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The evaluation of novel bio-ethanol derived co-products as potential feed ingredients for carp Cyprinus carpio and tilapia Oreochromis niloticus

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Copyright Statement

The evaluation of novel bio-ethanol derived co-products as potential feed ingredients for carp *Cyprinus carpio* and tilapia *Oreochromis niloticus*

By

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A thesis submitted to the University of Plymouth

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The evaluation of novel Bio-ethanol derived co-products as potential feed ingredients for carp *Cyprinus carpio* and Tilapia *Oreochromis nilitocus*

Samad S. Omar

Abstract

The nutritional value of novel yeast products were evaluated for warmwater fish species. A yeast co-product (yeast protein concentrate unrefined (YPCU)) obtained from a bio-ethanol process using wheat was tested using iso-nitrogenous (38% crude protein) and iso-lipidic (8%) diets for juvenile mirror carp (*Cyprinus carpio*). The fishmeal (FM) protein component of a basal diet was replaced by (YPCU) at 7.5, 15, 20, and 50% of total dietary protein. After an 8 week feeding trial, all fish fed YPCU yielded better growth performance than the control fed fish, with diets containing 15% and 20% YPCU being optimal. Whole body composition was unaffected by dietary treatment, however, ash levels were elevated in fish fed ≥15% YPCU. Hepatic alanine amino transferase (ALAT) and aspartate amino transferase (ASAT) were measured as bio-indicators of liver function in carp. Only ASAT activity was significantly lower for carp fed 20% and 50% YPCU. Additionally, histological assessment of liver and intestinal tissues gave no indication of impairment, but high YPCU inclusion (≥15%) elevated the number of goblet cells present in the posterior intestine. Molecular microbiological analysis using DGGE revealed no definitive changes in intestinal microbial communities.

In a second study, bio-ethanol yeast (refined YPC_R and unrefined YPC_U) and dried distillers grain with solubles (DDGS) a co-product of the bio-fuel process and distillery yeast from potable alcohol (whisky) production (YPC_{PA}) were evaluated as before for carp. FM was replaced with 30% of YPCU, YPCR and YPCPA and 15, or 30 % DDGS with a combination of 10% YPCR. Weight gain, and Apparent Net Protein Utilization

(ANPU%) were higher in fish fed YPC_U 30%, equivalent for fish fed FM, YPC_R 30%,DDGS 15% and DDGS 30%, and lower in fish fed YPC_{PA} 30% diets. Feed conversion ratio was significantly increased in carp fed YPC_U 30% and decreased for carp fed DDGS 30% and YPC_{PA} 30% compared with the control group.

A significant improvement of net mineral retention was seen for carp feed the yeast supplementation diets compared to the fishmeal control group. The YPC_U 30% diet produced the highest mineral retention in fish fed yeasts and the YPC_{PA} 30% gave lowest retention.

The microvilli density of the intestinal tract decreased for carp fed YPC_R 30%, but microvilli length significantly increased in fish fed YPC_U 30% compared with other groups, thus indicating changes in gut integrity.

In the third study, four diets were formulated to replace 0, 10, 20 and 30% of the fishmeal with refined yeast protein concentrate (YPC_R) for Nile tilapia (*O. niloticus*) of mean weight 12.39g. Growth performance and feed efficiency were not affected with up to 20% replacement with YPC_R. There were no obvious changes in the liver structure, but high yeast inclusion showed higher numbers of intestinal goblet cells with increasing YPC_R dietary inclusion suggesting enhanced intestinal integrity. Microvilli density and length was significantly ($P = 0.025$) improved with up to 10% and 30% YPC_R inclusion in comparison to other dietary treatments.

It was generally concluded that YPC co-products were effectively viable for both juvenile carp and tilapia offering an option for partial fish meal replacement.

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All experimental work involving animals was carried out in accordance with the 1986 Animals Scientific Procedures Act under the Home office project licence # 30/2644 and personal licence # 30/8745

Author's Declaration

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

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Dedication

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List of Abbreviations

ADF	Acid detergent fibre
ADG	Average daily gain
ADIN	Acid detergent insoluble nitrigen
ALAT	Alanine amino transferase
ANOVA	Analyses of variance
ASAT	Aspartate amino transferase
ANMU	Apparent net mineral utilization
ANPU	Apparent net protein utilization
AU	Arbitrary units
BLAST	Basic local alignment search tool
DCP	Digestable crude protein
DE	Digestable energy
DDG	Dried distillers grain
DGGE	Density gradient gel electrophoresis
DDGS	Dried distillers grain with soluble's
DDS	Dried distillers solubles
DM	Dry matter
DNA	Deoxyribonnucleic acid
EDTA	Ethylenediaminetetraacetic acid
EAA	Essential amino acid
EM	Electron microscopy
FCR	Feed conversion ratio
FM	Fishmeal
FO	Fish oil
GDFPD	General Directorate of Fish Production and Development
GI	Gastrointestinal
h	hour
Hb	Haemoglobin

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICP-MS	Inductively coupled plasma mass spectrometry
Hct	Haemotocrit
HSI	Hepatosomatic index
K-facter	Condition factor
KJ	Kilo Joules
KRG	Kurdistan Regional Government
L	litre
MCH	Mean Cellular Haemoglobin content
ME	Metaboloic energy
MCHC	Mean Cellular Haemoglobin Concentration
MCV	Mean Cellular Volume
min	minutes
MOAWR	Ministry of Agriculture and Water Resources
MOS	Mannan oligisaccharides
MS222	Tricane Methyle Sulphonate
NDF	Neutural detergent fibre
NEAA	Non-Essential aminoacid
NPN	Non-protein nitrogen
NRC	National Research Council
PCR	Polymerase chain reaction
PCV	Packed cell reaction
PER	Protein efficiency ratio
PUFA	Polyunsaturated fatty acid
RBCC	Red blood cells counts
RNA	Ribonucleic acid
rpm	Revolution per minutes
sec	second
SBM	Soybean meal
SEM	Scanning electron microscopy

SGR	Specific growth rate
t	Tonnes
TAN	Total ammonia nitrogen
TEM	Transmission electron microscopy
TSE	Transmissible Spongiform Encephalopathies
WDGS	Wet distillers dried grain
YPC	Yeast Protein Concentrates
YPC_U	Unrefined Yeast Protein Concentrates
YPC_R	Refined Yeast Protein Concentrates
YPC_{PA}	Potable alcohol Yeast Protein Concentrates

Chapter 1

General Introduction

1.1 Status of Aquaculture

Globally fisheries and aquaculture production has grown dramatically from less than 1 million metric tonnes in 1950 to 142.3 million tonnes in 2008, in which 90 million tonnes were from capture fisheries and 52.5 million tonnes from aquaculture in 2008 which was worth 98.5 billion USD. As such, there has been a very rapid expansion of this sector during the last 50 years. Currently, aquaculture is a major sector of world agribusiness. Indeed, aquaculture continues to be the fastest growing food producing sector (FAO, 2010). Aquaculture production in 2009 stood at 55.1 million tonnes contributing to 38% of the total world production of sea food (145.1 million tonnes) with a total value of USD 106 billion.

The contribution of aquaculture will account for over 50% of the global fish supply by 2020 if this sector continues to expand at the present rate. The global aquaculture system is dominated by the Asia – Pacific region 89% by weight and 79% by economic value, with China being the major player in this region. North and South America are accounted as second for 4.6% (production) with 9% (value), Europe 4.4% (production) with 9% (value), Africa 1.8 (production) with 2% (value) and Oceania 0.3% (production) with 1% (value). In terms of worldwide production, China dominated by accounting 62% in terms of weight and 51% of global value in 2008 (Bostock et. al., 2010).

Approximately 310 aquatic species (excluding aquatic plants) were reported by FAO as cultured in 2008 (Bostock et. al., 2010). It should be noted that the freshwater species play an important role in the world aquaculture industry as well as in annual regional finfish production. The total aquaculture production of fish was estimated to be 33,756, 303 tonnes with a value of 55,391,293 USD, from that, Cyprinids (i.e. carps, barbells, etc.) represent the largest group by output 20,593,403 tonnes (26,694,905 USD) followed by miscellaneous fresh water fish with a production of 5,359,290 tonnes (9, 799,669 USD) and cichlids (i.e. tilapias) with production placed at being the third

largest aquacultured fish group with 2,797,819 tonnes (4,021,164 USD) (FAO 2010). The world total carp production was accounted for 14,984,259 tonnes (19,801,433 USD) in 2008 (FAO 2010).

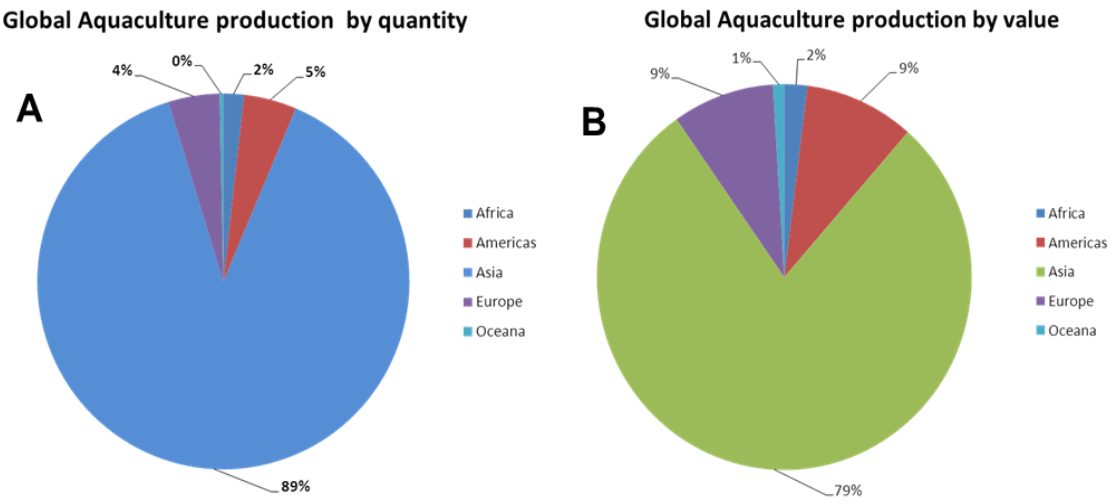


Figure 1.1 Global Aquaculture productions by region. A) Aquaculture by quantity 2008 (excluding aquatic plants). B) Aquaculture by value 2008 (excluding aquatic plants). Source (Bostock et al., 2010)

1.2 The status of fisheries and aquaculture in the Middle East

One of the major food sources for humans was fish in Mesopotamia 5000 years ago. Fish were considered to be one of the staple one of the foods of early human civilization in the Middle East (Sahrhage, 1999). There is historical evidence of traditional small-scale aquaculture activity based mainly on freshwater pond culture. Old earth fish ponds have been located in Jordan, Iraq, Iran and Yemen. However, in most cases the practices were limited to just holding fish or ongrowing wild-caught fry (FAO 1990).

In Mesopotamia (nowadays Iraq) aquatic animals including fish, crustaceans, molluscs and turtles were important commodities apart from milk and grain. They have been used as important sources of food in the early human civilization in Euphrates and Tigris

with their tributaries and Persian Gulf (Sahrhage, 1999). Cuneiform clay tablets from ancient Mesopotamia mentioned many kinds of fish in administrative documents in the period between the third millennium B. C. up until the first dynasty in Babylon. A Sumerian text described the habits and appearance of many species of fish in some detail in 2000 B. C. (Saggs, 1962).

At present, fisheries and aquaculture production in the Middle East countries (Bahrain, Iran, Iraq, Jordan, Kuwait, Oman, Qatar, Saudi Arabia, United Arab Emirate and Yemen) have been rapidly rising over the last five decades; the production of fish was estimated at 169,751 tonnes in 1970 (almost all of the production was by capture) and increased to 646,635 tonnes in 1990 and to 1.1 million tonnes in 2008. The largest producer of fisheries and aquaculture is Iran with a production of more than 50% of the region (562,281 tonnes), followed by Oman with a production of 13.3% (145,751 tonnes) and Yemen11.6% in production (127,132 tonnes) in 2008.

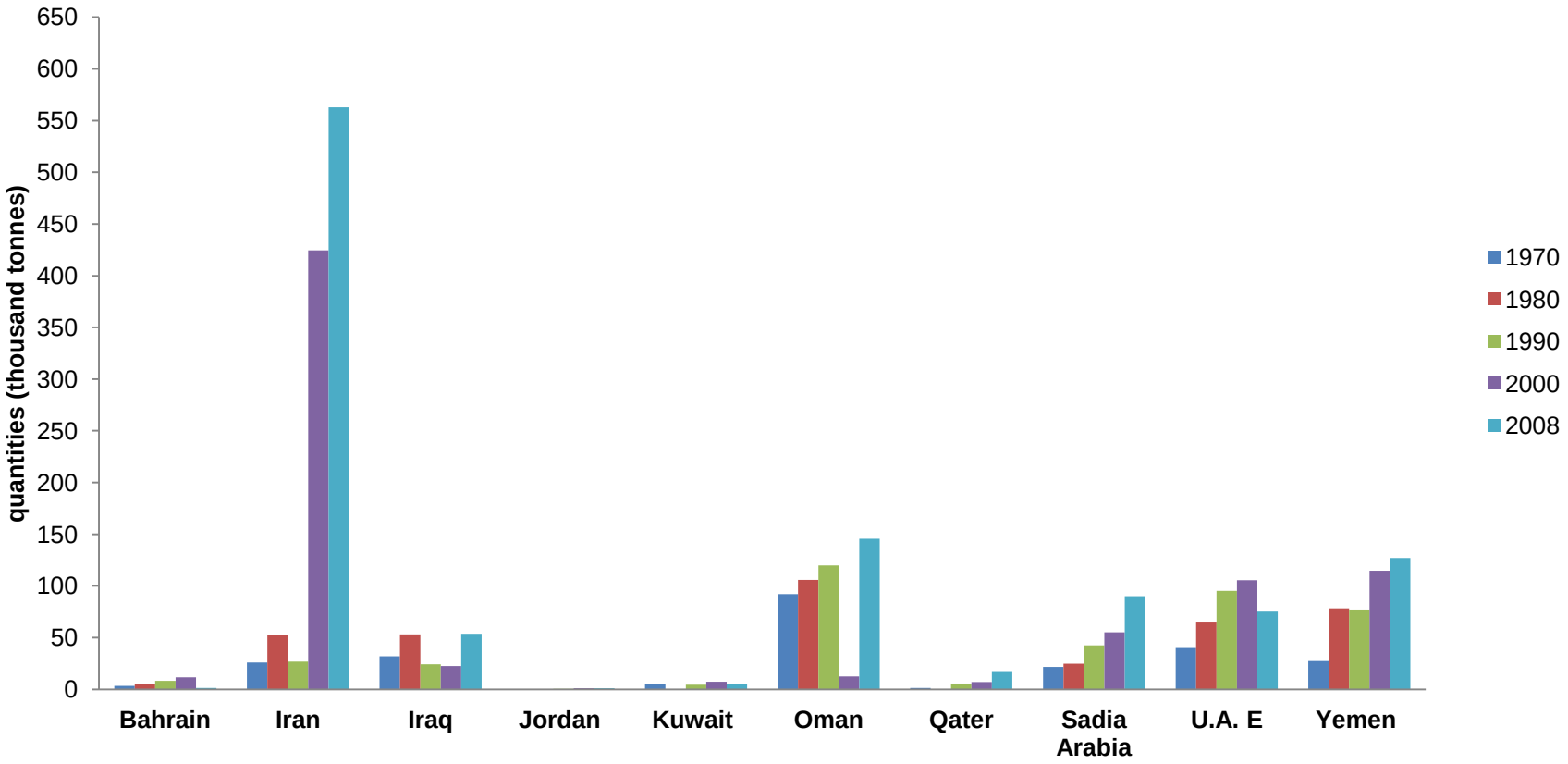


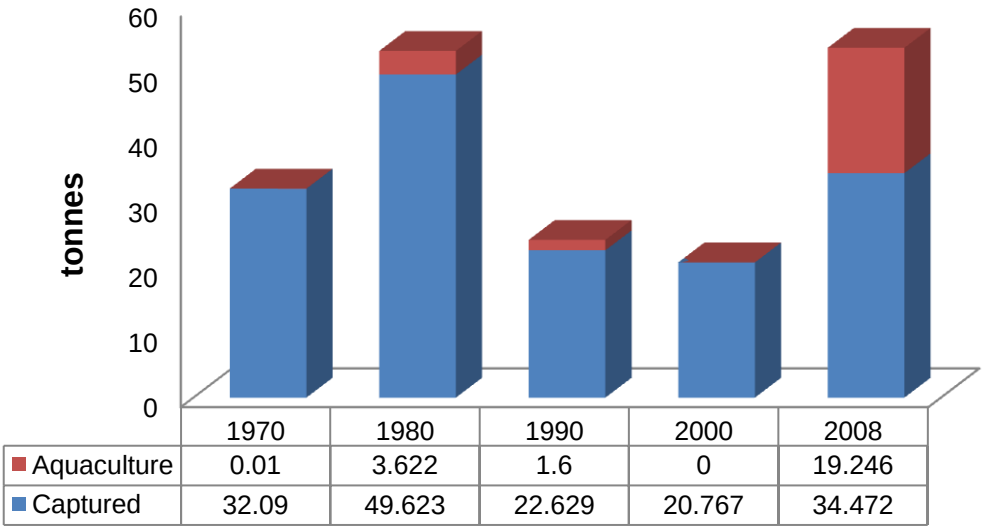
Figure 1.2 Middle East countries capture and aquaculture production from 1970 to 2008; (data are collected from FAO, FIGIS, 2008)

1.3 Aquaculture in Iraq and Kurdistan Region

The Euphrates and Tigris rivers are the main sources of water in Iraq, with fresh water bodies covering between 6 -7 million ha, from this 39% are natural lakes, 13.3% dams and reservoirs, 3.7% rivers and their branches and 44% marshes. The total area with employed for aquaculture consists of about 7500 ha consisting at around 2000 farms (Kitto and Tabish 2004).

In Iraq, fisheries and aquaculture output was estimated to be: 49,623 tonnes (captured) 3,622 tonnes (cultured) in 1980 but declined substantially to less than half 22,629 tonnes (capture) 1,600 tonnes (cultured) due to conflict and awareness crises in the region. This remained at the same level until 2000 due to continued unstable situations in Iraq for more than two decades (1980-2003). However recently the fish production has rapidly increased whereby capture fish was estimated at 34, 472 tonnes and cultured fish production at 19,246 tonnes in 2008 (FAO 2010). Currently, there are approximately 70 types of fish species available in Iraqi waters; the most important marine fish are *Tenualosa ilisha, Pampus argenteus, Epinephelus tauvina, Lieo sbuvlridis, Sparus aurata, Lethrlrus nebulous, Arius thalassinus, Acanothicybium solandri*; and freshwater are *Cyprinus carpio, Cyprinus sharpy* and *Barbus xanthopterus, Barbus grypus, Barbus luteus, Silurus triostegus* and *Asalus eorase*. The main culture species are *Cyprinus carpio, Hypophthalmichthys molitrix* and *Ctenopharyngodon idellus*. The average annual production of common carp for 1998 was 7,500 tonnes and decreased to 2,183 tonnes in 1999, this decline was due to the consistency drainage of the majority of Iraqi wetlands. In 2008 production of common carp was recorded to have been elevated significantly to approximately 10,700 tonnes. The aquaculture sector in Iraq is totally based on freshwater production. The rearing system of aquaculture is mainly earthen ponds, with some cage farming being used for culture as a common way for commercial production (Fan, 1996).

In the Kurdistan region, aquaculture is solely based on freshwater production. The sources of freshwater are the Tigris and its tributaries (i.e: Khabur River, Greater zab River, Lesser zab River, Awa Spi River and Sirwan River), and water reservoirs in the form of dams (Dukan dam, Darbandikhan dam, Dohuk dam and Mosul dam) (Plate 1.1). The water from these tributaries feed into the Tigris annually and are estimated to be at 16 billion m^3 which equals ~ 33.45% of the Tigris water (Tigris water is estimated at 48 billion m^3 per year) (Ismael, 2004).



	1970	1980	1990	2000	2008
Aquaculture	0.01	3.622	1.6	0	19.246
Captured	32.09	49.623	22.629	20.767	34.472

Figure 1.3 Aquaculture productions in Iraq (capture and culture) during last 40 years. (Data are collected from FAO, FIGIS, 2008)

Since the early 1960's, common carp has been placed into the Dukan and Darbandikhan lakes in the region for improving fish production but this strategy has not successfully elevated production and contributed to the economic value in these lakes. Subsequently in 1998, the Food and Agriculture Organization (FAO) established two stations; Erbil (Enkawa) and Sulaimnia (Dukan) for fish production. After that, in 2001, the FAO

offered the stations to the Ministry of Agriculture and Water Resources-Kurdistan Region-Iraq (Gowdet, 2007).

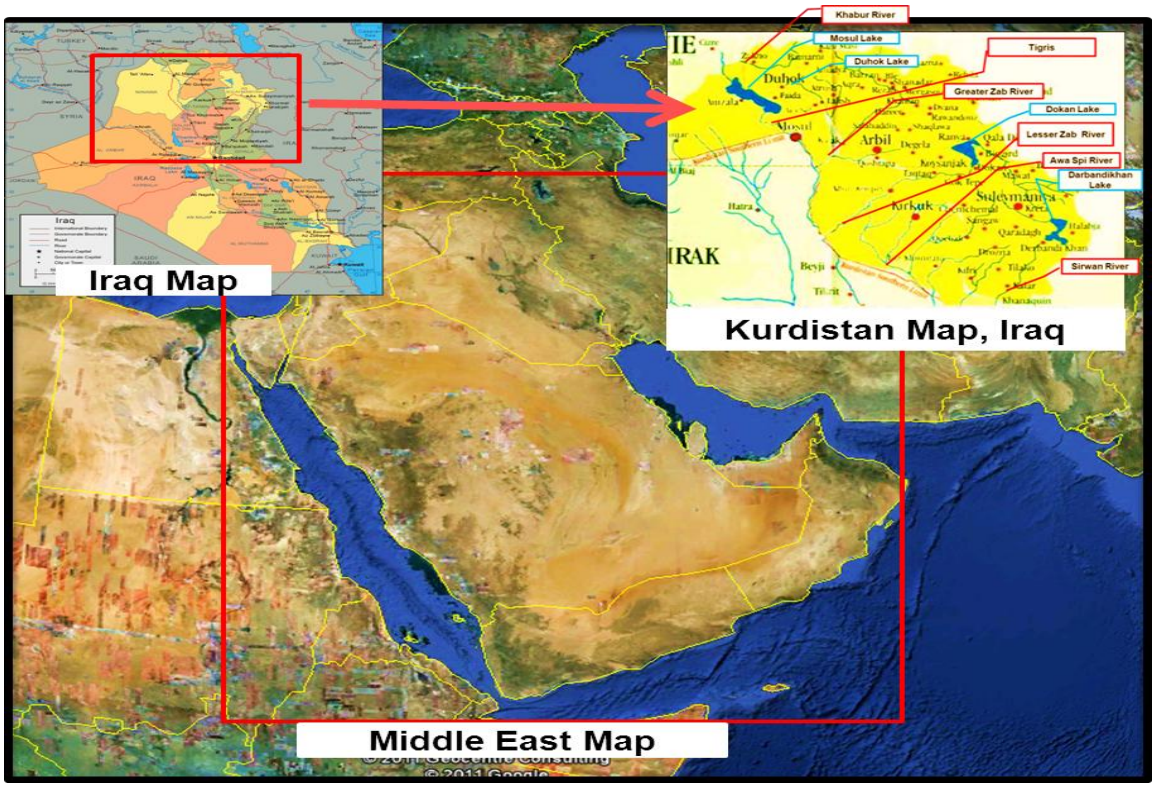
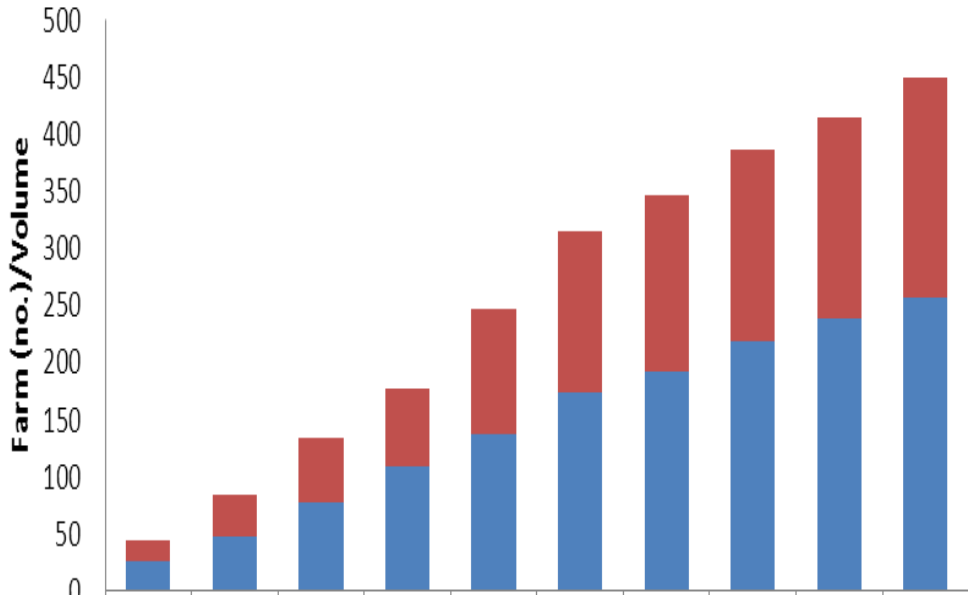


Plate 1.1 Middle East map, Iraq and Kurdistan Regional map, the sources of freshwater fish are shown in the Kurdistan Regional / Iraq map. (Source Middle East Map; Google Map2011 http://maps.google.co.uk/ maps? Hl = en &tab=wl ; Iraq Map; Istanbul – city - guide.com/Map/country/Iraq/Map/asp; Kurdistan Map; Kurdistan Regional Government.

