±0.08 | 19.05±0.04 | 19.5±0.01 |

### 3.3.2 Fish rearing

The experiment was carried out with juvenile common carp. Fish were obtained from Hampshire carp Hatcheries, UK. Fish were transported to the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth, UK. Fish were acclimatized to the experimental conditions for 88 days before starting the experiments. During that time, fish were fed EWOS Sigma 50, diet at 1-2 % body weight per day as a maintenance diet. After initial grading and weighing carp was randomly distributed into 12 fiberglass tanks and fed (BSD) based diet at 2% of body weight daily in three rations and three times per day for two days. All carp were subsequently re-weighted. After initial sampling 24 fish which an average weight 16.28±0.35g was stocked into fiberglass tanks. Each treatment was conducted in duplicates. Fish were fed the experimental diets at 3% biomass per day (equal rations at 09.00, 13.00 and 17.00 h) manually for 10 weeks. Daily feed was adjusted on a weekly basis following batch weighing after a 24h feed-deprivation period.

### 3.3.3 Rearing conditions

Described in section 2.2 and 2.4.

### 3.3.4 Sampling

Before starting the experiment, random samples of diets and ingredients were collected for chemical analysis. Twelve fish at the start of experiment were randomly sampled for carcass analysis. At the end of the experiment, 6 fish per tank (12 per treatment) were euthanized and individually weighted for carcass composition, and also, 2 fish per tank 4 fish per treatment were sampled for doing histology.

### 3.3.5 Growth and feed utilization calculations

Specific growth rate (SGR), final weight (FW), weight gain (WG), survival rate, feed conversion ratio (FCR), feed conversion efficiency (FCE), protein efficiency ratio (PER) and apparent net protein utilization (ANPU) were assessed as described in section 2.5.

Table 3.2: Formulation and proximate analysis of the experimental diets (dry weight)

| Ingredient g | BSD | BSDs | L12.5 | L12.5s | L25 | L25s |
|---|---|---|---|---|---|---|
| BSD[a] | 733.0 | 734.5 | 857.5 | 857 | 735 | 734.5 |
| SBM[b] | 245.0 | 245.0 | - | - | - | - |
| WL[c] | - | - | 122.5 | 122.5 | 245 | 245 |
| Oil[d] | 20 | 20 | 20 | 20 | 20 | 20 |
| CMC[e] | 2.00 | | | | | |
| Synergen™[e] | - | 0.5 | - | 0.5 | - | 0.5 |
| Proximate analysis (%) | | | | | | |
| Moisture | 5.8±0.00 | 6.75±0.00 | 6.06±0.01 | 8.11±0.16 | 8.13±0.00 | 5.76±0.16 |
| Protein | 56.05±0.30 | 55.7±0.21 | 53.72±0.15 | 52.64±0.07 | 50.02±0.14 | 52.12±0.27 |
| Lipid | 8.93±0.03 | 8.91±0.12 | 10.95±0.02 | 10.7±0.13 | 12.46±0.06 | 11.45±0.02 |
| Ash | 8.26±0.15 | 8.1±0.13 | 7.93±0.18 | 8.06±0.15 | 6.92±0.12 | 7.22±0.27 |
| NFE | 20.96±0.00 | 20.54±0.00 | 21.34±0.01 | 20.49±0.00 | 22.74±0.00 | 23.45±0.02 |
| Gross energy (MJ $kg^{-1}$) | 20.2±0.01 | 19.93±0.04 | 20.02±0.00 | 19.95±0.05 | 20.84±0.35 | 20.41±0.04 |

[a]= Basal skretting diet (royale horizon skretting, 4.5mm pelleted the origin specification of BSD contains 44% of protein and 28% of oil but pre-extracted and defatted version was used in the current study), [b] = Soybean meal, [c]= White Lupin meal, Terrena Lup Ingredients in France, [d]= Fish oil /corn oil [1:1], [e]=Carboxyl-methyl-cellulose, [f]= (SSF, Alltech, Irland),

BSD= Diet based on Basal skretting diet (BSD), BSDs= Diet based on Basal skretting diet (BSD) with addition 0.05% of Synergen™, L12. 5= Diet included 12.5% of white lupin meal, L12. 5s= Diet included 12.5% of white lupin meal with addition 0.05% of Synergen™, L25= Diet included 25 of white lupin meal, L25s= Diet included 25 of white lupin meal addition 0.05% of Synergen™

### 3.3.6 Chemical analysis

Diets and fish samples (initial and final) from the feeding trial were analysed for proximate composition as described in section 2.6.

### 3.3.7 Histology

The procedure for histological sample preparation was followed as described in section 2.7 and plate 2.5.

### 3.3.8 Light microscopy

Described in section 2.7.1 and plate 2.5.

### 3.3.9 Statistical analysis

The statistical analysis was performed as described in section 2.10

## 3.4 Results

### 3.4.1 Growth and feed utilization

Common carp juvenile readily accepted all the experimental diets. The survival rate was nearly 98% for all treatments. The initial and final body weights, weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), feed conversion efficiency (FCE), protein efficiency ratio (PER), apparent net protein utilization (ANPU) and survival rate of common carp fed the six experimental diets are presented in (Table 3.3). All groups received the experimental diets grew well and body weight increased between 3.3 and 3.9 folds at the end of the 10 week trial. The growth performance and feed utilization were significantly ($P<0.05$) improved by supplementing Synergen™ to BSD based diet and diet that included 12.5% of white lupin but this trend was not significant with diet that contained 25% of white lupin. On the other hand, including 12.5% of white lupin significantly

(*P<0.05*) improved growth performance and feed utilization. On contrary, including 25% of white lupin significantly (*P<0.05*) decreased growth performance and feed utilization expect apparent net protein utilization. Final weight ranged between 53.93±2.26g and 64.43±0.73g. Final specific growth rate (SGR) ranged between (1.7) and (1.95). Feed conversion ratio (FCR) ranged between (1.33) and (1.52). The highest feed conversion efficiency (FCE) (0.74) was obtained in the fish fed L12.5s flowed by fed received BSDs, L12.5, L25s and L25 respectively. The PER values further confirmed the above trend with respect to the productive value of dietary protein. Apparent net protein utilization (APNU) ranged between 19.33 and 21.77.

Table 3.3 Growth performance and feed utilization of common carp fed the experimental diets for 10 weeks. (*n*=2)

| Parameters | Diet | 0 | S | LSD |
|---|---|---|---|---|
| IW (g) | BSD | 16.01±0.06$^{a1}$ | 15.92±0.11$^{a1}$ | 0.29 |
| | L12.5 | 16.83±0.24$^{c2}$ | 16.42±0.04$^{b1}$ | |
| | L25 | 16.36±0.03$^{b1}$ | 16.09±0.11$^{a1}$ | |
| FBW (g) | BSD | 56.88±0.31$^{b1}$ | 59.8±0.63$^{b2}$ | 1.92 |
| | L12.5 | 62.13±0.07$^{c1}$ | 64.43±0.29$^{c2}$ | |
| | L12.5 | 54.13±0.26$^{a1}$ | 53.93±0.92$^{a1}$ | |
| WG (g) | BSD | 40.86±0.42$^{b1}$ | 43.73±0.84$^{b2}$ | 1.98 |
| | L12.5 | 45.29±0.13$^{c1}$ | 48.00±0.34$^{c2}$ | |
| | L25 | 37.77±0.31$^{a1}$ | 37.84±0.80$^{a1}$ | |
| SGR (%day$^{-1}$) | BSD | 1.81±0.01$^{b1}$ | 1.88±0.02$^{b2}$ | 1.97 |
| | L12.5 | 1.86±0.00$^{c1}$ | 1.95±0.00$^{c2}$ | |
| | L25 | 1.7±0.00$^{a1}$ | 1.72±0.01$^{a1}$ | |
| FCR | SPC | 1.45±0.00$^{b2}$ | 1.36±0.00$^{a1}$ | 0.05 |
| | L12.5 | 1.38±0.00$^{a2}$ | 1.33±0.00$^{a1}$ | |
| | L12.25 | 1.52±0.00$^{c1}$ | 1.49±0.00$^{b1}$ | |
| FCE (%) | BSD | 68.40±0.40$^{b1}$ | 73.00±0.40$^{b2}$ | 2.53 |
| | L12.5 | 71.00±0.40$^{c1}$ | 74.4±0.40$^{b2}$ | |
| | L25 | 65.00±0.40$^{a1}$ | 66.76±0.24$^{a1}$ | |
| PER | BSD | 1.22±0.00$^{a1}$ | 1.30±0.03$^{a2}$ | 0.04 |
| | L12.5 | 1.37±0.01$^{c1}$ | 1.41±0.01$^{b2}$ | |
| | L25 | 1.31±0.00$^{b1}$ | 1.28±0.02$^{a1}$ | |
| ANPU (%) | BSD | 19.33±0.15$^{a1}$ | 21.13±0.17$^{a2}$ | 0.58 |
| | L12.5 | 21.43±0.24$^{b1}$ | 21.77±0.10$^{b1}$ | |
| | L25 | 21.07±0.11$^{b1}$ | 20.59$^{1}$±0.11$^{a1}$ | |
| Survival (%) | BSD | 97.92±1.2$^{a1}$ | 97.92±1.2$^{ab1}$ | 2.51 |
| | L12.5 | 95.84±0.00$^{a1}$ | 95.84±0.00$^{a1}$ | |
| | L25 | 97.92±1.2$^{a1}$ | 100±00$^{b1}$ | |

0= Diet without Synergen™, S= diet with Synergen

Data presented as mean ± S.E.; a, b data with the same superscripts with the same column are not significantly different (*P>0.05*) and data with the different superscripts with the same column are significantly different (*P<0.05*). 1, 2 data with the same superscript with the same row are not significantly different (*P>0.05*) and data with the different superscript with the same row are significantly different (*P<0.05*).

### 3.4.2 Carcass composition

The whole body proximate composition (moisture, protein, lipid, ash and energy) at the beginning and after 10 weeks of feeding on the experimental diets is shown in (Table 3.4). At the end of the growth trial, fish received all experimental diets exhibited significant (*P <0.05*) decrease in moisture, ash and protein contents, with a significant (*P* <0.05) increase in lipid and gross energy contents compared to the initial body composition. Supplementing Synergen™ and including white lupin did not significantly (*P>0.05*) affect whole body moisture and protein contents. Nevertheless, including white lupin significantly (*P*<0.05) increased lipid content. Furthermore, supplementing Synergen™ to BSD based diet significantly increased lipid content, but had not significant (*P*>0.05) effect in case of diets that included 12.5% and 25% of white lupin. Including 25% of white lupin and adding Synergen™ to diet that included 25% of white lupin significantly (*P*<0.05) increased ash content. Including 25% of white lupin significantly increased whole body energy content. Furthermore, supplementing Synergen™ to diet that contained 12.5% of white lupin significantly (*P*<0.05) increased whole body energy content, but this trend was not significant (*P*>0.05) in the case of diet that contained 25% of white lupin.

Table 3.4 Proximate composition of initial fish carcasses and fish after 10 weeks feeding on the experimental diets (n = 4)

| Parameters | Diet | Initial | 0 | S | LSD |
|---|---|---|---|---|---|
| Moisture (%) | BSD | 75.78±0.49 | 72.48±0.31$^{a1}$ | 71.99±0.24$^{a1}$ | 0.88 |
| | L12.5 | | 72.02±0.22$^{a1}$ | 71.65±0.17$^{a1}$ | |
| | L25 | | 71.78±0.21$^{a1}$ | 71.91±0.26$^{a1}$ | |
| Crude protein (%)* | BSD | 57.76±0.24 | 55.42±0.39$^{a1}$ | 55.65±0.62$^{a1}$ | 2.64 |
| | L12.5 | | 55.03±0.93$^{a1}$ | 53.07±0.53$^{a1}$ | |
| | L12.5 | | 54.85±0.55$^{a1}$ | 55.01±1.05$^{a1}$ | |
| Crude lipid (%)* | BSD | 25.36±0.61 | 35.17±0.43$^{a1}$ | 38.62±1.09$^{b2}$ | 2.52 |
| | L12.5 | | 37.77±0.65$^{b1}$ | 36.62±0.33$^{ab1}$ | |
| | L25 | | 38.47±0.68$^{b1}$ | 36.05±0.70$^{a1}$ | |
| Ash (%)* | BSD | 14.03±0.15 | 7.92±0.08$^{a1}$ | 7.8±0.18$^{a1}$ | 0.37 |
| | L12.5 | | 7.7±0.08$^{ab1}$ | 7.7±0.07$^{a1}$ | |
| | L25 | | 7.46±0.11$^{b1}$ | 8.22±0.013$^{ab2}$ | |
| Gross energy | BSD | 23.12±0.02 | 25.48±0.09$^{ab1}$ | 25.13±0.12$^{a1}$ | 0.59 |
| (MJ kg$^{-1}$)* | L12.5 | | 25.2±0.02$^{a1}$ | 26.32±0.17$^{b2}$ | |
| | L25 | | 26.06±0.08$^{b2}$ | 25.63±0.13$^{a1}$ | |

Data presented as mean ± S.E.; a, b data with the same superscripts with the same column are not significantly different (*P>0.05*) and data with the different superscripts with the same column are significantly different (*P<0.05*). 1, 2 data with the same superscript with the same row are not significantly different (*P>0.05*) and data with the different superscript with the same row are significantly different (*P<0.05*).

*Dry matter basis.

## 3.4.3 Histological examination

### 3.4.3.1 Intestinal histology

Histological appraisal of the posterior intestine revealed that all groups displayed complex intestinal mucosal folding. The histology of posterior gut in fish received BSDs, L12.5s and L25s diets slightly better than fish received BSD, L12.5 and L25 diets. Atrophy, necrosis epithelial of cells and infiltration of mucosal epithelium into the lamina properia and submucosa were slightly observed especially in the fish received L12.5 and L25 diets. No significant (*P>0.05*) difference was recorded in the villus length among fish fed all experimental diets. Supplementing Synergen™ did not significantly affect the number of goblet cells in the posterior gut. However, including 25% of white lupin significantly (*P<0.05*) increased the number of goblet cells in the posterior gut (Table 3.5; Figure 3.1).

Table 3.5 Posterior Intestinal morphology of fish fed on the experimental diets for 10 weeks (n = 4)

| Parameters | Diet | 0 | S |
|---|---|---|---|
| Villi length (µm) | BSD | $629.74\pm11.43^{a1}$ | $662.23\pm4.49^{a1}$ |
| | L12.5 | $588.69\pm20.82^{a1}$ | $681.89\pm4.89^{a1}$ |
| | L25 | $626.32\pm2.44^{a1}$ | $683.98\pm7.34^{a1}$ |
| Goblet cells per (100 µm) | BSD | $5.92\pm0.27^{a1}$ | $6.62\pm0.22^{a1}$ |
| | L12.5 | $6.22\pm0.30^{ab1}$ | $6.07\pm0.18^{a1}$ |
| | L25 | $7.65\pm0.54^{b1}$ | $6.93\pm0.40^{a1}$ |

Data presented as mean ± S.E.; a, b data with the same superscripts with the same column are not significantly different (*P>0.05*) and data with the different superscripts with the same column are significantly different (*P<0.05*). 1, 2 data with the same superscript with the same row are not significantly different (*P>0.05*) and data with the different superscript with the same row are significantly different (*P<0.05*)

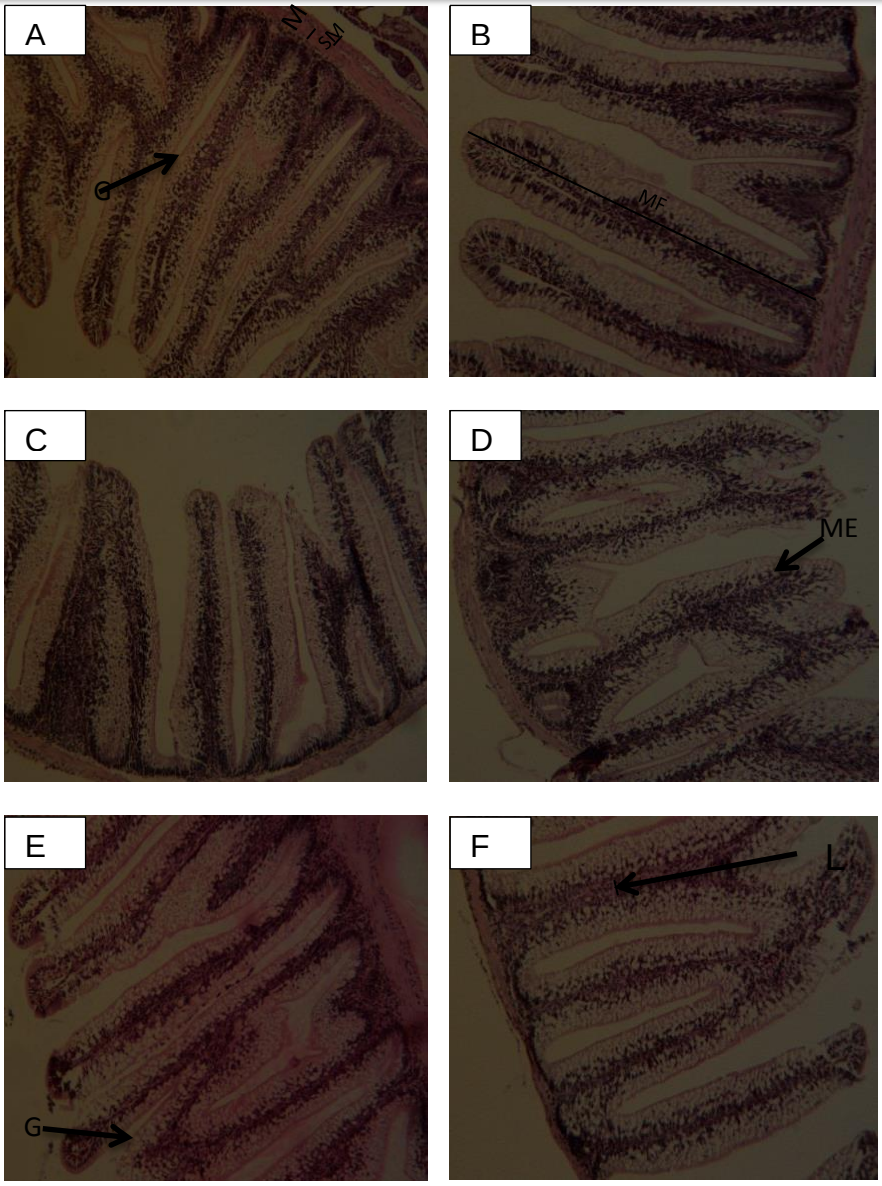



Figure 3.1 Posterior Intestinal bulbs of the common carp stained by haematoxylin and eosin (Scale bar 100 µm). (A) Fish fed BSD diet, (B) fish fed BSDs, (C) fish fed L12.5, (D) Fish fed L12.5s, (E) fish fed L25 and (F) fish fed L25s: Mucosal epithelium (ME), Lamina properia (LP), Goblet cells (G), Muscularis (M), Serous membrane (SM), Lumen (L).

### 3.4.3.2 Liver histology

Accumulation of lipid in the cytoplasm, nuclear atrophy, necrosis and atrophy of hepatic cells with vascular degeneration were generally observed. Supplementing Synergen™ and including white lupin did not significantly (*P>0.05*) affect hepatocyte size. Nevertheless, adding Synergen™ to BSD based diet and diets that contained 12.5% and 25% of white lupin significantly (*P<0.05*) increased the nucleus size. Supplementing Synergen™ to BSD based diet and diet that contained 12.5% of white lupin significantly (*P<0.05*) increased nucleus diameter to hepatocyte diameter but this trend was not significant in the case of diet that contained 25% of white lupin. Finally, including 12.5% and 25% of white lupin for common carp BSD based diet did not significantly (*P>0.05*) affect hepatocyte size, nucleus size and nucleus diameter to hepatocyte diameter.

Table 3.6 Liver histological analyses of fish fed the experimental diets for 10 weeks. (n = 4)

| Parameters | Diet | 0 | S |
|---|---|---|---|
| Hepatocyte size (µm) | BSD | $11.82\pm0.23^{a1}$ | $11.65\pm0.15^{a1}$ |
| | L12.5 | $11.58\pm0.25^{a1}$ | $11.73\pm0.10^{a1}$ |
| | L25 | $11.08\pm0.08^{a1}$ | $11.75\pm0.19^{a1}$ |
| Nucleus size (µm) | BSD | $4.39\pm0.05^{ab1}$ | $4.73\pm0.14^{b2}$ |
| | L12.5 | $4.11\pm0.01^{a1}$ | $4.37\pm0.01^{a2}$ |
| | L25 | $4.21\pm0.09^{a1}$ | $4.4\pm0.03^{a2}$ |
| Ratio of nucleus diameter to hepatocytes diameter (µm) | BSD | $37.5\pm0.36^{ab1}$ | $41.02\pm0.98^{b2}$ |
| | L12.5 | $35.83\pm0.93^{a1}$ | $38.12\pm0.50^{a2}$ |
| | L25 | $38.8\pm1.06^{b1}$ | $38.55\pm0.62^{a1}$ |

Data presented as mean ± S.E.; a, b data with the same superscripts with the same column are not significantly different (*P>0.05*) and data with the different superscripts with the same column are significantly different (*P<0.05*). 1, 2 data with the same superscript with the same row are not significantly different (*P>0.05*) and data with the different superscript with the same row are significantly different (*P<0.05)*

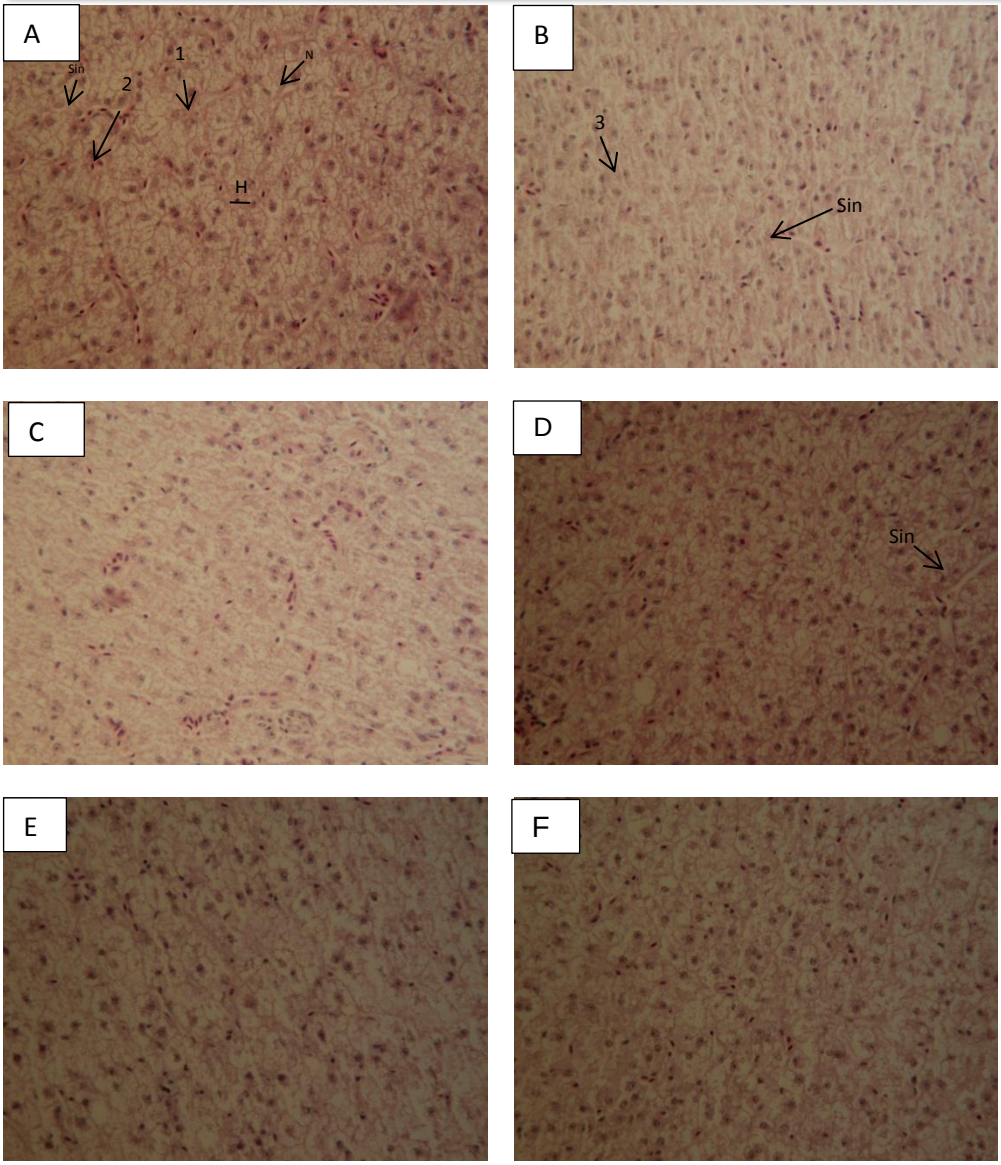



Figure 3.2 Haematoxylin and eosin stained sections of livers of common carp. 1- Necrosis of hepatic cells 2- Atrophy of hepatic cells 3- Necrosis of hepatic cells (A) Fish fed BSD diet, (B) fish fed BSDs, (C) fish fed L12.5, (D) Fish fed L12.5s, (E) fish fed L25 and (F) fish fed L25s**.** H: Hepatocytes, Sin: Sinusoid, V: vacuolization, N: hepatic nuclei. (40x) (Scale bar = 100 µm)

## 3.5 Discussion

The results of the present study demonstrated that inclusion of 12.5% of white lupin to juvenile common carp BSD based diet significantly improved growth performance and feed utilization. However, most parameters of growth performance and feed utilization significantly decreased by inclusion of 25% of white lupin to BSD based diet. It is likely due to carp fed diets which were unbalanced in nitrogen and energy contents because the diets were formulated as summit- dilution trial type which is unique and not commonly used in standard fish feeding trials although often used by commercial companies as a prerequisite step before commencing more traditional balanced trial with fish. Furthermore, it is possibly reflective of the relatively high levels of oligosaccharides in the lupin meal compared with soybean meal and basal skretting diet (BSD) meal. Glencross, Boujard & Kaushik (2003a) indicated that lupin oligosaccharides reduce the protein digestibility and hence the nutritional value of lupin meals. In contrast to the growth and feed utilization parameters protein efficiency ratio (PER) and apparent net protein utilization (ANPU) were significantly increased by including 12.5% and 25% of white lupin compared to BSD diet in the present study, which may be due to highly digestible protein in white lupin compare with soybean meal. Glencross *et al*. (2004b) reported that protein digestibility of white lupin, narrow-leaf lupin and yellow lupin kernel meals is higher than that of the solvent extracted high-protein soybean meal for rainbow trout and red seabream diets. Although, the present study is the first study to evaluate the potential of white lupin in diets for common carp, the findings of the current study can be compared with previous findings which have been obtained with different species of fish. The findings of the present study are in agreement with some previous

findings. Bórquez *et al*. (2011b) reported that whole grain white lupin can be incorporated up to 20% in extrude diet for rainbow trout without negative effect on growth performance and feed utilization. Nevertheless, the findings of the present study disagree with some previous findings. For example, Hernández *et al*. (2012) investigated that inclusion 25% of white lupin into juvenile rainbow trout fishmeal based diet decrease growth performance and feed utilization but not significantly. Burel *et al*. (1998) did not find any deleterious effects on growth performance, feed intake with incorporation white lupin up to 50% in the diet for juvenile.

There is much less information available on the replacement of soybean meal with white lupin meal. The results of the present study are in contrast with results found by Viola, Arieli & Zohar (1988) who indicated that total replacement of defatted soybean meal (45% protein) by whole-seed blue lupin meal based on 30% inclusion of blue lupin in common carp diet that contained 18% of defatted soybean meal significantly improved growth performance and feed utilization.

Hughes (1988, 1991) in two series studies showed that full fat soybean meal can be totally replaced with lupin flour in diets for rainbow trout. Robaina *et al*. (1995) also reported that soybean meal can be totally replaced by blue lupin meal based on 10%, 20% and 30% inclusion level in juvenile gilthead seabream diet without any deleterious effect on growth performance and feed utilization. Chien & Chiu (2003) reported that partial replacement of soybean meal (33, 67%) by blue lupin seed meal in plant based diet for juvenile tilapia resulted in better or at least equal, growth performance and feed performance.

As we mentioned above white lupin contains a high level of non-starch polysaccharides including oligosaccharides. The non-starch polysaccharide has been shown to reduce the digestion of the other nutrients, produce gas resulting

of bacterial digestion of the starch in the colon to produce carbon dioxide, methane and hydrogen, and also abdominal discomfort, cramps, gut distension, flatulence and diarrhea. The negative impacts of non-starch polysaccharide and oligosaccharide cannot be decreased by removing the seed coat (de-hull), heat treatment and other processing because they are heat stable anti-nutritional factors (Van Barneveld, 1999; Petterson, 2000; Francis *et al*., 2001; Glencross, Boujard & Kaushik, 2003).

The present study is the first study to examine Synergen™ supplementation for common carp BSD based diet and diets that contained 12.5% and 25% of white lupin. In the present study, dietary supplementation of Synergen™ for common carp BSD based diet and the diet containing 12.5% of white lupin significantly improved growth performance and feed utilization, while this improvement was very slight with adding Synergen™ to diet that included 25% of white lupin. It is suggested that dietary supplementation of Synergen™ to fish diets containing a high level of plant protein sources can reduce the negative effects of anti-nutritional factors in plant sources by ferment fibre and subsequently increasing nutrient and energy digestibility with improved growth performance and feed utilization. The benefits of dietary supplementation of exogenous enzymes to enhance growth performance and feed utilization for Tilapia (warm water fish species) plant based diets has been shown to date. For instance, Ng *et al*. (2002) reported that supplementation of commercial feed enzyme (Allzyme Vegpro™) led to enhanced growth performance and feed utilization in red hybrid tilapia. Similarly, Lin, Mai & Tan (2007) stated that supplementing only 0.1% of commercially exogenous enzyme (neutral protease, β-glucanase and xylanase) into plant-based diets for juvenile hybrid tilapia can significantly improve growth

performance and feed utilization as well as promote the secretion of the endogenous enzymes. The findings of current study confirmed that exogenous enzymes work very well with warm water fish species plant based diet such as carp and tilapia. Work with Atlantic salmon (Carter *et al*., 1994) has also confirmed that supplementing proteolytic and carbohydrases enzymes in Atlantic salmon diets that contained 34% of soybean meal improve growth and food conversion efficiency.

Nevertheless, the result of this study is in contrast with some previous findings with cold water fish species. Stone *et al*. (2003) stated that dietary supplementation of Natugrain-blend® [β-glucanase and β-xylanase at three nominal concentrations (0.75, 150 or 300 μL $kg^{-1}$) to silver perch diet that contained 30% of de-hulled blue lupin was ineffective. Similarly, Ogunkoya *et al*. (2006) investigated that supplement commercial enzyme cocktail (Superzyme CS) for rainbow trout soybean meal based diet containing up to 20% of soybean meal did not improve growth performance and feed efficiency. Recently, Farhangi & Carter (2007) did not find any enhancement in growth performance by supplementing Energex™, Bio-Feed™Pro, α-galactosidase™ and mixed of these enzymes to the rainbow trout diet that contained 50% of de-hulled blue lupin.

The results of the present study showed that including 12.5% and 25% of white lupin for common carp BSD based diet does not have any significant effects on whole body moisture, protein and energy contents. This is agreement with (Burel *et al*., 1998; Borquez *et al*., 2011b) findings with white lupin, (Gomes, Rema & Kaushik, 1995; Farhangi & Carter, 2001) findings with blue lupin and with (Glencross *et al*., 2004a) finding with yellow lupin on rainbow trout. However, these results disagree with Farhangi & Carter (2007) finding with narrow leafed

lupin on rainbow trout. The whole body lipid content was significantly increased by including 12.5% and 25% of white lupin for BSD based diet it is likely due to higher level of lipid in diets that contained 12.5% and 25% of white lupin compared with BSD based diets. This is in agreement with Burel *et al*. (1998) findings with a white lupin on rainbow trout and Farhangi & Carter (2007) findings with narrow leafed lupin on rainbow trout. However, it is disagreement with Bórquez *et al*. (2011a) findings with white lupin on rainbow trout, the reason for this disagreement may that they used isonitrogenous and isoenergetic in these two studies compared with the unbalanced diets in the present study. The whole body ash content significantly decreased with inclusion 25% of white lupin to BSD based diet. This is likely due to lower content of ash in the diet included 25% of white lupin compare with BSD based diet. On the other hand, the whole body moisture and protein and ash contents were not significantly changed by the supplementing Synergen™ to BSD based diet and diets that contained 12.5% and 25% of white lupin in the present study. However, adding Synergen™ to BSD based diet significantly increased whole body lipid content in the present study, while adding Synergen™ to diets containing 12.5% and 25% of white lupin did not affect whole body lipid content. This is indicated that Synergen™ is beneficial to increase lipid digestibility in agreement with (Farhangi & Carter, 2007; Lin, Mai & Tan, 2007; Dalsgaard *et al*., 2011). Supplementing Synergen™ to diet that contained 12.5% of white lupin significantly increased the whole body energy content compare with BSD diet it is may be due to ferment fibre and increase fibre digestibility thus release more energy.

Atrophy, necrosis of epithelial cells as well as infiltration of mucosal epithelium into the lamina properia and submucosa were slightly observed by doing

experiment especially in the fish receiving diets without Synergen™. Bórquez *et al*. (2011a) found that inclusion of 40% and 50% of white lupin in the rainbow trout diet led to histological changes in the mid intestine such decrease the number of basophil granulocytes, distal displacement of enterocyte nucleus and an increment in lipid drops. On contrary, Glencross *et al*. (2004a) did not find any negative effect of the dietary inclusion of yellow lupin on histology intestine in rainbow trout. Villi length generally decreased by inclusion of white lupin, this trend was not significant. On the other hand, supplementing Synergen™ increased villus length, although this trend was not significant. Farhangi & Carter (2001) found that increasing dietary inclusions of blue lupin can slightly shorten the villus length in rainbow trout. The number of goblet cells in the posterior intestine was significantly increased by including 25% of white lupin onto BSD based diet. This may be due to increase fibre concentration in the diet by including 25% of white lupin because lupin contains high levels fibre especially soluble and non-soluble non-starch polysaccharides (NSP) which may be caused to increase mucus secretion.

Accumulation of lipid in the cytoplasm, nuclear atrophy, necrosis and atrophy of hepatic cells with vascular degeneration in liver was generally observed. These cannot be ascribed exclusively to the use of lupin because they were also noted in the fish fed BSD based diets. It is possibly reflective of the anti-nutritional factor content in the both soybean meal and lupin meal which is led to damage liver in the fish as well as high level of lipid content especially in the diets that contained white lupin. Pereira & Oliva-Teles (2004) showed a small increase in liver lipid droplets in fish fed the diets with inclusion and also indicated that lupin seed meal protein should not exceed 20% of total dietary protein to prevent lipid deposition

in the liver. Bórquez *et al*. (2011a) found that inclusion white lupin into rainbow trout diet leads slight lipid infiltration in the hepatocyte. On contrary, Robaina *et al*. (1995) did not find any alterations in lipid and glycogen storage in hepatocytes from gilthead seabream hepatocytes inclusion of up to 30% of de-hulled blue lupin seed meal. Bórquez *et al*. (2011b) did not find any changes in liver histology by inclusion up to 20% of white lupin in the rainbow trout diet. Compares the results of different studies are further complicated by differences in lupin species and cultivars, level of inclusion, fish species, fish age, feeding system, trail type, type of enzyme, and the level of supplement enzyme and/or experimental condition used in this study.

## 3.6 Conclusion

The findings from the present study demonstrated including 25% of white lupin for common carp BSD based diet significant negative effect on growth performance, feed utilization, carcass composition, liver histology and gut histology. On contrary, the growth performance and feed utilization significantly decreased by inclusion of 25% of white lupin to BSD based diet. In addition, supplementing Synergen™ for common carp diet containing lupin meal and soybean meal is useful to reduce the negative effect of anti-nutritional factors in both plant protein sources and hence improve growth performance and feed utilization. Further research is needed to investigate the effect of different supplemental levels of Synergen™ to different types of lupin such as yellow lupin, narrow-leafed lupin and other species of fish.

# Chapter 4

## Partial replacement of soya protein concentrate (SPC) meal by white lupin seed meal and Synergen™ supplementation in complete diets for juvenile mirror carp (*Cyprinus carpio*)

### 4.2 Introduction

Plant proteins represent the major dietary protein source used within feeds for lower trophic level fish species (tilapias, carps, catfishes) and the second major source of dietary protein and lipid source after fishmeal and fish oil for shrimps and European high trophic level fish species (Tacon, Hasan & Metian, 2011). Among plant proteins soybean meal is the most common used in compound aquafeeds and the most prominent protein ingredient substitute for fishmeal in aquaculture feeds, with feeds for herbivorous and omnivorous fish species and crustaceans usually containing 15–45 percent of soybean meal, due to its high protein content and favourable amino acid profile (Kaushik *et al*., 1995; Dersjant-Li, 2002; Chien & Chiu, 2003; Gatlin III, 2003; Glencross & Australia, 2003; Ringø *et al*., 2009; Tacon, Hasan & Metian, 2011). Soybean meal has been previously tested in carp by Pongmaneerat *et al*. (1993), Escaffre *et al*. (1997), Sahar, Ali & Naqvi (2003). Generally, these workers have reported good performance when the fishmeal protein contribution was replaced up to 50% with soybean meal. More recently, Marković *et al*. (2012) stated that partial replacing of fishmeal with a mixture of soy ingredients, maize gluten, wheat gluten and yeast does not have any significant adverse effect on growth performance and feed utilization in the diet for juvenile common carp.

Increase using soybean meal protein as a human food and terrestrial animal feeds has resulted in an increase market price of soybean globally. This is

especially true in a western country and developing region that import soybean for agriculture and human food. On the other hand, soybean is deficient in lysine and methionine concentration and it contains a wide variety of anti-nutritional factors (ANFs) (Rana, Siriwardena & Hasan, 2009; Kumar *et al*., 2011; Gunnar, 2011). For these reasons, over depends on the use of soybean in aquafeeds may become less favourable and could increase the production costs. Therefore, partial replacement of soybean protein with more economical, alternative sources of protein is imperative to sustain aquaculture production.

Lupin is regarded as one of the legumes that having promising potential as an aquaculture feed ingredient, due to its high protein content and low market price (Glencross *et al*., 2002a; Glencross *et al*., 2004b; Glencross & Hawkins, 2004; Glencross, 2004; Sweetingham *et al*., 2008). Lupins have been successfully used as a partial replacement for fishmeal in the diet for most aquaculture species. The earliest reported study was that by Viola, Arieli & Zohar (1988) who stated that inclusion of 45% of whole-seed blue lupin as a fishmeal replacement in diet for common carp does not have any significant adverse effects on growth performance and feed utilization. Workers with rainbow trout (Burel *et al*., 1998; Farhangi & Carter, 2001; Glencross *et al*., 2002a; Glencross *et al*., 2004a; Bórquez *et al*., 2011a; Glencross, Rutherford & Hawkins, 2011) have reported that incorporation of between 40% and 50% of dietary lupin as fishmeal replacement did not affect growth performance and nutrient utilization. Furthermore, in the study by Bransden, Carter & Nowak (2001) the possibility of inclusion up 40% of de-hulled blue lupin for Atlantic salmon diet without any adverse effects on growth, immune function or blood chemistry and disease resistance were also reported. In the other study, Gouveia *et al*. (1993) reported

that inclusion 20 % of white lupin in the diet for wrainbow trout does not adversely affect growth performance. Recently, Bórquez *et al*. (2011b) indicated that including whole seed white lupin meal up to 20% in extruded diets for rainbow trout does not have any adverse effect on growth performance and feed performance. More recently, Hernández *et al*. (2012) investigated that including whole seed white lupin meal up to 25% in extruded diets for rainbow trout does not have any adverse effect on growth performance and feed performance. In another study, Pereira & Oliva-Teles (2004) investigated that incorporates up to 30% of narrow-leafed lupin seed meal into the diet for juvenile gilthead sea bream does not have any negative effects on growth performance and whole-body composition. In the further study by Burel *et al*. (2000a) it was reported that extruded white lupin can be incorporated into the diets of turbot up to a level of 50% without any adverse effects on growth performance and body composition.