In Kurdistan small-scale fish farms are operated by individual farmers in rural lake and river areas. Again the main important fish species is carp due to efficient production, wide range of tolerance, and high resistance for diseases, ease to hatch naturally or artificially and their ability to be cultured in different systems (El-Sayed, 1994a; El-Khalil, 2005). The General Directorate of Fish Production and Development (GDFPD) presently owns Enkawa station which comprises 20 fish ponds of different sizes with an annual capacity of over 2,000 tonnes and a hatchery to produce common silver and grass carp fingerlings for distribution to farmers to practice and develop fish farming as a profitable food source. In addition there are another three hatcheries in Kurdistan

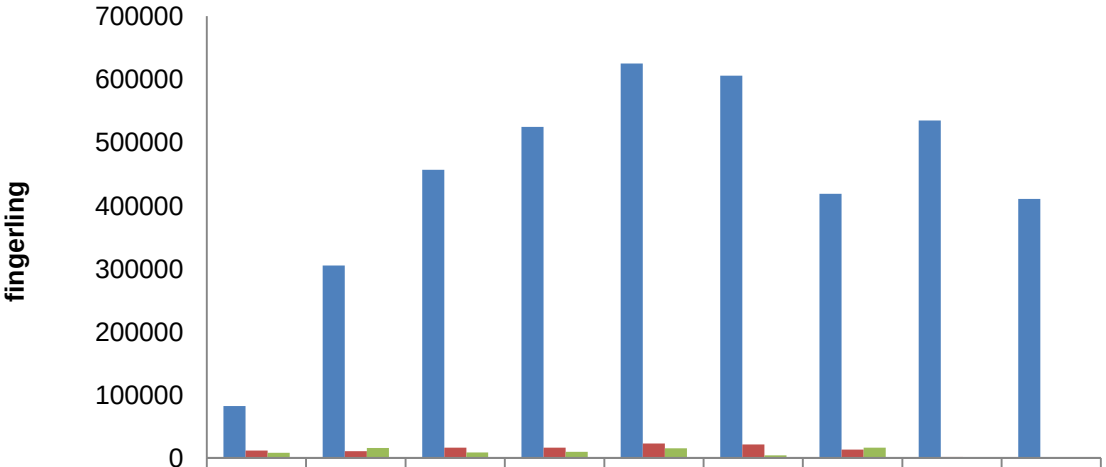
producing more fingerlings. In 2000 (GDFPD) of the Ministry of Agriculture and Water Resources (MOAWR) have started to issue licences to 25 new fish farms to be set up in the region (see Figure 1.4). In 2001, large numbers (331,100) of carp fingerlings were produced for restocking purposes which greatly contributed to the inland fisheries catch. The majority of that number was common carp (304,850 fingerlings) supplied to independent fish farmers. The production of fingerlings increased to 631,150 in 2005 and then decreased to 410,500 in 2008 (see Figure1.5). In Erbil Enkawa station, a fish feed factory with a capacity of 3 tonnes per hour was established by (MOAWR) in 2007 to produce the diets for carp feeds. These diets are produced as a pellet and distributed to famers in the region. Indeed, the number of fish farms increased rapidly to 260 by 2008. The average size of most of these farmers is comparatively small, about 1-3 ha. The priority plan for the Kurdistan Regional Government (KRG) is to regenerate agriculture production in the State. From 2009-2014 it is the policy for the agriculture sector to embrace new technology improving production. In the fish sector, MOAWR has decided to support all the 260 farms with new areas for farming to be leased and further licences will be issued thus rising to 500 farms by 2013. Furthermore, (GDFPD) will choose new ways of farming like closed system (water recirculation systems), cage systems in the lakes as well as raceway systems. In addition, new portable hatcheries will be provided with complete equipment and technology capabilities. The rate of development has, to some extent, been constrained by the geography of the area which is not particularly suitable for aquaculture. Also economic and political changes may impede development in specific sector such as the importation of raw materials and support infrastructure eg: feed production. As mentioned above, where there are comparatively large freshwater resources, large-scale freshwater fish culture has been developed and has capacity to grow; it is also feasible to carry out large-scale restocking

programmes for lakes, and river bodies. Therefore, in Kurdistan where freshwater is a major resources the development will be faster.



	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Area/ Hectares	19.25	35.75	55.5	68.5	109	141.25	154.5	168	176.5	192.75
Fish farm	25	48	78	109	137	174	192	219	239	257

Figure 1.4 Fish farms and water area used for fish farming in Kurdistan Region during 2000 – 2008.



	2000	2001	2002	2003	2004	2005	2006	2007	2008
Common carp	81950	304850	456350	524500	624750	605750	418650	534700	410500
Silver carp	11550	10550	16005	16050	22900	21350	13100	1500	0
Grass carp	7900	15700	8350	9600	15000	4150	16400	0	0

Figure 1.5 Fingerlings production (common carp, silver carp and grass carp) in the Kurdistan region, Iraq between 2000 – 2008.

1.4 Aquafeed production

Aquaculture production needs a consistent supply of feed ingredients to support the growth and consolidation of a global sea-food producing industry and to remain as the largest and fastest food sector production for the world population.

In typical extensive fish culture, several types of supplementary feeds are commonly used. The majority of feedstuffs are simple blends of agriculture by-products (Pillay 1990; Pillay and Kutty 2005). These are easy to obtain at relatively low prices and are widely available, ranging from chicken wastes, fishery waste, oil cake, wheat and rice bran, brewery waste (DDGS), bean residues, silk worm pupae, poultry by-products and slaughterhouse wastes, trash fish, offal and bakery wastes (Pillay and Kutty 2005; Gatlin et al., 2007). The majority of farmers tend to employ a limited range of supplementary feed, which could be either a single ingredient or a mixture of two or

three, at most. The amounts of feed and quantity of individual ingredients used in the feed mixes tend to vary greatly (Lucas and Southgate 2003).

In the preparation of modern dry fish diets for intensive aquaculture, the principle aim is to ensure that fish receive a balanced diet that meets their full nutritional requirements, for instance; adequate in the quality and availability of nutrients to enhance growth and production of stock. Feeds are classified according to nutrient specifications and the product quality can be maintained at a uniform standard for different fish species (Pillay 1990; Pillay and Kutty 2005).

Traditionally, fish meal has been used as a main source of protein in aquafeeds due to its high protein content, excellent amino acid profile, as well as high nutrient digestibility (Gatlin et al., 2007). Consequently most aquaculture formulations still use more than 50% fishmeal in the diet. However, being too dependent on any one ingredient presents considerable risk associated with supply, price and quality fluctuations; finding replacements for FM and fish oil (FO) has become an international research priority (Naylor et al., 2000; Edwards et al., 2004; Fournier et al., 2004; Glencross et al., 2007; Tacon and Metian 2008a). On the other hand, ensuring feeds are safe and meet the dietary requirements of the cultured species can be challenging and this must be considered as the ultimate objective of an aquaculture feed (Tacon and Metian 2008b). Indeed, aquafeeds can account for over 50 percent of the total cost of culture in some aquaculture practices. With much interest in the partial replacement for FM and FO with alternative protein and lipid sources, research efforts are focused on definite levels of substitutions that can be made without impairing growth and health of fish. A number of ingredients have been suggested to be viable replacements for FM for different fish species. In order to reduce diet costs and increase the production profitability, the nutritionist must consider using less expensive protein sources as a

replacer for fish meal because the most expensive dietary components are mainly protein, although dietary energy and oil is also costly.

Alternative protein derived from plant sources often provides reasonably good growth and are advantageous for diet manufacture (Davis and Stickney1978; Jackson et al., 1982; El-Sayed 1990; Coyle et al., 2004). Soybean meal (SBM) containing high quality protein and oils (Kaushik et al., 1995; Refstie et al., 1998,1999) canola meals protein concentrates and oils (Higgs et al., 1982; Mwachireya et al., 1999; Burel et al., 2000; Forster et al., 2000; Glencross et al., 2003a, 2004a,b) lupin seed meal and pea seed meal (Gouveia and Davies 1998, 2000), as well as lupin meals and protein concentrates (Burel et al., 1998; Booth et al., 2001; Farhangi and Carter 2001; Glencross et al., 2003b, 2004c) have all shown varying degrees of success when included in aquafeed.

Soybean meal has been one of the common sources of plant protein used in the formulation of aquaculture feeds, as a substitution of FM (Carter and Hauler 2000; Peres et al., 2003; Evans et al., 2005, Patnaik et al., 2005, Úran et al., 2008). Typically, SBM has been accepted due to its low cost and availability (Patnaik et al., 2005) also for its relatively high protein content and good balance of amino acids (Carter and Hauler 2000). However, in recent years there are suggestions querying some of SBM's valued assets. The increasing cost of proteins have declined the economic benefit and it is proposed that SBM has become relatively expensive and is not commercially available in some geographical regions, such as the tropics (Zerai et al., 2008). In recent years, SBM prices have risen to up to 500 USD per metric tonne (FAO 2009). Therefore restrictions to its overall use as a feed in the global expansion of aquaculture have been encountered. This further hampers the problem of supplying protein in formulated feeds as not only does it appear FM may need to be replaced but the use of plant proteins which have been shown to be potentially good substitutes may also need to be

reassessed. This highlights the need to find multiple sources of protein to meet an expansive global demand for fish feeds.

Much research has focused on evaluating the viability and nutritional value of animal by-product protein; meat and bone meal (MBM), meat meal (MM), blood meal (BM), hydrolysed feather meal (HFM), poultry by-product meal (PBM), hydrolysed poultry feather meal (HPFM), chicken concentrate (CC) and chicken (CE) as substitutes for fishmeal. The use of animal by-products (PAP) as protein sources to replace fishmeal in formulated fish diets are due to their high protein content, good nutrient profile with respect to minerals as well as low costs are reasons for their successful inclusion in aquafeeds (Sealey et al., 2011) . On the other hand, use of these alternative sources in aquafeeds faces some restriction. These are due to possible deficiency of essential amino acids EAAs (lysine, isoleucine and methionine), palatability, availability in the local market and impairment of health status (Hasan 2000; Gatlin et al., 2007; Zerai et al., 2008; FAO 2009).

These PAP ingredients have been widely used in feeds for major fish species such as Chinook salmon *Onchorhynchus tshawytscha* (Fowler 1990, 1991), coho salmon *Oncorhynchus kisutch* (Higgs et al., 1979), rainbow trout *Onchorhynchus mykiss* (Gouveia 1992; Steffens 1994; Bureau et al., 2000; Cheng and Hardy 2002; Sealey et al., 2011), European eel *Anguilla anguilla* (Gallaghar and Degani 1988) Nile tilapia *Oreochromis niloticus* (El-Sayed 1998), African catfish *Clarias gariepinus* (Goda et al., 2007), red drum *Sciaenops ocellatus* (Moon and Gatlin 1994; Kureshy et al., 2000), Cuneat drum *Nibea miichthioides* (Guo et al., 2007), gilthead sea bream *Sparus aurata* (Davies et al., 1991; Nengas et al., 1999; Davies and Gouveia 2009), European sea bass *Dicentrarchus labrax* (Davies and Gouveia 2009), silver sea bream *Rhabdosargus sarba* (El-Sayed 1994b), turbot *Scophthalmus maximus* (Davies and Gouveia 2009), Australian snapper *Pagrus auratus* (Quartararo et al., 1998), Australian silver perch

Bidyanus bidyanus (Allan et al., 2000), grouper *Epinephelus coioides* (Millamena 2002), Indian major carp *Labeo rohita* (Hasan et al., 1997), gibel carp *Carrassius auratus gibelio* (Yang et al., 2004, 2006; Hu, et al., 2008) and sunshine bass *Morone chrysopes x M. saxatilis* (Webster et al., 1999, 2000).

Nowadays, the using of processed animal protein in fish diets is restricted and that is due to prion disease. However, blood meal can be used in the fish diets. Proteinaceous infections agents called prions which affect the central nervous system of humans and other mammals include scrapie in sheep, spongiform encephalopathy (BSE) in cows and Creutzfeldt-Jakob disease in human (CJD). The first divergent group was found in 1982 which believed that the responsible agent of the transmissible spongiform encephalopathy (TSE) (Joly et al., 2001).

Friedland et al. (2009) demonstrated the prion protein TSE homology in zebrafish, pufferfish, and salmonids (salmon and rainbow trout). However, in all cases the sequence homology is low. More recently, Valle et al. (2008) indicated that prion proteins TSE were unable to cross the intestinal barrier in trout force fed prion infected material, and concluded that prion proteins TSE like disease would be unlikely to affect freshwater fish. As a result of this risk a restriction on the use of PAP (except fishmeal) in feeds of animals for food production was announced in 2000 due to the EU applying additional security measures to prevent the amplification of prion related disease. Currently, the situation in Europe with regards to using PAP is legalised by the animal by-product regulation (ABPR 1774/2002) together with the TSE regulation (999/2001) which defines conditions and restrictions attached to the current and future use of protein animal by product (PAP). The handling, processing and marketing of PAP including categorisation of raw materials, no intra-species recycling and traceability have been explained by this regulation (Woodgate 2004). Therefore, the nutritionists need to focus on searching for novel ingredients that are acceptable for promoting

growth performance and optimum health of fish. The use of these ingredients in fish feed will make fish a sustainable food supply using feeds derived from animals fit for human consumption (category 3) (ABPR 1774/2002).

Nutritionists have often used single cell proteins (algae, bacteria and yeasts) as good candidates for new alternative protein sources in diets for aquafeed. This is mainly due to their wide-spread availability even in tropical countries where the lack of sufficient plant proteins (i.e soybean) occurs (Zerai et al., 2008).

There are also many complementary protein sources available, such as single cell proteins including micro-organisms such as algae, bacteria and yeasts. Also distillers dried grains and solubles (DDGS) are available as one of the main co-products obtained from fermentation of cereal grains for the production of ethanol, carbon dioxide, as well as syrup in some ethanol plants (Williams et al., 2007).

1.5 Distillers dried grains and soluble (DDGS) and Yeasts

1.5.1 Distillers dried grains and soluble (DDGS)

Distiller's dried grains with solubles (DDGS) is a valuable feed ingredient which is a by-product of the dry-grind or wet mill ethanol production resulting from the yeast fermentation of cereal grains (Firkins et al., 1985; Ham et al., 1994; Spiehs, et al., 2002; Lan et al., 2008; Klopfenstein et al., 2008; Ganesan et al., 2008). Conventionally, distillers dried grains have been widely used for ruminants and non-ruminants as a protein source but drying distiller's by-products is costly and can account for around 40% of the energy costs incurred by the alcohol plant (Stock and Klopfenstein 1982). The typical crude protein content topically averages about 30% and the product is relatively high in fibre.

It is likely that the continual expansion of the fuel ethanol industry will provide a steady and growing DDGS output and improving processing technologies will provide interesting economic opportunities for low level inclusion in animal feeds.

The typical crude protein content averages about 30% and fibre content ranges from 7-11% (refer to Table 1.1). On the other hand, DDGS contains a good level of energy and is a rich source of vitamins and minerals. The physical appearance, chemical composition and nutrient digestibility can vary considerably depending on sources, processing and different drying procedures (Cromwell et al., 1993).

A number of workers have reported the nutrient content of DDGS in considerable detail. For example, Cromwell et al. (1993) investigated the characteristics and composition of nine DDGS sources (7 from brewery production and 2 from ethanol plants) and found that the average dry matter (DM) content was 90.5%, crude protein ranged from 23.4 to 28.7%, fat ranged from 2.9 to 12.8%, acid detergent insoluble nitrogen (ADIN) ranged from 8.8 to 36.9%, neutral detergent fibre (NDF) ranged from 28.8 to 40.3%, acid detergent fibre (ADF) ranged from 10.3 to 18.1% and ash ranged from 3.4 to 7.3% (see Table 1.1). The amino acids profiles also varied greatly with source, particularly lysine concentrations, which ranged from 0.43 to 0.89 percent. In addition, the 240 samples of DDGS originating from new ethanol plants in Minnesota and South Dakota, older ethanol plants and published reference values analysed by Spiehs et al. (2002) indicate that the dry matter content average of 89 % was lower than the value of 93% listed by NCR (1998). The crude protein levels with an overall average of 30.2% were higher than crude protein values in NRC (1998). The average fat values of 10.7% were higher than the fat value of 9% listed by NRC (1998). The crude fibre content of 8.6% and acid detergent fibre (ADF) levels of 16.1%, were considerably lower than ADF values in NRC (1998). The average neutral detergent fibre (NDF) content of 41.45%, was higher than NDF value of 37.2% in NRC (1998). The essential amino acid concentrations of

the DDGS sources were higher and lysine, methionine and threonine were extremely variable (see Table 1.2). The average levels for lysine of 0.85%, methionine of 0.55% and threonine of 1.13% were higher than lysine, methionine and threonine values listed by NRC (1998). The total ash content of the DDGS (6.1%) was considerably higher than the values (4.8%) in Feedstuff References Issue (1999). The most important element in feed formulation is phosphorus and it is the third most expensive nutrient; an average phosphorus level of 0.90% was reported to be higher than the phosphorus values of 0.83% listed by NRC (1998) but lower than the phosphorus values of 1.02% published in Feedstuff References Issue (1999).

Furthermore, the same results by Spiehs et al. (2002) found that the variation in dry matter (DM), crude protein (CP), and gross energy (GE) in the ten sources of DDGS agree with the findings that have been reported more recently by Pedersen et al. (2007), but the values for NDF (16.1%) were current lower than the values listed by the NRC (1998), Speihs et al. (2002) Stein et al. (2006) found that the concentration of phosphorus was lower than those values reported by NRC (1998) but the benefit for adding DDGS in diets fed to growing swine, a higher portion of organic phosphorus will be digested and utilized, hence increasing the need for adding inorganic phosphorus to the diets (see Table 1.3).

Moreover, a study undertaken by Fastinger and Mahan (2006) showed that the five sources of DDGS from corn gave CP values of 28.1% and NDF values of 32.2%; those values were greater than the values reported by Cromwell et al. (1993), and the average lysine values of 0.60% were lower than the lysine value of 0.67% listed by NRC (1998). The colour of the DDGS sources ranged from being light to dark and odour varied from normal to burnt/smokey (Cromwell et al. 1993). Authors stated that the colour was highly correlated to the nutritional values of DDGS (Cromwell et al., 1993; Noll et al., 2005; Fastinger and Mahan 2006; Stein et al., 2006; Ganesan et al., 2006) in which the

darkest colour represented lower nutritional value and the lightest colour represented higher nutritional value. Ganesan et al. (2006) indicated that lightness (L*) of DDGS colour reduced with increased soluble percentage addition (r^2 = 0.76) redness (a*) and yellowness (b*) values increased with increased soluble percentage addition (r^2 = 0.63 and 0.72, respectively). Hunterlab colour scores were used to give a nutritional value degree for a DDGE samples based on its lightness or darkness (L*), redness (a*) and yellowness (b*). These results showed that the colour of DDGS depended on the original grain colour the amount of solubles added to the grains to make DDGS, the drying time and temperatures used. It has also been suggested that the colour of DDGS is not a reliable measure due to the lack of good correlations between L*, a* and b* values and the digestible energy (DE) and metabolic energy (ME) in the samples (Fastinger and Mahan 2006). The results of a study conducted by Fastinger and Mahan (2006) imply that the darker coloured DDGS sources may have lower lysine and essential amino acid digestibility, compared to lighter coloured DDGS sources.

Table 1.1 Mean chemical compositions of the DDGS from previous investigations.

	Cromwell et al. (1993)	Spiehs et al. (2002)	Stein et al. (2006)	Pedersen et al. (2007)
DM (%)	90	89	88.9	87.6
CP (%)	26.9	30.2	27.4	29.2
Fat (%)	9.7	10.7	ND	10.5
Fibre (%)	ND	8.6	6.8	7.8
Ash (%)	4.8	6.1	ND	3.86
ADF (%)	14.4	16.1	10.9	10.15
NDF (%)	35.1	41.45	40.13	24.2
DE MJ kg^{-1}	ND*	15.7	20.13	19.6

* ND = not determined

Table 1.2 Amino acid composition of DDGS from previous investigations.

	Cromwell et al. (1993)	Fastinger and Mahan (2006)	Stein et al. (2006)	Pedersen et al. (2007)
Essential AA				
Arg	1.06	1.00	1.10	1.28
His	0.72	0.65	0.77	0.76
Ile	1.00	0.98	1.02	1.09
Leu	3.33	3.07	3.11	3.54
Lys	0.70	0.64	0.79	0.81
Met	0.51	0.48	0.62	0.69
Phe	1.45	1.34	1.3	1.40
Thr	1.03	0.95	0.99	0.95
Trp	0.19	0.25	0.17	0.19
Val	1.35	1.3	1.41	1.44
Non-essential AA				
Ala	ND*	1.88	1.76	2.14
Asp	ND	1.66	1.94	2.02
Cys	0.53	0.48	0.70	0.39
Glu	ND	3.98	3.52	5.38
Gly	ND	0.94	0.98	1.16
Pro	ND	2	1.95	2.34
Ser	ND	1.05	1.08	1.34
Tyr	ND	0.97	0.97	1.18

* ND = not determined

Table 1.3 Mineral analyses of DDGS from previous investigations.

Minerals	Concentration	References
Ca (%)	0.25 - 0.40	Spiehs et al. (2002); Robinson et al. (2008)
P (%)	0.79 - 0.90	Spiehs et al. (2002); Stein et al. (2006); Robinson et al. (2008)
K (%)	0.97 - 1.12	Spiehs et al. (2002); Robinson et al. (2008)
Mg (%)	0.32 - 0.37	Spiehs et al. (2002); Robinson et al. (2008)
S (%)	0.48 - 0.64	Spiehs et al. (2002); Robinson et al. (2008)
Na (%)	0.18 -0.26	Spiehs et al. (2002); Robinson et al. (2008)
Zn (ppm)	91.4 -254	Spiehs et al. (2002); Robinson et al. (2008)
Mn (ppm)	17 - 32.4	Spiehs et al. (2002); Robinson et al. (2008)
Cu (ppm)	4.9 – 9.7	Spiehs et al. (2002); Robinson et al. (2008)
Fe (ppm)	85.0 – 169.3	Spiehs et al. (2002); Robinson et al. (2008)
Mo (ppm)	0.7	Robinson et al. (2008)
Se (ppm)	0.28	Robinson et al. (2008)
Co (ppm)	< 0.1	Robinson et al. (2008)

1.5. 2 Yeasts; source and characterizations

There are many types of yeasts available such as yeast flakes, engitive yeast, brewer's yeast, debittered brewer's yeast, torula yeast and yeast extract spreads. These may also be in two forms either as active or inactive. Yeasts are primary used from four species; *Saccharomyces cerevisiae*, *Candida utilis, Candida saitoana* and *Candida pintlopsii.* The most commonly used yeast by humans, for baking and brewing which is also applied in ruminant and mono-gastric animal nutrition due to their relatively high content of protein and B vitamins. It can be grown on various by-products such as: citrus press liquor, potato pulp, apple pomace, molasses, sugar cane, sulphite waste liquor wood, and fruit wastes (Fuller eds. 2004).

Yeasts are characterized by their relatively high protein content and vitamin levels. Generally, yeast contain dry matter of 89.1% and the typical nutrient composition; crude protein of 49.9%, crude fibre of 1.5%, and ash of 8.5% as well as, calcium of 0.13% and phosphorus of 1.56% (Fuller eds. 2004). These results are in accordance with the results of Brown et al. (1996) who reported the marine yeast *Saccharomyces cerevisiae* contains 89% of dry mater (DM %), 35% of crude protein (CP %), 7.7% lipid and 6.4% of ash (Brown et al. 1996). However, the protein content of brewer's yeast (42.6%) or torula yeast (49) is higher, and that is may be due to the strain of the yeast and the effect associated with process in producing yeast.

More recently new types of yeast (ethanol yeast) have been derived from bioethanol production with 42% of protein, 6.45% lipid and relatively low in ash content (2.36%). The protein content (50%) of yeast published by Premier Nutrition Atlas (2005) are generally higher than typical levels reported from traditional sources. The lipid and ash contents

Table 1.4 Chemical composition of the yeast (*Saccharomyces cerevisiae*) from previous investigations.

	Brown et al. (1996)	Fuller ed. (2004)	Gause and Trushenski (2011a)	Premier Nutrition (2005)	NRC 1993, 2011 brewer's yeast	NRC 1993, 2011 torula yeast
DM (%)	89	89.1	ND	90	93	93
CP (%)	35	30.2	42	50	42.6	49
Fat (%)	7.7	1.3	6.45	4	1	1.5
Carbohydrate (%)	39	38.8	39.23	26.6	39.6	32.6
Fibre (5%)	ND	1.5	ND	1.9	3.2	2.2
Ash (%)	6.4	8.5	2.32	7.50	6.6	7.70

* ND = not determined.

were estimated at (4%) and values were reported to fluctuate (see Table 1.4). These yeasts contain a complete amino acid profile (18 amino acids) such as argnine, leucine, methionine phenylalanine and threonine. The highest level for arginine of 2.48%, leucine of 4.67% methionine of 0.82% phenylalanine of 2.36% and threonine of 2.14% were higher than argnine, leucine, methionine, phenylalanine and threonine values listed by NRC. So far, very little information is known on mineral content of yeast, however there are two sets of data available which are published by NRC (1993, 2011) and Premier Atlas (2005).