However, the possibility of a partial substitution soybean meal by lupin meal has been evaluated in a few studies. In the earlier two studies (Hughes, 1988; 1991) indicated that full fat soybean meal can be totally replaced with lupin flour in complete diet for rainbow trout and the economic value of using lupin meal over soybean meal was also reported. In another study, Jenkins *et al*. (1994) also reported that soybean meal can be totally replaced by blue lupin meal in the diet for juvenile snapper. In the further study, Robaina *et al*. (1995) reported that soybean meal can be totally replaced by blue lupin meal in the diet for juvenile gilthead seabream. However, despite the widespread use of lupins and the many studies undertaken examining lupins nutritional qualities there is much less information available on the use of lupin in Cyprinids diets especially for carp.

There are, however, still challenges associated with the use of lupin and soybean meals at high concentration in diets for fish. These include imbalanced essential amino acid (EAA) composition (Glencross & Australia, 2001; Glencross, 2004; Petterson *et al*. 1997) and the presence of anti-nutritive factors (ANFs) (Petterson, 2000; Francis *et al*., 2001; Glencross, 2004; Hardy, 2010). Furthermore, lupins have low energy density due to high contents of carbohydrates such as starch, indigestible oligosaccharides and non-starch polysaccharides (Petterson, 2000; Glencross, Boujard & Kaushik, 2003). Balanced amino acid composition can be obtained by supplementing limiting EAA in diets for rainbow trout (Zhang *et al*., 2012). Ingredient processing, such as de-hulling (Glencross *et al*., 2007) and enzyme treatment (Farhangi & Carter, 2007) can, to some extent, reduce the ANFs in the seed. Therefore, supplement Synergen™ to mirror carp diet contains soya protein concentrate and lupin meals would be beneficial to improve nutrient utilization, reduce feed cost and decrease excretion of nutrients into the environment.

The effects of the substitution of 12.5% and 25% of the soya protein concentrate as the principal protein ingredient with white lupin and the addition of Synergen™ in complete diet for juvenile mirror carp was determined on several growth performances and feed utilization parameters with health status under defined condition.

## 4.3 Materials and Methods

### 4.3.1 Diets

Experimental diets were prepared in 5 kg batches; diets were formulated to contain (38% crude protein and 8% crude lipid) using FeedSoft© (Feedsoft Corporation, USA) linear least cost formulating software, with the restrictions used representing the NRC (2011) guidelines for the appropriate nutrient class. Six isonitrogenous (38%) and isolipdic (8%) diets were formulated by substitution 12.5% and 25% of soya protein concentrate (SPC) meal with white seed meal and 0.1% Synergen™ supplementation. Of the dietary protein, except for 3.8% (10% in diet composition) from fishmeal, (38% crude protein), the rest was from vegetable protein sources. Composition and source of all ingredients used are presented in Table 4.1. There were two soy protein concentrate (SPC) based diets with and without supplemental Synergen™. Four experimental diets based on the substitute of 12.5% and 25 % of soya protein concentrate (SPC) meal with white lupin with and without adding 0.1 % of Synergen™ were formulated. The dry milled ingredients were mixed uniformly to ensure homogeneous distribution of the diet components and then mixed for approximately 30 min using a Cater-Bake food mixer (Cater–Bake UK). After the initial mixing, fish oil and corn oil were gradually added in a continuous flow. After further mixing, water (~ 3.5 $L^{-1}$) was added to form light dough of each diet. The pastes were passed through an extruder (PTM Extruder System, Plymouth, Devon, UK: Model= P6, year 2006) and an appropriate aperture die (7) was used to achieve the desired pellet size (2-mm pellets). The resulting strands were carefully broken up and spread onto trays lined with aluminium foil. These trays were subsequently transferred into a

warm air oven (Genlab, MINO/ 200/ SS/F, Cheshire, UK) where they were left for 24h at 40 ºC. Diets were crushed very well and put in a plastic vessel then labelled and kept stored in a dry dark place until used (see section 2.1). Dietary formulations and proximate composition and energy content of the experimental diets are presented in (Table 4.1). The estimate essential amino acid profiles in the experimental diets are presented in the (Table 4.2).

Table 4.1 Dietary formulations and proximate composition and energy content of the experimental diets

| | Diets | | | | | |
|---|---|---|---|---|---|---|
| | SPC | SPCs | L12.5 | L12.5s | L25 | L25s |
| Ingredient g $kg^{-1}$ | | | | | | |
| Soybean (Hamlet HP100) [a] | 498.08 | 498.08 | 414.75 | 414.75 | 331.42 | 331.42 |
| White lupin[b] | - | - | 125.00 | 125.00 | 250.00 | 250.00 |
| Corn Starch[c] | 283.24 | 282.24 | 255.22 | 254.22 | 227.19 | 226.19 |
| Hearing Meal LT94 [d] | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| Fish Oil[e] | 30.00 | 30.00 | 30.00 | 30.00 | 26.38 | 26.38 |
| Corn Oil[f] | 23.66 | 23.66 | 10.02 | 10.02 | - | - |
| Glutalys [g] | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 |
| Lysamine Pea protein [h] | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 |
| Vitamin Premix [i] | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 |
| Carboxyl-methyl-cellulose (CMC) [j] | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| Synergin™ [k] | - | 1.00 | - | 1.00 | - | 1.00 |
| Proximate composition (%) | | | | | | |
| Moisture(%) | 6.53±0.06 | 5.27±0.03 | 6.64±0.01 | 5.12±0.04 | 6.45±0.01 | 6.18±0.00 |
| Protein (%) | 40.02±0.09 | 43.26±0.19 | 41.74±0.35 | 42.16±0.05 | 41.66±0.20 | 41.62±0.09 |
| Lipid (%) | 7.52±0.09 | 7.32±0.08 | 7.39±0.00 | 7.62±0.06 | 7.43±0.03 | 7.72±0.00 |
| Ash (%) | 6.2±0.02 | 6.27±0.00 | 6.12±0.01 | 6.09±0.03 | 5.73±0.11 | 6±0.01 |
| NFE (%) | 39.72±0.11 | 37.87±0.15 | 38.09±0.28 | 39±0.05 | 38.72±0.13 | 38.45±0.03 |
| Gross energy (MJ $kg^{-1}$) | 19.29±0.03 | 19.4±0.02 | 18.9±0.01 | 19.38±0.08 | 19.2±0.03 | 19.07±0.01 |

[a] Soybean (Hamlet HP 100): soya protein concentrate (crude protein 57.5%, crude fibre 3%) produced from genetically modified soja production in Denmark by Hamlet production A/S DK [b]White lupin (Moisture 5.16, Protein 38 %, Lipid12. 58±0.17Ash3.51%), Terrena Lup Ingredients in France, [c] Corn starch: Sigma-Aldrich Company Ltd, [d] Hearing Meal LT94 Scottish fish meal 70, United Fish Products Ltd, UK., [e]Fish Oil, [f]Corn oil, [g] Glutalys (Maize Gluten meal) = Roquette, [h] Lysamin pea protein: ESMC, [i] Vitamin Premix, Premier Nutrition vitamin, [j] Carboxyl-methyl-cellulose (CMC), Sigma –ALDRICH, [k] Synergen™ Powered by SSF Technology Alltech Irland, (Analytical construction: Crude protein 21.0%, Crude fibre 13.0%, Crude oils and fats 4.2), Nitrogen-free extracts (NFE %) = 100-(Ash+ moisture+ crud fat+ crude protein)

SPC= soya protein concentrates based diet, SPCs= diet based soya protein concentrate with adding 0.1% of Synergen™ L12. 5= Diet replaced 12.5% of soybean with white lupin meal, L12. 5s= Diet replaced 12.5% of soybean with white lupin meal and supplemented 0.1 % of Synergen™, L25= Diet replaced 25% of soybean with white lupin meal, L25s= Diet replaced 25 % of soybean with white lupin meal and supplemented 0.1 % of Synergen™.

Table 4.2 Estimated essential amino acid profile in the experimental diets g amino acid $kg^{-1}$ diet

| Amino acid | SPC | SPCs | L12.5 | L12.5s | L25 | L25s |
|---|---|---|---|---|---|---|
| Lysine | 24.5 | 24.5 | 21.6 | 21.6 | 18.7 | 18.7 |
| Methionine | 6.1 | 6.1 | 5.5 | 5.5 | 4.9 | 4.9 |
| Met+Cys | 2.8 | 2.8 | 2.8 | 2.8 | 2.8 | 2.8 |
| Arginine | 26.9 | 26.9 | 23.5 | 23.5 | 20.1 | 20.1 |
| Histidine | 9.7 | 9.7 | 8.5 | 8.5 | 7.2 | 7.2 |
| Threonine | 15.2 | 15.2 | 13.3 | 13.3 | 11.4 | 11.4 |
| Tryptophan | 4.8 | 4.8 | 4.2 | 4.2 | 3.5 | 3.5 |
| Leucine | 30.5 | 30.5 | 26.8 | 26.8 | 23.1 | 23.1 |
| Isoleucine | 17.5 | 17.5 | 15.3 | 15.3 | 13.1 | 13.1 |
| Phenylalanine | 18.7 | 18.7 | 16.3 | 16.3 | 13.9 | 13.9 |
| Valine | 19.4 | 19.4 | 17.1 | 17.1 | 14.8 | 14.8 |

### 4.3.2. Rearing condition

Described in section 2.2 and 2.4

### 4.3.3 Fish

The trial was performed with mirror carp. Fish were obtained from Bowlake fish farm, Hampshire, UK. Fish were transported to the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth, UK. Fish were acclimatized to the experimental conditions for 75 days before starting the experiment. During that time, fish were fed EWOS Sigma 50, diet at 1-2 % body weight per day as a maintenance diet. After initial grading and weighing the carp were randomly distributed into 12 fiberglass tanks and fed SPC diet at 3-4% of body weight daily in three rations and three times per day for two days. After initial sampling all carp were subsequently re-graded uniformly at a stocking density of 25 fish per tank an average weight (15.35±0.57g) in duplicate groups randomly assigned. A ration level of 3-4% of live body weight daily was adjusted on the basis of a weekly record of biomass from each tank. The ration was fed by hand three times daily and total feed intake for each group of fish recorded together with a total biomass gain for the complete growth trial period.

### 4.3.4 Sampling

Before starting the experiment, random samples of diets and ingredients were collected for chemical analysis. Twelve fish at the start of the experiment were randomly sampled for initial carcass composition. At the end of the experiment, 6 fish per tank (12 per treatment) were euthanized and individually weighted for carcass composition, and also, 3 fish per tank 6 fish per treatment were sampled for doing histology and haematology. Furthermore, 2 fish per tank 4 fish per treatment were sampled for doing microbiology.

### 4.3.5 Growth and feed utilization calculations

Specific growth rate (SGR), final body weight (FBW), weight gain (WG), feed intake (FI), protein intake (PI) survival rate, feed conversion ratio (FCR), feed conversion efficiency (FCE), protein efficiency ratio (PER), apparent net protein utilization (ANPU), lipid efficiency ratio (LER), energy retention (ER) and condition factor (K) were assessed as described section (2.5).

### 4.3.6 Chemical analysis

Diets and fish samples (initial and final) from the feeding trial were analysed for proximate composition as described in section 2.6.

### 4.3.7 Histology

The procedure for histological sample preparation was followed as described in section 2.7 and plate 2.5.

### 4.3.8 Light microscopy

Histological appraisal of the posterior gut, mid gut and liver from 6 fish per experimental group was conducted at the end of the trial by using light

microscopy. Tissue samples for light microscopy were fixed at 4% formal buffered saline for 24 h, dehydrated in graded ethanol concentrations and embedded in paraffin wax. Haematoxylin and eosin were used to stain slides to count villi length, villi width and lamina properia width. Alcian blue staining manually XL or alecian blue periodic acid schiff staining (AB-PAS) was used to count the number of goblet cells. Micrographs were produced using an Olympus Vanox-T microscope model (AHBT) and Olympus digital camera (E-620). Hepatocyte size, nucleus size and ratio of nucleus diameter to hepatocyte diameter were measured manually: 10 cells were randomly counted in each slide as described (Omar, 2011). Intestinal images taken from light microscopy were analysed to determine the length and width of the mucosal folding (villi) and width of lamina properia. Additionally, the number of goblet cells was counted.

### 4.3.9 Electron Microscopy

Samples for SEM were taken in the posterior region of gut from six separate fish per treatment as described in section 2.7. Microvilli density was assessed, sampling and processing protocols is described in section 2.7.2.1 Samples from the posterior region of the gut were observed from six fish per treatment for TEM and microvilli density were measured as described in section 2.7.2.2.

### 4.3.10 Haematological parameters

At the end of the trial, fish were scarified and blood collected from 6 fish per treatment as described in section 2.8.

#### 4.3.11.1 Haematocrit

Haematocrit determination was assayed using heparinized capillary tubes as described in section 2.8.1.

#### 4.3.11.2 Haemoglobin (Hb)

See section 2.8.2

#### 4.3.11.3 Determination of differential leukocyte counts

See section 2.8.3

### 4.3.12 Microbiology

Described in section 2.9

### 4.3.13 Statistical analysis

The statistical analysis was performed as described in section 2.10.

## 4.4 Results

#### 4.4.1 Growth and feed utilization

During the study all fish readily accepted experimental diets and fish exhibited normal behaviour throughout the trial. No mortality occurred during the feeding period and no signs of stress or disease were observed. The initial and final body weights, specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), feed conversion efficiency (FCE), protein intake (PI), protein efficiency ratio (PER), apparent net protein utilization (ANPU), lipid efficiency ratio (LER), energy retention (ER) and body condition factor (K) are presented in Table 4.3. No significant difference in initial body weight for all treatments showed that the fish were homogeneously distributed among treatments and replicates at stocking.

Supplementing Synergen™ to soya protein concentrated (SPC) based diet and diets substituting 12.5% and 25% of soya protein concentrate (SPC) with white lupin meal significantly (*P<0.05*) increased final weight, weight gain, specific growth rate (SGR), feed intake (FI), feed conversion efficiency (FCE), protein efficiency ratio (PER), energy retention (ER) and apparent net nitrogen utilization (ANPU) and decreased feed conversion ratio (FCR). On the other hand, including 12.5% of white lupin significantly (*P<0.05*) improved growth performance and feed utilization affect growth performance and feed utilization but including 25% of white lupin did not significantly (*P<0.05*) affect growth performance and feed utilization.

The final mean body weights of fish at the end of the 10 weeks feeding trial ranged from 42.12 g (L25 diet) to 62.24 g (L12.5s Diet). This amounted to a >300% increase in biomass gain. Mean feed intake (FI) and protein intake (PI) per fish over the course of the experiment were (*P<0.05*) significantly better by fish fed the L12.5s diet, than fish fed SPC, L12.5, L25, L25 diets.

The percentage of WG, SGR, FCR and FCE reflected growth performance across treatments. Carp received the L12.5s diet showed the highest weight gain (WG), specific growth rate (SGR), feed conversion efficiency (FCE) and protein efficiency ratio (PER) which were significantly (*P<0.05*) higher than carp received SPC, L12.5, L25 and L25s diets even significantly higher than carp received diet SPCs. The highest final body weight (62.24g) was recorded in carp received (L125s) diet which was significantly (*P<0.05*) higher than carp received (SPC, SPCs, L12.5, L25 and L25s) diets.

Carp receiving (L12.5s Diet) showed the highest weight gain (46.84g fish$^{-1}$) which was significantly (*P<0.05*) higher than carp received SPC, SPCs, L12.5, L25 and L25s diets. Specific growth rate (SGR) value for all groups ranged from 2% day$^{-1}$ (L12.5s Diet) to 1.43% day$^{-1}$ (L25Diet). The highest SGR was recorded in carp received (L125s) which was significantly (*P<0.05*) higher than carp received (SPC, SPCs, L12.5, L25 and L25s diets). The highest feed efficiency ratio (65.24%) was recorded in carp received (L125s) which was significantly (*P<0.05*) higher than carp received (SPC, SPCs, L12.5, L25 and L25s) diets. Feed conversion ratio values for all groups ranged from 2.14 (SPC Diet) to 1.53 (L12.5s Diet). The lower FCR of 1.53 was recorded in the carp received L12.5s diet which was significantly (*P<0.05*) superior compared with carp received SPC, SPCs, L12.5, L25 and L25s diets. The PER values further confirmed the above trend with respect to the productive value of dietary protein. Carp fed the L12.5s diet produced a PER of 1.52 which was significantly higher (*P<0.05*) than the fish fed the SPC, SPCs, L12.5, L25 and L25s diets. Apparent net protein utilization (ANPU) seems to corroborate the other parameters measured; no appreciable effect was evident on protein retention efficiency for all dietary treatments fed to carp. The highest (ANPU) of was recorded on the fish received L12.5s diet which was significantly (*P<0.05*) higher than fish received SPC, SPCs, L12.5, L25 and L25s diets. The lowest ANPU was recorded in fish received L25 which was significantly (*P<0.05*) lower than fish received SPCs, L125s, L25s, L12.5 diets, but was not significantly different (*P>0.05*) from fish received SPC diet. The condition factor (K) value for all groups ranged from 2.3% (L12.5sDiet) to 2.17% (L25Diet). No significant (*P>0.05*) difference was found among all groups of in the condition factor (K). Substitution of soya protein concentrate (SPC) up to 25% with white lupin meal

did not significantly (*P>0.05*) affect final weights, specific growth rate (SGR), feed conversion ratio (FCR), feed efficiency ratio (FER), protein efficiency ratio (PER), energy retention and apparent net nitrogen utilization (ANPU). The highest energy retention was recorded in the fish received L12.5s diet which was significantly (*P<0.05*) higher than fish received SPC, SPCs, L12.5, L25 and L25s diets. The lowest energy retention was recorded in fish received L25 which was significantly (*P<0.05*) lower than fish received SPCs, L125s, L25s, L12.5 diets, but was not significantly different (*P>0.05*) from fish received SPC diet.

Table 4.3 Growth performance and feed utilization of carp fed the experimental diets. (n=2)

| Parameters | Diet | 0 | S | LSD |
|---|---|---|---|---|
| IW (g) | SPC | 15.68±0.36$^{a1}$ | 15.4±0.1$^{a1}$ | 1.08 |
| | L12.5 | 15.2±0.09$^{a1}$ | 15.4.0.06$^{a1}$ | |
| | L25 | 15.36±0.27$^{a1}$ | 15.06±0.54$^{a1}$ | |
| FBW (g) | SPC | 43.92±1.01$^{a1}$ | 53.84±0.73$^{a2}$ | 3.18 |
| | L12.5 | 44.2±0.76$^{a1}$ | 62.24±0.41$^{b2}$ | |
| | L25 | 42.12±0.94$^{a1}$ | 52.36±1.17$^{a2}$ | |
| WG (g) | SPC | 28.24±0.64$^{a1}$ | 38.44±0.62$^{a2}$ | 2.34 |
| | L12.5 | 29±0.85$^{a1}$ | 46.84±0.34$^{b2}$ | |
| | L25 | 26.76±0.66$^{a1}$ | 37.3±0.63$^{a2}$ | |
| SGR (%day$^{-1}$) | SPC | 1.47±0.00$^{a1}$ | 1.78±0.00$^{a2}$ | 0.05 |
| | L12.5 | 1.52±0.03$^{b1}$ | 2.00±0.00$^{b2}$ | |
| | L25 | 1.44±0.00$^{a1}$ | 1.78±0.01$^{a2}$ | |
| FI (g fish$^{-1}$) | SPC | 60.71±1.54$^{a1}$ | 68.48±1.01$^{ab2}$ | 3.74 |
| | L12.5 | 58.38±0.61$^{a1}$ | 71.79±0.06$^{b2}$ | |
| | L25 | 57.32±1.25$^{a1}$ | 64.80±0.98$^{a2}$ | |
| FCR | SPC | 2.14±0.00$^{b2}$ | 1.77±0.00$^{b1}$ | 0.05 |
| | L12.5 | 2.01±0.03$^{a2}$ | 1.53±0.01$^{a1}$ | |
| | L25 | 2.14±0.00$^{b2}$ | 1.73±0.00$^{b1}$ | |
| FCE (%) | SPC | 46.51±0.11$^{a1}$ | 56.13±0.06$^{a2}$ | 1.56 |
| | L12.5 | 49.64±0.94$^{b1}$ | 65.24±0.42$^{b2}$ | |
| | L25 | 46.67±0.14$^{a1}$ | 57.55±0.10$^{a2}$ | |
| PI (g fish$^{-1}$) | SPC | 24.29±0.61$^{a1}$ | 29.62±0.44$^{ab2}$ | 1.54 |
| | L12.5 | 24.36±0.25$^{a1}$ | 30.26±0.02$^{b2}$ | |
| | L25 | 23.88±0.52$^{a1}$ | 26.97±0.41$^{a2}$ | |
| PER | SPC | 1.15±0.00$^{ab1}$ | 1.29±0.00$^{b2}$ | 0.046 |
| | L12.5 | 1.18±0.02$^{b1}$ | 1.52±0.02$^{c2}$ | |
| | L25 | 1.11±0.00$^{a1}$ | 1.37±0.00$^{a2}$ | |
| ANPU | SPC | 17.86±0.04$^{a1}$ | 20.06±0.02$^{a2}$ | 0.55 |
| | L12.5 | 18.69±0.33$^{b1}$ | 24.06±0.13$^{c2}$ | |
| | L25 | 17.32±0.05$^{a1}$ | 21.27±0.04$^{b2}$ | |
| LER | SPC | 6.19±0.01$^{a1}$ | 7.67±0.00$^{b2}$ | 0.21 |
| | L12.5 | 6.72±0.12$^{b1}$ | 8.57±0.06$^{c2}$ | |
| | L25 | 6.28±0.017$^{a1}$ | 7.45±0.014$^{a2}$ | |
| ER (%) | SPC | 2.48±0.00$^{a1}$ | 2.98±0.00$^{a2}$ | 0.07 |
| | L12.5 | 2.59±0.04$^{b1}$ | 3.43±0.01$^{c2}$ | |
| | L25 | 2.46±0.01 $^{a1}$ | 3.05±0.00$^{b2}$ | |
| K- Factor (%) | SPC | 2.26±0.05 $^{a\ 1}$ | 2.29±0.02 $^{a1}$ | 0.14 |
| | L12.5 | 2.21±0.02 $^{a\ 1}$ | 2.30±0.02 $^{a1}$ | |
| | L25 | 2.17±0.02 $^{a1}$ | 2.28±0.06 $^{a1}$ | |
| Survival (%) | SPC | 100.00$^{a1}$ | 100.00$^{a1}$ | |
| | L12.5 | 100.00$^{a1}$ | 100.00$^{a1}$ | |
| | L25 | 100.00$^{a1}$ | 100.00$^{a1}$ | |

Data are presented as mean ± S.E. a, b data with the same superscripts with the same column are not significantly different (*P>0.05*); 1, 2 data with the same superscript with the same row are not significantly different (*P>0.05*).

## 4.4.2 Carcass composition

The terminal carcass composition of fish fed the experimental diets is presented in Table 4.4. Compared with the initial values, the whole-body composition of fish at the end of the trial showed higher lipid and energy contents, and lower moisture, ash and protein contents. Supplementing Synergen™ did not significantly (*P>0.05*) affect whole-body moisture and protein contents. The lowest moisture content (75.36*%*) was recorded in the fish received L12.5s diet which was significantly (*P<0.05*) lower than fish received L25 diet and fish fed other experimental diets did not display any significant difference in body moisture content. No significant (*P<0.05*) difference was found in whole-body protein content of fish fed the different experimental diets. Supplementing Synergen™ to diets substituting 12.5% and 25% of soya protein concentrate (SPC) with white lupin meal significantly (*P<0.05*) increased whole body lipid content but this trend was not significant (*P>0.05*) with supplement Synergen™ to SPC based diet. The highest whole body lipid content was recorded in fish received L12.5s diet which was significantly higher than fish received L12.5 and L25 diets. Supplementing Synergen™ to soya protein concentrated (SPC) based diet significantly (*P<0.05*) decreased whole body ash content but this trend was not significant (*P>0.05*) with supplementing Synergen™ to diet that substituted 12.5% and 25% of soya protein concentrate (SPC) with white lupin meal. The lowest whole body ash content was recorded on the fish received SPCs diet which was significantly (*P<0.05*) lower than fish fed SPC, L12.5, L25 and L25s diets. Whole body energy content was significantly (*P<0.05*) increased by supplement Synergen™ to diet that substituted 12.5% of soya protein concentrate (SPC) by white lupin meal but this trend was not significant (*P>0.05*)

with supplement Synergen™ to SPC based diet and diet contained 25% of white lupin. Finally, there were no significant (*P>0.05*) differences between SPC and L25 in whole body moisture, protein, lipid, energy and ash contents.

Table 4.4: Carcass composition of initial and final carp fed the experimental diets

| Parameters | Diet | Initial | 0 | S | LSD |
|---|---|---|---|---|---|
| Moisture (%) | SPC | 77.97±0.13 | 76.02±0.19$^{a1}$ | 75.64±0.08$^{a1}$ | 0.80 |
| | L12.5 | | 76.09±0.21$^{a1}$ | 75.36±0.19$^{a1}$ | |
| | L25 | | 76.40±0.20$^{a2}$ | 75.58±0.35$^{a1}$ | |
| Crude protein (%) | SPC | 14.47±0.02 | 15.25±0.09$^{a1}$ | 15.17±0.03$^{a1}$ | 0.42 |
| | L12.5 | | 15.25±0.09$^{a1}$ | 15.28±0.12$^{a1}$ | |
| | L12.5 | | 15.02±0.20$^{a1}$ | 15.09±0.08$^{a1}$ | |
| Crude lipid (%) | SPC | 4.74±0.02 | 7.03±0.20$^{a1}$ | 7.62±0.09$^{a1}$ | |
| | L12.5 | | 6.65±0.11$^{a1}$ | 7.65±0.15$^{a2}$ | 0.71 |
| | L25 | | 6.58±0.22$^{a1}$ | 7.49±0.30$^{a2}$ | |
| Ash (%) | SPC | 2.45±0.02 | 1.96±0.05 $^{a2}$ | 1.72±0.02 $^{a1}$ | 0.12 |
| | L12.5 | | 1.90±0.03 $^{a1}$ | 1.82±0.02 $^{ab1}$ | |
| | L25 | | 1.95±0.01 $^{a1}$ | 1.92±0.04$^{b1}$ | |
| Gross energy (MJ kg$^{-1}$)* | SPC | 23.76±0.01 | 25.09±0.14$^{b1}$ | 25.22±0.07$^{a1}$ | 0.5 |
| | L12.5 | | 24.39±0.15$^{a1}$ | 25.06±0.21$^{a2}$ | |
| | L25 | | 24.76±0.12$^{ab1}$ | 24.87±0.05$^{a1}$ | |

Data are presented as mean ± S.E. a, b data with the same superscripts with the same column are not significantly different (*P>0.05*); 1, 2 data with the same superscript with the same row are not significantly different (*P>0.05*).

*Dry matter basis.

## 4.4.3 Histological examination

### 4.4.3.1 Gut histology

Normal morphology was observed in carp mid and hind gut. However, some slides showed slight autolysis of the mucosa (Figure 4.1). The results of the histological examinations of the villus length, villi width, number of goblet cells and lamina properia width of the mid gut and posterior gut as well as microvilli density and microvilli length of posterior gut are presented in Table 4.5. Substituting 12.5% and 25 % of soya protein concentrate with white lupin marginally increased villi length in the posterior and mid gut but this increase was not deemed significant. Substituting 12.5% and 25 % of soya protein concentrate with white lupin did not significantly affect the number of goblet cells in the posterior gut. However, substituting 25 % of soya protein concentrate with white lupin significantly increased the number of goblet cells in the mid gut. On the other hand, no statistical ($P<0.05$) differences in the villi width, lamina properia width and micro villi density were observed in the either of mid gut or posterior gut regarding with substitution 12.5% and 25% of soya protein concentrate with white lupin. On the other hand, substituting 25% of soya protein concentrate with white lupin significantly increased microvilli length. Villi length in the mid gut was significantly ($P<0.05$) increased by supplement Synergen™ to SPC based diet but this trend was not deemed significant with supplement Synergen™ to diets that contained 12.5% and 25% of white lupin. However, supplementing Synergen™ to SPC based diet and diets containing 12.5% and 25% of white lupin marginally increased villus length in the posterior gut but this increase was not deemed significant. Villi width in the posterior and mid gut was significantly

(*P<0.05*) increased by supplementing Synergen™ to diet including 25% of white lupin but this increase was not deemed significant with supplementing Synergen™ to SPC based diet and diet that included 12.5% of white lupin. Lamina properia width in the posterior gut was significantly (*P<0.05*) increased by supplement Synergen™ to diets that included 12.5% and 25% of white lupin but this increase was not deemed significant with supplement Synergen™ to SPC based diet. Additionally, supplementing Synergen™ to SPC based diet and diets containing 12.5% and 25% of white lupin significantly increased lamina properia width in the mid gut. Supplementing Synergen™ to SPC based diet and diets that contained 12.5% and 25% of white lupin increased the number of goblet cells in mid gut and posterior but this increase was not deemed significant. Supplementing Synergen™ generally increased microvilli density but this increase was not deemed significant. Supplementing Synergen™ to diet that included 25% of white lupin did not significantly affect the microvilli length.

Table 4.5 Intestinal morphology of fish fed on the experimental diets for 10 weeks. (*n = 6*)

| Parameters | Region | Diet | 0 | S | LSD |
|---|---|---|---|---|---|
| Villi length (µm) | Posterior gut | SPC | 507.21± 26$^{a1}$ | 608.41±80$^{a1}$ | 163.5 |
| | | L12.5 | 509.08±15$^{a1}$ | 644.56±64$^{a1}$ | 9 |
| | | L25 | 602.06±64$^{a1}$ | 603.09±24$^{a1}$ | |
| | Mid gut | SPC | 440.71±12$^{a1}$ | 612.78±14$^{a2}$ | 100.7 |
| | | L12.5 | 530.50±23$^{a1}$ | 596.86±34$^{a1}$ | 6 |
| | | L25 | 517.53±44$^{a1}$ | 598.04±30$^{a1}$ | |
| Goblet cells per (100 µm) | Posterior gut | SPC | 3.60±0.26$^{a1}$ | 3.77±0.28$^{a1}$ | 0.99 |
| | | L12.5 | 3.08±0.40$^{a1}$ | 3.19±0.27$^{a1}$ | |
| | | L25 | 2.98±0.09$^{a1}$ | 3.59±0.21$^{a1}$ | |
| | Mid gut | SPC | 3.7±0.30$^{a1}$ | 3.11±0.23$^{a1}$ | 0.73 |
| | | L12.5 | 3.77±0.16$^{a1}$ | 4.31±0.16$^{b1}$ | |
| | | L12.5 | 4.61±0.14$^{b1}$ | 4.61±0.14$^{b1}$ | |
| Villi width (µm) | Poterior gut | SPC | 111.42±3.19$^{a1}$ | 111.72±2.83$^{a1}$ | 13.08 |
| | | L12.5 | 111.99±5.43$^{a1}$ | 123.08±2.36$^{ab1}$ | |
| | | L25 | 106.91±4.15$^{a1}$ | 125.32±3.86$^{b2}$ | |
| | Mid gut | SPC | 106.90±3.14$^{a1}$ | 120.85±4.73$^{a1}$ | 18.47 |
| | | L12.5 | 117.53±3.51$^{a1}$ | 119.53±6.91$^{a1}$ | |
| | | L25 | 107.10±7.28$^{a1}$ | 128.53±4.99$^{a2}$ | |
| Lamina Properia Width (µm) | Poterior gut | SPC | 27.44±1.87$^{a1}$ | 31.03±1.47$^{a1}$ | 5.92 |
| | | L12.5 | 26.37±1.53$^{a1}$ | 35.22±1.81$^{a2}$ | |
| | | L25 | 26.76±1.57$^{a1}$ | 32.68±1.92$^{a2}$ | |
| | Mid gut | SPC | 29.99±1.13$^{a1}$ | 35.99±0.46$^{a2}$ | 4.54 |
| | | L12.5 | 33.54±1.48$^{a1}$ | 40.64±0.97$^{b2}$ | |
| | | L25 | 31.28±1.44$^{a1}$ | 38.97±1.62$^{ab2}$ | |
| Microvilli density* | Poterior gut | SPC | 1.52±0.04$^{a1}$ | 2.21±0.34$^{a1}$ | 0.72 |
| | | L12.5 | 1.85±0.2$^{a1}$ | 1.59±0.19$^{a1}$ | |
| | | L25 | 1.18±0.08$^{a}$ | 1.66±0.18$^{a1}$ | |
| Microvilli length (µm) | Poterior gut | SPC | 1.12±0.02$^{a}$ | | |
| | | L12.5 | | | |
| | | L25 | 1.3±0.06$^{b}$ | 1.34±0.06$^{b}$ | |

Data presented as mean ± S.E.; a, b data with the same superscripts with the same column are not significantly different (*P>0.05*) and data with the different superscripts with the same column are significantly different (*P<0.05*). 1, 2 data with the same superscript with the same row are not significantly different (*P>0.05*) and data with the different superscript with the same row are significantly different (*P<0.05).*

* Arbitrary unit.

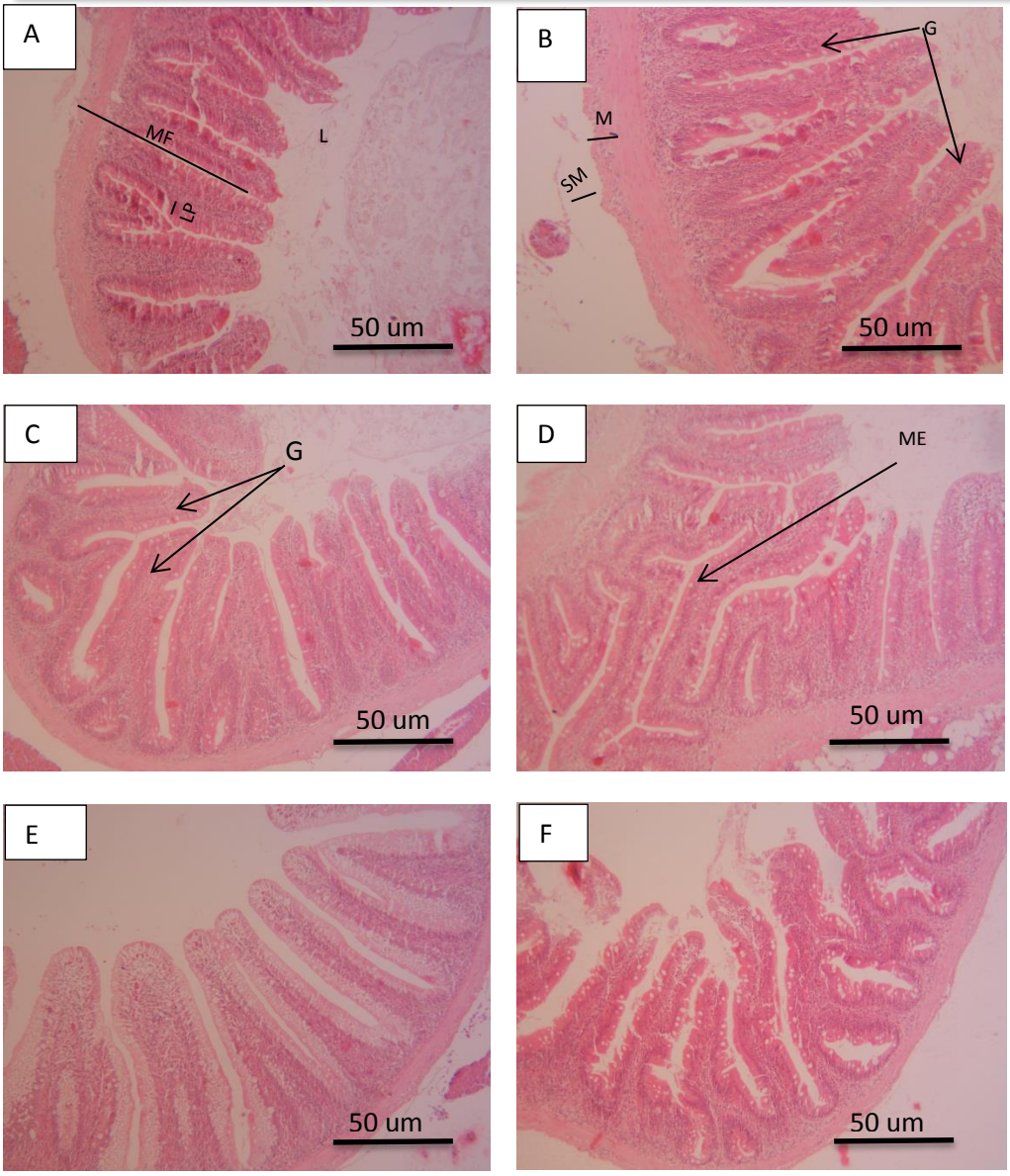



Figure 4.1 Haematoxylin and eosin stained section of the mid gut of mirror carp (Scale bar 50µm). (A) fish fed SPC diet, (B) fish fed SPCs, (C) fish fed L12.5, (D) Fish fed L12.5s, (E) fish fed L25 and (F) fish fed L25s (Scale bar = 50µm). L: Lumina, LP: Lamina propria, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells

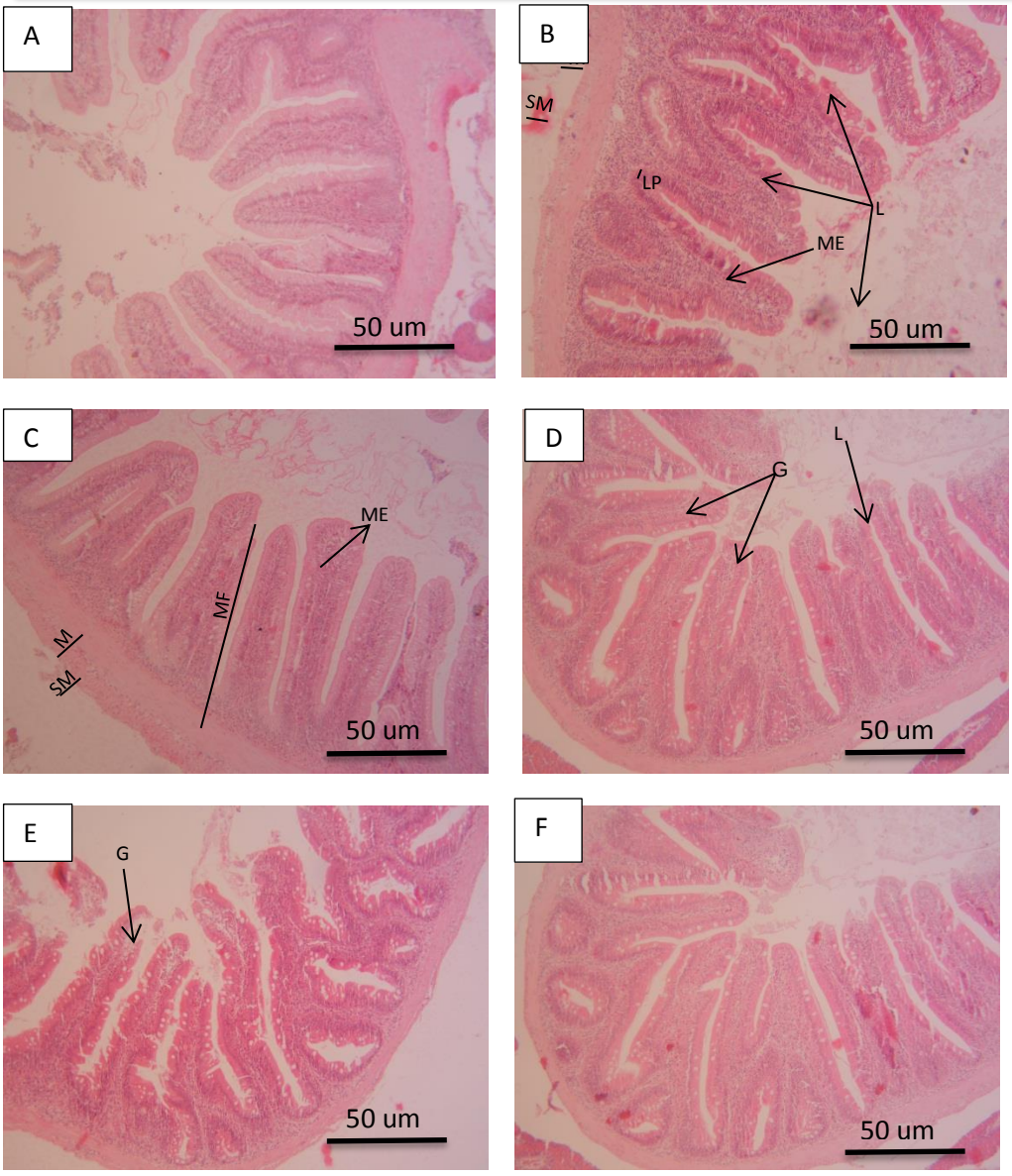



Figure 4.2 Haematoxylin and eosin stained section of the posterior gut of mirror carp (Scale bar 50µm). (A) fish fed SPC diet, (B) fish fed SPCs, (C) fish fed L12.5, (D) Fish fed L12.5s, (E) fish fed L25 and (F) fish fed L25s (Scale bar = 50µm). L: Lumina, LP: Lamina properia, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells

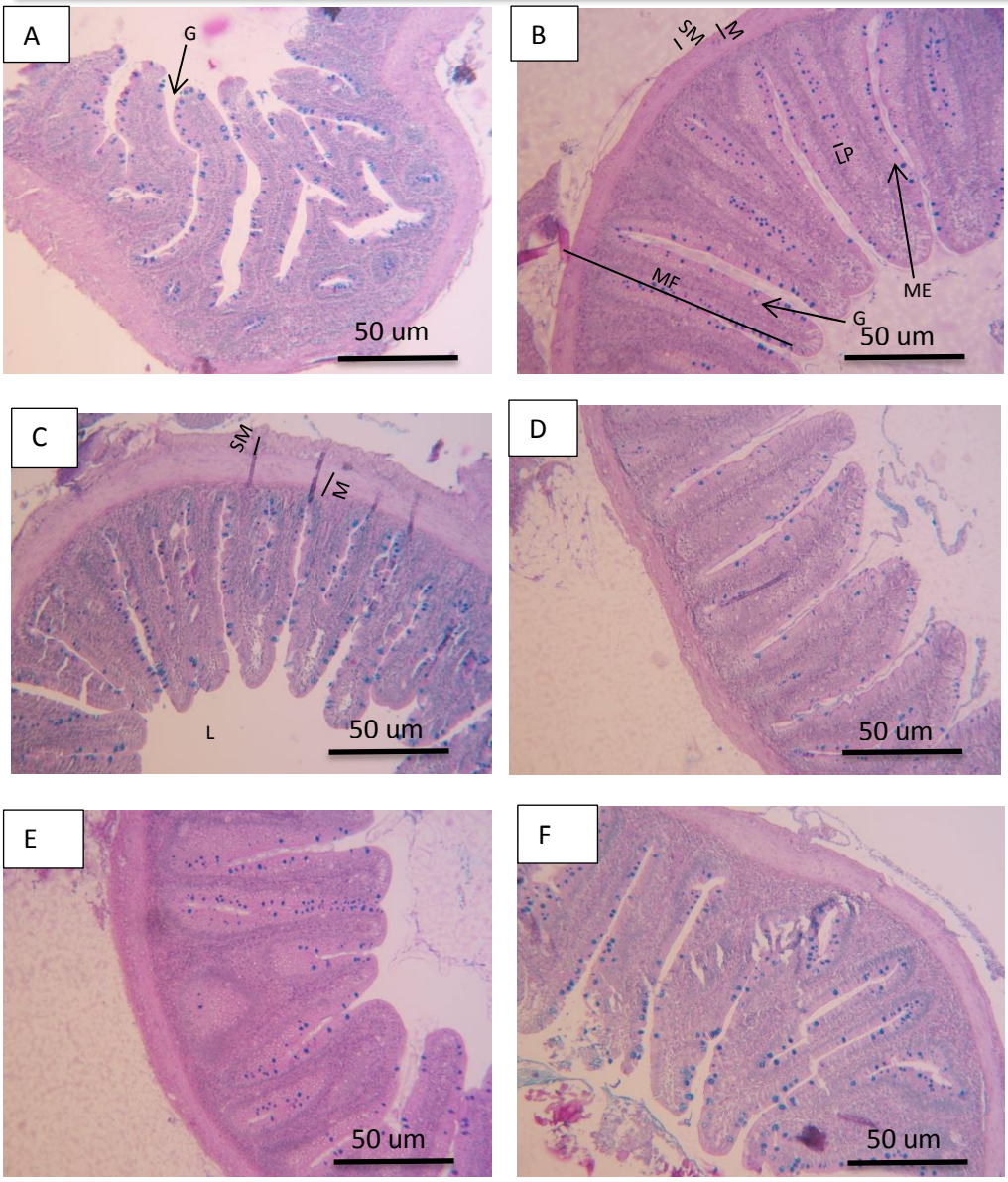



Figure 4.3 Alcian blue and PAS stained section of the mid gut of mirror carp (x10) (Scale bar 50µm). (A) fish fed SPC diet, (B) fish fed SPCs, (C) fish fed L12.5, (D) Fish fed L12.5s, (E) fish fed L25 and (F) fish fed L25s (Scale bar = 50µm). L: Lumina, LP: Lamina properia, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells.

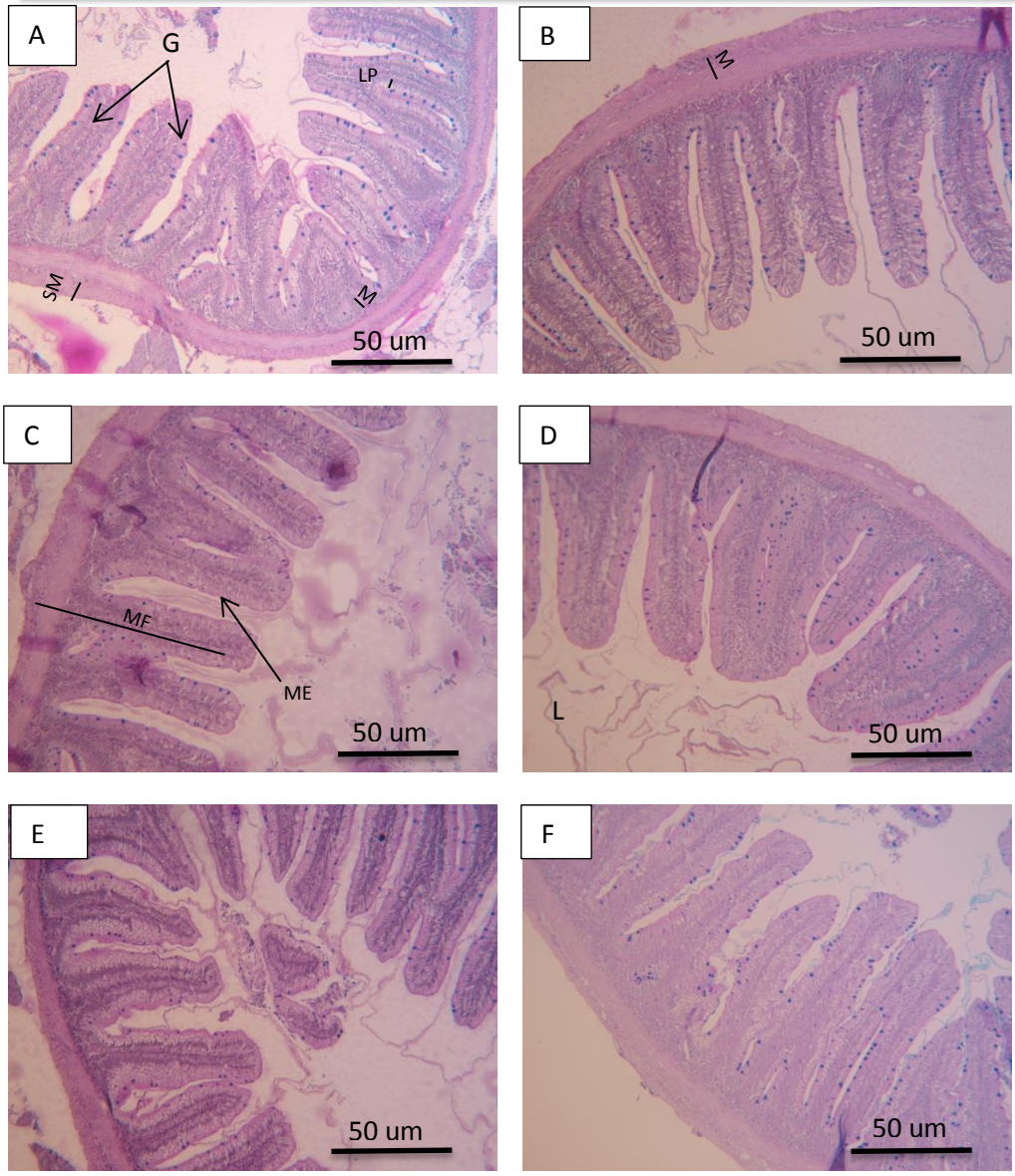



Figure 4.4 Alcian blue and PAS stained section of the hind gut of mirror carp (x 10). (Scale bar 50µm). (A) fish fed SPC diet, (B) fish fed SPCs, (C) fish fed L12.5, (D) Fish fed L12.5s, (E) fish fed L25 and (F) fish fed L25s (Scale bar = 50µm). L: Lumina, LP: Lamina properia, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells

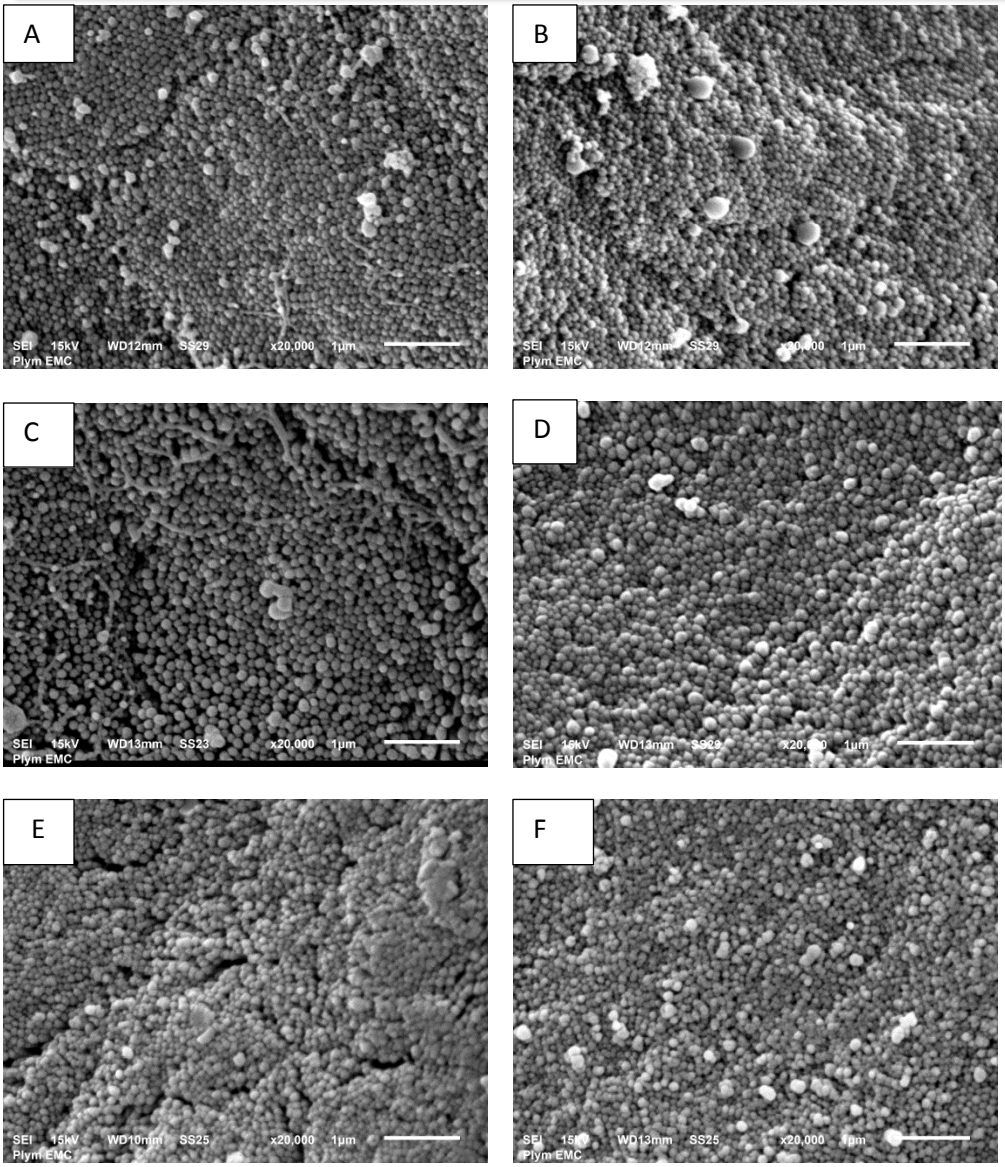



Figure 4.5 Comparative SEM micrographs of posterior intestine of mirror carp fed. (A) fish fed SPC diet, (B) fish fed SPCs, (C) fish fed L12.5, (D) Fish fed L12.5s, (E) fish fed L25 and (F) fish fed L25s. (Scale bar = 50 μm)

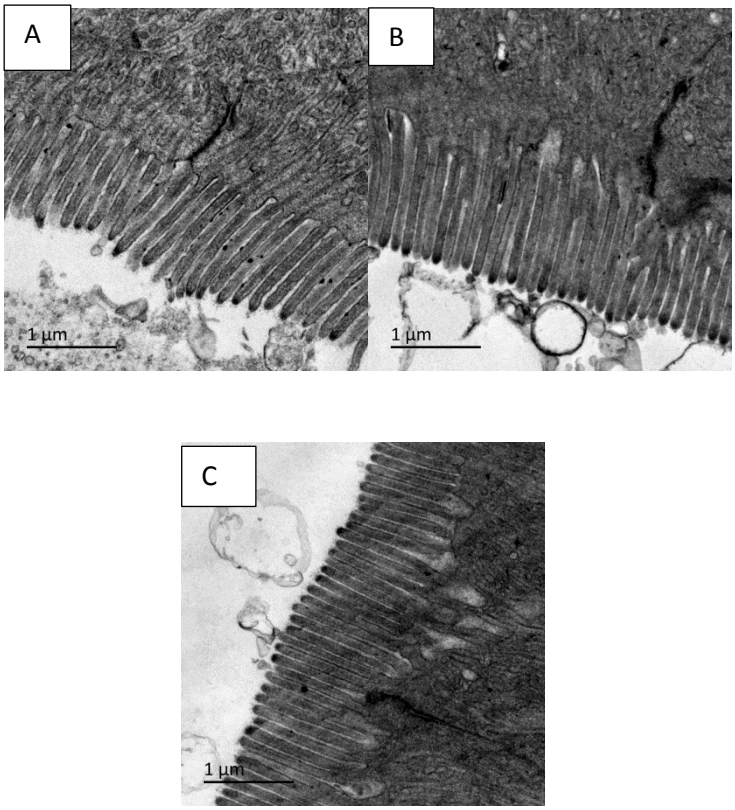



Figure 4.6 Comparative TEM micrographs of the posterior intestine of (A) fish fed SPC diet, (B) fish fed L25 and (C) fish fed L25s. MV: Microvilli.

### 4.4.3.2 Liver histology

The histology of liver tissues taken from the fish fed on the experimental diets is presented in Figure 4.5. Atrophy and necrosis of hepatic cells, vascular and fatty degeneration were generally observed. Hepatocyte size significantly increased by substitution 25% soya protein concentrate with white lupin but this trend was not significant (*P>0.05*) with 12.5% substitution level. Supplementing Synergen™ to diet containing 12.5% of white lupin significantly (*P<0.05*) increased hepatocyte size but this trend was significant in terms of soya protein concentrate based diet and diet containing 25% of white lupin. No significant (*P>0.05*) differences in hepatic nuclei size and the ratio of nucleus diameter to hepatocyte diameter were observed between groups (Table 4.6).

Table 4.6 Liver histological analyses of fish fed the experimental diets for 10 weeks. (*n* = 6)

| Parameters | Diet | 0 | S | LSD |
|---|---|---|---|---|
| Hepatocyte size (µm) | SPC | $11.76\pm0.24^{a1}$ | $12.27\pm0.44^{a1}$ | 1.13 |
| | L12.5 | $12.45\pm0.16^{ab1}$ | $13.65\pm0.30^{b2}$ | |
| | L25 | $12.94\pm0.29^{b1}$ | $13.02\pm0.41^{ab1}$ | |
| Nucleus size (µm) | SPC | $5.42\pm0.34^{a1}$ | $5.66\pm0.13^{a1}$ | 0.71 |
| | L12.5 | $5.9\pm0.14^{a1}$ | $5.94\pm0.11^{a1}$ | |
| | L25 | $5.85\pm0.18^{a1}$ | $5.76\pm0.17^{a1}$ | |
| Ratio of nucleus diameter to hepatocytes diameter (µm) | SPC | $46.05\pm2.41^{a1}$ | $46.69\pm2.32^{a1}$ | 6.04 |
| | L12.5 | $46.13\pm1.16^{a1}$ | $43.07\pm0.79^{a1}$ | |
| | L25 | $44.62\pm0.67^{a1}$ | $43.93\pm1.69^{a1}$ | |

Data presented as mean ± S.E.; a, b data with the same superscripts with the same column are not significantly different (*P>0.05*) and data with the different superscripts with the same column are significantly different (*P<0.05*). 1, 2 data with the same superscript iwith the same row are not significantly different (*P>0.05*) and data with the different superscript with the same row are significantly different (*P<0.05).*

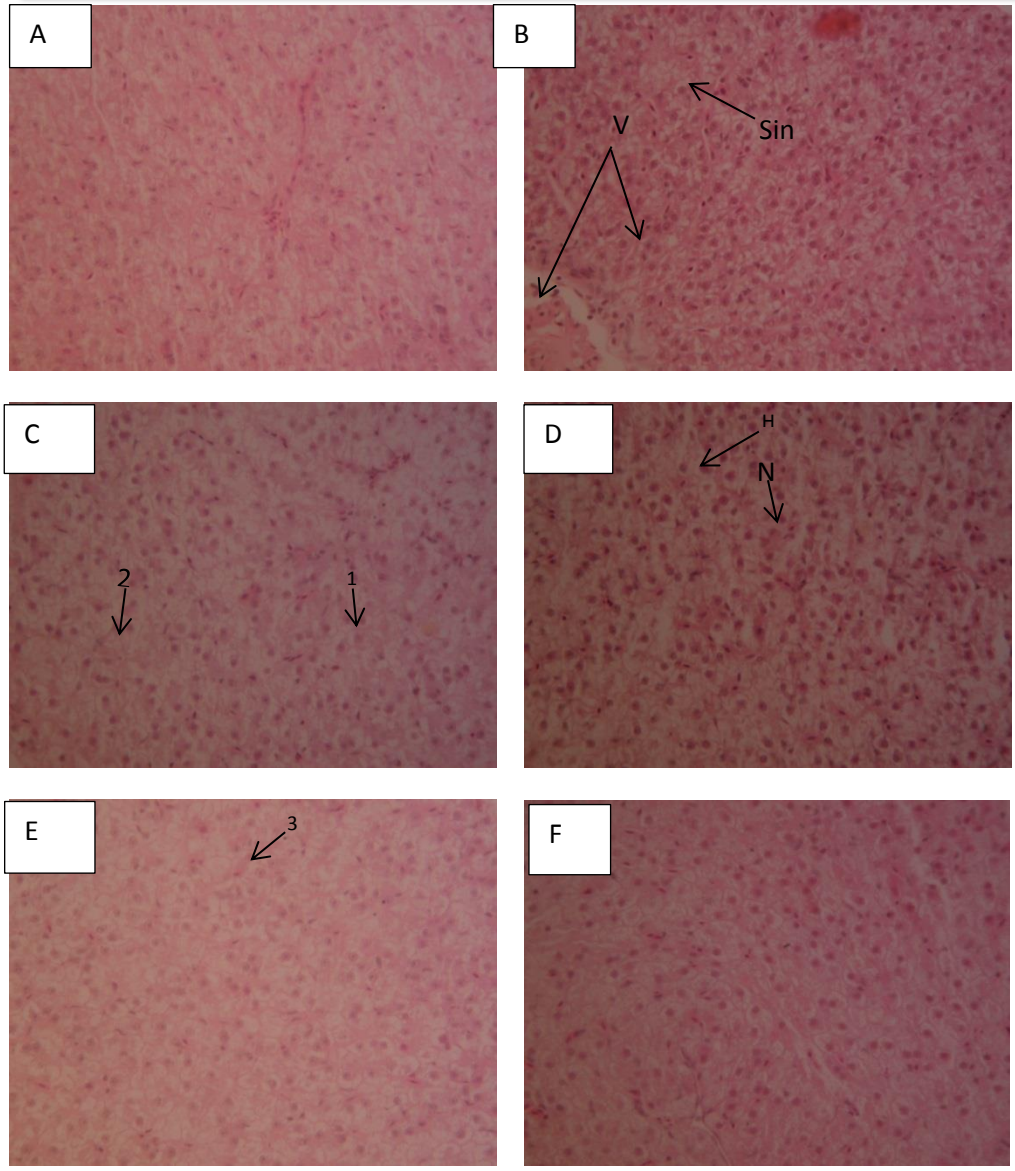



Figure 4.7 Haematoxylin and eosin stained sections of livers of mirror carp.1- Atrophy of nuclei 2- Atrophy of hepatic cells 3- Necrosis of hepatic cells (A) Fish fed SPC diet, (B) fish fed SPCs, (C) fish fed L12.5, (D) Fish fed L12.5s, (E) fish fed L25 and (F) fish fed L25s**.** H: Hepatocytes, Sin: Sinusoid, V: vacuolization, N: hepatic nuclei. (40x) (Scale bar = 100 µm)

### 4.4.4 Blood parameters

Haematological measurements for the different groups of fish are shown in Table 4.7. Haematocrit (Hct) is used as an indicator of animal health and is the percentage of packed blood cells to plasma volume. Haematocrit (Hct), significantly (*P<0.05*) increased by substituting soya protein concentrate with white lupin. Haemoglobin (g/dl) was significantly (*P<0.05*) increased with increasing substitution level of soya protein concentrate with white lupin. However, substituting 12.5% and 25 % of soya protein concentrate with white lupin did not significantly (*P>0·05*) of lymphocytes, neutrophils, monocytes, eosinophiles and basophils. Dietary supplementation of Synergen™ did not significantly affect haematocrit, haemoglobin and the number of neutrophils, monocytes, eosinophiles and basophils. However, the number of lymphocytes was significantly (*P<0.05*) increased by supplementing Synergen™ to diet that contained 12.5% of white lupin.