Table 1.5 Amino acid composition of Yeast from previous investigations.

	Brown et al. (1996)	Gause and Trushenski (2011)	NRC (2011)	Premier Nutrition (2005)
Essential AA				
Arg	1.86	2.48	2.25	2.05
His	0.74	1.03	1.09	1.02
Ile	1.72	2.17	1.98	2.20
Leu	2.84	4.67	2.85	3.15
Lys	1.75	2.41	2.97	3.40
Met	0.74	0.82	0.67	0.75
Phe	1.75	2.36	1.62	ND
Thr	2.14	1.97	2.04	2.25
Trp	0.53	0.30	0.52	0.55
Val	1.89	2.67	2.36	2.45
Non-essential AA				
Ala	2.31	3.12	ND	ND
Asp	4.01	ND	ND	ND
Cys	0.25	0.72	0.49	0.45
Glu	5.25	6.41	ND	ND
Gly	1.96	1.87	ND	ND
Pro	1.33	2.54	ND	*
Ser	2.38	2.06	ND	ND
Tyr	1.61	1.75	1.50	*

*(Proline+Tyrosine) = 3.35
ND: Not determined.

Table 1.6 mineral content of yeast from previous investigations.

Mineral	Brewer's yeast*	Yeast brewer's dried**	Yeast torula dried**
Ca (%)	0.25	0.14	0.58
P (%)	1.25	1.52	1.52
K (%)	1.70	1.69	1.94
Mg (%)	0.20	0.24	0.0.20
S (%)	0.40	0.43	0.55
Na (%)	0.10	0.07	0.07
Zn (ppm)	50	39	99
Mn (ppm)	30	6.70	13
Cu (ppm)	35	38.40	17
Fe (ppm)	150	109	222
Se (ppm)	0.50	0.91	0.02

*Premier Atlas (2005).
**NRC (1993, 2011).

The concentration of phosphorus is high (1.23 – 1.52%) in the yeasts and calcium content is between 0.14 - 0.25%. In terms of trace minerals the iron content (109-222 ppm) is the highest and manganese content (6.7 – 30 ppm) which is relatively high (see Table 1.6).

1.6 The use of DDGS and yeasts in animal nutrition

Conventionally, DDGS have been widely used for ruminants (beef and dairy cattle) and increasingly for non-ruminant terrestrial animals (mainly and swine and poultry) because of the relatively high protein and low fibre content. A number of studies have focused on growth and carcass parameters but also the effect on milk yield of dairy cattle has been investigated. For example, Powers et al. (1995) stated that a satisfactory replacement at up to 26% in dietary dry matter (DM) in cow diet can be provided by DDGS for soybean meal. They indicated that the cows fed higher quality DDGS sources produced marginally more milk yield than cows fed soybean meal supplement. More recently, Kleinschmit et al. (2006) conducted a study to determine the effect of feeding diets with 20% DDGS inclusion from three different sources compared with DDGS-free control diets; dairy cows fed diets containing DDGS produced higher

amount of milk, approximately 3.4 kg d^{-1} more than cows fed diets containing no DDGS. Also they found that the feed efficiency was higher in cows fed DDGS compared with the control ration.

Klopfenstein et al. (2008) concluded that the various levels of wet dried grains and solubles (WDGS) and distiller's dried grain and solubles (DDGS) fed to feedlot cattle produced higher average daily gain (ADG) than cattle fed corn-based diets without dried grains solubles (DGS). Also they observed that the feeding value of DGS is greater than dry rolled-corn and high-moisture corn. However, the feeding value of DGS tends to be lower when fed in finishing diets based on steam-flaked corn than based on dry-rolled corn or high-moisture.

The Use of distiller's dried grains with solubles in poultry diets has historically been about a 5-10% inclusion level because it was hard to find locally (Waldroup et al., 1981) and variability in nutrient content and digestibility (Noll et al., 2001). In past decades, DDGS has been used in poultry diets primarily as a source of alternate protein that promoted growth and egg production. Distillers' dried solubles (DDS) or DDGS were added to poultry diets at levels of 10% of the diet. Couch et al. (1957) showed that adding 5% DDGS improved turkey growth rates by 17-32%. Day et al. (1972) reported improvements in broiler body weights when 2.5% DDS or 5% DDGS were added to the diet in one of three trials. Alenier and Combs (1981) reported that chicken layer hens preferred diets containing 10% DDGS or 15% DDS over corn-soybean meal diet without DDGS. However, Cantor and Johnson (1983) were unable to document a feed preference effect for diets containing DDGS compared to corn-soybean meal diets.

Currently suggested maximum dietary inclusion rates for DDGS are 10-15% for chicken. Higher levels of DDGS can be used successfully with appropriate diet formulation adjustments for energy and amino acids (Waldroup et al., 1981; Noll et al., 2004).

During the last century less than 3% of total productions of DDGS have been used in swine diets. Much research has been conducted to evaluate three types of distiller's co-products in swine diets – distiller's dried solubles (DDS), distiller's dried grains (DDG) and distiller's dried grains with solubles (DDGS) (Wahlstrom et al., 1970; Smelski and Stothers 1972; Cromwell et al., 1985).

The recommended maximum dietary levels of DDGS in swine diets are 20-50% depending on the goal of rearing, the diets are formulated on a digestible amino acid and available phosphorus basis. The quality of DDGS is very important as it assumes that high quality DDGS is free of mycotoxins. Growth improvement has been documented by many researchers for swine of various ages (Monegue and Cromwell 1995; Spiehs et al., 2000; Wilson et al., 2003; Whitney and Shurson 2004; Gaines et al., 2006). Also other factors which have been indicated to potentially affect feed quality, in respect of nutrient composition, amino acid profile, digestibility and general protein quality; are refining methods used to process DDGS into suitable protein concentrates (Urzúa 2010; Chiesa and Gnansounou 2011). Another processing factor that needs to be considered is the form in which DDGS based feeds are presented as well as feed ingredient and extrusion conditions such as ingredient moisture content and screw speed; these parameters have been shown to be important factors which determine and impact upon extrudate characteristics (e.g. moisture content, durability, brightness and redness) when using feed formulations containing DDGS (Chevanan et al., 2009). Further work is required to optimize processing conditions and technologies in order to produce more consistent feed products.

Yeasts could be used as supplements in animal feed nutrition. Yeasts have great potential to improve growth production and enhance health status in animals, much research has showed this in livestock. Magalhães et al. (2008) demonstrated that feeding yeast culture in grain enhances health and performance of dairy calves, minimized the

frequency of health disorders and reduced the risk of morbidity and numerically decreased mortality. Consequently the response to yeast products on performance of dairy calves has been beneficial to date. However, a previous study by Lesmeister et al. (2004) reported that the young calves performance has been variable when fed diets containing two levels (1% and 2%) of yeast culture in the ration; the 2% of yeast culture in the grain of dairy calves improved dry matter intake, BW and feed efficiency but 1% inclusion of yeast culture did not influence on performance. In addition, the use of live yeast in diets for piglets was tested in a study undertaken by Bontempo et al. (2006) where piglets were fed a control and live yeast supplementation (2g kg^{-1}) diets during the weaning stage. Live yeast produced better live weight and higher average daily gain of piglets as well as improved intestinal gut flora. In some studies, similar findings have been reported in piglet average daily gain fed with yeast supplementation (Jurgens et al., 1997; Maloney et al., 1998; Mathew et al., 1998); however other workers have found that yeast supplementation to weanling piglets had no effect on average daily gain (Kornegay et al., 1995).

Furthermore in lamb production, yeast has been used as a growth promoting feed additive in diets, for example Tripathi and Karim (2011) using three types of yeast *Kluyveromyces marximanus*, *Saccharomyces cerevisiae* and *Sacchromyces uvarum* culture or mixed yeast concluded that the lambs fed yeast had higher growth rate, positive rumen fermentation and better microbial population. However, Kawas et al. (2007) and Chaucheyras-Durand (2008) recommended that rate of response of yeast supplementation depended on the strain of yeast culture, the nature of diet, and the physiological state of ruminants.

Haldar et al. (2011) revealed that feeding YPC and pelleted YPC (1g kg^{-1}) supplemented diets to broiler chickens resulted in increased production performance and improved health status. Also, other studies conducted by Silva et al. (2009); Pelícia et al.

(2010) reported that the use of yeast as a supplementation for diets elevated growth performance and maintained broiler health.Over all, yeasts as probiotics can be supplemented to a range of terrestrial animal diets. Recommended levels are as follows; for cattle ranged from 1-2 kg day^{-1}, lambs up to1.25%, and steers up to 1.85%, calves 3-5% or 2g day^{-1} of starter, pigs 5% (sows 100-400 g day^{-1}), and poultry at 9% for rooster (male chicks) and 2-3% for hens (female chicks) (Fuller eds. 2004). Therefore, adding yeast products to the animal feed has mainly positive effects on growth performance and microbial population.

1.7 The use of DDGS and yeasts in fish nutrition

The exploitation of DDGS and yeasts for aquaculture diets is now major significance as a strategy to use alternative proteins and high energy sources. Recently, a few studies have focused on the feeding of diets containing DDGS and yeasts to freshwater fishes and prawns (Tidwell et al, 1993; Birtz 1996; Ghosh et al., 2005; Wu et al., 1996; Stone et al., 2005).

Coyle et al. (1996) demonstrated that DDGS can be consumed directly by juvenile prawn (over 2 g in weight), and that the DDGS may be used as both a food supply and a pond fertilizer. Webster et al. (1993) showed that the diets containing 0, 10, 20 or 30% DDGS to partially replace corn or soybean meal fed to cage juvenile Channel catfish (*Ictalurus punctatus*) did not alter individual fish weights, survival, feed conversion, carcass composition, carcass waste (head, skin, viscera) or organoleptic properties of the fillets. They also suggested that more than 30% DDGS can be added to the diets with no negative impact on growth performance, carcass composition or flavour qualities of the fillets.

Wu et al. (1996) showed that the levels of DDGS at 30% of the diet fed to tilapia provide good growth performance and food utilization. In agreement, Coyle et al. (2004)

indicated that diets containing 30% DDGS in combination with meat bone meal (MBM) and soybean meal (SBM) provided good growth in hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*). However, a diet without animal protein did not support acceptable growth in the same species. The same result was found by Robinson and Menghe (2008) in Channel catfish.

Thiessen et al. (2003) conducted a series of experiments to evaluate thin distillers solubles diets containing 3.3% or 3.9% (dry matter basis) as a palatability enhancer in diets containing 15% of canola meal or 30.5% of air-classified pea protein for rainbow trout (*O. mykiss*). However, feed intake did not increase when using 1.9% or 6.6% thin distiller's solubles. Also, supplementation of the diets with 1% of a commercial palatability enhancer did not increase feed intake. These workers also found that different levels of air-classified pea protein and canola meal combined with thin distiller's solubles (4.0 dry matter basis), did not impact the feed intake or growth over the course of 12-weeks.

Cheng et al. (2004) observed that up to 50% of dietary FM could be replaced by a mixture of SBM and DDGS without affecting weight gain or feed conversion. Inclusion levels beyond this led to reduced performance which could not be rectified with supplementation of dietary methionine hydroxyl analogue MHA (2.75 g kg^{-1}). Kukačka and Mareš (2008) showed that upto 30% dietary inclusion of DDGS can be used in carp diets without affecting growth performance and feed utilisation parameters. Contrary to these findings 17.5 - 27.5% DDGS dietary inclusion significantly reduced the growth performance of Nile tilapia *Oreochromis niloticus* compared to the reference diet (0% DDGS) (Schaeffer et al., 2010). However, fish fed the 20% DDG diet did not show reduced feed conversion ratio and protein efficiency ratio whereas higher inclusion levels did. In Channel catfish, diets containing 10% distillers solubles (DS) or 30% DDGS has been shown to improve feed consumption and weight gain compared to the

control diet (Li et al., 2010). However, feed conversion ratio was lower. DDGS in diets for sunshine bass (*Morone chrysops* X *M. saxatalis*) at 30% inclusion have displayed lower protein efficiency ratio compared to other FM protein sources, poultry by-products and SBM (Thompson et al., 2008).

A study by Zerai et al. (2008) showed that up to 50% brewer's waste could replace fish meal in the diet without a negative impact on growth and feed utilization when fed to Nile tilapia for a ten week period. In a nine week experiment by Olvera-Novoa et al. (2002) tilapia (*O. mossambicus*) fry were fed diets in which up to 45% of animal protein with a mixture of plant ingredient was replaced with torula yeast (*Candida utilis*), with 20% soybean meal and 15% with Alfalfa Leaf Protein Concentrate (CLA). It was concluded that a replacement of up to 65% of animal proteins, including 30% from torula yeasts was without adverse effects on fish growth and the efficacy of production.

Barnes et al. (2006) showed that supplementation the dried yeast culture at (DVAqua from Diamond V. Mills, Cedar Rapids, IA, USA) fed to McConaughy strain rainbow trout diets (0.125, 0.25%), resulted in increased survival and growth through the first four weeks of feeding, with continued improvements over the remaining productive period. Therefore, limited levels of DDGS and yeasts are considered to be an acceptable source of protein in fish diets. Ethanol yeast was produced as a new product which derived from ethanol by-product using a novel technology (AB-Agri Patent # 109203A1, 2010). To the authors knowledge, the only study so far to evaluate the potential of similar yeast co-products derived from the biofuel sector for use in aquafeeds was that of Gause and Trushenski (2011a,b) on sunshine bass. However, these authors' only reported fundamental growth and feed performance for this species and did not give any detailed evidence as to the effects of yeast co-products on health criteria and general status.

As stated previously, warm water fish such as carp and tilapia are excellent fish for aquaculture and prime candidates for the development of novel feeds. These species have unique characteristics and are described accordingly prior to their choice in this research programme on fish nutrition and feed technology.

1.8 The carp (Cyprinus carpio)

The common carp (*Cyprinus carpio*) belongs to the family of fresh water fish known as *Cyprinidae*. In nature, carp inhabit in rivers, reservoirs and marshes. They are omnivorous in nature as they feed on a wide variety of plants and animals such as; zooplankton, insects, worms, aquatic plants, algae and seeds. Common carp in the nature thrive on decayed vegetable matter containing bottom-dwelling organisms tubificids, molluscs, chinomids. The common carp can tolerate a very wide range of conditions. Carp can live in a wide range of temperatures between 1-40 $^{\circ}$C. The best growth occurs at a temperature of above 13 $^{\circ}$C, and they reproduce at temperatures up to 20 ^{o}C. They originally live in fresh or brackish water with a 7.0-7.5 pH (Swift 1993; Billard, 1995; Takeuchi et al., 2002; Fuller eds. 2004). Carp are distributed all over Asia, most parts of Europe and in some African countries. Recently, they have also been introduced in the USA and Australia (Fuller ed. 2004; Pillay and Kutty 2005). The required protein levels in the diets of carp are between 30-38% crude proteins. Carbohydrates and lipids are easily utilized by common carp as a dietary energy sources. Common carp has to be feed more frequently (4 times a day) due to absence of a stomach (Fuller eds. 2004).

1.9 The Nile tilapia (*Oreochromis niloticus*)

Tilapia comes from the *Cichlidae* family of warm water fish; tilapia can live successively in freshwater, brackishwater and salt water. Tilapia are herbivorous and

opportunistic omnivores and they feed on a wide range of natural food organisms, including plankton, succulent green leaves, benthic organisms, aquatic invertebrates, larval fish, detritus and decomposing organic matter (Pillay and Kutty 2005; Shelton and Popma 2006). Tilapia are highly tolerant of a wide range of environmental conditions. The upper lethal limit temperature of tilapia is 42 $^{\circ}$C and lower lethal limit is 8-12 $^{\circ}$C, the preferred range of tilapia growth is 26-35 $^{\circ}$C with the optimal growth of 29-31 $^{\circ}$C. The growth of tilapia will be very slow under 16 $^{\circ}$C. They normally live in fresh, brackish and saltwater within the range of pH (3.5 -12). Tilapia are native to Africa, Egypt and Israel, and have emerged from mere obscurity to one of the most commercially important culture species. Globally, there are more than 70 species (McAndrew 2000) most of them are endemic to rivers of Western Africa (Anon, 1984). As a consequence of transplantation tilapia became established as a potential farmed species in the 1950s (Modadugu 2004). In 1960s, Tilapia have been introduced to tropical and sub-tropical countries, either accidentally or deliberately (Pillay and Kutty 2005). Recently, in monosex culture supermales have been obtained through genetic manipulation using novel selection techniques. Based on the theory of predominantly monofactorial sex determination, it has proved possible to manipulate the sex ratio using a combination of sex reversal and progeny testing to identify sex genotypes. In a breeding program in *O. niloticus*, the developed technology produces genetically male tilapia (GMT) with an average sex ratio of >95% male and 40%increse in yield (Mair et al., 1997). The Developed Improved Tilapia (GIFT) technology based on traditional selective breeding and meant to improve commercially important traits of tropical farmed fish is a major milestone in the history of tilapia aquaculture (Modadugu 2004). Through combined selection technology, the GIFT program attained 12-17% average genetic gain per generation over five generations and cumulative growth, and elevation in growth rate of 85% in *O. niloticus* (Eknath and Acosta 1998). The major culture

species of tilapia is *O. niloticus*, the preferred sex for culture is male tilapia because they have faster growth and reach optimum harvest weight compared to females.

1.10 Nutrient requirements of carp and tilapia

In general the nutritional requirements of fish are well documented in the recent NRC (2011) guidelines. The nutritional requirements of carp and tilapia include protein, amino acids, lipids, fatty acid, carbohydrates, vitamins, minerals. The optimum energy and protein/energy ratio have been investigated and reviewed (Kaushik 1995; Takeuchi 1999; Pillay and Kutty 2005; Lim and Webster 2006).

1.10.1 Protein and amino acid requirements

Protein is the most expensive dietary nutrient in fish diets (Jauncy 2000; Lim and Webster 2006) and essential for maintenance, growth and reproduction as well as energy (Lim and Webster 2006). A deficiency of protein will cause impairment and retardation or cessation of growth or loss of weight because of withdrawal of protein from less important tissues to retain the function of more vital organs. Carp and Tilapia, do not have an absolute requirement of protein but need to meet a good balanced mixture of essential and non-essential amino acids (Lim and Webster 2006). Ogino and Chen (1973) indicated that the common carp needs 1g kg^{-1} body weight of protein to meet their daily requirement and for 12g kg^{-1} body weight for maximum protein retention. The protein requirements of *C. carpio* have been demonstrated with crude protein levels ranging from 30-42% as being adequate for fish to grow well. Siddiqui et al. (1988) showed that tilapia fingerlings (50 g mean weight) required only 30% proteins in the diet compared to fry (0.8 g) requiring 40%. Protein requirement therefore depends on the fish age, size, type of rearing, protein quality presence of natural feed

and feed allowance (NRC 1993; Lovell 1998; Watanabe 1988; Parker 2002; Siddhuraju and Becker 2002; Takeuchi et al. 2002; Sardar et al. 2007). Proteins usually contain over 20 amino acids; 10 of these amino acids are essential amino acids (EAA) for all fish species which are arginine, histidine, isoluecine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The remaining are called non-essential amino acids NEAA which can be synthesized by fish.

Generally, the dietary protein requirement should contain the EAA up to 60% and NEAA around 40% (Jauncey 2000). Carp and tilapia do not have an absolute protein requirement however, they need to meet a well-balanced mixture of essential and nonessential amino acids in the dietary protein (Jauncey 2000; Lim and Webster 2006). The efficiency of EAA will negatively affect growth response and health status. The recommended levels of EAA in the dietary protein in the diet have been investigated by many workers for carp and tilapia see Table 1.7 (NRC, 2011).

Table 1.7 Recommended essential amino acids* requirements tested for carp and tilapia.

Essential amino acids (EAA)	Species	Estimated requirement of diet (%)
Arginine	Common carp (*Cyprinus carpio*)	1.70
	Mrigal carp (*Cirrhinus mrigala*)	1.80
	Nile tilapia (*Oreochromis niloticus*)	1.20
	Tilapia (*Sarotherodon mossambicas*)	1.60
Histidine	Common carp (*Cyprinus carpio*)	0.80
	Mrigal carp (*Cirrhinus mrigala*)	0.90
	Nile tilapia (*Oreochromis niloticus*)	1.0
Isoluecine	Common carp (*Cyprinus carpio*)	1.0
	Mrigal carp (*Cirrhinus mrigala*)	1.30
	Rohu carp (*Labeo rohita*)	1.50 – 1.60
	Nile tilapia (*Oreochromis niloticus*)	1.80
Leucine	Common carp (*Cyprinus carpio*)	1.30 (with 1.0% isoleucine)
	Mrigal carp (*Cirrhinus mrigala*)	1.50
	Rohu carp (*Labeo rohita*)	1.50 – 1.60
	Nile tilapia (*Oreochromis niloticus*)	1.90
Lysine	Common carp (*Cyprinus carpio*)	2.20
	Grass carp (*Ctenopharyrngodon idella*)	2.10 2.30
	Mrigal carp (*Cirrhinus mrigala*)	1.30 – 1.44
	Nile tilapia (*Oreochromis niloticus*)	1.60
	Tilapia (*Sarotherodon mossambicas*)	
Methionine	Common carp (*Cyprinus carpio*)	0.80(with 2.0% cysteine)
	Mrigal carp (*Cirrhinus mrigala*)	1.0-1.20 (with 1.0% cysteine)
	Nile tilapia (*Oreochromis niloticus*)	0.80-1.10 (with2.0% cysteine or total sulphur amino acid)
	Mossambique tilapia (*Oreochromis mossambicuss*)	0.50 (with 0.7% cysteine)
Phenylalanine	Common carp (*Cyprinus carpio*)	1.30 (with 2.9% tyrosine)
	Mrigal carp (*Cirrhinus mrigala*)	1.30 (with 1.0% tyrosine)
	Rohu carp (*Labeo rohita*)	1.20 (with 1.0% tyrosine)
	Nile tilapia (*Oreochromis niloticus*)	1.10(with 0.5% tyrosine)
Threonine	Common carp (*Cyprinus carpio*)	1.50
	Mrigal carp (*Cirrhinus mrigala*)	1.80
	Rohu carp (*Labeo rohita*)	1.5-1.7
	Nile tilapia (*Oreochromis niloticus*)	1.10
Tryptophan	Common carp (*Cyprinus carpio*)	0.30
	Mrigal carp (*Cirrhinus mrigala*)	0.40
	Nile tilapia (*Oreochromis niloticus*)	0.28
Valine	Common carp (*Cyprinus carpio*)	1.40
	Mrigal carp (*Cirrhinus mrigala*)	1.52
	Rohu carp (*Labeo rohita*)	1.50
	Nile tilapia (*Oreochromis niloticus*)	1.60

*(NRC) 2011.

1.10.2 Energy requirements

Energy is released during the metabolic oxidation of carbohydrate, protein and lipid; energy does not strictly account as a nutrient component but is a property of nutrients (Parker 2002; Lim and Webster 2006; NRC 2011). However, almost of all dietary energy is derived from protein and lipid (Takeuchi et al. 2002). Parker (2002) reported that energy requirements for common carp (expressed as concentration of diet) at 13.4 MJ kg^{-1} DM agrees with the study by Takeuchi et al. (2002) which states dietary energy requirement values for the common carp of 12.97-15.06 MJ kg^{-1}. The growth performance of carp required for optimum growth based on the total gross energy intake (100%), the maintenance and activity, productive and heat increment energy as well as, faecal and non-faecal energy values was; 12.6, 24, 31.9, 30 and 1.5, respectively (Ohta and Watanabe 1996). In growth of Nile tilapia, the maximum ratio of a P/ME of 0.314 MJ kg^{-1} was required for optimum growth rate when fed a diet of 30% protein content cultured in brackish water (Fineman-Kalio and Camacho 1987; Lim and Webster 2006). Also, El-Sayed and Teshima, 1992) observed optimum performance of fry Nile tilapia (12 mg) with a diet containing 35% protein with a P/DE ratio of 0.460 MJ kg^{-1}. In other study done by Lupatsch et al. (2010) it was determined that the maximum maintenance energy requirement for the growth of Nile tilapia is 0.251 MJ kg^{-1} day^{-1}.

1.10.3 Lipid requirements

Each gram of lipid contains 2.5 fold the energy content compared to a gram of carbohydrate or protein (Jauncy 2000; Parker 2002). It has been established that common carp can utilize lipids as a effective dietary energy source (Beveridge and McAndrew 2000; Takeuchi et al., 2002). Dietary lipids have two primary functions: the first of which is that they are a source of metabolic energy. Secondly, as phospholipids they maintain biological structure and normal functions of cell membranes (Jauncey

1982; Sargent et al., 1999; NRC, 2011). The requirements of EFA for freshwater and diadromous fish (juveniles and subadult) species can be met by C_{18} PUFA, 18:3n-3 and 18:2n-6 at around 1% of the diet dry weight. Traditionally, freshwater and diadromous species are divided into three groups in respect of EFA: higher levels of 18:3n-3 (compare to 18:2n-6) are required for cold water species including salmonids in a comparison to warm water species (tilapia) that require higher levels of 18:2n-6 (compare to 18:3n-3) and common carp which require a significant amount of both (NRC 2011). The optimum growth and feed efficiency was obtained in juvenile common carp when fed diets that supplied 1% of n-3 and n-6 fatty acids (Takeuchi and Watanabe 1977). Growth performance for hybrid tilapia was considerably elevated by feeding cod liver oil compared to corn oil, for optimum growth of tilapia are require n-3 fatty acid, at least n-3 LC-PUFA (Chou and Shiau 1999).