Table 4.7 Haematological parameters of common carp after 10 weeks of feeding on experimental diets. (*n*=6)

| Parameters | Diet | 0 | S | LSD |
|---|---|---|---|---|
| Haematocrit (%) | SPC | 33.83±2.89$^{a1}$ | 32.33±3.13$^{a1}$ | 8.49 |
| | L12.5 | 43.00±1.83$^{b1}$ | 43.33±0.65$^{b1}$ | |
| | L25 | 46.33±2.98$^{b1}$ | 41.5±1.38$^{b1}$ | |
| Haemoglobin (g/dl) | SPC | 7.66±0.26$^{a1}$ | 7.95±0.41$^{a1}$ | 1.43 |
| | L12.5 | 8.89±0.43$^{ab1}$ | 8.45±0.43$^{ab1}$ | |
| | L25 | 9.2±0.48$^{b1}$ | 9.45±0.24$^{b1}$ | |
| Lymphocytes (%) | SPC | 87.16±1.81$^{a1}$ | 87.66±1.40$^{b1}$ | 2.21 |
| | L12.5 | 88.83±1.91$^{a2}$ | 84.16±1.99$^{a1}$ | |
| | L25 | 87.16±1.02$^{a1}$ | 88.11±1.71$^{b1}$ | |
| Neutrophiles (%) | SPC | 3.25±0.88$^{a1}$ | 3.91±0.66$^{a1}$ | 3.04 |
| | L12.5 | 4.16±1.16$^{a1}$ | 5.08±0.89$^{a1}$ | |
| | L125 | 3.33±0.65$^{a1}$ | 4.08±0.62$^{a1}$ | |
| Monocytes (%) | SPC | 4.75±0.89$^{a1}$ | 4.66±0.62$^{a1}$ | 2.41 |
| | L12.5 | 3.25±0.35$^{a1}$ | 4.75±0.47$^{a1}$ | |
| | L25 | 4.5±0.85$^{a1}$ | 3.16±0.6$^{a1}$ | |
| Eosinophiles (%) | SPC | 3.58±0.61$^{a1}$ | 2.08±0.26$^{a1}$ | 2.45 |
| | L12.5 | 2.00±0.44$^{a1}$ | 4.08±1.00$^{a1}$ | |
| | L25 | 3.41±0.80$^{a1}$ | 3.05±0.64$^{a1}$ | |
| Basophile (%) | SPC | 1.25±0.30$^{a1}$ | 1.66±0.42$^{a1}$ | 1.65 |
| | L12.5 | 1.75±0.60$^{a1}$ | 1.91±0.59$^{a1}$ | |
| | L25 | 1.58±0.35$^{1a}$ | 1.58±0.35$^{a1}$ | |

Data presented as mean ± S.E.; a, b data with the same superscripts with the same column are not significantly different (*P>0.05*) and data with the different superscripts with the same column are significantly different (*P<0.05*). 1, 2 data with the same superscript with the same row are not significantly different (*P>0.05*) and data with the different superscript with the same row are significantly different (*P<0.05).*

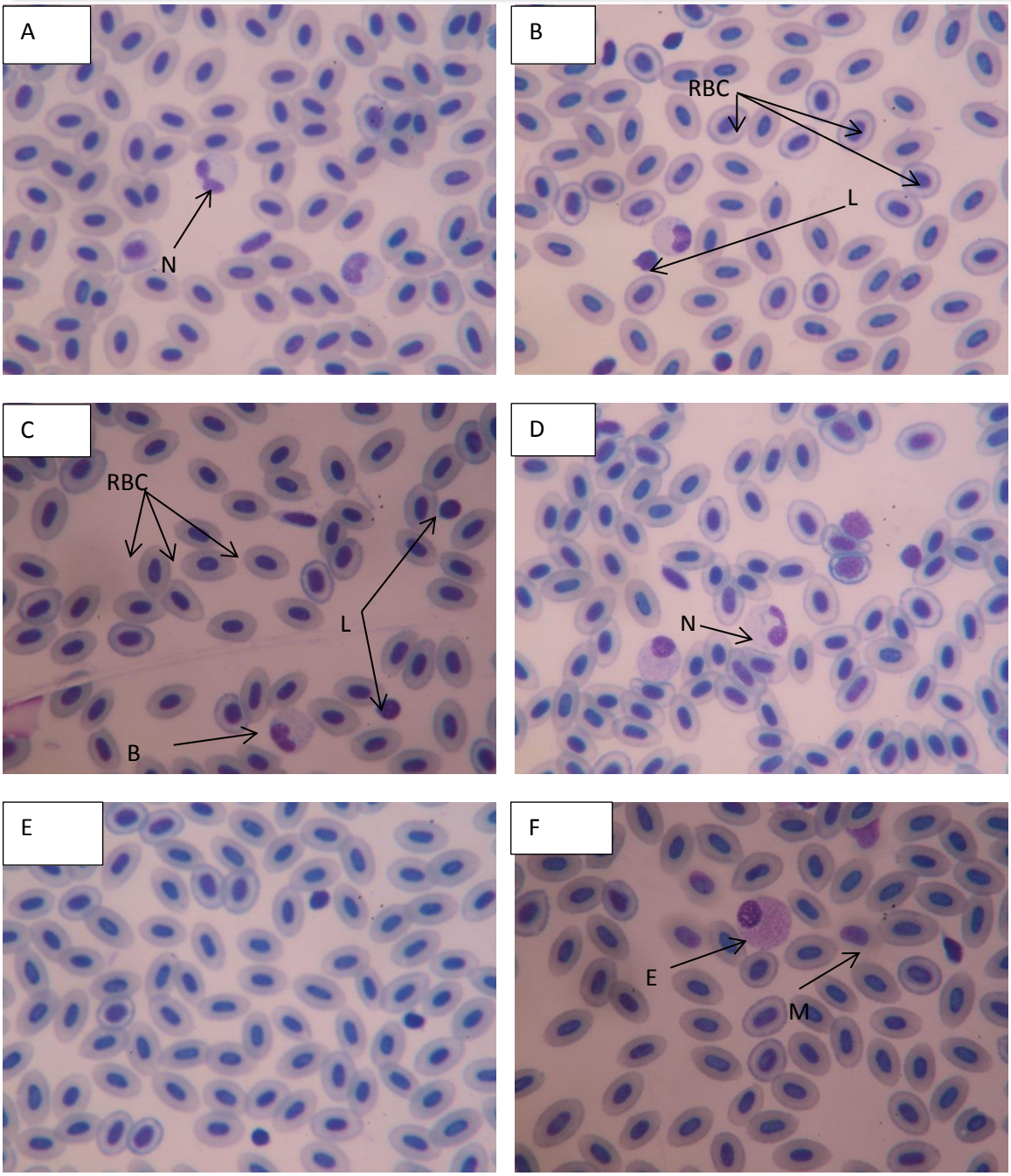



Figure 4.8 Blood cells of mirror carp. A: Red blood cells (RBC), lymphocytes (L), Neutrophil (N), Monocytes (M), Eosinophil (E) and Basophil (B): May Grunwald Giemsa stains. Scale bars: 50µm (A) fish fed SPC diet, (B) fish fed SPCs, (C) fish fed L12.5, (D) Fish fed L12.5s, (E) fish fed L25 and (F) fish fed L25s (100x)

## 4.4.5 Culture-based analysis

Log total viable counts and lactobacillus from digesta are shown in Tables 4.8. In general the lactobacillus populations were found to be higher in the fish fed diet with Synergen™ than the fish fed diet without Synergen; however, these differences were only between 0.1-0.29 log units in all cases, which were not significant (*P >0.05*). No significant differences of viable populations between the dietary groups were found.

Table 4.8 Log viable counts (CFU $g^{-1}$) from the digesta mirror carp. (n=4)

| Parameters | Diet | 0 | S | LSD |
|---|---|---|---|---|
| *Lactobacillus Sp* (CFU $g^{-1}$) | SPC | $5.46\pm0.20^{a1}$ | $5.74\pm0.20^{a1}$ | 0.78 |
| | L12.5 | $5.6\pm0.097^{a1}$ | $5.89\pm0.15^{a1}$ | |
| | L25 | $5.67\pm0.32^{a1}$ | $5.77\pm0.16^{a1}$ | |
| Total Viable counts ($CFUg^{-1}$) | SPC | $8.9\pm0.18^{a1}$ | $8.71\pm0.17^{a1}$ | 0.69 |
| | L12.5 | $8.91\pm0.19^{a1}$ | $8.28\pm0.09^{a1}$ | |
| | L25 | $8.65\pm0.24^{a1}$ | $8.83\pm0.19^{a1}$ | |

Data are presented as mean ± S.E. a, b data with the same superscripts with the same column are not significantly different (*P>0.05*); 1, 2 data with the same superscript with the same row are not significantly different (*P>0.05*).

## 4.5 Discussion

The results of this study demonstrated that soya protein concentrate (SPC) can be substituted with white meal up to 25% in the diet for juvenile mirror carp without any significant adverse effect on growth performance, feed utilization, body composition and general health of fish. Although most parameters of growth performance and feed utilization were slightly decreased with substitution of 25% of soya protein concentrate meal with white lupin meal, but this decrease was not significant. Reasons for this can be explained by lower organic matter digestibility of the white lupin meal relative to the soya protein concentrate. Furthermore, it is possibly reflective of the relatively high levels of non-starch polysaccharides in the lupin meal compare with soya protein concentrate meal. The results of the current study disagree with previous study with common carp was by Viola, Arieli & Zohar (1988) who indicated that total replacement of defatted soybean meal (45% protein) by whole-seed blue lupin meal based on 30% inclusion of blue lupin in common carp diet that contained 18% of defatted soybean meal significantly improved growth performance and feed utilization. This discrepancy may be due to higher protein content (57.5%) in soya protein concentrate that used in the current study compare with defatted soybean meal that used in pervious study. Indeed, Chien & Chiu (2003) were able to demonstrate that juvenile tilapia fed plant based diet performed well when up to 67% of soybean meal was replaced with blue lupin meal. On the other hand, Hughes, (1988; 1991) in two studies with rainbow trout found that full fat soybean meal can be totally replaced with lupin flour without any adverse effects. In the further study with seawater fish species was by Robaina *et al*. (1995) stated that soybean meal can be totally replaced by blue lupin meal based on the 10%, 20% and 30% inclusion level in diets for

juvenile gilthead seabream without any deleterious effect on growth performance and feed utilization.

This study showed that the growth performance and feed utilization of juvenile mirror carp significantly improved with the dietary supplementation of Synergen™ to soya protein concentrated (SPC) based diet and diets substituting 12.5% and 25% of soya protein concentrate (SPC) with white lupin meal. These results indicate that dietary supplementation of Synergen™ to a complete fish diet containing high level of plant protein sources can reduce the negative effects of anti-nutritional factors in plant based diets by ferment fibre and increase digestibility thus increase growth performance and feed utilization. It should be noted that the non-starch polysaccharides (NSP) constitute essentially the entire carbohydrate content of plant derived meals such as lupins and soybean, which are typically poorly utilized by fish. The improvement of growth performance and feed utilization by supplement Synergen™ to diets that substituted 12.5% and 25% of soya protein concentrate (SPC) with white lupin were higher than by supplementing Synergen™ to soya protein concentrated (SPC) based diet. The reasons for this can be explained by relatively high levels of non-starch polysaccharides in the lupin meal compare with soya protein concentrate meal. A lupin kernel meal contains about twice the amount of NSP as the relatively more than soybean meal (Van Barneveld, 1999).

The benefits of adding exogenous enzyme to improve growth performance and feed utilization for several warm water fish species plant based diets has been demonstrated to date. For example, Ng *et al*. (2002) demonstrated that supplementing commercial enzyme (Allzyme Vegpro™) to plant based diets that included 20% and 40% of palm kernel meal (PKM) for juvenile red hybrid tilapia

(*Oreochromis Sp*.) improved growth performance and feed utilization. Additionally, Sardar *et al*. (2007) reported that microbial phytase supplementation could improve growth, weight gain, feed utilization and survival in common carp soybean-based diet. Lin, Mai & Tan (2007) reported that supplement 0.1% commercially exogenous enzyme (neutral protease, b-glucanase and xylanase) into plant-based diets for juvenile hybrid tilapia can significantly improve growth performance and feed utilization. Moreover, these workers indicated that supplement exogenous enzymes can promote the secretion of the endogenous enzymes. Furthermore, these researchers investigated that solid- state fermentation of palm kernel meal PKM with cellulolytic fungus *Trichoderma koningii* (*Oudemans*) in plant based diets for juvenile red hybrid tilapia do not improve growth performance and feed utilization.

Indeed, the use of exogenous enzyme in cold water fish species has also received good attention. For example, Carter *et al*. (1994) observed favourable growth rates with supplementing proteolytic and carbohydrases enzymes for Atlantic salmon diets containing 34% of soybean. Additionally, Ai *et al*. (2007) in study with Japanese sea bass plant based diets found significant improve in growth performance and feed utilization with supplementing NSP enzymes (400 mg VP mainly includes glaucoma, pentosanase and cellulose, each with 50 IU per gram, 800 mg WX mainly includes xylanase, 1000 IU per gram, or the combination of 800 mg WX and 400 mg VP per kilogram diet). These later authors also confirmed the importance of supplementing exogenous enzyme to degrade the anti-nutritional effects of non-starch polysaccharide (NSP).

Nevertheless, the findings of this current study are in contrast with some findings. For example, Stone *et al*. (2003) did not observe any significant improvement in

dry matter, protein and energy digestibility with supplementing Natugrain-blend® [β-glucanase and β-xylanase at three nominal concentrations (0.75, 150 or 300µL $kg^{-1}$)] to silver perch diet that contained 30% of de-hulled blue lupin. Ogunkoya *et al*. (2006) investigated that supplement commercial enzyme cocktail (Superzyme CS) to soybean meal based diet containing up to 20% of soybean meal for rainbow trout does not improve growth performance and feed efficiency. Recently, Farhangi & Carter (2007) in study with rainbow trout were not able to find any significant improvement in growth performance by adding (Energex™), (Bio-Feed™ Pro), (Alpha galactosidase™) and (mixed of them) enzymes to the diet that contained 50% of de-hulled blue lupin.

Body composition of mirror carp juveniles was not significantly affected by substitute 12.5% and 25% of soya protein concentrate with white lupin in the present study. This coinciding with the results of other studies where white lupin meals were included in the rainbow trout diet (Burel *et al*., 1998; Bórquez *et al*., 2011a; Bórquez *et al*., 2011b; Zhang *et al*., 2012) and turbot (Burel *et al*., 2000a).

Whole body moisture, protein and lipid contents of mirror carp juveniles were not significantly affected by substituting 12.5% and 25% of soya protein concentrate with white lupin in the present study. This coinciding with the results of other studies where white lupin meals were included in the rainbow trout diet (Burel *et al*., 1998; Bórquez *et al*., 2011a; Bórquez *et al*., 2011b; Zhang *et al*., 2012) and turbot (Burel *et al*., 2000a). However, the whole body ash content significantly decreased by substitution 25% of soya proteins concentrates with white lupin. This observation is in agreement with (Burel *et al*., 1998; Bórquez *et al*., 2011a; Bórquez *et al*., 2011b; Zhang *et al*., 2012) findings with rainbow trout. On the

other hand, supplementation Synergen™ to soya protein concentrate based diet and diets that substituted 12.5% and 25% of soya protein concentrated by white lupin did not affect the whole body moisture and protein contents. This observation is in agreement with (Farhangi & Carter, 2007) who indicated that supplement (EnergexTM), (Bio-FeedTM Pro), (Alpha galactosidaseTM); and (Mix)) exogenous enzymes to diet that contained 50% of de-hulled blue lupin for rainbow trout does not significantly affect whole body composition. However, whole body lipid content was significantly increased by supplement Synergen™ to diets that substituted 12.5% and 25% of soya protein concentrate (SPC) with white lupin meal. On the other hand, whole body ash content was significantly increased by adding Synergen™ to diets that substituted 12.5% and 25% of soya protein concentrate (SPC) by white lupin meal.

Histological analysis of the fish fed diets substituting 12.5% and 25% of soya protein concentrate with white lupin did not show any significant differences compared with soya protein concentrate based diet even slightly improved with substitution of white lupin. However, in the present study the length and width of villi, the width of lamina properia, and the number of goblet cells in the mid and hind gut and the microvilli density and length were generally increased by adding Synergen™ to soya protein concentrated based diet and diets substituting 12.5% and 25% of soya protein concentrate with white lupin. These results suggest that dietary supplementation of Synergen™ increase absorptive area in the mid and hind gut subsequently improve growth performance and feed utilization. This is in contrast with Marković *et al*. (2012) who found an inverse relationship between mucosal fold length and growth rate. Several authors have observed histological alterations in the intestine of fish fed high level of a plant based diet. Uran *et al*.

(2008) stated that common carp show signs of enteritis when fed high levels of soybean.

In the case of lupin meal, Farhangi & Carter (2001) observed that increasing dietary inclusions of blue lupin for rainbow trout (diet can slightly shorten the villus length. Furthermore, Bórquez *et al.* (2011a) found that inclusion of 40% and 50% of white lupin to rainbow trout diet led to histological changes in the mid intestine such as decrease the number of basophil granulocytes, distal displacement of enterocyte nucleus and an increment in lipid drops. On contrary, Glencross *et al.* (2004a) did not find any negative effect of the dietary inclusion of yellow lupin into rainbow trout on histology intestine. Furthermore, Serrano *et al*. (2012) did not find any lesions or abnormalities in the middle and distal intestine in rainbow trout fed with lupinine alkaloid.

Atrophy and necrosis of hepatic cells, vascular and fatty degeneration were generally observed. This is in agreement with, Bórquez *et al*. (2011a) who investigated that increasing levels of dietary white lupin in the rainbow trout diet leads a slight lipid infiltration into hepatocytes and enterocytes. On contrary, Robaina *et al.* (1995) did not find any alterations in lipid and glycogen storage in hepatocytes from gilthead seabream hepatocytes inclusion of up to 30% of de-hulled blue lupin seed meal. Bórquez *et al.* (2011b) did not find any changes in liver histology by inclusion up to 20% of white lupin in the diet for rainbow trout. Substitution of 12.5% and 25 % of soy protein concentrate with white lupin in the present study did not affect hepatocyte size, nucleus size and the ratio of nucleus size to hepatocyte size. On the other hand, supplementation Synergen™ to diets contained 12.5% and 25% of white lupin soya protein concentrate based diet generally increased hepatocyte size.