1.10.4 Carbohydrate requirements

Warmwater fish species such as carp and tilapia typically do not have requirements for carbohydrates per se. Fish are able to grow satisfactory and without any impact on health status when fed diets with low levels or even without carbohydrates. This is due to the ability of fish to obtain their glucose requirement by synthesis from amino acids (Jauncey 2000; NRC 2011). Although, carbohydrates are not essential dietary components in aquafeeds they are constantly included in fish feeds because they are relatively cheap sources of energy, spare dietary protein for growth, and function as pellet binder and act as filling agents in making diets (Jauncey 2000; Lim and Webster 2006). Jauncey (1982) pointed out a great deal more research is required in order to fully understand the benefit of carbohydrates. A study by Takeuchi et al. (2002) showed that carbohydrates can be easily utilized in common carp and used as dietary energy sources. They found that amylase activity in the digestive tract and the digestibilities 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. It has been reported that the optimum range of dietary carbohydrate for common carp is between 30-40% (Takeuchi et al. 2002). Several studies have been shown that tilapia can also utilize carbohydrates as energy source more efficiently than other finfish species i.e. Wee and Ng (1986) showed that tilapia has a superior ability to utilize carbohydrates more efficiently than salmonids, seabass, seabream, and yellowtail as a source for energy. Also they obtained a trend of elevated growth and feed utilization with increased level of cassava in the diet up to 60%. Shiau (1997) reported weight gain and protein efficiency ratio (PER) was not reduced in tilapia when the dietary level of starch and dextrin was elevated from 37% to 41% while dietary protein were decreased from 28% to 24%, this study indicated that starch and dextrin could function to spare protein sources when protein in the diet was low.

1.10.5 Vitamins requirements

Other dietary nutrient requirement that are essential in the diets, which are required in small quantities for normal growth, reproduction and good health are vitamins (Jauncey 2000; Lim and Webster 2006; NRC 2011). In general, vitamins are divided into two distinct groups, which are water soluble vitamins (B-complex vitamins, inositol, Choline and vitamin C) and fat soluble vitamins (vitamin A, D, E and K) (Lim and Webster 2006; NRC 2011). The recommended levels of vitamins in the diet have been investigated by many workers for carp and tilapia (Table 1.8).

Table 1.8 Recommended vitamin requirement estimates for carp and tilapia.

Vitamin and fish	Requirement (mg kg^{-1})	Reference
Thiamin		
Carp	0.5	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	2.5 - 4.00	(Lim et al., 1991, 2000)
Riboflavin		
Carp	6 - 7	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	4.1 - 6	(Soliman and Wilson 1992; Lim et al., 1993; NRC 2011)
Pyridoxine		
Carp	5 - 11.4	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	1.7 - 16.5	(Shiau and Hsieh 1997; NRC 2011)
Pantothenic Acid		
Carp	23 - 50	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	10	(NRC 2011)
Niacin		
Carp	20 - 28	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	26	(NRC 2011)
Biotin		
Carp	1	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	0.06	(Shiau and Chin 1999; NRC 2011)
Vitamin B12		
Carp	0 - 0.09	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	NR*	(NRC 2011)
Folate		
Carp	0 - 4.3	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	0.82	(NRC 2011)
Choline		
Carp	500 - 3000	(Shiau and Lo 2000; NRC 2011), (Shiau and Lo 2000; NRC 2011)
Tilapia	1000	
Inositol		
Carp	166 - 440	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	0 - 400	(Perres et al., 2004; NRC 2011)
Vitamin A		
Carp	0.12 - 6	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	1.76 - 2.09	(NRC 2011)
Vitamin E		
Carp	100 - 200	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	25 - 100	(Shiau and Shiau 2001; NRC 2011)
Vitamin K		
Carp	1.9	(Satoh 1991; NRC 1993; NRC 2011)
Tilapia	19 - 79	(Shiau and Jan 1992; Shiau and Hsu 1999)
Vitamin K		
Carp	ND**	
Tilapia	0.001	(NRC 2011)

*NR = Not required.

**ND = Not determined.

A deficiency of vitamins occurs in animals when diets do not contain required levels and leads abnormal physiological process and organ dysfunction (Lall 2000; NRC 2011). Vitamin deprivation in diets will cause morphological changes and functional alteration as reported in numerous fish species (Lim and Webster 2006; NRC 2011). They concluded that many factors may impact vitamin requirements of finfish including carp and tilapia, such as fish size, water temperature and diet composition. Takeuchi et al. (2002) stated that juvenile and adult carp do not require vitamin C because they can synthesize ascorbic acid from D-glucose.

1.10.6 Mineral requirements

There is small amount of information are available on the mineral requirement of fish and crustaceans. Minerals play a vital role in the body of aquatic animals for tissue formation and basic metabolic functions including osmoregulation, proper function of muscles and nerves and acid-base balance (Lim and Webster 2006; NRC 2011). Fish are able to directly absorb minerals through their gills and skin which are required in normal metabolism from their external aquatic environment, thus not all elements that are used in metabolism are required in the fish diet (Lovell 1989; De Silva and Anderson 1995; Pillay and Kutty 2005; NRC 2011). The recommended levels of mineral in the diet have been investigated by many workers for carp and tilapia (Table 1.9).

Table 1.9 Recommended macro and micro mineral requirements for carp and tilapia.

Mineral and fish	Requirement	Refernce
Macromineral	g kg^{-1}	
Calcium		
Carp	Dispensable	(NRC 2011)
Tilapia	3.5 – 7.5	(Robinson et al., 1987; O'Connel and Gatlin 1994; NRC 2011)
Potassium		
Carp	ND*	
Tilapia	2 - 3	(NRC, 2011)
Magnesium		
Carp	0.4 - 0.6	(Takeuchi et al., 2002; NRC 2011)
Tilapia	0.59 - 0.77	(Reigh 1991; NRC 2011)
Sodium		
Carp	ND	
Tilapia	1.5	(Watanabe et al.,1988; NRC 2011)
Phosphorus		
Carp	6 - 8	(Takeuchi et al., 2002; NRC 2011)
Tilapia	5	(Robinson et. al. 1987; NRC 2011)
Micromineral	mg kg^{-1}	
Copper		
Carp	3	(Satoh 1991; NRC 1993; Kim et al., 1998; Takeuchi et al., 2002; NRC 2011)
Tilapia	4	(Watanabe et al.,1988; NRC 2011)
Iron		
Carp	150 - 199	(Satoh 1991; NRC 1993; Kim et al., 1998; Takeuchi et al., 2002; NRC 2011)
Tilapia	85	(Watanabe et al.,1988; NRC 2011)
Manganese		
Carp	12 - 13	(Satoh 1991; NRC 1993; Kim et al., 1998; Takeuchi et al., 2002; NRC 2011)
Tilapia	7 - 12	(Watanabe et al.,1988; NRC 2011)
Zinc		
Carp	15 -30	(Satoh 1991; NRC 1993; Kim et al., 1998; Takeuchi et al., 2002; NRC 2011)
Tilapia	20 -30	(Watanabe et al.,1988; NRC 2011)

*ND = Not determined.

1.11 Aims and objective of research programme

The aim of this research programme was to evaluate the optimum inclusion of DDGS and YPC in a series of balanced diets for juvenile carp and tilapia under controlled laboratory conditions.

The principle aims being to greatly reduce the use of fishmeal and other high value expensive components of the diet with DDGS and YPC as reliable substitutes.

Objective

The specific objectives were to:

1- Determine the effects of DDGS and YPC on growth, feed utilization efficiency when tested at graded inclusion levels at the expense of fishmeal.

2- Quantify effects of carcass composition, protein retention and also changes in gross carcass mineral status.

3- Examine the potential fundamental properties of both DDGS and YPC as modulators of the gut microbiota in these species. Namely, assess the effects on gut integrity, gut and liver ultrastructure and aspects of overall fish health status.

4- Also, to measure the response of selected physiological and biochemical indicators of carp and tilapia in response to diets containg DDGS and YPC.

5- Evaluate the potential of DDG and YPC as first generation Co-products for general use in the aquafeed formulation and to capture their economic value in such diet.

Chapter 2

General Materials and Methods

2.1Overview

Experimental analyses conformed to the following protocols unless otherwise indicated. Specific methods for individual trials such as diet formulation and preparation are described in the relevant chapters. Chemicals, materials and reagents were sourced from Fisher Scientific Ltd. (Loughborough, Leicestershire, UK) and Sigma-Aldrich Ltd. (Poole, Dorset, UK) or Telford Ltd. (Telford Shropshire, UK). All experimental work involving fish was performed under the Home office project licence # 30/2644 and personal licence #30/8745.

2.2 Specification and nutrient composition of the major ingredients used in this study

2.2.1. Fishmeal

Generally, fishmeals are made by two different methods, direct drying (or cooking), pressing to remove liquid and oil before drying and grinding the whole fish of the same species (brown fishmeal). The fishmeals produced from by-catch of mixed species or by-product processing is called white fishmeal. The various types of fishmeals are graded on the basis of the species and area of production (Norwegian and American fishmeals). The quality of fishmeal relies on the manufacturing processes such as cooking times as well as cooking temperature (Guillaume et. al., 1998; Lim and Webster, 2006).

Within the raw ingredients in the feed, fishmeal has and continues to receive the most attention due to excellent nutritional profile and high palatability (Fin 2007; Laporte 2008). In fact, fishmeal is an excellent source of essential amino acids, minerals and essential fatty acid (Guillaume et. al., 1998; Lim and Webster 2006). Norwegian fishmeal LT 94 (high quality and low temperature) purchased from Skretting

Aquaculture, a Nutreco company (Preston, UK) was used as a control diet. For nutrient composition see Table 2.1 and Table 2.2.

2.2.2 Dried distiller's grain and solubles (DDGS)

Distiller's dried grain with solubles (DDGS) is the spent residue from typically corn and wheat fermentation after producing ethanol. DDGS is a valuable feed ingredient which is a by-product of the dry-grind or wet mill ethanol production resulting from the yeast fermentation of cereal grains (Belyae et. al, 2004). Corn (maize) is often the cereal of choice for ethanol plants because of its highly fermentable starch content. Each tonne of corn is converted into approximately 303 kg of DDGS, 323 kg of ethanol and 323 kg of CO_2 (Jacques et al., 2003). The continual expansion of the fuel ethanol industry will provide a steady and growing DDGS output and continually improving processing technologies provides interesting economical opportunities for low level inclusion in animal feeds.

The protein content is generally around 30% of the Dry Matter (DM). On the other hand, DDGS contains a modest level of digestible energy and is also a rich source of vitamins and minerals. The physical appearance, chemical composition and nutrient digestibility can vary considerably depending on sources, processing and different drying procedures see Chapter 1 Table 1.1.

2.2.3 Yeast protein concentrate unrefined (YPCu)

The yeast concentrate used in this experiment is bio-fuel yeast derived by product from dried distiller's grains and solubles obtained by a novel separation technique. In the bio-ethanol process, grain is milled into wholemeal flour, blended with water to form a mash, cooked and the gelatinized starch hydrolysed to fermentable sugars. Yeast is added and the mash is fermented to a beer of 12% alcohol strength. The process takes up to 100 h and the apparent substrate-alcohol conversion is a round 84%. The process produces a beer which is distilled, concentrated and dried to produce 99.7% ethanol. In the conventional process the remaining grain is dried to produce distiller's dark grains and solubles (see Plate 2.1).

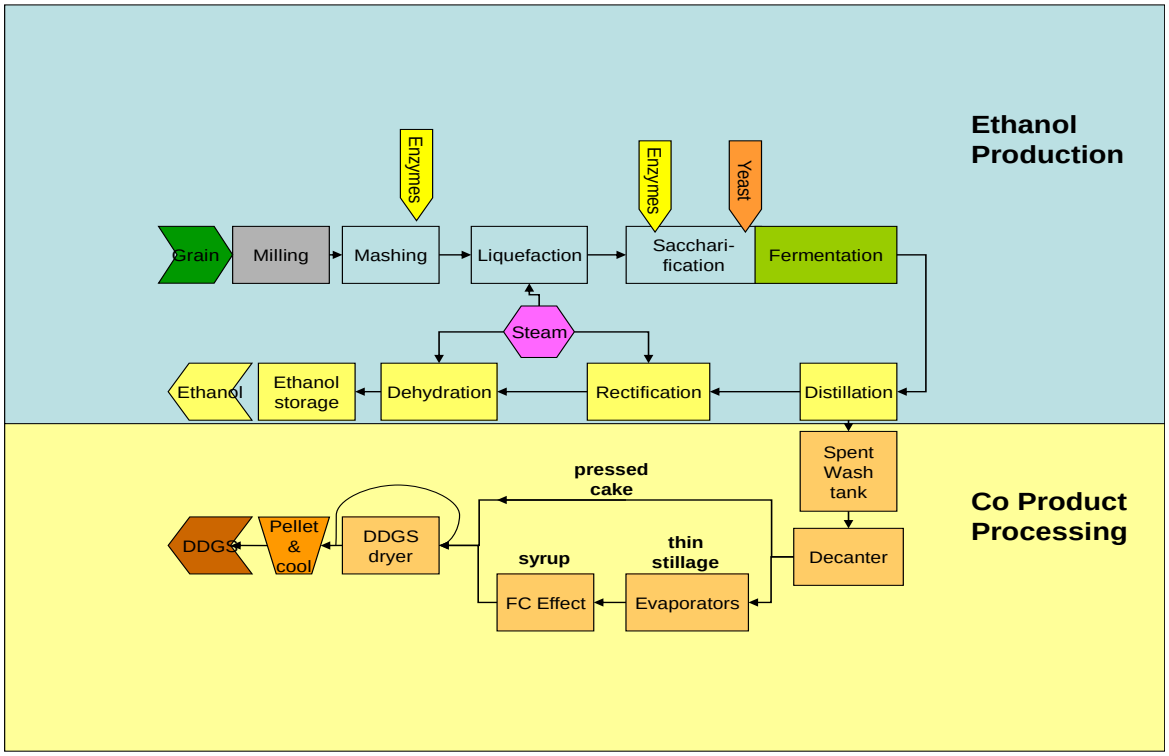


Plate 2.1 A conventional dry grind bio-ethanol process (Source AB-Agri Ltd).

Approximately 16% loss in starch to ethanol conversion may be due largely to the energy required for yeast growth but whilst the quantity of yeast set is well documented

the quantity of yeast generated in the process and hence present in the final co-product has not been measured.

The bio fuel yeast (yeast protein concentrate unrefined (YPCU)) is produced from the concentration of the yeast fraction following fermentation. In the modified process, the dry YPCU is separated by a three stage process; whole stillage is fed into a first stage separation which separates the fibrous components of the whole stillage from the suspended and dissolved solid. The suspended and dissolved solid (thin stillage) is then separated into a high concentrate yeast cream and a supernatant. The yeast cream may then be dried to produce YPCU. The YPCU may represent up to 15 % of the total co-product dry matter. The protein content of the yeast is approximately 34% of the dry matter (see Plate 2.2).

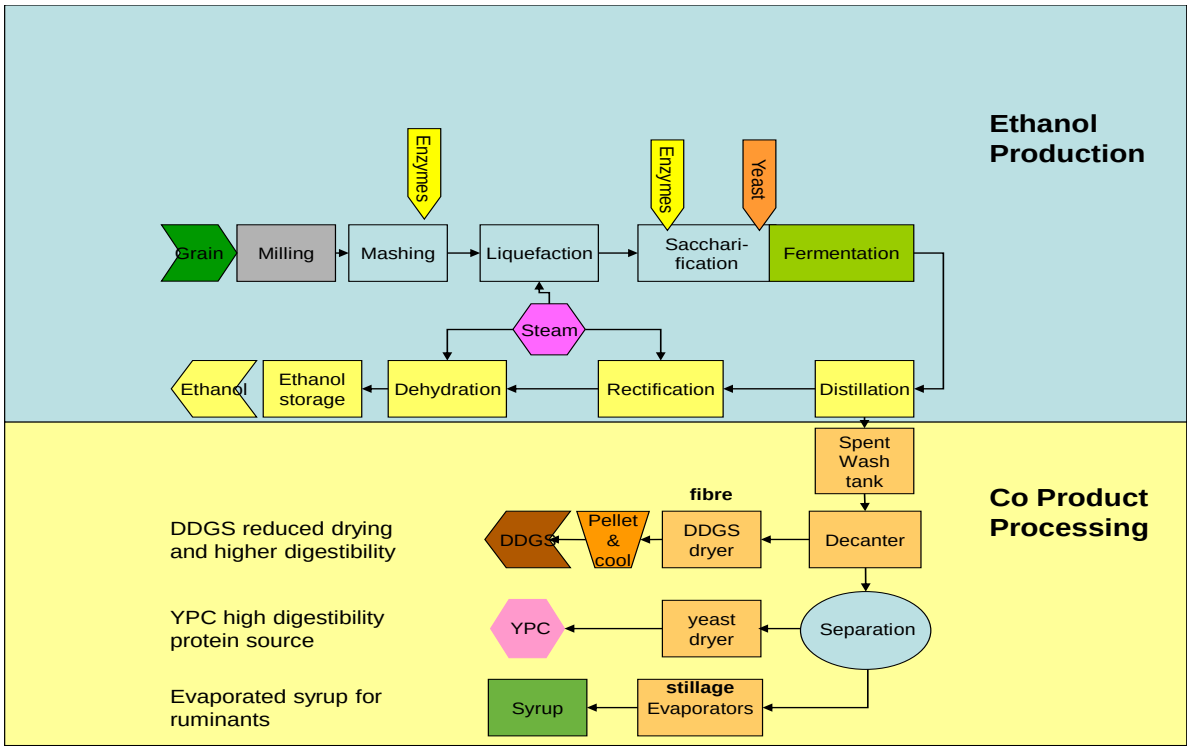


Plate 2.2 Modified co-product processing for producing yeast protein concentrate (Source AB-Agri Ltd).

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Table2.1 Proximate composition, amino acid profiles of the ingredients used in the experimental diets.

Proximate composition (%)	FM	YPC_U	YPC_R	SCY_{PA}	DDGS	WM
Moisture	7.55	11.76	6.77	13.91	7.82	10.59
Dry matter	92.18	88.24	93.23	86.09	92.18	89.41
Protein	67.14	33.99	54.62	52.85	31.79	13.42
Lipid	9.66	3.62	6.68	0.38	5.75	2.80
Ash	12.80	5.65	2.63	4.01	4.40	3.88
NFE	2.84	43.98	18.78	28.85	50.25	69.32
Gross Energy(MJ kg^{-1})	20.40	18.37	21.19	19.37	19.59	19.74
Essential amino acids (%)						
Arginine	5.31	1.35	2.03	2.11	1.21	1.19
Histidine	1.56	0.78	1.20	1.06	0.97	0.55
Iso-Leucine	2.97	1.25	2.14	2.46	1.2	0.58
Leucine	5.13	2.19	3.82	3.72	2.13	0.99
Phenylanine	2.72	1.47	2.60	2.18	1.5	0.69
Lysine	5.09	0.99	1.59	3.51	0.83	0.78
Threonine	2.77	1.07	1.56	2.37	1.05	0.53
Valine	3.34	1.49	2.43	2.84	1.51	0.84
Methionine	1.86	0.49	0.85	0.89	0.45	0.23
Tryptophan*	ND	ND	ND	ND	ND	ND
Non-essential amino acids (%)						
Alanine	3.84	1.23	1.77	3.04	1.28	0.74
Aspartic acid	6.09	1.87	2.49	4.76	1.74	1.14
Cysteine	0.7	0.66	1.07	0.6	0.61	0.38
Glutamine	8.86	8.75	18.32	5.47	8.70	2.97
Glycine	3.43	1.32	1.91	2.05	1.45	0.81
Proline	2.2	2.58	4.61	1.73	2.57	0.74
Serine	2.66	1.34	2.06	2.27	1.58	0.75
Tyrosine	1.61	0.87	1.45	1.45	0.86	0.41

All data are expressed as mean ± Standard deviation. *Not determined, Nitrogen-free extracts (NFE) = dry matter – (crude protein + crude lipid + ash), FM = Fish Meal, YPC_U = Yeast Protein Concentrate Unrefined, YPC_R = Yeast Protein Concentrate Refined, YPC_{PA} = Yeast Protein Concentrate Potable Alcohol, DDGS = Dried Distiller's Grain and Solubles, WM = Wheat Meal.

Table 2.2 Mineral profile of the ingredient used in the experimental diets.

Proximate composition (%)	FM	YPC_U	YPC_R	YPC_{PA}	DDGS	WM
Macro Mineral (g kg^{-1})						
Ca	16.38±0.45	1.12±0.07	1.79±0.15	0.69±0.16	1.11±0.09	1.11±0.06
P	15.44±0.46	9.00±0.22	5.57±0.24	7.35±0. 10	6.14±0.06	6.74±0.33
K	9.05±0.11	11.38±0.22	4.47±0.20	2.29±0.03	7.23±0.08	8.74±0.29
Mg	2.21±0.03	2.51±0.06	1.03±0.05	0.76±0.02	2.18±0.02	2.69±0.14
Na	12.02±0.15	0.34±0.03	1.00±0.04	7.09±0.11	2.24±0.06	0.23±0.02
Micro mineral (mg kg^{-1})						
Cr	0.52±0.26	0.60±0.17	0.37±0.19	0.15±0.02	0.43±0.11	0.30±0.07
Cu	7.00±1.55	11.25±0.87	14.61±1.68	79.45±2.18	12.49±0.35	14.80±0.40
Fe	196.45±3.42	188.54±11.49	203.50±13.02	37.09±7.16	106.32±11.09	147.52±34.62
Mn	3.58±0.06	74.95±1.65	33.10±1.35	40.44±0.67	43.08±0.17	54.11±2.81
Zn	66.56±0.99	51.25±2.50	30.24±3.40	26.17±4.22	49.47±1.64	57.15±2.81

All data are expressed as mean ± Standard deviation, *Not determined, FM = Fish Meal, YPC_U = Yeast Protein Concentrate Unrefined, YPC_R = Yeast Protein Concentrate Refined, YPC_{PA} = Yeast Protein Concentrate Potable Alcohol, DDGS = Dried Distiller's Grain and Solubles, WM = Wheat Meal.

2.2.4 Yeast protein concentrate refined (YPC_R)

The washed form of yeast protein concentrate YPC_R is YPC_U washed to produce a pure yeast product which contains a higher amount of protein. The protein content of washed yeast (YPC_R) is considerably higher (~55%). The unwashed form (YPC_U) contains yeast and non-starch polysaccharides.

2.2.5 Yeast protein concentrate Potable Alcohol (Diageo) YPC_{PA}

The yeast protein concentrate from potable alcohol (YPC_{PA}) is produced by fermentation of yeast fraction for process of making alcohol. The process involves an enzymatic hydrolysis of the *S. cerevisiae* cells by yeast autolytic enzymes with additional copper added for enzyme activation. The spent wash (containing yeast) was produced by fermentation of a commercial strain of wheat or corn hydrolysate by baker's yeast (*S. cerevisiae*) which was retained at a temperature close to 50 ºC for 5 - 10 h. Then the water soluble fraction was separated by centrifugation or filtration and concentrated by evaporation at 90 ºC to produce YPC_{PA}. The protein concentration of YPC_{PA} is relatively high (~53%) and contained a high amount of copper (80 mg kg^{-1}) compared to the copper content of YPC_U (11 mg kg^{-1}) and YPC_R (15 mg kg^{-1}).

2.3 Feed formulation and Diet preparation

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 formulation software, with the restrictions used representing the NRC (2011) guidelines for the appropriate nutrient class. All the raw materials were weighed in plastic containers and placed into a food bench mixer. The ingredients were blended uniformly to ensure homogeneous distribution of the diet components and then mixed for approximately 25 min using a Hobart food mixer (Hobart Food Equipment, Australia). After the initial blending, fish oil and corn oil were gradually added in a continuous flow. After further mixing, water (~ 2 L) was added to form light dough of each diet. The pastes were passed through an extruder (PTM Extruder System, Plymouth, UK) and an appropriate aperture die was used to achieve the desired pellet size (2-mm pellets). The resulting strands were carefully broken up and spread onto trays lined with tinfoil. These trays were subsequently transferred into a warm air oven (Genlab, MINO/ 200/ SS/F, Chashire, UK) where they were left for 24 h at 60 $^{\circ}$C. Diets were all stored in polythene containers and kept in a dry dark cabinet prior to their use in the respective feeding trials.

2.4 Fish and experimental facilities

During course of the programme of investigations three different groups of fish were obtained for the separate experimental trials. Juvenile mirror carp and common carp (*Cyprinus carpio*) were obtained from Bowlake fish farm, Hampshire, UK and Nile tilapia were obtained from FishGen Ltd, Swansea, Wales, UK (Courtesy of Mr. Eric Roderick). In both cases, fish were transported from the fish farm directly to the aquarium facility in a 1000 L tank supplied with pure oxygen (BOC, UK). Total transportation time did not exceed 24 h at any stage. Fish for each group were

subsequently acclimatized in plastic tanks (each measuring 30x45x60 cm^3) with a mean fish weight of ~5 g for approximately six weeks prior to the start of the trials. Both carp and tilapia were fed a commercial diet (2% of the body weight) twice daily. The fish were subsequently re-graded and distributed randomly into experimental tanks to proceed with experimental trials. The light regime was 12 h lightness and 12 h darkness and was applied throughout the experimental period (8 weeks) (see Plate 2.3).

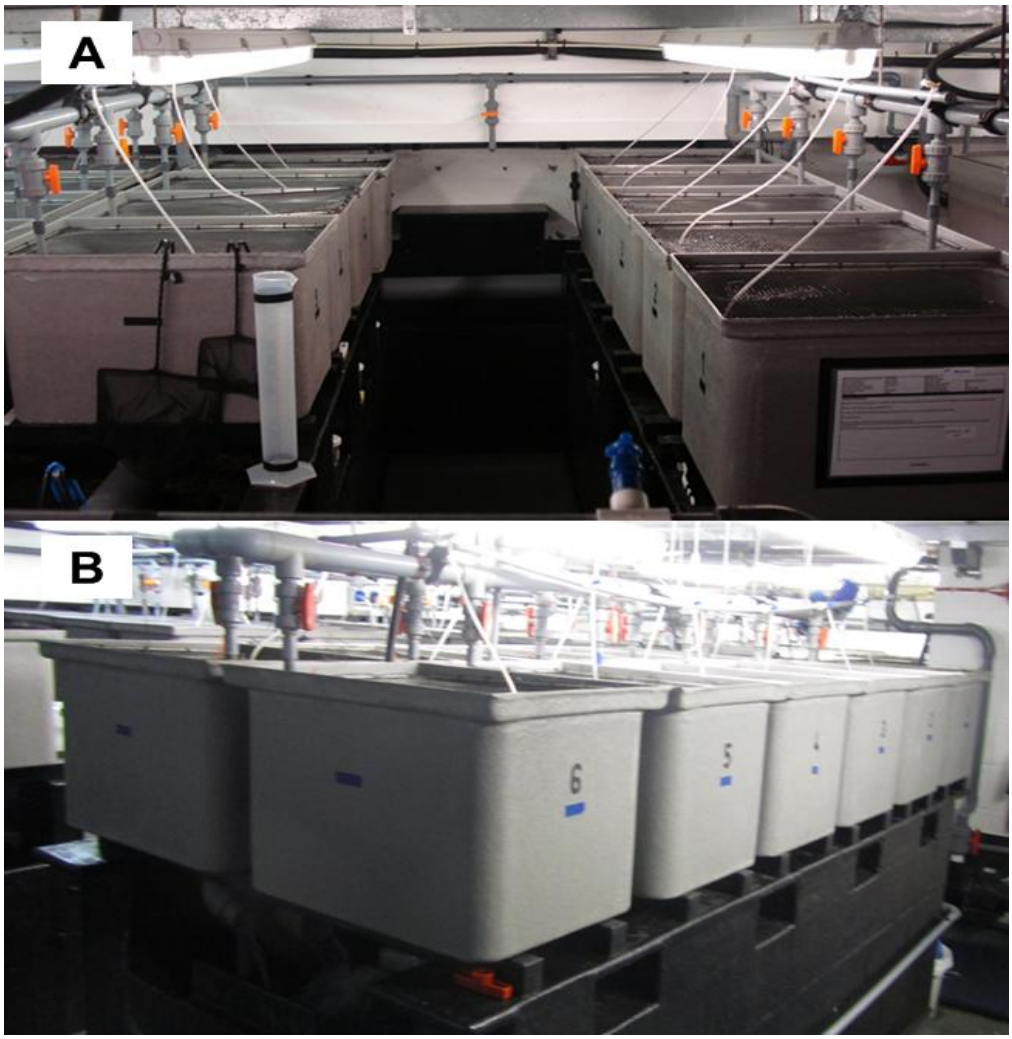


Plate 2.3 A and B are Recirculation systems (B and C) located at University of Plymouth fish nutrition research unit, West aquarium, which used in this program of study.

2.5 Feed and Weighing

All fish in each trial were fed 3.5-4% 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 on six consecutive days per week. Fish were deprived of feed one day prior to weighing (7^{th} day). Feeding rates were adjusted accordingly on the basis of the new total biomass in each tank.

2.6 Water quality

All the experiments were undertaken at the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth. Trials were conducted in experimental system B and C (Plate 2.1 A, B), the facility used was a freshwater recirculation system with a total water capacity of 3933 L and 2300 L respectively. Fish were randomly distributed into 80 L fibreglass tanks, each provided with 99% re-circulated aerated freshwater at a rate of 300 L h^{-1}. Biological filtration was provided by a submerged biological filter bed containing *Nitrosomonas* and *Nitrospira* spp. Water samples were collected three times a week from the aquarium system. Dissolved oxygen, temperature and pH were measured daily by HQ 40d multiparameter meter (HACH Company, Loveland, USA). The water temperature was measured daily and held at an average value of 24.26 ± 0.29 $^{\circ}$C throughout the experiment with an immersed heater. Dissolved oxygen (D.O) was recorded and averaged at 7.37 ± 0.27 mg L^{-1}. The pH of was maintained at an average at 6.71 ± 0.29 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 ammonia concentration. Each filter was also cleaned daily. Total ammonia nitrogen (TAN) (0.089 ± 0.051) mg L^{-1}, nitrite (0.046 ± 0.023) mg L^{-1} and nitrate (29.42 ± 11.57) mg L^{-1} 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 tilapia and carp as proposed by Balarin and Haller (1982). These are dissolved oxygen >3.0 mg L^{-1}, pH 4-12, TAN < 12 mg L^{-1} and nitrite < 2 mg L^{-1}.

2.7 Fish performance and feed utilization assessments

Growth performance and feed utilization parameters were determined using the following standard expressions and equations as well as calculations to assess nutrient utilization and organ; body mass indices.

Specific Growth Rate (SGR %) $= \frac{(\ln \mathrm{FW} - \ln \mathrm{IW})}{\mathrm{T}} \times 100$

Weight Gain (WG g $fish^{-1}$) = FBW-IBW

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

Protein Efficiency Ratio (PER g) $= \frac{\mathrm{WG}}{\mathrm{PI}}$

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

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

Condition Factor (K %) = $\frac{\mathrm{FBW}}{\mathrm{FL\^{}3}} \times 100$

Mortality (%) = [(initial Nb – final Nb) / initial Nb] × 100

Survival (%) = 100 – Mortality (%) = (final Nb / initial Nb) × 100

Where;

lnFW = Logarithm of final weight, lnIW = Logarithm of initial weight, IBW = Initial body weight (g), FBW= Final body weight (g), IBP = Initial body protein (g), FBP = Final body protein (g), WG = Weight gain (g), FI= Food intake (g), FL =Final Length

(cm) T=Time (day), PI= Protein intake (g). Initial Nb is the initial number of fish and final Nb the final number of fish.

2.8 Chemical and proximate analysis

Raw materials (ingredients), complete test diet and whole fish bodies were subjected to analysis for the determination of moisture, ash, lipid, protein, amino acid, mineral and gross energy content in triplicate. Initial and final fish from each treatment were sampled to determine carcass composition. All samples were analysed according to the standard methods AOAC (2002) with slight modification for automatic equipment and analytical instrumentation.

2.8.1 Moisture

All samples were weighed and dried (in duplicate) at 105 $^{\circ}$C with a fan assisted oven (Gallenkamp Oven BS, Model; OV-160, England) until a constant weight was achieved. Percentage moisture was calculated by:

Moisture (%) = Wet weight (g) – Dry weight (g) / Wet weight (g) ×100

Dry matter or total solid was measured as; (100 - % moisture)

2.8.2 Ash

Ash (total mineral or organic matter) content was determined in duplicate by combusting known dry weight of sample (~500 mg) into to a pre-weighed crucible. The crucibles were then incinerated in a muffle furnace (Carbolite, Sheffield, England) at 550 $^{\circ}$C for 12 h until light grey ash results or to constant weight. Percentage ash was determined from the sample residue by:

Ash (%) = ((Sample residue (g) - crucible weight (g)) / (initial sample weight (g))) ×100

2.8.3 Lipid

Lipid content was determined in duplicate using the Soxhlet extraction method. Samples were weighed (~3 g) and placed into a cellulose thimble lightly plugged with cotton wool and inserted into the condensers of a SoxTec^{TM} extraction system (Tecator Systems, Högnäs, Sweden; model Soxtec 1043 and service unit 1046). Pre-weighed cups containing 40 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 (see Plate 2.4). Lipid content was determined as:

Total lipid (%) = Final weight of cup (g) – Initial weight of cup (g) / Initial weight of cup (g) ×100.

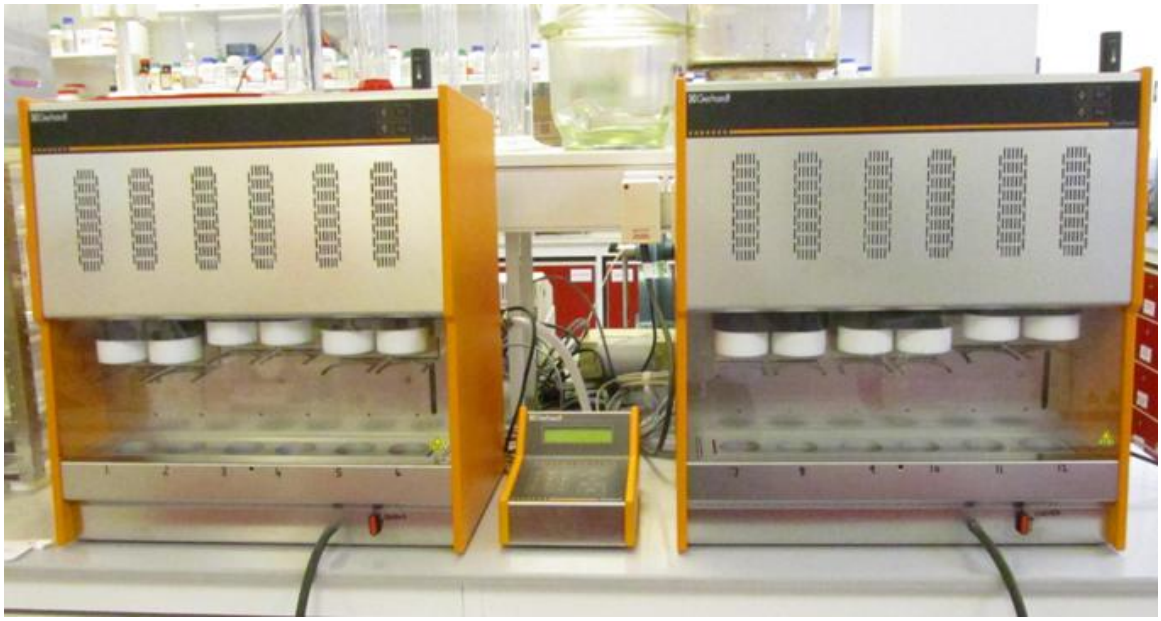


Plate 2.4 Soxhlet system operated in the nutrition laboratory of the University of Plymouth.

2.8.4 Protein

Determination of crude protein (CP) in diets, whole fish carcass and feed ingredients was done 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 (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 , 105 mg $CuSO_4.5H_2O$ 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 $^{\circ}$C for 30 min, 225 $^{\circ}$C for 45 min and at 380 ^{o}C 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) (see Plate 2.5). The distillate was neutralised with concentrated H_2SO_4 and from the titration value crude protein

determined as;

Crude protein (%) = (((Sample titre (mL) – Blank titre (mL) ×0.10×14×6.25) / Sample weight (mg) × 100

Where 0.10 is the molarity of acid, 14 the relative atomic mass of nitrogen and 6.25 a constant relationship between N and the animal (plant) protein of the sample.

2.8.5 Amino acids

The samples (ingredients, feed diets and initial carcass fish) were analysed by an external private Laboratories (Sciantec Analytical Services Ltd, Stockbridge Technology Centre, Cawood North, Yorkshire; UK). Analyses were achieved using high performance liquid chromatography (HPLC) for Amino Acids. As three replicates were pooled together, a single value per treatment was obtained.

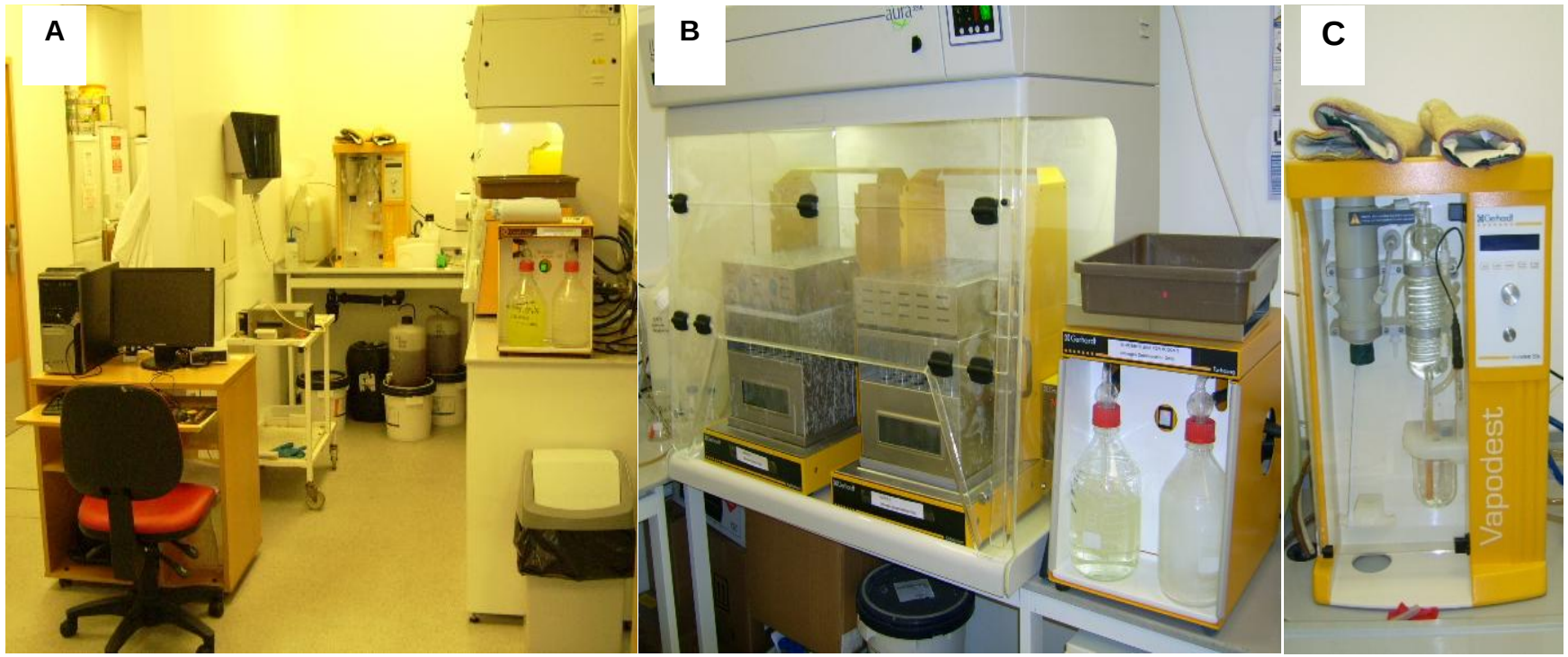


Plate 2.5 A, B and C are computerized digestion block and distillation unit of the Kjeldahl system utilized (Gerhardt Laboratory instruments) at the University of Plymouth.

2.8.6 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, USA). Ground and dried sample was first compressed into a 1 ± 0.1 g pellet and weighed. The pellet was then loaded into a nickel crucible with a 10 cm 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 litres of water was used with the instrument to determine released heat energy. This was weighed as 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 2.6).

Plate 2.6 Bomb calorimeter in the nutrition laboratory at the University of Plymouth.

2.8.7 Mineral analysis

Mineral analysis was carried out in triplicate nitric acid digested samples according to the Standard method AOAC (2002). Feed ingredients, diets and whole body samples were dried and percentage moisture determined (see 2.5.1 Moisture). For the analysis of elements samples (1.5 ± 0.5 g) were weighed and directly placed into micro Kjeldahl tubes and digested in 10 mL of (ANALAR , State Quality) nitric acid. Samples were digested using a Gerhardt Kjeldatherm 40 tube digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) at 60 $^{\circ}$C for 1 h, 90 $^{\circ}$C for 1 h, 110 $^{\circ}$C for min and 4 h at 135-140 $^{\circ}$C. After a 1 h cooling phase, the samples were then transferred into pre-weighed 50 ml polypropylene vials and diluted to exactly 50 ml with ultra-pure Milli-Q water (Millipore Corp., MA, USA).

Macro elements were determined by preparing 5 standards (0, 0.5, 1, 5, 10 mg) and (0, 0.05, 0.1, 0.5 and 1 mg) for micro elements and along with the diluted nitric acid digests were spiked with 0.1% Yttrium as the internal reference standard. Using the software provided with the ICP mass spectrophotometer, standard curves were created and then the samples run through the spectrometer. Simply, the sample is atomized into the plasma flame which causes the elements present to release energy at a specific wavelength for that element. The machine will produces a concentration of each element in mg kg^{-1}. Mineral analyses were conducted by using ICP-MS (ISO 9001-2000SGC) classification.

2.9. Histology

Two fish per tank (six per treatment) were euthanized with tricane methane sulphate (MS222) at 120 mg L^{-1} followed by destruction of the brain, on termination of each experimental trial. The intestinal and liver samples from nine fish per treatment (n=3) were retained for histological examination by both light and electron microscopy. Intestinal sections from the anterior and posterior regions (ca 2 mm^2) were excised for both light and electron microscopy. Liver samples were analysed using light microscopy (LM).

2.9.1 Light microscopy

Sample for LM were fixed in 4% saline formalin 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 7 µm thickness with a RM2235 microtome and stained using either haematoxylin and eosin staining for liver samples with a Lica Autostainer XL or alecian blue periodic acid-Schiff staining (AB-PAS) (Kiernan, 1981) for intestine samples. Slides were mounted with cover slip and DPX. A Photograph of slides at appropriate magnification were taken with an Olympus e-620 digital camera mounted Vanox Olympus research microscope model AHBT.

2.9.2 Electron microscopy

2.9.2.1 Scanning electron microscopy (SEM)

Fish were dissected as described in section 2.9. SEM samples were taken from nine fish per per treatment unless otherwise stated. Samples for SEM were washed thoroughly in 1% S-carboxymethyl-L-cysteine for 30 sec prior to fixation in order to remove epithelial mucus. Then, samples were fixed using 2.5% glutaraldehyde with 1% M cacodylate acid sodium salt (1:1) solution at pH 7.2 with 2.5% NaCl. Fixative was removed from the samples by rinsing samples 3-times with 1% M sodium cacodylate buffer for 15 min. Dehydration of the samples were attained by positioning in graded ethanol solutions (30%, 50%, 70%, 90%) and twice in 100% for at least 15 min each. All processed samples were dried using a critical point dryer (Emitech K850; Kent, UK) to a critical dried status with ethanol as the intermediate fluid and CO_2 as the transition fluid. Subsequently, dried samples were mounted on aluminium stubs and gold coated using a K550 sputter coater (Kent, UK). Samples were then screened with a Jeol JSM 5600 LV electron microscope at 15 kV (Jeol, Tokyo, Japan). 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.

2.9.2.2 Transmission electron microscopy (TEM)

Fish were dissected as described in section 2.9 Samples for TEM were collected from nine fish per treatment unless otherwise stated. The samples were excised and fixed in 2.5% glutaraldehyde with 1% M cacodylate acid sodium salt (1:1) solution at pH 7.2 with additional 2.5% NaCl. Samples were rinsed again twice 15 min in order to remove fixative. The samples were then rinsed and post-fixed in osmium tetroxide for 1 h (O_SO_4). Afterwards, they were rinsed again twice with 1% M cacodylate sodium buffer in order to remove the residual osmium. Samples were then dehydrated with graded alcohol solutions 30%, 50%, 70%, and 90% respectively (for 15 min) and twice 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 5 h, 70% resin: 30% ethanol for a minimum of 5 h, and finally in 100% resin for 24 h. Samples were placed in beam capsules and the resin was polymerised at 70 ºC overnight. 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 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 (Lewis and Knight 1977). Final examination of the ultrathin sections were made on a Jeol JSM 1200 EX transmission electron microscope at 120 kV (Jeol, Tokyo, Japan). Transmission electron microscopy (TEM) images were analysed using Image J 1.43 (Magnification x 20, 000) to calculate the length of microvilli (Hu et al., 2007). Ten well orientated individual microvilli were calculated per image, with typically three images per sample.

2.10 Haematological parameters

Blood samples were collected from nine fish per treatment (three from each tank) at the end of the growth trials. Fish were anaesthetized with buffered tricane methanesulphate (MS222) at 120 mg L^{-1} (Sodium bicarbonate 240 mg L^{-1}) followed by destruction of brain. Blood was sampled from the caudal vein using a 25-gauge needle and 1-ml syringe and placed into eppendrof tubes.

2.10.1 Haematocrit (Hct)

Haematocrit (packed cell volumes) of fresh blood was determined in triplicate as described by Klontz (1997) Hct was calculated by drawing fresh blood into heparinised haematocrit tubes filled three quarters full, with Cristaseal, sealed and centrifuged at 3600 g for five min in a microhaematocrit centrifuge. Haematocrit values were measured as the total percentage packed cell volume (PCV) using a Hawksley reader.

2.10.2 Haemoglobin (Hb)

Haemoglobin (Hb) concentration was calculated based on Drabkin's reagent solution (0.1 g sodium bicarbonate, 50 mg potassium cyanide, 200 mg potassium ferrycyanide). Assay was performed by adding 20 µL of whole blood to 5 ml of Drabkin's reagent, and vortexed immediately. The haemoglobin was measured at 540 nm using a spectrophotometer against a blank containing 5 ml Drabkin's reagent and 20 µL distilled water. Haemoglobin absorbance were measured from a curve prepared from reference standards (cyanmethaemoglobin; Sigma diagnostic kit Nº 525 A). The values obtained are expressed in g dL^{-1}.

2.11 Hepatic enzyme activity

At the end of the experiment two fish per tank (six per treatment) were euthanized with tricane methane sulphate (MS222) at 120 mg L^{-1} followed by destruction of the brain. Liver samples were removed and immediately frozen in liquid nitrogen and stored at -80 $^{\circ}C$ until analysis of enzymatic activities. In order to measure alanine aminotransferase (ALAT; EC 2.6.1.2) and aspartate aminotransferase (ASAT; EC 2.6.1) activity, a frozen sample of liver was homoginzed (dilution 1/10) in ice-cold-buffer (30 mM HEPES, 0.25 mM saccharose, 0.5 mM EDTA, 5 mM K_2HPO_4 , 1 mM dithiothreitol, pH=7.4). After centrifugation 900 g for 10 min, the resultant supernatant was sonicated and centrifuged again at 13000 g for 5 min (Enes et al., 2008). Resultant supernatant was separated for ALAT and ASAT activities which were performed in triplicate at 340 nm using a micro plate reader (Dynatech Laboratories MRX plate, Billingshurt, UK) according to Bergmeyer et al. (1983) with some modification; a reduction of the final volume of the reaction cocktail to 250 µL and 10 µL of sample was used. The assay temperature was maintained at 25 ºC and the activity was expressed in µmol min^{-1} g^{-1} wet weight.

Total protein content of each supernatant fraction was calculated according to Bradford (1976) using a Sigma protein assay kit with bovine serum albumin as a standard. The specific activity of the enzymes was defined as activity unit per mg protein. One unit of enzyme activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 µmol of substrate per minute at assay temperature.

2.12 Microbiology and microbiota identification

2.12.1 Fish dissection

Under strict aseptic conditions, nitrile gloves regularly wiped down with 99% ethanol, were used for dissecting carp and tilapia. 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 and 70% ethanol and the peritoneal cavity was opened with a sterile scalpel blade. Nine 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 120 mg L^{-1} and Sodium bicarbonate 240 mg L^{-1} followed by destruction of the brain. The time between termination and dissection did not exceed 2h 30 mins. The whole intestine was removed and the digesta from three fish per tank were pooled together to avoid interfish variation (Hovda et al., 2007; Merrifield et al., 2009a). The samples were stored at -20 ºC for further use.

2.12.2 Bacterial DNA extraction and 16S rRNA amplification

All molecular methodologies were conducted in a Labcaire PCR work station (Labcaire Systems Ltd, Clevedon, UK). The sample was suspended in 200 µL distilled water subjected to 10 min boiling pre-treatments. DNA was extracted using a modified protocol of the QIAamp® Stool Mini Kit (Qiagen).

Concentration of DNA was determined spectrophotometrically at 260 nm (Thermo Scientific Nano Drop™ 1000, DE, USA) and standardized.

PCR amplification of the 16S rRNA was undertaken using the forward primer P3 with a GC clamp (5'- CGC CCG CCG CGC GCG GCG GGG GCG GGG GCA GGG GGG

GCC TAC GGG AGG CAG CAG-3') and the reverse primer P2 (5'-ATT ACC GCG GCT GCT GG-3') after Muyzer et al.(1993). These primers correspond to position 341 and to position 534 in the 16S rDNA of *E.coli*, which release a fragment of 233 bp. The following reagents were included in each PCR tube: 1µL of each primer P2 and P3 (50 pmol μL^{-1}; MWG-Biotech AG, Germany), 5µL DNA Template, 25µL ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma) and PCR grad water to a final volume of 50 µL. Giving a final concentration of 1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCI, 1.5 mM $MgCl_2$, 0.001% gelatine, 0.2 mM dNTPs. The touchdown cycling was conducted using a GeneAmp® PCR System 9700 (Perkin-Elmer, CA., USA), under the following conditions: 94 ºC for 10 min, then 30 cycles starting at 94 ºC for 1 min, 65 ºC for 2 min, 72 ºC for 3 min. The annealing temperature decreased by 1 ºC for the remaining cycle until 55 ºC and then remained at 55 ºC for the remaining cycles. The PCR products were stored at 4 ºC until used.