No abnormalities were observed in the morphology of blood cell in the present study. This is confirmed that the experimental fish were healthy. Haematologic evaluation can be useful in monitoring the health status of fish (Clauss, Dove & Arnold, 2008). Determining the packed cell volume (PCV) or haematocrit (Hct) can be useful in diagnosing disease. Haematocrit (Hct) and haemoglobin (g/dl) were significantly increased by substitution of 12.5% and 25% of soya protein concentrate with white lupin. On the other hand, supplementation Synergen™ to white lupin based diet slightly increased haematocrit (Hct), but did not affect haemoglobin. The results obtained in the present study is higher than the results was obtained by Sardar *et al*. (2007) with common carp soybean-based diet in case of haematocrit (Hct), and haemoglobin. Present observation is in agreement with finding by Bransden, Carter & Nowak (2001) who reported that inclusion 40% of de-hulled blue lupin for Atlantic salmon diet does not have any significant adverse effects on growth, immune function or blood chemistry and disease resistance. However, substitution of 12.5% and 25% of soya proteins concentrate with white lupin and dietary of supplementation Synergen™ did not significantly affect the number of lymphocytes, neutrophils, monocytes, eosinophiles and basophils.

## 4.6 Conclusion

In conclusion that soya protein concentrate (SPC) can be substituted by white lupin meal up to 25% in the mirror carp diet without any adverse effect on growth performance and feed utilization, body compositions haematological, histological and microbial status. Supplementing Synergen™ to a complete (balanced) diet containing plant protein sources significantly improves growth performance and feed utilization in carp (a warm water fish species). This auger well for consideration towards exploiting plant protein concentrates and cereal grains for other fish species of high economic importance such as sea bass, sea bream, turbot and tilapia.

# Chapter 5

## General discussion and conclusion

There have been numerous studies examining the use of lupins when fed to a variety of fish species as extensively reported in the literature by de la Higuera *et al*. (1988), Burel *et al*. (1998); Burel *et al*., (2000b), Farhangi & Carter (2001), Glencross *et al*. (2002b), Glencross *et al*. (2004a), Farhangi & Carter (2007), Glencross *et al*., (2007), Glencross *et al*. (2008a), Bórquez *et al*. (2011a), Bórquez *et al*. (2011b), Hernández *et al*. (2012) and Zhang *et al*. (2012) with rainbow trout . Carter & Hauler (2000) and Bransden, Carter & Nowak (2001) investigated the potential of lupins with Atlantic salmon. However, for marine species there have been limited investigations (Burel *et al*., 2000a; Burel *et al*., 2000b) with turbot, (Glencross *et al*., 2003; Pereira & Oliva-Teles, 2004) with seabream.

Only Viola *et al*. (1988) examined the use of whole seed blue lupin in the common carp diet. There have been relatively few studies examining fish growth directly comparing soybean meals and lupin meals when included in extruded diets for fish species. Furthermore, it should be noted that there is much less information available on the use of lupin in warm water fish species such as carp and tilapia.

On the other hand, there are several challenges associated with the use of lupins in fish feeds. These include imbalanced essential amino acid (EAA) composition (Glencross *et al*., 2003) and presence of anti-nutritive factors (ANFs) (Petterson, 2000; Francis *et al*., 2001). Furthermore, Glencross, Boujard& Kaushik (2003) reported that the presence of the ethanol-soluble fraction of the lupin meal has a

significant negative impact on the nutritional value of the organic matter and nitrogen-free extractive content of lupin meals. Ingredient processing, such as de-hulling (Glencross *et al*., 2007), ethanol extraction processing to remove the oligosaccharides and enzyme treatment (Glencross, Boujard & Kaushik, 2003; Farhangi & Carter, 2007) can, to some extent, reduce the ANFs in the seed. Additionally, genetic improvement of plants such as lupin has successfully produced varieties with lower contents of detractive alkaloids, which can lead to significant improvement of the palatability of these feed ingredients (Petterson, 2000). Indeed the use of selected varieties of lupin was the basis of this work and developments in genetics and crop science have helped to produce lupin varieties with lower ANFs levels and reduced alkaloid content.

Synergen™ is available on the market as a leading feed additive with many interesting properties that may go beyond its multi-enzyme characteristics. Adding Synergen™ is very economical because it is not relatively expensive (£9.70 per kg; £970 per tonne). The cost of adding it is 0.0048£ per kg diet at an inclusion level of 0.05% or 0.0097£ at an inclusion level 0.1%. It is produced by Alltech (USA) as a product of solid state fermentation biotechnology. By applying this exogenous enzyme (Synergen™) in plant protein-based fish diets, the digestibility of carbohydrate (soluble and non-soluble oligosaccharide) as well as availability of minerals, energy, protein and amino acids of fish diets was likely to have been increased, although no digestibility assessment was performed in this programme of research due to time and technical constraints associated with carp. Total-phosphorus discharged into water will also be reduced when total-phosphorus levels are lowered by changes in feed formulation and that the product contains an appreciable level of natural phytase expressed by the

*Aspergilus niger* inoculation process during the solid state fermentation process. In the present study, there was a marked improvement in fish growth and feed performance linked with the use of Synergen™.

Therefore, replacing portions of soybean with a lupin meal with supplementation of exogenous enzyme (Synergen™) in fish diets is a promising economical alternative for the aquaculture business. It will not only reduce fish diet costs, but also reduce environmental pollution.

Chapter 3 investigated the effect of total replacing soybean meal by white lupin based on inclusion 12.5% and 25% with the addition of 0.05% of Synergen™ into common carp BSD (Basal standard Diet) diet on growth performance, feed utilization and general fish health. The findings of this study indicated that extruded white lupin can be successfully incorporated up to a level of 25% as a soybean replacement into common carp BSD based diet. The decline in growth rate and feed conversion ratio observed with the inclusion of 25% of white lupin may be attributable to several factors. Firstly, it may be due to lower protein content in the diet included 25% of white lupin comparisons with BSD based diet because the diets used in this experiment were not formulated as isonitrogenous and isolipidic. Secondly, it is possibly reflective of the relatively high levels of oligosaccharides in the lupin meal compared with soybean meal and basal skretting diet (BSD) meal. Thirdly, reduced feed efficiency may be attributed to the low carbohydrate digestibility of the lupin seed meal.

Although diets that included lupin contained lower protein content compared with BSD diet, the protein efficiency ratio and apparent net protein utilization were significantly increased by including 12.5% and 25% of white lupin. The improving protein efficiency ratio and apparent net protein utilization by inclusion white lupin

could be attributed to high protein digestibility in lupin compare with soybean meal thus providing a more balanced essential amino acid (EAA) profile to carp. The higher level of protein digestibility of lupins compared with soybean meal when fed to rainbow trout has been investigated (Glencross & Hawkins 2004; Glencross, Rutherford & Hawkins, 2011).

Comparison of the results obtained in this study with previous studies to evaluate lupin inclusion in aquafeeds is more complicated because this current study is the first study to employ white lupin in carp experimental diets as a warm fresh- water fish species. Furthermore, the carp fed diets which were formulated as summit-dilution trial type in this study was unique and not commonly used in standard fish feeding trials although often used by commercial companies as a prerequisite step before commencing more traditional balanced trial with fish. The results of this study are disagreeing with some previous studies with different fish species. In rainbow trout, Hughes (1991) found that crude lupin could be used to replace equal amounts (40% of the diet) of full-fat soybean without negative effects on fish performance, while performance was better with de-hulled lupin than with the soybean diet. Glencross, Rutherford & Hawkins (2011) investigated the superiority of blue and yellow kernel lupin meals when included in the rainbow trout diet compare to soybean meal in growth performance and feed utilization.

On the other hand, the results of the present study, demonstrated that included 12.5% and 25% of white lupin to BSD based diet does not have any significant effect on whole body moisture, protein and energy contents, while whole body lipid content significantly increased by including 12.5% and 25% of white lupin into BSD based diet it is likely due to higher level of lipid in diets that included

12.5% and 25% of white lupin compare with BSD based diets. The whole body ash content significantly decreased with inclusion 25% of white lupin to BSD based diet. This is likely due to lower content of ash in the diet included 25% of white lupin compare with BSD based diet.

As we mentioned above lupin contains certain anti-nutritional factors which have negative effects on the nutritional value of lupins in fish feeds. In order to reduce the negative effects of anti-nutritional factors 0.05% of Synergen™ as an exogenous enzyme (Synergen™) was added to BSD based diet and diets that contained 12.5% and 25% of white lupin. Growth response and feed utilization were improved with exogenous enzyme (Synergen™) supplementation, this improvement was better with diet contained 12.5% of lupin compare with BSD based diet. It is suggested that the negative effects of anti-nutritional factors in plant ingredients were compensated to some extent by supplement Synergen™. The finding of this study is in agreement with some previous findings by several authors testing exogenous enzyme sources. Glencross, Boujard & Kaushik (2003) investigated that an addition of exogenous a-galactosidase to the blue lupin meal in rainbow trout diet significantly increases the digestibility of dry matter, nitrogen, organic matter and nitrogen-free extractive.

On the other hand, the whole body moisture and protein and ash contents were not significantly changed by the supplement Synergen™ to BSD based diet and diets that contained 12.5% and 25% of white lupin in the present study. This is a disagreement with (Farhangi & Carter, 2007) with rainbow trout.

Indeed, supplementation of Synergen™ to the BSD based diet in the summit dilution experiment significantly increased whole body lipid content; while supplement Synergen™ to diets that contained 12.5% and 25% of white lupin did

not affect whole body lipid content. This is indicated that Synergen™ is beneficial towards elevation of lipid digestibility in fish which is in agreement with (Farhangi & Carter, 2007; Lin, Mai & Tan, 2007; Dalsgaard *et al*., 2011) the findings. Supplementing Synergen™ to the diet that contained 12.5% of white lupin significantly increased the whole body energy content compare with BSD basal diet, and this may be due to the fermentation of fiber causing more release of energy.

Other more subtle benefits seemed to ensue related directly to gastro-intestinal integrity as demonstrated by the histological examination of the gut of carp fed the experimental diets. The feed additive may exert a positive effect on gut dynamics via interaction with the microbiota and enterocyte turnover in carp with enhancement of both mucosal fold depth and microvilli length.

Supplementation of diets with Synergen™ led to a measurable increase the villi length in the present study with juvenile carp and enhanced the absorptive area of the lumen/gut interface with a consequent likely increase in nutrient digestibility resulting in a significant improvement in growth performance and feed utilization. It should be noted however that although direct nutrient absorption was not determined using physiological methods, all experiments used a mass balance approach to assess the retention of energy, lipid and protein (Nitrogen) in carcasses fed each diet in turn and compared against feed intake (nutrient intake) and the initial nutrient status of fish at the start of the feeding trial.

Chapter 4 investigated the effect of replacing 12.5% and 25% of soya protein concentrate with white lupin with the addition of 0.1% of Synergen™ in more practical plant protein-based diet for mirror carp more representative of

commercial practice and essentially iso-proteic and iso- lipidic in terms of formulation protocol. This study served to evaluate the inclusion of lupin meal replacing soya protein concentrate with and without Synergen™. Effects on growth performance, nutrient utilization, body composition and haematological, histological and microbial status were reported.

Replacement 12.5% and 25% of soya protein concentrate (SPC) with white meal lupin did not significantly affect growth performance and feed utilization in the present study. Although it was not deemed to be significant, a slight decrease of the growth performance and feed utilization parameters were observed with substitution 25% soya protein concentrate meal by white lupin meal. This is possibly reflective of the relatively high levels of non-starch polysaccharides in the lupin meal compared with soya protein concentrate meal. The findings of the current study are in agreement with previous findings (Viola, Arieli & Zohar, 1988) with blue lupin in common carp, (Chien & Chiu, 2003) blue lupin on tilapia, (Hughes, 1988;1991) with rainbow trout and (Robaina *et al*., 1995) blue lupin with gilthead seabream.

On the other hand, the findings of the present study explored that supplementation with Synergen™ to a complete plant based diet for mirror carp did indeed improve growth performance and feed utilization markedly with much obvious benefits. The reasons for this improvement could be attributed to enhance fermentation of fibre and other non-starch polysaccharides (NSP's) and thus make them more digestible allowing greater release of nutrients such as protein, amino acids and digestible energy. Furthermore, addition of Synergen™ to complete plant based diets for mirror carp showed an elevated villi length and width, lamina properia width, increased number of goblet cells in the mid and hind

gut and improved microvilli density in the posterior gut. These will lead to increased absorptive area thus improving growth performance and feed utilization. Further evidence in this second trial demonstrated that supplementation of Synergen™ to a complete plant based diet for mirror carp increased hepatocyte size and nucleus size in the liver with improved liver architecture. This may explain a higher metabolic rate and liver performance also aiding the performance of fish.

Additionally, the population of lactobacillus which are used for fermentation of animal feeds in typical monogastric animals was increased with supplementing Synergen™. Increasing the population of lactobacillus in the digesta may assist in the increased efficiency of fibre fermentation within carp. These stomachless fish have a relatively long intestine and a rapid gut transit time for digesta thus limiting the capacity for nutrient digestion and absorption. Certainly the use of exogenous enzymes as feed additives could easily modulate the gut microbial ecology towards improved degradation of indigestible dietary fractions such as fibre and NSP's. It may even alter the viscosity of the gut digesta content with important consequences to intestinal transit time, digestion rate and nutrient assimilation efficiency. These in turn can affect appetite response and general feed intake, meal size and frequency in carp influencing growth rates and production.

Further work should address these points and we should also notice, that all experiments described in this thesis relate to juvenile pre-grow-out fish that are rapidly growing and appropriate to obtain reliable and fast performance data for comparing feeds and dietary compositional changes. It would be most important to extrapolate these preliminary findings to the full production cycle, leading to marketable weight category fish of several kilogrammes and also broodstock fish

of the same species. Globally there are other major carp species such as the Indian major carps, Chinese varieties as well as ornamental fish such as the Koi that must be tested for lupin inclusion and potential benefit of exogenous enzyme supplementation products. Synergen™ and other additives that contain a mixture of enzymes may operate in vivo at a preferred narrow temperature range and may not function adequately for temperate fish species at lower temperatures below 20ºC. It is therefore imperative that we evaluate many more scenarios that include a wide range of fish species such as tilapia, catfish spp and marine fish of importance such as sea bass, sea bream, turbot, various other flounders, cobia, barramundi, mahi mahi and tuna that are important for aquaculture. Work on salmon and trout should also be considered although these fish are reared under cold water conditions. There may be some benefit also of pre-treatment of plant by-products such a soybean meal and lupins with exogenous enzymes and also Synergen™ prior to their inclusion in compound feeds. The cost benefit analysis and technical problem associated with such processing methods must be critically assessed to make this a viable economic possibility. Presently, several laboratories are undertaking experiments with rainbow trout, salmon and tilapia to further extend this knowledge base for vastly improving the nutritional potential of vegetable derived protein concentrates and energy rich cereals for use in aquaculture feeds.

In summary, this program of research has shown the potential of using lupin meal as a soybean meal replacement in juvenile carp diets. Furthermore, this research program explored the potential of supplementary use of Synergen™ to reduce the negative effect of anti-nutritional factors, increase nutrient digestibility as well as improve growth performance and feed utilization of a plant based carp diet.

Further research is needed to illustrate the potential of supplementing Synergen™ to plant based diets for general use in aquaculture with information leading to their optimum deployment and application based on sound data from feeding trials and efficacy standards. This will serve to meet the necessary economic commercial and legislative requirements throughout the world that are now mandatory for transparency of the food chain and bi-security for modern consumer demands.

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# Appendix II

# Diploma supplement

# The University of Plymouth


# DIPLOMA SUPPLEMENT

**INFORMATION IDENTIFYING THE HOLDER OF THE QUALIFICATION**

Surname

Anwar

First Name(s)

Ayub

Date of Birth

15/06/1984

Student Reference Number

10340409

**INFORMATION ON THE LEVEL OF THE QUALIFICATION**

Level of the Qualification

Res.M (Research Masters) Degree

Mode of study (full time/part time/mixed mode)

Full time

Official length of the programme

2 years

Completion date

**INFORMATION IDENTIFYING THE QUALIFICATION**

Name of Qualification and title conferred

Res.M (Research Masters) Degree

Title of thesis

Inclusion of lupin meal and effect of exogenous enzyme supplementation in diets for carp, *Cyprinus carpio*

Names of Supervisors

Prof. Simon Davies

Dr. Daniel Merrifield

Name(s) of collaborating institution(s)

Language of thesis

English

**FURTHER INFORMATION**

Professional Status

**Official Stamp of the Graduate School**

**Diploma supplement Appendix 1**

**RESEARCH OUTPUTS**

Seminar/Conference/Performance presentations

| Title of Paper or Performance | Title of Meeting & location | Poster or Oral paper | Date | Published* or not published |
|---|---|---|---|---|
| Evaluation the effect of white lupin (*L. albus*) inclusion and dietary supplementation of Synergen™ on growth performance and feed utilization in diet for common carp (*Cyprinus carpio*) | Postgraduate Society Annual conference, Rolle Building, Plymouth University–UK | Poster | 11th March 2013 | Not published |
| Partial replacement of soya protein concentrate (SPC) meal by white lupin seed meal and Synergen™ supplementation in complete diets for juvenile mirror carp (*Cyprinus carpio*) | CARS (Centre for Agricultural and Rural Sustainability) Annual Conference, Duchy College, UK | Poster | 19th June 2013 | Not published |
| Evaluation the effect of white lupin (*L. albus*) inclusion and dietary supplementation of Synergen™ on growth performance and feed utilization in diet for common carp (*Cyprinus carpio*) | LUKAA Project Management Board meeting, Roland Levinsky Building, Plymouth University-UK | Oral Presentat-ion | 20th Februar y 2013 | Not published |

## RESEARCH SKILLS

### Subject Specific Skills Training completed

| Course Title | Module Code | Date Attended | Performance Pass/Dist or % | Credit rating and level |
|---|---|---|---|---|
| Postgraduate Research Skills & Methods | BIO5124 | Oct. 2011-Feb. 2012 | 66.50 | 20 |
| Health and Production in Aquaculture | BIO5129 | Oct. 2011-Dec. 2011 | 53.60 | 20 |
| Principles and Applications in Electron Microscopy | BIO5102 | Oct. 2011-Dec. 2011 | 61.00 | 10 |
| Academic English Programme for Research Students | ELC006 | Jan 2011-Sep. 2011 | | A |

### Other Skills Training

| Title | Location | Organisers | Date Attended |
|---|---|---|---|
| Postgraduate English language support | Business School (Cookworthy Building) Plymouth University- UK. | English Language Centre | Oct. 2012-Jun. 2013<br>1h per week |

Verified by Director of Studies

Name …….. Signature …… Date ……

**University of Plymouth Transcript**

PIONEER WITH PLYMOUTH UNIVERSITY

Date: 27 June 2012
Date of Birth 15/06/1984
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Institution responsible for programme delivery: University of Plymouth
Language(s) of Instruction/Assessment: English
**ResM Biological Sciences Stage 1 - 2011/2012**

Credit achieved in this academic year: 50

Programme/Stage Aggregate Final Mark:

Award Board: 21-Jun-2012
Progression: A2/ Module results and any credit awarded are as indicated on the transcript.

| Module Code | Module Title | Credit | Level | Mark (%) | Att. | Result | Mod. Decn. |
|---|---|---|---|---|---|---|---|
| BIO5102 | Principles and Applications in Electron Microscopy | 10 | 7 | 61.00 | 1 | A | |
| | Coursework | | | 61.00 | | | |
| BIO5124 | Postgraduate Research Skills and Methods | 20 | 7 | 66.50 | 1 | A | |
| | Coursework | | | 66.50 | | | |
| BIO5129 | Health and Production in Aquaculture | 20 | 7 | 53.60 | 1 | A | |
| | Coursework | | | 53.60 | | | |

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Faculty Business Manager

# Evaluation of White lupin (*L.albus*) Inclusion and Synergen™ Supplementation on Growth Performance and Feed Utilization in Common carp (*Cyprinus carpio*)

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POSTGRADUATE SOCIETY WITH PLYMOUTH UNIVERSITY

## Introduction

Increasing demand, uncertain availability, and the high price of fishmeal with expansion of aquaculture have made it necessary to search for alternative protein sources. Alternatives to fishmeal are available from plant and animal protein sources as well as single–celled proteins (e.g. microalgae, bacteria and yeast. Lupin (*Lupinus Sp.*) is regarded as one of the legumes with potential, due to high protein content and low market price. Several recent studies have found that lupin seed meal (LSM) may be a good alternative plant protein source to fishmeal in diet for variety of aquaculture species. Lupins like all members of the legume plant family contain certain anti-nutritional factors (ANFs) that may have adverse effects on nutritional value and palatability of lupin in aquaculture diets. The positive effects of supplemental exogenous enzymes to reduce the negative effect of ANFs and improve growth performance and feed utilization in the fish diets containing plant ingredient have been investigated in many aquaculture species.