2.12.3 Denaturing gradient gel electrophoresis DGGE

DNA extract/PCR products were run on agarose gels to assess quality. Unless otherwise stated, all standard electrophoresis was conducted using 1.2 % agarose (Cabrex Bio Sciences, ME, USA). 5µL of a standardized PCR product were run on 8% acrylamide gels with a denaturing gradient of 40-60% (where 100% denaturant is 7 M urea and 40% formamide). 5µL of the sample was loaded with 1µL of loading buffer (Bioline). 5µL of HyperLadder IV (Bioline) was used to assess the size of DNA products containing ethidium bromide (EtBr, 0.5 mg mL^{-1}) run with 1 x Tris-borate-EDTA (TBE) buffer in a Pharmacia electrophoresis tank. 5 µL of HyperLadder IV (Bioline) was used to assess the size of DNA products. Visualization of denaturing agarose gels bands was achieved with a Bio-Rad universal hood II (Bio-Rad Laboratoris, Italy).

PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions. DNA products were diluted to 10 – 50 ng μL^{-1} with molecular grade water and sequenced by GATC laboratories (GATC-biotech laboratories, Germany). A Blast search in the GenBank (http://blast.ncbi. nlm. nih. gov/Blast.cgi) was run to retrieve the taxonomic groups for which the sequences displayed the highest identities.

2.13. Statistical analysis

All data were subjected to one way analysis of variance (ANOVA) using SPSS statistics version 16.0 Statistical analyses (i.e. growth parameters, body composition, Enzyme assay, histology) were conducted using an ANOVA (SPSS V17.0) for windows (SPSS, Chicago, USA). The post-hoc test was used to determined significant differences between control and treatments. Data are presented as mean ± standard deviation (SD). It should be noted that the percentage data were converted to Arcsine prior for statistical analyses. DGGE banding patterns were transformed into presence/absence matrices for assessment between treatments using Quantity one® V4.6.3 analyses software (Bio-Rad laboratories, CA, USA). Dice coefficients of similarity were calculated to compare fingerprints and band intensities were measured. Similarity percentages (SIMPER), a one-way analysis of similarity (ANOSIM) and Bray Curtis similarity matrix was used to represent the relative similarities between treatments and replicates using Primer V6 (Clarke and Gorley 2006). The species richness was assessed using Margalef's D measure of richness and species diversity was assessed using the Shannon–Weaver index. All other Significance was accepted at $P < 0.05$.

Chapter 3

Bio-fuel derived unrefined yeast protein concentrate (YPCU) as a novel feed ingredient in carp diets

3.1 Introduction

Finding novel sustainable protein sources has become a major drive in the aquaculture sector in order to reduce dependency on fish meal as the main protein component in aquafeeds. In the aquaculture industry the cost of feed represents more than 50% of variable costs, depending on the level of intensity of the operation (NRC 2011). The current fishmeal usage in aquafeeds is becoming unsustainable as the aquaculture production output continues to expand. This exacerbates pressures on wild fisheries which cannot be sustained to meet such demands. Traditionally, alternatives to protein meals have been sought from vegetable sources such as soybean meals and cottonseed meals due to their wide-spread availability, relatively favourable amino acid profiles, reduced cost and sustainable nature (Hardy 2010). 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 (Oliva-Teles and Gonçalves 2001; Gatlin et al., 2007).

Single cell protein (micro algae, bacteria and yeast) have also been examined as alternative non-conventional protein sources in aquafeeds (Sanderson and Jolly 1994; Tacon 1994). Among single cell protein sources, yeasts have received a great deal of attention because they contain moderately high levels of protein and are a good source of nucleotides and RNA, ß-glucans, vitamins, arabinoxylan and mannan oligosaccharides (MOS) (Tacon, 1994; Oliva-Teles and Gonçalves 2001; Gatlin 2003; Ghoshk et al., 2005; Abdel-Tawwab et al., 2008, 2010; He et al., 2009).

The development of biofuels has occurred as part of the world-wide search for sustainable and alternative sources of energy which have a lower contribution to global warming than fossil fuels. First generation ethanol production employs enzymes produced by yeast to ferment the starch from grains to ethanol. The replication of yeast

during the process results in the formation of a major additional source of biomass. Recovery of the microbial biomass from an ethanol bio refinery is a new concept to produce a valuable feed ingredient that can be used as a protein supplement in animal nutrition including aquaculture. Although previous studies demonstrated that good fundamental growth and feed performance of sunshine bass (female white bass *Morone chrysops* x male striped bass *Morone saxatalis*) could be maintained with a biofuel derived yeast protein source (Gause and Trushenski 2011a, b), no information is available on other important aquaculture species, and no information is available on the effects on fish gastrointestinal (GI) health.

The GI microbiota of fish has received much attention in recent years as the importance of their interactions with the host at the intestinal mucosa has become increasingly more apparent (Merrifield et al., 2010a). It is generally accepted that the indigenous microbial populations provide a defensive barrier against intestinal pathogens. Maintaining microbial balance is key to maintaining host health and nutrition. Recent studies have shown that these complex microbial communities may be disrupted and altered by different dietary protein sources, such as soybean meal and bioprocessed soybean meal (Ringø et al., 2006a; Merrifield et al., 2009a). More recently, yeast as well as cell wall components of the yeast have been shown to modulate gut microbial communities and impart health benefits to fish under intensive production (Gatesoupe 2007; Sweetman et al., 2010).

In addition to changes in the gut microbiota, diet composition will likely affect the physiological, biochemical and overall nutritional status of fish. It is therefore important to examine the effect of increased inclusion of YPCU on key metabolic enzymes such as alanine aminotransferase (ALAT) and Aspartate aminotransferase (ASAT). These enzymes indicate normal hepatic function and may indicate the health of carp fed the experimental diets. Also, the measurement of blood parameters such as haematocrit and

haemoglobin would confirm any deleterious effects of yeast such as impaired iron metabolism and general protein status. These were all determined on carp fed each experimental diet.

The aim of the present study was to determine the efficacy of a novel yeast protein concentrate unrefined (YPCU) derived from bio-fuel production (Williams et al., 2009). The product is reported to be an excellent source of protein and nucleotides in specialist animal nutrition particularly in starter diets for poultry, diets for aquaculture, companion animal nutrition and the nutrition of young animals (AB-Agri Patent # 109203A1, 2010). Therefore, its nutritional value in a series of experimental diets for juvenile mirror carp *Cyprinus carpio* was evaluated during the course of a feeding trial to assess effects on the growth performance, body composition, mineral analysis, liver function, intestinal histology and intestinal microbiology of this important farmed species.

3.2 Materials and method

3.2.1 Diets and feed preparation

Five isonitrogenous (38% protein) and isolipidic (8% fat) diets were formulated by replacing part of the FM protein with different levels of yeast protein concentrate unrefined (YPCU); YPCU was included at levels effectively replacing 7.5% (YPCU7.5), 15% (YPCU15), 20% (YPCU20) or 50% (YPCU50) of dietary protein from fish meal (Table 3.1). Dietary ingredients were mixed in a Hobart food mixer (Hobart Food Equipment, Australia, model no: HL1400 –10STDA) with warm water until a soft slightly moist consistency was achieved. This was subsequently cold press extruded (La Monferrina P6, La Monferrina, Asti, Italy) to produce a 2 mm pellet see section 2.3. The dietary composition and chemical analyses of the experimental diet is shown in Table 3.1.

Table 3.1 Dietary formulations and proximate composition of experimental diets and protein sources.

Ingredient g kg^{-1}	Control	YPCu7.5	YPCu15	YPCu20	YPCu50
Herring Meal LT92[1]	474.5	430	400	370	230
Yeast (YPCu)[2]	0	60.6	140.3	196.7	460.3
Wheat Carrier Flour [3]	465.5	433.8	389.5	359	216.4
Fish oil[4]	0	0.6	5.2	9.3	28.3
Vegetable oil[5]	15	20	20	20	20
Viten[6]	20	30	20	20	20
Vitamin and mineral Premix[7]	20	20	20	20	20
Molasses[8]	5	5	5	5	5
Proximate composition (%)					
Moisture (%)	5.59	4.61	4.71	5.34	6.92
Protein (%)*	36.68	35.80	35.74	35.62	35.21
Lipid (%)*	6.11	6.47	6.90	7.20	6.70
Ash (%)*	11.31	10.60	10.40	10.07	9.05
NFE (%)*[9]	40.31	42.52	42.25	41.77	42.12
Gross Energy (MJ kg^{-1})	17.85	18.35	18.21	18.69	19.24

*Dry matter basis

[1] Scottish Fishmeal 70, United Fish Products Ltd, UK.

[2] AB Vista (AB-Agri Ltd)

[3] Ewos-Bathgate Scotland

[4] Epanoil, Sevenseas Ltd, UK.

[5] Corn oil.

[6] Roquette Frêres, France.

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

[8] Holland and Barret

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

ND – not determined.

3.2.2 Fish, experimental facilities and nutrition trial

Mirror carp (*Cyprinus carpio*) were obtained from Bowlake fish farm, Hampshire, UK. Carp fry were transported to the Aquaculture and Fish Nutrition Research Aquarium, the University of Plymouth, UK. After 7 weeks acclimation and on-growing, 20 fish (12.29 ±0.11 g) were randomly distributed into 15 x 80 L fibreglass tanks, each provided with 99% re-circulated aerated freshwater at a rate of 300 L h^{-1} see section 2.4 for more details. Each treatment was conducted in triplicate. Fish were fed the experimental diets at 4% biomass per day (equal rations at 09.00, 13.00 and 17.00 h) for 8 weeks. Daily feed was adjusted on a weekly basis following batch weighing after a 24 h feed- deprivation period see section 2.5 for details.

3.2.3 Water quality

Water temperature was maintained at 23.93 ± 0.40 $^{\circ}C$ and dissolved oxygen maintained at 7.44 ± 0.22 mg L^{-1}. The pH was maintained at 6.84 ± 0.31 and adjusted with $NaHCO_3$ as necessary, total ammonia nitrogen (TAN) was maintained at 0.144 ± 0.070 mg L^{-1}, Nitrite was maintained at 0.043 ± 0.021 mg L^{-1} and nitrate was maintained at 16.5 ± 10.04 mg L^{-1} see section 2.6 for details.

3.2.4 Growth parameters and feed utilisation

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 utilisation (ANPU) were assessed as described in section 2.7.

3.2.5 Chemical composition analysis of the diets and fish carcasses

Diets and fish samples (initial and final) from the feeding trial were analysed according to AOAC (2002) standard methods for proximate composition. Fish sampled for whole body analysis (18 fish sampled at the start and 4 fish per tank at the end of trial) were ground and homogenized in a blender prior to chemical assays. All samples were analyzed in triplicate (except Lipid in duplicate).

Moisture content (dry matter) was determined using drying oven (105 ºC for 24 h). Crude protein was calculated from sample nitrogen content was determined using Kjeldahl apparatus (Gerhardt Kjeldatherm method, N % x 6.25) and crude lipid using

ether extraction in multi-unit extraction Soxtec apparatus (dicloromethane extraction by Soxlhet method). Ash Content was analysed using a muffle furnace (incineration at 550 ºC for 12 h). Gross energy analysed using (Parr bomb calorimeter). All protocols concerning analytical chemistry are described in section 2.8. Amino acids analysed (except tryptophan) carried out by Sciantec Analytical Services Ltd. Laboratories (Yorkshire; UK) Table 3.2. Mineral compositions were conducted using spectrophotometer (ICP-MS) on a dry basis Table 3.3 as described in section 2.8.7.

Table 3.2 Amino acid profiles of the experimental test diets.

Amino Acid	Control	YPCu 7.5	YPCu 15	YPCu 20	YPCu 50	Carp requirement*
Essential AA (%)						
Arginine	2.23	2.39	2.07	2.06	1.84	1.6
Histidine	0.91	0.82	0.85	1.11	0.87	0.8
Iso-Leucine	1.37	1.46	1.43	1.51	1.34	1.0
Leucine	2.32	2.35	2.33	2.4	2.32	1.30
Phenylanine	1.5	1.44	1.49	1.59	1.54	2.50[a]
Lysine	2.31	2.16	1.97	2.06	1.7	2.20
Threonine	1.18	1.07	1.31	1.23	1.12	1.50
Valine	1.54	1.95	1.82	1.5	1.66	1.40
Methionine	0.74	0.68	0.65	0.63	0.6	0.80
Tryptopan	ND[1]	ND	ND	ND	ND	0.30
Non-Essential AA (%)						
Alanine	1.99	2.02	2.17	1.93	1.68	ND
Aspartic acid	2.08	1.89	2.72	1.98	1.72	ND
Cysteine	0.5	0.43	0.49	0.51	0.58	ND
Glutamine	6.23	6.05	6.43	6.82	7.9	ND
Glycine	3.41	3.2	3.12	3.27	2.32	ND
Proline	2.29	2.95	2.05	1.91	2.59	ND
Serine	1.43	1.29	1.4	1.9	1.48	ND
Tyrosine	0.84	0.87	0.89	1.06	0.94	ND

ND Not determined.

*% values obtained from references as cited by the *NRC (2011).

[a] with 1% Iso-leucine.

Table 3.3 Mineral composition of the experimental diets. (*n=3*)

Mineral	Control	YPCu 7.5	YPCu 15	YPCu 20	YPCu 50
g kg^{-1}					
Ca	27.68 ± 0.82	27.12 ± 0.23	24.66 ± 0.87	23.36 ± 0.86	16.69 ± 0.64
K	5.07 ± 0.18	5.76 ± 0.40	6.4 ± 0.21	7.13 ± 0.14	9.61 ± 0.15
Mg	1.14 ± 0.03	1.29 ± 0.02	1.47 ± 0.07	1.63 ± 0.06	2.19 ± 0.02
Na	5.96 ± 0.18	5.65 ± 0.01	5.08 ± 0.19	5.12 ± 0.12	3.62 ± 0.07
P	16.96 ± 0.54	17.32 ± 0.19	17.01 ± 0.70	16.45 ± 0.66	15.11 ± 0.02
mg kg^{-1}					
Cr	3.3 ± 0.30	2.49 ± 0.21	2.49 ± 0.17	2.62 ± 0.22	1.86 ± 0.32
Cu	5.24 ± 0.16	4.5 ± 0.29	5.20 ± 0.54	7.21 ± 1.40	6.27 ± 0.05
Fe	187.88 ± 16.38	173.47 ± 10.01	167.8 ± 13.48	181.78 ± 13.79	186.54 ± 6.63
Mn	70.34 ± 2.23	75 ± 0.96	79.23 ± 3.96	94.00 ± 3.84	122.18 ± 3.37
Zn	118.93 ± 15.67	114.01 ± 7.32	104.89 ± 3.58	107.02 ± 1.60	107.07 ± 1.60

Data are presented as mean ± S.D.

3.2.6 Blood parameters

At the end of the growth trials, nine fish per experimental groups were anaesthetized and blood collected as described in section 2.10

3.2.6.1 Haemotocrit

Haemotocrit determination was assayed using heparinized capillary tubes as described in section 2.10.1.

3.2.6.2 Haemoglobin

Total blood haemoglobin concentration was determined using Drabkins spectrophotometric method as described in section 2.10.2.

3.2.7 Enzyme assays

At the end of experiment six fish per treatment were euthanized and liver were freezed as described in section 2.11. Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were assayed using the micro plate reader (Molecular Devices) as described in section 2.11.

The total protein content protein of the supernatant was determined as described in section 2.11.

3.2.8 Intestinal microbiological analysis

At the end of the trial three fish per tank were sampled to isolate intestinal material from fish fed the control diet, and fish fed 20% and 50% YPCU diets. After dissecting aseptically, Samples were pooled by tank resulting in three replicates per treatment (200 mg) each constituting material from three fish. DNA extraction, PCR amplification of the 16S rRNA V3 region, DGGE and subsequent phylotype isolation and sequencing were conducted as described in section 2.12.

3.2.9 Light microscopy

Histological appraisal of the liver, anterior intestine and posterior intestine from three fish per experimental group was performed at the end of the trial using light microscopy as described in section 2.9.1. Micrographs were produced using an Olympus Vanox-T microscope 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 by Smith et al. (2007). Additionally, the number of leucocytes was counted on at x 400 magnification within a surface area of 728 mm^2.

Intestinal images taken from light microscopy were analysed to determine the length of the mucosal folding (villi). Additionally, the number of goblet cells and the number of leucocytes (histologically stained cells) infiltrated in the epithelium across a standardised distance of 100 enterocytes (only nucleated cells) was calculated by averaging the cell numbers from all specimens (Ferguson et al., 2010).

3.2.10 Statistical application

Statistical analyses (i.e. growth parameters, body composition, enzyme activity, and histology) were conducted using a One-Way ANOVA (SPSS 17.0).). *Post hoc* LSD test was used to determine significant differences between means Percentage data was arcsine transformed prior to subsequent analysis. DGGE banding patterns were transformed into presence/absence matrices for assessment between treatments using Quantity one® V4.6.3 analyses software (Bio-Rad laboratories, CA, USA). Dice coefficients of similarity were calculated to compare fingerprints and band intensities. Similarity percentages (SIMPER), a one-way analysis of similarity (ANOSIM) and Bray Curtis similarity matrix was used to represent the relative similarities between treatments and replicates using Primer V6 (Clarke and Gorley 2006). The species richness was assessed using Margalef's measure of richness and species diversity was assessed using the Shannon–Weaver index. Significance was accepted at $P \leq 0.05$.

3.3 Results

3.3.1 Growth and feed utilisation

All groups fed the experimental diets grew well and body weights increased by between 3.2 and 3.9 fold at the end of the 8 week nutrition trial (Table 3.4). During the study, all the fish readily accepted the experimental diets. No mortalities were recorded during the experimental period. Final weights and protein efficiency ratios were higher in fish fed YPCu- containing diets (YPCu7.5, YPCu15, YPCu20 and YPCu50) when compared to fish fed the fish meal control diet (Table 3.4). Furthermore, weight gains of fish fed the YPCu15 and the YPCu20 diets was significantly ($P<0.05$) higher than carp fed the YPCu7.5 and the YPCu50 diets. Although trends towards elevated SGR were observed

in all fish fed YPCU- diets, this was only significantly (*P<0.05*) higher at 15% and 20% levels. The highest FCE was observed in the group YPCU20 (81.01±0.88), followed by groups YPCU15 (76.93±0.71), YPCU7.5 (71.40±1.36) and YPCU50 (69.99±3.36), which were all significantly (*P<0.05*) higher than the control group (66.50±3.21) (*P*<0.001). Compared to the control group, the FCR was significantly (*P<0.05*) improved with yeast protein concentrate (YPCU) inclusion up to 20% included.

Table 3.4 Growth performance and feed utilization of carp fed the experimental diets for 8 weeks. (*n* = 3)

Variable	Control	YPCU 7.5	YPCU 15	YPCU 20	YPCU 50
Initial weight (g)	12.15 ± 0.26	12.23 ± 0.08	12.27 ± 0.10	12.42 ± 0.06	12.37 ± 0.20
Final Weight (g)	39.22 ± 1.40^{a}	41.38 ± 0.65^{b}	45.92 ± 0.23^{c}	48.00 ± 1.10^{c}	41.73 ± 1.53^{b}
Weight Gain (g)[1]	27.07 ± 1.66^{a}	29.15 ± 0.64^{ab}	33.65 ± 0.29^{c}	35.58 ± 1.15^{c}	29.36 ± 1.69^{b}
SGR (% day^{-1})[2]	2.13 ± 0.10^{a}	2.21 ± 0.03^{a}	2.40 ± 0.02^{b}	2.46 ± 0.05^{b}	2.21 ± 0.09^{a}
FCE	66.50 ± 3.26^{a}	71.40 ± 1.36^{bc}	76.93 ± 0.71^{d}	81.01 ± 0.88^{d}	69.99 ± 3.36^{ab}
FCR	1.50 ± 0.08^{a}	1.40 ± 0.03^{b}	1.30 ± 0.01^{c}	1.23 ± 0.01^{c}	1.43 ± 0.07^{ab}
PER [3]	1.81 ± 0.09^{a}	1.99 ± 0.04^{b}	2.15 ± 0.02^{c}	2.27 ± 0.02^{d}	1.96 ± 0.01^{b}

Data are presented as mean ± S.D.
Data in the same row with different superscript are significantly different (*P<0.05*)
[1]$Y = 26.29 + 0.6699X-0.01213X^2$ (R-sq = 82.20%) (Y = Weight Gain and X = YPCU levels)
[2]$Y = 2.100 + 0.02672X-0.0004893X^2$ (R-sq = 75.03%) (Y = SGR and X = YPCU levels)
[3]$Y = 1.796 + 0.03448X-0.000623X^2$ (R-sq = 87.18%) (Y = PER and X = YPCU levels)

3.3.2 Carcass composition and mineral profiles

The body composition of fish is presented in Table 3.5 No significant (*P<0.05*) differences were found in whole-body moisture, protein or lipid contents of fish fed the different experimental diets. However, whole-body ash was significantly (*P<0.05*) higher in carp receiving dietary YPCu levels of ≥15%. However, body gross energy level was significantly (*P<0.05*) lower in fish fed 50% YPCu inclusion than in fish fed the control or low levels of YPCu (7.5% and 15%).

Carcass mineral levels are displayed in Table 3.6. Also clear trends of elevated carcass calcium levels was observed with increasing YPCu inclusion leading to significant (*P<0.05*) differences at YPCu inclusion levels of ≥15%. Similar results were observed with respect to phosphorous whereby significantly (*P<0.05*) higher levels were observed in all YPCu fed groups. Additionally, elevated levels of carcass potassium and magnesium were observed in carp fed the YPCu20 and YPCu50 diets. Manganese levels were also significantly (*P<0.05*) higher in fish fed YPCu50. In contrast, zinc levels were significantly (*P<0.05*) lower in all fish fed YPCu diets except YPCu50. Sodium, chromium, copper and iron levels were not affected by dietary inclusion of YPCu.

Table 3.5 Proximate composition of initial fish carcasses and fish after 8 weeks feeding on the experimental diets. (n = 3)

Whole Body composition	Initial	Control	YPCu 7.5	YPCu 15	YPCu 20	YPCu 50
Moisture (%)	76.06 ± 0.34	72.43 ± 0.50	73.02 ± 0.70	73.37 ± 0.35	73.36 ± 0.54	72.78 ± 1.10
Protein (%)*	53.57 ± 0.46	49.06 ± 0.15	50.15 ± 1.15	49.06 ± 1.94	50.87 ± 1.26	48.66 ± 2.88
Lipid (%)*	29.66 ± 0.84	36.01 ± 1.39	34.51 ± 2.74	35.19 ± 0.46	33.86 ± 0.18	34.42 ±1.30
Ash (%)*	9.98 ± 0.23	7.47 ± 0.24^{a}	8.33 ± 0.88^{ab}	8.56 ± 0.25^{b}	8.59 ± 0.25^{b}	9.82 ± 0.77^{c}
NFE (%)*	6.79 ± 0.73	7.46 ± 1.05	7.01 ± 0.72	7.19 ± 1.67	6.68 ± 1.17	7.10 ± 2.93
Gross energy (MJ kg^{-1})	23.68 ± 0.25	25.47 ± 0.26^{a}	25.57 ± 0.59^{a}	25.04 ± 0.41^{a}	24.87 ± 0.43^{ab}	24.11 ± 0.51^{b}

* Dry matter basis.Data are presented as mean ± S.D.
Data in the same row with different superscript are significantly different (*P<0.05*).
Nitrogen-free extracts (NFE) = dry matter – (crude protein + crude lipid + ash).

Table 3.6 Mineral composition of initial fish carcasses and fish after 8 weeks feeding on the experimental diets. (*n* = 3)

Mineral	Initial	Control	YPCu 7.5	YPCu 15	YPCu 20	YPCu 50
g kg^{-1}						
Ca	20.74 ± 1.01	16.31 ± 0.80^{a}	17.7 ± 0.40^{ab}	18.7 ± 2.24^{b}	18.85 ± 0.15^{b}	22.67 ± 1.07^{c}
K	1.11 ± 0.06	1.07 ± 0.07^{a}	1.07 ± 0.03^{a}	1.12 ± 0.05^{ab}	1.20 ± 0.04^{b}	1.19 ± 0.04^{b}
Mg	1.11 ± 0.70	0.88 ± 0.40^{a}	0.94 ± 0.04^{a}	0.94 ± 0.05^{a}	0.99 ± 0.04^{a}	1.21 ± 0.19^{b}
Na	3.53 ± 0.15	3.42 ± 0.27	3.46 ± 0.07	3.63 ± 0.24	3.67 ± 0.04	3.72 ± 0.17
P	17.75 ± 1.03	14.04 ± 0.53^{a}	15.23 ± 0.31^{b}	15.62 ± 0.81^{bc}	16.63 ± 0.21^{c}	18.84 ± 0.96^{d}
mg kg^{-1}						
Cr	0.39 ± 0.26	1.17 ± 0.83	0.77 ± 0.40	1.12 ± 0.55	1.73 ± 45	0.51 ± 0.22
Cu	3.05 ± 0.26	2.70 ± 0.83	1.61 ± 0.16	1.73 ± 0.22	2.13 ± 0.33	1.78 ± 0.46
Fe	72.15 ± 3.89	54.42 ± 15.67	50.45 ± 16.00	57.89 ± 10.97	56.73 ± 4.40	46.73 ± 2.40
Mn	4.75 ± 0.04	7.56 ± 2.92^{a}	9.92 ± 6.09^{ab}	15.35 ± 6.19^{ab}	16.31 ± 4.18^{b}	23.59 ± 0.21^{c}
Zn	327.88 ± 6.28	208.33 ± 18.89^{a}	184.52 ± 2.92^{a}	187.60 ± 1.40^{a}	189.50± 1.65^{ab}	199.58 ± 10.34^{b}

* Dry matter basis.
Data are presented as mean ± S.D.
Data in the same row with different superscript are significantly different (*P<0.05*).