## Objectives

The purpose of the current study was to evaluate the effect of inclusion 12.5, 25% of white lupin (*L.albus*) as a soybean replacement and Synergen™ supplementation on growth performance, feed utilization and general health in juvenile common carp (*Cyprinus carpio*) diet.

## What is synergen™?

Synergen™ is a product of solid state fermentation of Aspergillus niger that contains residual enzyme activity. Synergen allows for a more flexible approach to feed formulation through the inclusion of by-products and alternative raw materials, or by reducing t he nutrient density and reducing nutrient constraints such energy, amino acid, phosphorus and calcium in the diet. It is used for pig, poultry and aquaculture diets.

## Materials and Methods

### Rearing conditions

- 12 fiberglass tanks (measuring 40×45×55 cm3), each provided with 99% re-circulated aerated freshwater at a rate of 300 L h-1freshwater with a total water capacity of 2223 L.
- Dissolved oxygen, temperature and pH were measured daily by HQ 40d Multi-parameter meter (HACH) Company, Loveland, USA).
- Temperature :25° C ±0.2
- Photoperiod:12 h light/12 h dark

Total ammonia nitrogen (TAN), nitrite and nitrate were measured weekly by the discrete automatic analyser (HACH LANGE, DR 2800 Germany).

### Experiment protocol

| | |
|---|---|
| Acclimation period | 88 days: Fish were fed by EWO 8 Sigma 60, fed at 1-2 % body weight per day as a maintenance diet during acclimation period. |
| Trial type | Summit dilution |
| Feeding regime | Restricted regime 3 %of body weight |
| Feeding frequency | 3 times manually |
| Number of treatments | Six treatments (two replicates) |
| Initial weight (g)<br>Final weight (g) | 18.28±0.36<br>68.66±4.2 |
| Statistic | SPSS statistics version 20 for windows ( SPSS Inc., IBM company, copy right 1989-2010). One-way ANOVA. Duncan's multiple ranged ad- Post hoc LSD test w 95 %confidence level (associated probability < 0.05). Standard deviation SD |
| Stocking density | 24 fish per tank |
| Duration of | 10 weeks |

### Parameters

(SGR %) =(lnFBW-ln IBW)/D×100
Feed Conversion Ratio (FCR g) =(FC ( g))/(IFW (g))
Feed Conversion Efficiency (FCE %) = WG/FI×100
Protein efficiency ratio (PER %)=WG/PI ×100
Apparent Net Protein Utilization (ANPU %) =((FBP-FBW)-(IBP-IBW))/PI×100
Mortality (%) =(Intial Nb-Final Nb)/Intial Nb×100
Survival (%)= 100–Mortality (%)=Final Nb/Intial Nb×100

Table 2 Formulation of experimental diets (% as DM) based on g/kg

| Ingredient | R1 | R2 | L12.5 | L12.5s | L25 | L25s |
|---|---|---|---|---|---|---|
| BSD | 733 | 734.5 | 867.5 | 867 | 735 | 734.5 |
| SBM | 245 | 245 | - | - | - | - |
| LPM | - | - | 122.5 | 122.5 | 245 | 245 |
| OIL | 20 | 20 | 20 | 20 | 20 | 20 |
| CMC | 2 | - | - | - | - | - |
| Synergen™ | - | 0.5 | - | 0.5 | - | 0.5 |

BSD= Basal starting diet , SBM= Soybean meal, L= Lupin meal, Oil= Fish oil/corn oil (1:1), Synergen ™, CMC= Carboxyl-methyl-cellulose, R1= Reference diet , R2= Reference diet with adding Synergin™, L12.5 =Diet contained 12.5% lupin, L12.5s= Diet contained 12.5% lupin with adding 0.05% Synergin™, L25= Diet contained 25% lupin

Table 3: Nutrient composition of experimental diets(%)

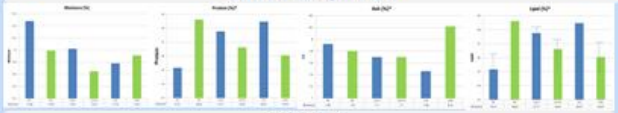
| Proximate analysis (%) | R1 | R2 | L12.5 | L12.5s | L25 | L25s |
|---|---|---|---|---|---|---|
| Moisture | 5.8±0.02 | 6.75±0.04 | 6.06±0.04 | 8.11±0.4 | 8.13±0.02 | 5.76±0.4 |
| Protein | 56.05±0.74 | 55.7±0.52 | 53.72±37 | 52.64±0.18 | 50.02±0.36 | 52.12±0.68 |
| Lipid | 8.93±0.09 | 8.91±0.3 | 10.95±0.05 | 10.7±0.33 | 12.46±0.15 | 11.45±0.06 |
| Ash | 8.26±0.38 | 8.1±0.32 | 7.93±0.45 | 8.06±0.38 | 6.92±0.3 | 7.22±0.67 |
| Gross Energy (MJ kg-1) | 20.2±0.04 | 19.93±0.11 | 20.02±0.007 | 19.95±0.13 | 20.84±0.86 | 20.41±0.11 |

## Results

Table 4:Growth performance and feed utilization

| Parameters | Experimental diets R1 | R2 | L12.5 | L12.5s | L25 | L25s |
|---|---|---|---|---|---|---|
| Initial weight (g) | 16.01±0.17$^{a}$ | 15.92±0.28$^{a}$ | 16.83±0.6$^{a}$ | 16.42±0.1$^{ab}$ | 16.36±0.09$^{ab}$ | 16.09±0.28$^{a}$ |
| Final weight (g) | 66.88±0.87$^{abc}$ | 69.8±1.56$^{bc}$ | 62.13±0.18$^{ab}$ | 64.43±0.73$^{a}$ | 64.13±0.66$^{a}$ | 63.93±2.26$^{a}$ |
| Weight gain (g) | 40.86±1.05$^{abc}$ | 43.73±2.06$^{bc}$ | 45.29±0.34$^{bc}$ | 48±0.84$^{c}$ | 37.77±0.76$^{a}$ | 37.84±1.98$^{a}$ |
| SGR(%) day | 1.01±0.04$^{abc}$ | 1.08±0.06$^{cd}$ | 1.86±0.01$^{cd}$ | 1.95±0.02$^{d}$ | 1.7±0.02$^{b}$ | 1.72±0.03$^{bc}$ |
| FCR | 1.46±0.02$^{b}$ | 1.36±0.02$^{a}$ | 1.38±0.02$^{a}$ | 1.33±0.02$^{a}$ | 1.62±0.02$^{c}$ | 1.49±0.02$^{bc}$ |
| FCE (%) | 68±1$^{ab}$ | 73±1$^{c}$ | 71±1$^{bc}$ | 74±1$^{c}$ | 65±1$^{a}$ | 66.5±0.5$^{a}$ |
| PER | 1.22±0.028$^{a}$ | 1.3±0.021$^{b}$ | 1.3±0.021$^{b}$ | 1.41±0.021$^{c}$ | 1.3±0.021$^{b}$ | 1.27±0.021$^{ab}$ |
| ANPU (%) | 19.33±0.37$^{a}$ | 21.13±0.43$^{bc}$ | 21.43±0.59$^{bc}$ | 21.77±0.26$^{c}$ | 21.07±0.28$^{bc}$ | 20.59±0.29$^{b}$ |
| Survival (%) | 97.92 | 97.92 | 95.84 | 95.84 | 97.92 | 100 |

### Carcass composition



### Histology

Table 5: Posterior Intestinal morphology of fish fed on the experimental diets for 10 weeks (*n = 4*

| | R1 | R2 | L12.5 | L12.5s | L25 | L25s |
|---|---|---|---|---|---|---|
| Villi length (µm) | 629.74±28 | 662.23±112 | 588.69±51 | 681.89±121 | 626.32±60 | 683.98±184 |
| Goblet cells (per 100 um) | 5.92±67$^{a}$ | 6.62±0.54$^{ab}$ | 6.22±0.75$^{ab}$ | 6.07±0.45$^{ab}$ | 7.65±1.34$^{b}$ | 6.93±0.99$^{ab}$ |

Table 6 Liver histological analyses of fish fed the experimental diets for 10 weeks. (*n = 4*)

| Parameter | R1 | R2 | L12.5 | L12.5s | L25 | L25s |
|---|---|---|---|---|---|---|
| Hepatocyte | 11.82±0.23 | | 11.65±0.39 | 11.58±0.62 | 11.73±0.25 | 11.08±0.22 | 11.75±0.48 |
| Nucleus | 4.39±0.14$^{ab}$ | | 4.73±0.35$^{b}$ | 4.11±0.03$^{a}$ | 4.37±0.03$^{ab}$ | 4.21±0.24$^{a}$ | 4.4±0.09$^{ab}$ |
| Ratio of nucleus diameter to hepatocytes diameter (µm) | 37.5±0.9$^{ab}$ | | 41.02±2.41$^{b}$ | 35.83±2.3$^{a}$ | 38.12±1.24$^{ab}$ | 38.8±2.61$^{ab}$ | 38.55±1.52$^{ab}$ |

## Conclusion

*Including white lupin (*L.albus*) up to 25% with supplement 0.05% syneregen™ do not have any significant negative effect on growth performance, feed utilization, carcass composition and gut and liver histology in juvenile common carp (*Cyprinus carpio*) diet.

* Supplement synergen™ is beneficial to improve growth performance and feed utilization of lupin in common carp.

**Acknowledgment:**

Special thanks for my supervisor Professor Simon Devis

Special thanks to my sponsor- Ministry of Higher Education, Kurdistan Regional Government.

**Reference**

Petterson, D. (2000) The use of lupins in feeding systems', *ASIANAUSTRALASIAN JOURNAL OF ANIMAL SCIENCES*, 13 (6), pp. 861-882

POSTGRADUATE SOCIETY WITH PLYMOUTH UNIVERSITY

RESEARCH WITH PLYMOUTH UNIVERSITY

# Certificate of Participation

This is to certify that

## Ayub Anwar

Presented a poster at the Postgraduate Society Annual Conference
11th March 2013
Plymouth University

Professor Mick Fuller
Head of Graduate School and
Director of Graduate Studies

Imane El Hakimi
Chair of the Postgraduate Society

POSTGRADUATE SOCIETY WITH PLYMOUTH UNIVERSITY

RESEARCH WITH PLYMOUTH UNIVERSITY

# Award Certificate

Best Poster

2nd prize

Ayub Anwar

Winner of the best poster at the
Postgraduate Society Conference
11th March 2013
Plymouth University

Professor Mick Fuller
Head of Graduate School

Imane El Hakimi
Chair of the Postgraduate Society

# Partial replacement of soya protein concentrate (SPC) meal by white lupin (*L.albus*) seed meal and Synergen™ supplementation in complete diets for juvenile mirror carp (*Cyprinus carpio*)

Ayub Anwar, Simon Davies, Denial Merrifield
School of Biomedical and Biological Science, Plymouth University, PL4 8AA, UK
ayub.anwar@plymouth.ac.uk

## Introduction

Plant proteins represent the major dietary protein source used within feeds for lower trophic level fish species (tilapias, carps, catfishes). Among plant proteins soybean meal is the most common used in compound aquafeeds, due to its high protein content and favourable amino acid profile. However, an increase use of soybean meal protein as a human food and terrestrial animal feeds has resulted in an increase market price of soybean globally. Therefore, replacement soybean protein with more economical, alternative sources of protein is imperative to sustain aquaculture production.

Lupin (*Lupinus Sp.*) is regarded as one of the legumes that having exciting potential as an aquaculture feed ingredient, due to high protein content and low market price. There are, however, still challenges associated with the use of lupin and soybean meals at high concentration in diets for fish. These include imbalanced essential amino acid (EAA) composition and presence anti-nutritive factors. Furthermore, lupins have low energy density due to high contents of carbohydrates such as starch, indigestible oligosaccharides and non-starch. Balanced amino acid composition can be obtained by supplementing limiting EAA to diet and ingredient processing, such as de-hulling and enzyme treatment can, to some extent, reduce the ANFs in the seed.

## Lupins

Lupins are plants belong to (*Lupinus*) genus, tribe Genisteae and family Leguminosae.

Blue lupin Yellow lupin White lupin

## Objectives

The aim of this study was to evaluate the effect of replacement 12.6%and 26%of soya protein concentrate (SPC) withe white lupin (*L.albus*) with addition of Synergen™ on growth performance, feed utilization and general health in complete diet for juvenile mirror carp (*Cyprinus carpio*).

## Synergen™

Synergen™ is a product of solid state fermentation of Aspergillus nigert that contains residual enzyme activity.

## Materials and Methods

### Rearing conditions

- 12 fibergiass tanks (measuring 40×45×55 cm3), each provided with 99% re-circulated aerated freshwater at a rate of 300 L h-1freshwater with a total water capacity of 2223 L.
- Dissolved oxygen, temperature and pH were measured daily.

- Temperature :25° C ±0.2 - Photoperiod:12 h light/12 h dark

Total ammonia nitrogen (TAN), nitrite and nitrate were measured weekly

- Stoking density: 25 fish per tank - Acclimation period : 75 days
- Duration of trial: 10 weeks - Feeding regime: Restricted regime 3 -5% of body weight - Feeding frequency: 3 times manually
- Number of treatments : Six treatments (two replicates)

## Diet Composition Chart


## Nutrient composition of experimental diets (%)

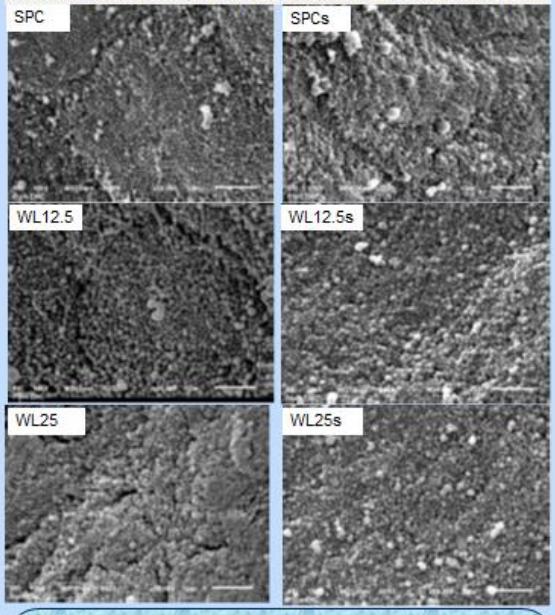
| Proximate composition (%) | SPC | SPCs | WL12.5 | WL12.5s | WL25 | WL25s |
|---|---|---|---|---|---|---|
| Moisture (%) | 6.53±0.16 | 5.27±0.09 | 6.64±0.03 | 5.12±0.1 | 6.45±0.03 | 6.18±0.02 |
| Protein (%) | 40.02±0.24 | 43.26±0.48 | 41.74±0.86 | 42.16±0.14 | 41.66±0.49 | 41.62±0.23 |
| Lipid (%) | 7.52±0.24 | 7.32±0.2 | 7.39±0.02 | 7.62±0.16 | 7.43±0.09 | 7.72 |
| Ash (%) | 6.2±0.07 | 6.27±0.02 | 6.12±0.04 | 6.09±0.08 | 5.73±0.27 | 6±0.03 |
| NFE (%) | 39.72±0.29 | 37.87±0.39 | 38.09±0.7 | 39±0.14 | 38.72±0.34 | 38.45±0.08 |
| Gross Energy (MJ kg-1) | 19.29±0.09 | 19.4±0.07 | 18.9±0.04 | 19.38±0.21 | 19.2±0.08 | 19.07±0.04 |

## Parameters

FBW (g)= final body weight (SGR %)= Specific growth rate, (FCR)= Feed conversion ratio, (FCE %)= Feed conversion efficiency, WG= weight gain, (PER %)= Protein efficiency ratio, (ANPU %) =Apparent Net Protein Utilization, LER= Lipid efficiency ratio, (ER %) = energy retention

## Results


## Scanning electron microscopy of posterior of fish fed experimental



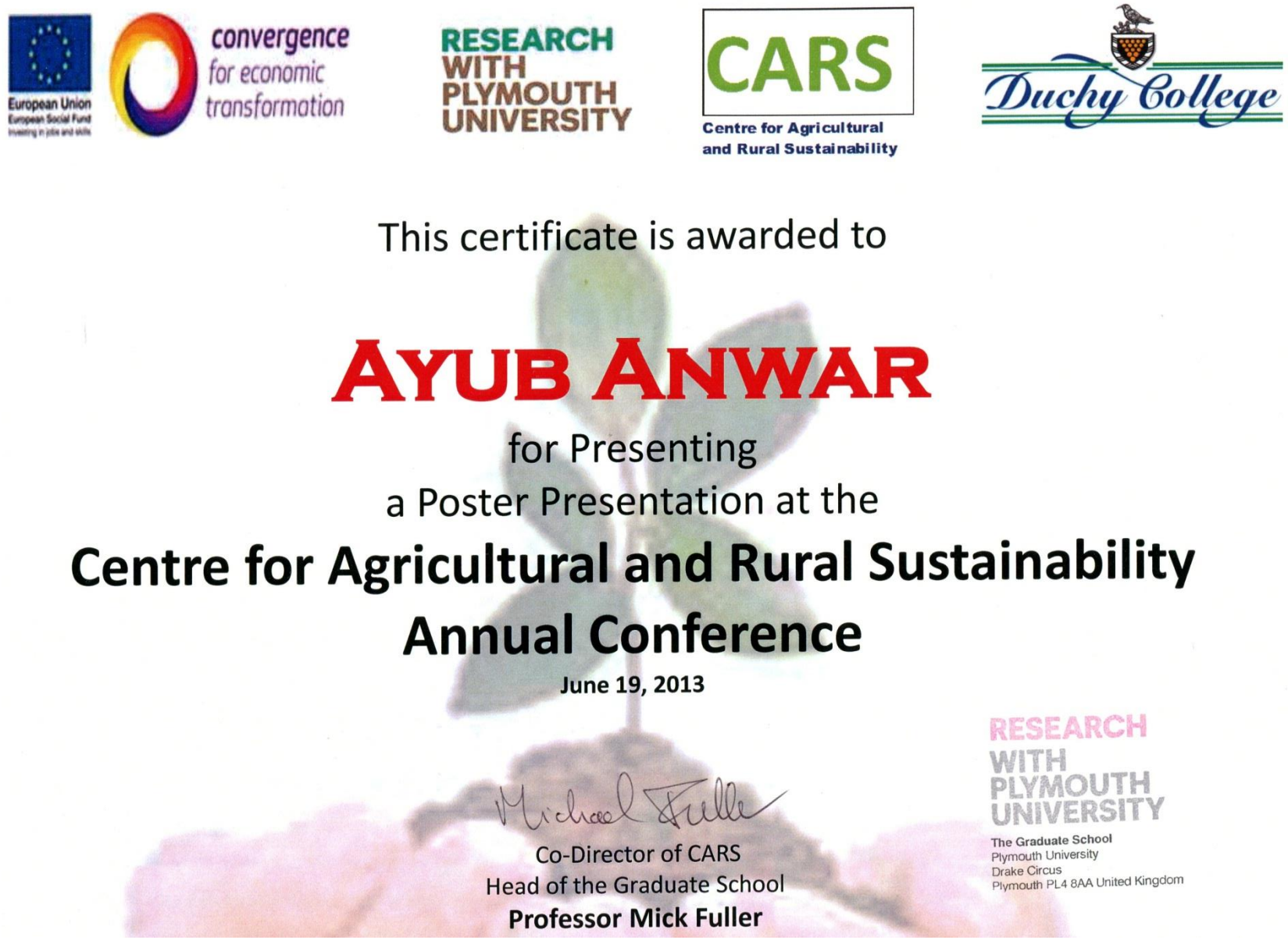

## Conclusion

1- The results of this study demonstrated that soya protein concentrate (SPC) can be substituted by white meal up to 25% in the diet for juvenile mirror carp without any significant adverse effect on growth performance, feed utilization, body composition and general health of fish.

2- Adding Synergen™ to a complete fish diet containing high level of plant protein sources can reduce the negative effects of anti-nutritional factors in plant sources by ferment fiber and increase digestibility with improve growth performance and feed utilization

## Acknowledgment:

Special thanks for my supervisor Professor Simon Davis
Special thanks to my sponsor- Ministry of Higher Education, Kurdistan Regional Government.



This certificate is awarded to

# AYUB ANWAR

for Presenting

a Poster Presentation at the

## Centre for Agricultural and Rural Sustainability Annual Conference

**June 19, 2013**

Co-Director of CARS

Head of the Graduate School

**Professor Mick Fuller**

RESEARCH WITH PLYMOUTH UNIVERSITY

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