3.3.3 Hepatic histology and enzymatic profiles

Inclusion of dietary YPCu did not induce histological changes in the liver; no differences of hepatocyte size, hepatic nuclei size, the ratio of nuclei diameter to hepatocyte diameter or the number of leucocytes were observed between the groups (Table 3.7, Figure 3.1). Hepatopancreas ALAT activities of carp fed YPCu diets showed a general trend towards lower activity with increasing YPCu inclusion but these differences were not significant (*P<0.05*). Similarly, ASAT activities decreased with increasing YPCu levels and compared to the control were statistically ($P<0.05$) lower as YPCu levels increased from 20% to 50% inclusion. Haemotocrit values and haemoglobin levels remained unaffected by the YPCu treatment. However the value haemotocrit increased slightly in fish fed YPCu 20% compared to control fish fed.

Table 3.7 Haemotological and enzymatic analyses of fish fed the experimental diet for 8 weeks.

	Control	YPCu7.5	YPCu15	YPCu20	YPCu50
Haematocrit (%)	35.78 ± 5.30	36.62 ± 4.69	39.78 ± 4.52	41.28 ± 6.72	36.67 ± 4.44
Haemoglobin (g dL^{-1})	6.67 ± 2.08	6.89 ± 0.84	6.51 ± 0.97	7.33 ± 0.92	6.35 ± 0.79
ALAT (U mg^{-1} protein)	1.80 ± 0.50	1.63 ± 0.25	1.51 ± 0.15	1.51 ± 0.43	1.48 ± 0.43
ASAT (U mg^{-1} protein)	3.82 ± 0.43[a]	3.63 ± 0.34[a]	3.27 ± 0.32[a]	2.57 ± 0.32[b]	2.24 ± 0.50[b]

Data are presented as mean ± S.D.
Data in the same row with different superscript are significantly different (*P<0.05*).

Table 3.8 Liver histological analyses of fish fed the experimental diets for 8 weeks. (*n* = 6)

	YPCu7.5	YPCu15	YPCu20	YPCu50	YPCu7.5
Hepatocytes size (µm)	17.15 ± 0.65	16.79 ± 0.36	16.77 ± 0.14	17.71 ± 0.58	16.73 ± 0.63
Nucleus size (µm)	4.23 ± 0.17	4.18 ± 0.06	4.14± 0.17	4.31 ± 0.24	4.05 ± 0.17
Ratio of nucleus diameter to hepatocytes diameter (µm)	25 ± 1.31	25.13 ± 0. 94	24.86 ± 0.93	24.51 ± 1.71	24.4 ± 0.53
Number of leucocytes	111 ± 10.12	131.5 ± 20.11	131.83 ± 3.54	133.17 ± 27.19	130 ± 52.30

Data are presented as mean ± S.D.
Data in the same row with different superscript are significantly different (*P<0.05*).

Table 3.9 Intestinal morphology of fish fed on the experimental diets for 8 weeks. (*n = 6*)

	Region	YPCu7.5	YPCu15	YPCu20	YPCu50	YPCu7.5
Villi length (µm)	Anterior intestine	747 ± 74	662 ± 77	786 ± 65	713 ± 71	648 ± 144
	Posterior intestine	694 ± 87	804 ± 115	826 ± 107	774 ± 124	741 ± 37
Epithelial leucocytes (per 100 enterocytes)	Anterior intestine	123 ± 18	132 ± 16	130 ± 11	131 ± 11	127 ± 9
	Posterior intestine	96 ± 18	94 ± 18	91 ± 16	98 ± 20	95 ± 7
Goblet cells (per 100 µm)	Anterior intestine	8.3 ± 0.90	8.1 ±1 .00	8.0 ± 1.60	8.4 ± 0.90	8.2 ± 0.60
	Posterior intestine	6.0 ± 1.60[a]	6.1 ± 0.70[a]	8.4 ± 2.30[b]	8.6 ± 1.60[b]	8.9 ± 0.70[b]

Data are presented as mean ± S.D.
Data in the same row with different superscript are significantly different (*P<0.05*).

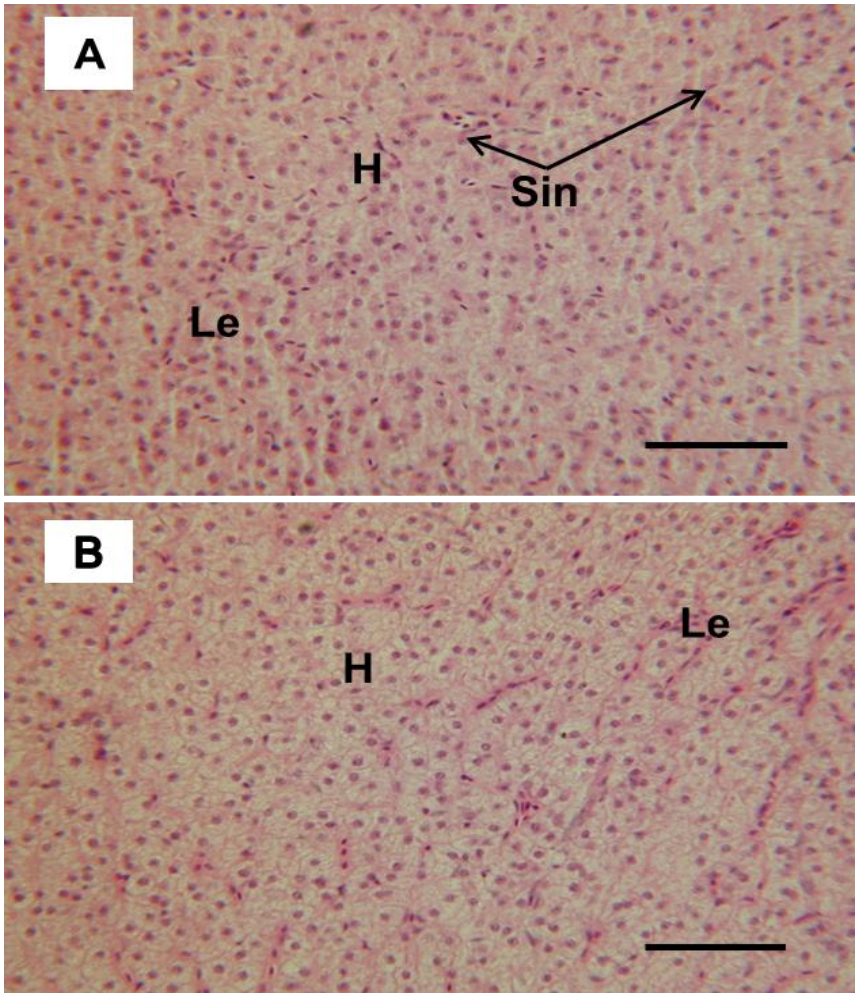


Figure 3.1 Haematoxylin and eosin stained sections of livers of Mirror carp. (A) control fishmeal fed group and (B) dietary inclusion of levels of 15% YPCu. (Scale bar = 50 µm) H: Hepatocytes, Le: Leukocytes, Sin: Sinusoid

3.3.4 Intestinal histology and microbiology

Histological appraisal of the intestine revealed that all groups displayed complex intestinal mucosal folding with no signs of tissue damage or necrosis. No statistical ($P<0.05$) differences in the villi length or the number of epithelial leucocytes were observed in either the posterior or anterior intestine of the respective dietary groups (Table 3.8, Figure 3.2 and 3.3). However, the number of goblet cells in the posterior intestine of fish fed the YPCu15, YPCu20 and YPCu50 diets were significantly ($P<0.05$) higher than fish fed the basal and YPCu7.5 dietary treatments (Table 3.9; Figure 3.4). There were no significant ($P<0.05$) differences in the anterior region however (Figure 3.5).

Intestinal microbial profiles were similar in all dietary groups (displaying 78%-80% similarity) and no significant ($P<0.05$) differences between the microbial ecological diversity indices (i.e: # of phylotypes, species richness, species diversity and similarity of within-group replicates) (Table 3.9, Figure 3.6). Minor variations between replicates were observed in regards to individual phylotypes present, but no clear differences were observed between dietary treatments. Indeed, no phylotypes were found uniquely present within the dietary groups (Table 3.10). A total of 10 bands/phylotyes (the most prominent, phylotypes or phylotypes which showed large variation between samples) were isolated for sequencing of which six yielded successful identifications from Blast searches and four failed to be sequenced successfully (Figure 3.6).

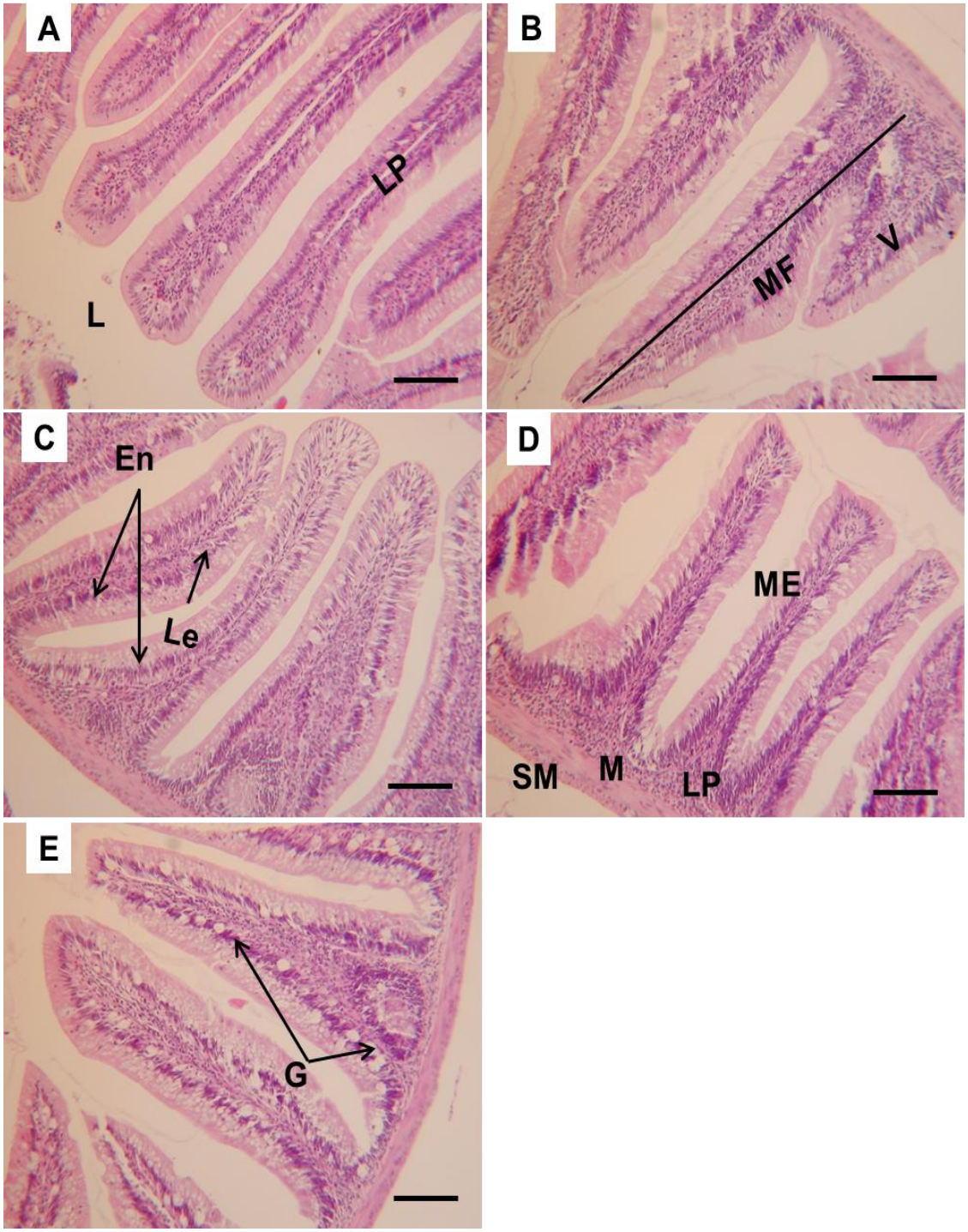


Figure 3.2 Observation of intestinal folds and epithelial leucocytes in Haematoxylin and eosin stained section of the posterior intestine of mirror carp (Scale bar 100 µm). (A) fish fed control diet, (B) fish fed YPCu7.5,(C) fish fed YPCu15, (D) Fish fed YPCu20 and (E) fish fed YPCu50. (Scale bar = 100 µm). L: Lumina, LP: Lamina properia, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells, V: Villi, Le: Leukocyte, En: Enterocyte.

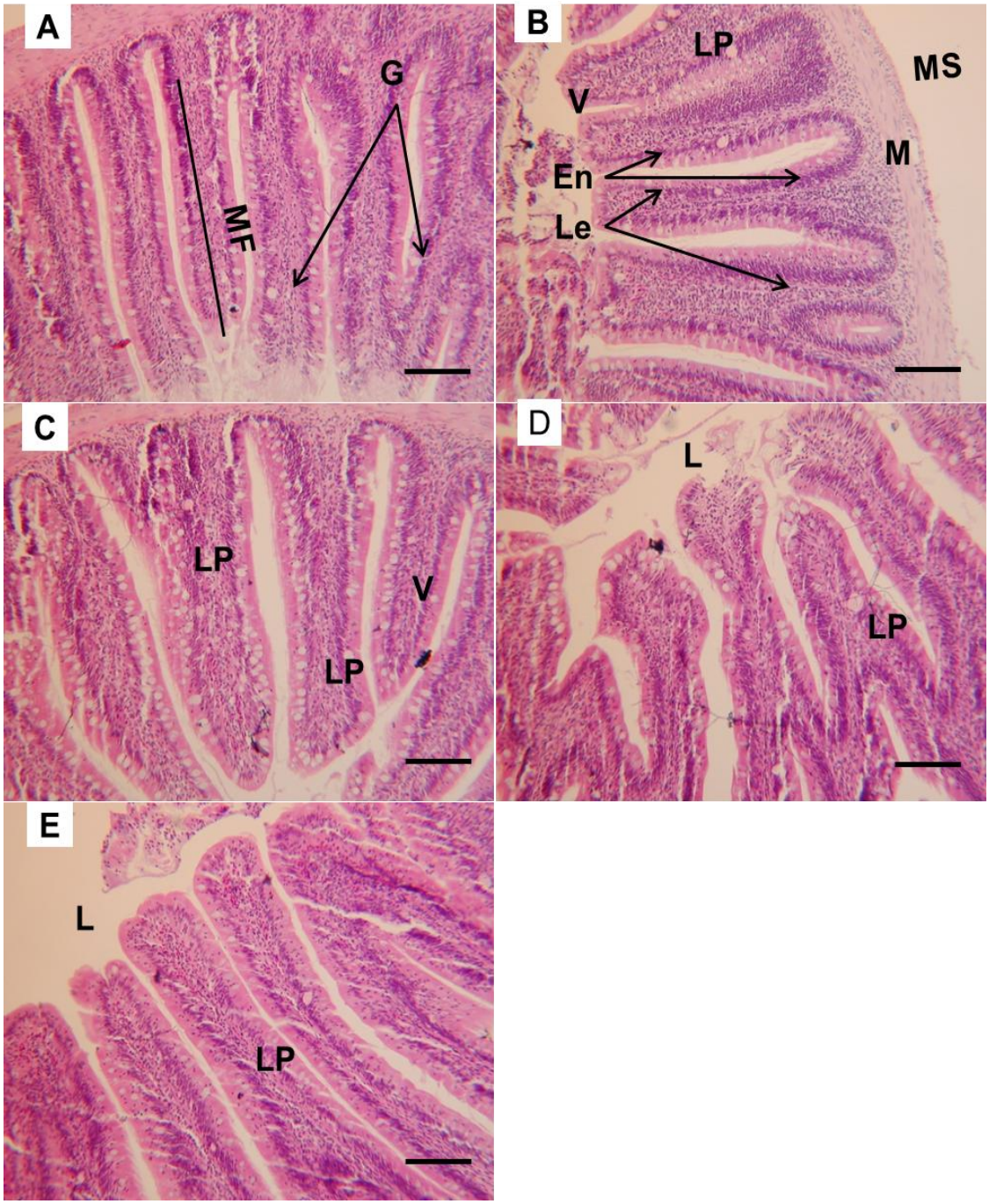


Figure 3.3 Observation of intestinal folds and epithelial leucocytes in Haematoxylin and eosin stained section of the anterior intestine of mirror carp. (A) fish fed control diet, (B) fish fed YPCu7.5, (C) fish fed YPCu15, (D) Fish fed YPCu20 and (E) fish fed YPCu50. (Scale bar = 100 µm). L: Lumina, LP: Lamina properia, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells, V: Villi. , Le: Leukocyte, En: Enterocyte.

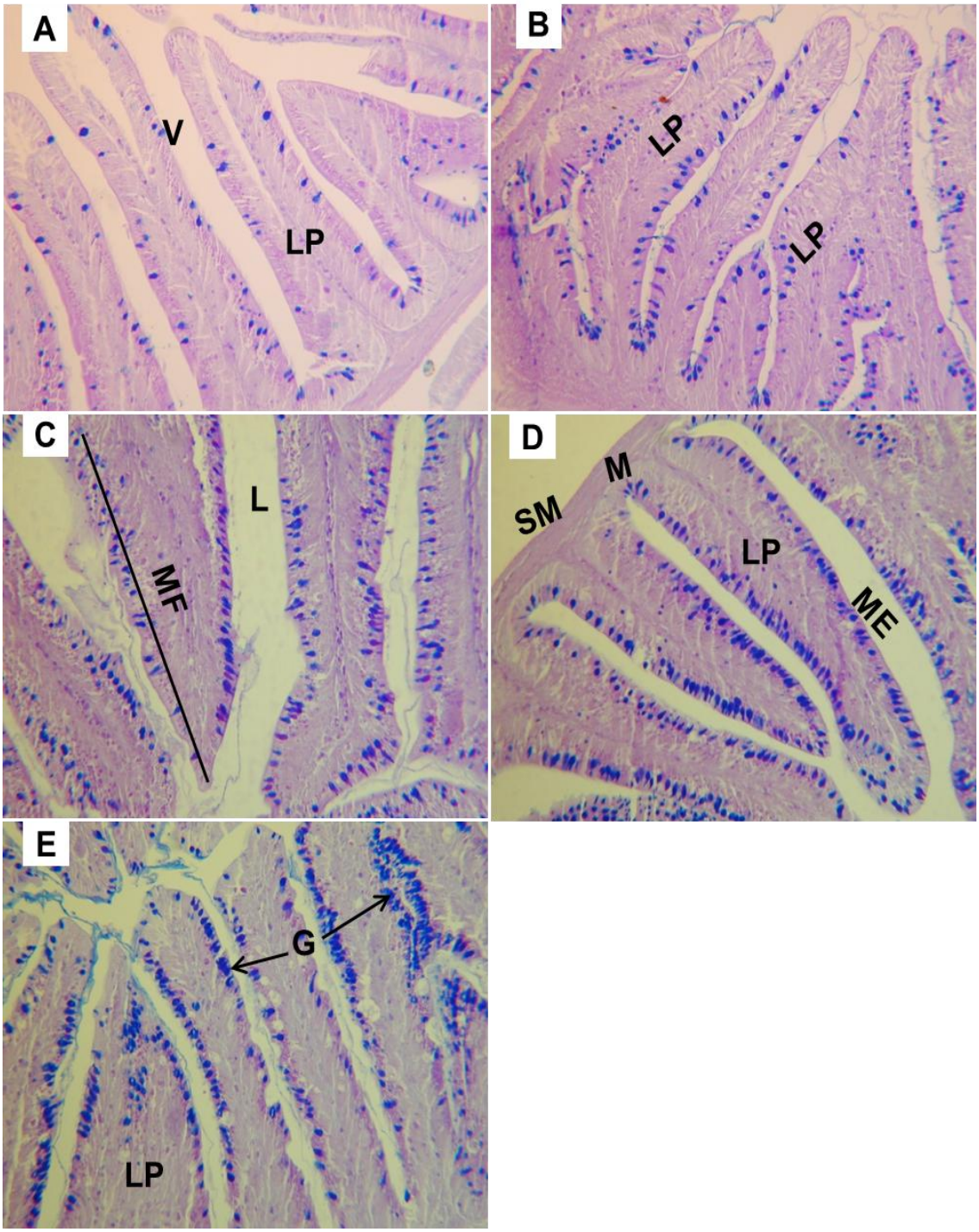


Figure 3.4 Posterior intestine histological details (alcian blue and PAS staining) of posterior intestine from fish fed, (A) control diet, (B) YPCu7.5 diet, (C) YPCu15 diet, (D) YPCu20 diet and (E) YPCu50 diet showing a significant improvement in the number of goblet cells with increasing YPCu inclusion level in the dietary treatment. (Scale bar = 100 µm). L: Lumina, LP: Lamina properia, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells, V: Villi.

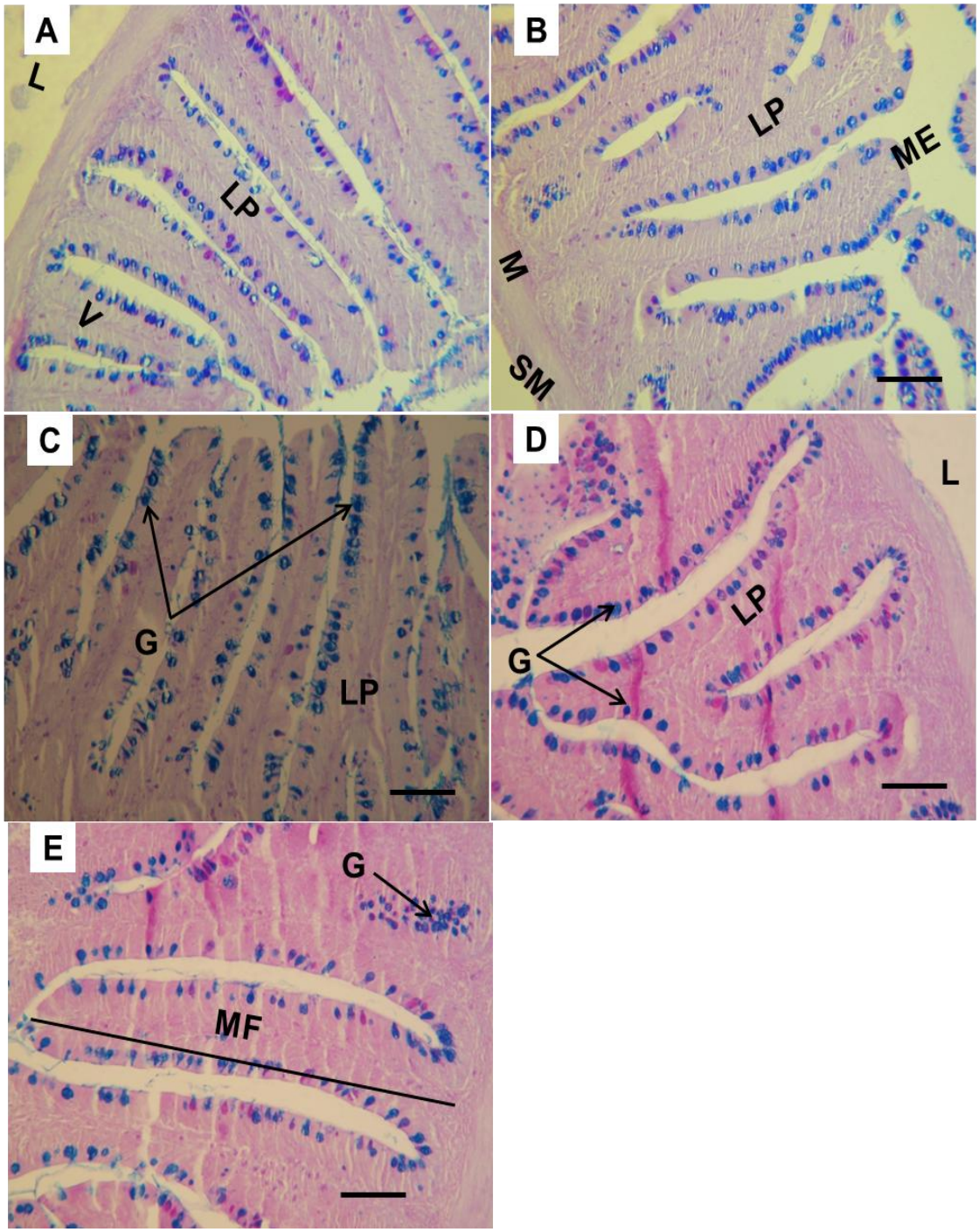


Figure 3.5 Anterior intestine Histological details (alcian blue and PAS staining) of anterior intestine from fish fed, (A) control diet, (B) YPCu7.5 diet, (C) YPCu15 diet, (D) YPCu20 diet and (E) YPCu50 diet. (Scale bar = 100 µm). L: Lumina, LP: Lamina properia, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells, V: Villi.

Table 3.10 Isolated bacterial bands and their closest relatives (BLAST) from PCR-DGGE of the intestinal communities of fish after feeding on the experimental diets for 8 weeks.

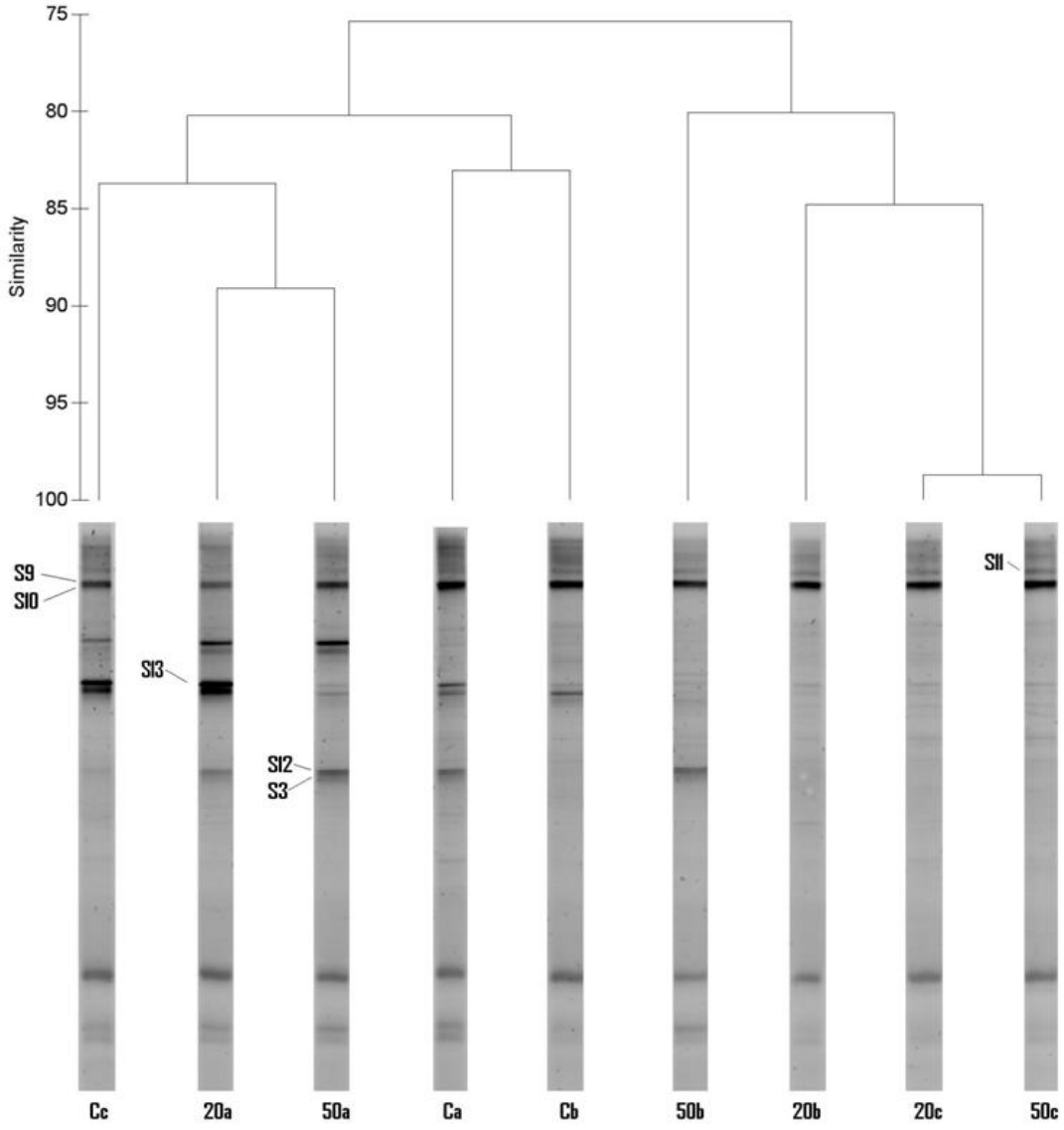
Band	Nearest neighbour(s)	Similarity to nearest neighbour(s)	Accession number of nearest neighbour(s)
S3	Uncultured bacterium from the gut of grass carp	92%	EU585890
		92%	DQ815298
	Uncultured bacterium from the gut of zebrafish *Cetobacterium somerae*	92%	AB353124
S9	Uncultured bacterium from the gut of tilapia	97%	GU117872
S10	*Weissella confusa*	97%	GU223369
	Weissela cibaria	97%	GU138616
S11	*Halomonas* sp.	99%	EU768828
	Cobetia sp.	99%	FJ984844
S12	Uncultured bacterium from the gut of grass carp	98%	EU585890
		98%	DQ815298
	Uncultured bacterium from the gut of zebrafish *Cetobacterium somerae*	98%	AB353124
S13	*Streptococcus parauberis* from the gut of goldfish	97%	FR873791
	Streptococcus iniae	97%	HM053435
	Streptococcus ictaluri	97%	DQ462421

Table 3.11 Microbial community analysis from PCR-DGGE fingerprints of the GI microbiota mirror carp from each group. (*n* = 3).Values expressed as means ± standard deviation.

						ANOSIM		
	N[1]	Richness[2]	Eveness[3]	Diversity[4]	SIMPER	*R*-value	*P*-value	Similarity (%)
Control	22.33 ± 1.53	2.00 ± 0.13	0.992 ± 0.000	3.08 ± 0.07	79.28 ± 3.57			
YPC20	19.67 ± 1.53	1.79 ± 0.12	0.993± 0.002	2.96 ± 0.07	76.21 ± 7.53			
YPC50	20.67 ± 1.15	1.88 ± 0.11	0.995 ± 0.002	3.01 ± 0.06	77.48 ± 7.39			
Control v YPC20						-0.037	0.60	78.16 ± 5.71
Control v YPC50						-0.037	0.70	78.81 ± 3.99
YPC20 v YPC50						-0.222	0.80	80.07 ± 9.90

[1] Average number of bands/presumed species.
[2] Margalef species richness: d = (S -1)/log (N).
[3] Pielou's evenness: J'=H'/log(S).
[4] Shannons diversity index: H' = -∑(pi(ln pi)).
Values expressed as means ± standard deviation.
SIMPER, similarity percentage within group replicates; ANOSIM, Analysis of similarities between groups.

Figures 3.6 PCR-DGGE dendrogram of the intestinal microbial communities of fish fed the experimental diets for 8 weeks. Labels indicate phylotypes isolated for sequence analysis. T reatment name: Control = Ca, Cb, Cc. 20% YPCU = 20a, 20b, 20c and 50% YPCU = 50a, 50b, 50c.



3.4 Discussion

The effectiveness of yeast to partially substitute FM in aquafeeds has been tested by several authors. Yeast has been shown to affect growth performance in many fish species (Lara-Flores et al., 2003; Li and Gatlin 2005; Abdel-Tawwab et al., 2008, 2010; Hoseinifar et al., 2011). The present study indicates that a yeast protein concentrate unrefined (YPCU) can replace up to 50% of FM protein in the diets for carp with appreciable improvement in performance and optimal performance at 15 and 20% inclusion. Olvera-Novoa et al. (2002), in studies with Mozambique tilapia, indicated that a 15% fishmeal inclusion and a combination of four other protein sources (i.e. meat and bone meal (MBM) at 5%, alfalfa protein concentrate (APC) at 15%, SBM at 20%, and torula yeast (yeast derived from wood sugar) at 45% produced a growth response with significant improvements over the control group fed a FM only diet. These results agree with the earlier results of Rumsey et al. (1991) who fed rainbow trout with dietary brewer's yeast at varying levels and found that the optimum level of substitution was 25%. Furthermore, Zerai et al. (2008), in trials with Nile tilapia (*O. niloticus*), also reported that there were no negative effects on growth or feed utilization when brewer's waste (obtained from microbrewing) replaced up to 50% of FM. Moreover, in a recent study on sunshine bass (female white bass *Morone chrysops* x male striped bass *Morone saxatalis*), the optimum level of growth was reported when FM protein was replaced by bio-ethanol yeast at 27-41% inclusion levels (Gause and Trushenski 2011a).

The amino acid profiles of all experimental diets were compared in this study and there were no obvious deviations from the known requirements for carp as expressed as percent of diet from the literature to date (NRC 2011). Although for lysine YPCU 50% inclusion resulted in a dietary lysine level of 1.7 falling below the 2.2% requirement for carp (NRC 2011). All other essential amino acids appeared to be in excess of stated requirements for this species, although it should be cautioned that yeast protein and

specific amino acid digestibility coefficients were not measured and so exact patterns and ratios in diets cannot be defined. Further, YPCU contains an appreciable level of non-protein nitrogen which, as is the case with all yeast protein studies, affects the 'true' protein and amino acid content of feeds tested.

This investigation also indicated that carcass moisture, protein and lipid content were not affected by the different dietary treatments. This is in agreement with the recent findings in tilapia (Ng and Chong 2002; Yigit and Olmez 2010). However, the ash content was significantly higher in carp fed high dietary YPCU inclusions (15%, 20% and 50%, respectively) when compared to the groups fed the control and YPCU7.5 diets. These results are consistent with those of Abdel-Tawwab et al. (2010) who fed gallilee tilapia (*Sarotherodon galilaeus*) on diets containing 10% of live yeast showing significantly higher level of body ash compare to fish fed a control diet. However, protein efficiency ratio was improved in carp fed at 15-20% YPCU inclusion levels and body ash levels were significantly increased in fish fed YPCU $\geq$ 15%.

The importance of trace elements in fish nutrition was reviewed by Watanabe et al. (1997). Since then, there has been renewed interest in redefining the mineral requirements of fish with particular concerns in meeting the metabolic needs of farmed fish under intensive conditions. Reports of increased susceptibility to stress, reduced disease resistance as a consequence of immunological impairment and reduced overall feed efficiency have focused largely on whether trace element premixes provide the correct balance of essential micronutrients including minerals (Davies et al., 2010). This has been reinforced by the wide use of plant by-products being increasingly used in feed formulations (Gatlin et al., 2007). The latter frequently contain a variety of anti-nutritional factors some of which can interfere with mineral availability within the digestive tract of mono-gastric animals. Fish are especially sensitive to the phytate levels in SBM meal and other such legumes which can considerably affect phosphorous

and zinc absorption and retention during growth. Either both calcium and phosphorus are more bio- available from the yeast source compared to fish meal or the slight dietary reductions in calcium and phosphorus with YPC inclusion promotes superior absorption of calcium and phosphorus from the gut. The decline in calcium can assist phosphorus absorption as reported in the literature since calcium/phosphorus ratio is deemed important in the absorption of both minerals (NRC 2011). The role of phosphorus in the health of farm fish is well established and reviewed by Sugiura et al. (2004). Phosphorus is very important for growth and mineralization of carp as reported by Nwanna et al. (2010) and must be of concern in feed formulations. An improved retention of phosphorous has environmental consequences and may lead to the reduced need to supplement diets with inorganic phosphorous sources.

Examination of liver ultrastructure showed no obvious detrimental changes associated with increased dietary inclusion of the novel yeast product. Light microscopy examination of hepatic tissue removed from representative fish taken at the end of the study from each group showed consistent hepatocyte density, size and nuclear to cytoplasmic ratio. This suggests no influence on liver cell synthesis in terms of hyperplasia or altered nutritional state. There were no obvious changes in liver lipid or glycogen accumulation as either vacuoles or enlarged cell dimension. Since the diets were formulated to be balanced (iso-nitrogenous and iso- caloric) then these results are consistent with the overall growth and feed performance recorded in this investigation with carp. Other researchers have reported increased hepatic glycogen or lipid deposition in a number of fish species fed diets containing excess energy. According to Takeuchi et al. (2002), carp are able to utilize different carbohydrates sources more effectively than strictly carnivorous fish and this would explain the more uniform composition and liver status in this study where yeast is the primary test ingredient also

providing non- starch carbohydrate at the expense of the wheat carrier flour in our diet formulations.

Hepatic enzyme function is a useful indicator of metabolic and nutritional status in fish diet evaluation (Glover et al., 2007; Liu et al., 2010). Excess deposition of energy as glycogen or lipid can impair the activities of several key enzymes involved in glycolysis, lipogenesis as well as protein and amino acid synthesis and degradation (Wolf and Wolf 2005). In this study, ALAT activity was reduced marginally, but not significantly with increasing YPCu inclusion in the diets. However, ASAT activity was significantly reduced in carp fed at levels YPCu15% and YPCu20% inclusion respectively compared to carp fed the other diets. In contrast to this finding, Abdel-Tawwab et al. (2010) found that a diet containing live baker's yeast did not affect the ALAT and ASAT of gallilee tilapia. The findings in the current trial indicate that dietary yeast inclusion may influence liver metabolism of carp at high inclusion level but not detrimentally, reductions in ASAT activity could also have been related to a decreased need for pyrimidine biosynthesis which involves aspartate as a substrate. YPC is a rich source of purines and pyrimidine bases within nucleic acids and may have reduced demand within the hepatocytes of carp fed higher levels of yeast protein concentrate thus sparing the need for *de novo* synthesis.

The findings in the current trial indicate that dietary yeast inclusion may influence liver metabolism of carp at high inclusion level but not detrimentally. In this study, the gastrointestinal (GI) tract of carp was also examined for ultra-morphological changes. The dietary modulation of the posterior gut area was of particular interest. The effect of diet on mucus production and goblet cell activity has been reported by several workers recently (Merrifield et al., 2011).

The numbers of goblet cells in the posterior section of the gut of carp increased with increasing of YPCu levels and that is may be due to some components (i.e. ß-glucans or

MOS) within the cell wall of the yeast being responsible for enhancing tolerance of the GI tract (Marchetti et al., 2006). For example, recent studies have shown that dietary MOS elevates the number of goblet cells in the intestine of European sea bass *Dicentrarchus labrax* (Torrecillas et al., 2011).

The complex GI microbial communities in fish, as with higher vertebrates, are implicated in a great number of host functions (Rawls et al., 2004, 2007; Bates et al., 2006). These include gastric development, mucosal tolerance, immunity and digestive function (Perez et al., 2010; Dimitroglou et al., 2011). The correct microbial balance is therefore paramount to have a good health and growth. These microbial systems are sensitive however with numerous reports of disruption or modulation as a result of dietary changes. Indeed, it has been previously reported that some components of the microbial populations are affected by different dietary protein sources, such as SBM (Refstie et al., 2006; Ringø et al., 2006a; Merrifield et al., 2009a) and krill meal (Ringø et al., 2006b). Contrary to these findings the present study showed that the inclusion of YPCu did not effect of microbial ecological parameters investigated using PCR-DGGE. These initial findings are positive in that no signs of disruption of the microbial balance were observed. The PCR-DGGE method is a rapid and informative method to study microbial communities from environmental samples, including the fish intestinal tract (Merrifield et al., 2009b; Ferguson et al., 2010; Dimitroglou et al., 2010) but is at best only semi-quantitative; future studies should incorporate quantitative methods to further elucidate the effects of higher levels of dietary YPCu on fish GI microbial communities.

3.5 Conclusion

The findings from the present study demonstrate that up to half the fishmeal protein component within experimental diets for mirror carp can be effectively replaced with an YPCU which supports good growth performance and feed utilization. There were no obvious adverse effects on health as measured by several physiological and biochemical indices and general tissue morphology.

Carp are important fish commercially cultured in many regions of the world where aquaculture depends on many cyprinid species such as in Central Europe, the Middle East, Asia and China. Therefore, YPCU could be strategically employed to replace up to half of the protein in practical diets for carp and is also likely to be effective for tilapia and catfish. It remains to be seen if this is also true for salmonid fish (salmon and trout) and even high value marine species like sea bass and gilthead sea bream.

Further investigations are warranted to establish the optimum inclusion levels in diets across the range of fish cultured intensively. With the growing expansion of the bio-fuel industries, we expect a number of emerging co-products with considerable potential for aquaculture feeds in the coming decades.

Chapter 4

Evaluation of a yeast protein co-product originating from a bio-ethanol process and distillery yeast as a dietary ingredient supporting growth, mineral retention, histological observations, biochemistry and haematological changes in common carp *Cyprinus carpio.*

4.1 Introduction

Globally, aquaculture output has been increasing at a rate of around 8% per annum and now supplies about 65 million tons whereas fisheries landings have remained constant at about 90 million tons of fish for the last decade (FAO 2010). Indeed, more than 50% of the fish products produced for human consumption come from commercial aquaculture (NRC 2011). However, the sustained growth of the industry has resulted in an increased need for specialised compound feedstuffs, at ca. 30 million tons per year in 2009, and is expected to increase two- fold by 2020 (Tacon 2010).

Feed preparation receives the most attention in the production of intensive aquaculture, the key aspect being to provide a balanced diet that meets the full nutritional requirements. Fish meal (FM) has typically been the main source of dietary protein in the commercial production of fish (Carter and Hauler 2000, Edwards et al., 2004, Naylor et al., 2000, Tacon and Metian 2008a). This is due to its high protein content, excellent amino acid profile, as well as its high nutrient digestibility (Gatlin et al., 2007). However, being too reliant on any one available ingredient considerable increases risks associated with supply, price and quality fluctuations (Naylor et al., 2000; Glencross et al., 2007). In fact, aquafeed production costs are very high (over 50%) in some aquaculture practices. In order to decrease dietary costs, increase profitability and obtain good growth, expensive ingredients may be substituted with lower cost ingredients. A number of ingredients have been suggested as viable replacements for FM, albeit at different levels of substitution, depending on the consumer species (NRC 2011). These include animal protein, meat meal, meat and bone meal, blood meal, poultry by-product meal, hydrolyzed poultry feather meal, offal and mortalities of poultry, swine and cattle (Li et al., 2006; Meeker 2006) and Plant protein SBM, canola meal, corn gluten meal, sunflower meal, cotton seed, peanut meal (Lim and Webster 2005; Gatlin et al., 2007). Single cell protein sources (SCP) (micro algae,

bacteria and yeasts) are good candidates to use as alternative non-conventional protein sources in aquafeeds (Sanderson and Jolly.1994; Tacon 1994; Oliva-Teles and Gonçalves 2001; Li and Gatlin 2003; Ghoshk et al., 2005; Abdel-Tawwab et al., 2008, 2010; He et al., 2009). Also Distillers dried grains with solubles (DDGS) have been used as alternative protein sources (Muzinic et al., 2004; Chevanan et al., 2009).

Soybean meal (SBM) has been a common source of plant protein used in the formulation of aquaculture feeds, as an substitute for FM (Carter and Hauler 2000, Evans et al., 2005, Patnaik et al., 2005, Peres et al., 2003, Úran et al., 2008). Generally, SBM has been popular due to its cost efficiency, availability, and for its relatively high protein content and good balance of amino acids (Carter and Hauler 2000). However, recent studies query some of SBM's valued assets. It is proposed that SBM has become unprofitable to use as alternative ingredients in formulated diets due to increasing prices and availability for aquafeeds use (Zerai et al., 2008, FAO 2009). Consequently, the increasing cost of feedstuffs including SBM can place limitations on its overall use as a feed in the global expansion of aquaculture. This highlights the need to find alternative protein sources to meet an expansive global demand.

The use of DDGS and yeast has been the focus of recent interest in feed development. As industrial waste from ethanol manufacturing, these products may provide a cost-effective potential source of protein in aquaculture feeds (Muzinic et al., 2004). DDGS is a source of relatively high protein, fat and fibre, and has been widely used in agricultural livestock feeds, especially in beef production (Chevanan et al., 2009, Muzinic et al., 2004). The high protein conversion efficiency of fish in comparison to livestock means dried distitillers grain and soluble DDGS is potentially a good source of alternative protein in aquaculture (Chevanan et al., 2009). DDGS may also be beneficial to consumer species as it is a good source of concentrated micronutrients, present as a result of the process of starch fermentation in ethanol production (Chevanan et al.,

2009). The essential amino acids lysine and methionine are however lower in DDGS than FM, and this may limit its inclusion in fish diets (Cheng and Hardy 2004; Shelby et al., 2008). The substitution of FM by DDGS in the diets has already been recommended at varying substitution levels in a number of different species such as rainbow trout (*Oncorhynchus mykiss*), Channel catfish (*Ictalurus punctatus*) Nile tilapia (*Oreochromis niloticus*), sunshine bass and carp (Thiessen et al., 2003; Cheng and Hardy 2004; Robinson and Menghe 2008; Zerai et al., 2008; Shelby et al., 2008; Thompson et al., 2008; Kukačka and Mareš 2008).

Single cell proteins such as micro algae, bacteria and yeast have been used in formulated diets as an alternative ingredient (Oliva-Teles and Gonçalves 2001). The suitability of brewer's yeast, *Saccharomyces cerevisiae*, as an additional ingredient in feeds for a number of different animals has been investigated (Fuller eds. 2004). Within SCPs yeast have received the greatest interest because they contain moderately high levels of protein and are a good source of nucleotides and RNA, ß-glucans, vitamins, arabinoxylan and mannan oligosaccharides (MOS) (Tacon 1994; Oliva-Teles and Gonçalves 2001; AB-Agri Patent # 109203A1, 2010). Its experimental use has been put into practice in diets for freshwater and saltwater fish with varying nutritional needs, such as cobia (*Rachycentron canadum*) (Lunger et al., 2006), European seabass (*Dicentrarchus labrax*) (Oliva-Teles and Gonçalves 2001) and tilapia (*Oreochromis mossambicus* x *O.niloticus*) (Olvera-Novoa et al., 2002; Zerai et al., 2008), sunshine bass (*Moron chrysops* x *M. Saxatalis*) (Gause and Trushenski 2011a,b), mirror carp (*Cyprinus carpio*) (Chapter 3) to name but a few. *S .cerevisiae* has also been studied to assess its ability to provide beneficial immunostimulating compounds such as β-glucans and chitin (Li et al., 2005); and has been shown to enhance immune responses and disease resistance in species such as hybrid striped bass (*Morone chrysops* x

M.saxatilis) (Li and Gatlin 2003, Li and Gatlin 2004) and gilthead sea bream (*Sparus aurata*) (Ortuño et al., 2002).

Normally protein in DDGS contains around 50% yeast (Belyea et al., 2004). A novel technology has recently been patented, which separates the YPC from first generation (DDGS) product in biofuel production. Wheat is fermented to produce ethanol using yeast. The resulting wheat protein then undergoes further processing to produce a valuable feed ingredient that can be used as a protein replacement in animal nutrition, particularly aquaculture. This product contains approximately 34% protein, and is termed yeast protein concentrate unrefined (YPC_U). In order to produce high protein yeast based product, the YPC_U is washed with water and the new dry yeast protein increased to (55%). This product is termed yeast protein concentrate refined (YPC_R). The DDGS that are used in this study are relatively high in protein content (32%). The third type of yeast which used in this study derived from the alcohol distillery using a novel way to refine the yeast from the remaining DDGS in alcohol production in Scotland and is called yeast protein concentrate potable alcohol (YPC_{PA}) with a protein level of (53%) (AB-Agri Patent # 109203A1, 2010).

The aim of the present study was to investigate the efficacy of various novel yeast protein concentrates (YPC_U, YPC_R and YPC_{PA}) and DDGS derived from bio-fuel production. Their nutritional value was evaluated in a series of experimental diets for juvenile common carp, *Cyprinus carpio* during the course of a short-term feeding trial to assess their effects on the growth performance, body composition, mineral analysis, liver function, intestinal histology, haematology and enzyme activity of this important farmed species.

4.2 Materials and methods

4.2.1 Dietary preparation

Six isonitrogenous (38%) and isolipidic (8%) diets were formulated by partial replacement of FM protein with three different types of derived yeast protein concentrates (*S. cerevivisiae*) which was substituted at 458.02 g kg^{-1} (30%) yeast protein concentrate unrefined (YPC_U), 214.42 g kg^{-1} (30%) yeast protein concentrate refined (YPC_R) and 286.81 g kg^{-1} (30%) yeast protein concentrate potable alcohol (YPC_{PA}). Distillers dried grains and solubles (DDGS) were also used to formulate two more diets with combination of (YPC_R) 100 g kg^{-1} (10%) in each, 150 g kg^{-1} (15%) DDGS and 300 g kg^{-1} (30%) DDGS substituted with FM. Dietary ingredients were mixed in a Hobart food mixer (Hobart Food Equipment, Australia, model no: HL1400 – 10STDA) with warm water until a soft slightly moist consistency was achieved. This was then cold press extruded (La Monferrina P6, La Monferrina, Asti, Italy) to produce a 2 mm pellet see section 2.3. Dietary chemical composition is shown in Table 4.1.

4.2.2 Fish rearing

Common carp (*Cyprinus carpio*) were provided by Bowlake fish farm, Hampshire, UK. Carp fry were transported to the Aquaculture and Fish Nutrition Research Aquarium, the University of Plymouth, UK. After 4 weeks acclimation and on-growing, 25 fish (15.21 ±0.07 g) were randomly distributed into 80 L fibreglass tanks, each provided with 99% re-circulated aerated freshwater at a rate of 300 L h^{-1} see section 2.4. Each treatment was conducted in triplicate. Fish were fed the experimental diets at 4% tank biomass per day (equal rations at 09.00, 13.00 and 17.00 h) for 8 weeks.

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

Ingredient g kg^{-1}	Control	YPC_U30	YPC_R30	$YPC_{PA}30$	DDGS15	DDGS30
Herring Meal LT92[1]	419.54	250	250	250	297.65	243.03
Wheat Carrier Flour[2]	508.02	214.6	457.07	371.24	380.06	276.58
Yeast (YPC_U)[3]	-	458.02	-	-	-	-
Yeast Washed (YPC_R)[3]	-	-	214.42	-	100	100
Scottish Yeast (YPC_{PA})[3]		-	-	286.81	-	-
DDGS[3]	-	-	-	-	150	300
Fish Oil[4]	-	20	20	20	5	10
Vegetable Oil [5]	27.44	12.38	13.51	26.95	22.29	15.39
Vitamin and mineral Premix[6]	20	20	20	20	20	20
Viten[7]	20	20	20	20	20	30
Molasses[8]	5	5	5	5	5	5
Proximate composition (%)						
Moisture (%)*	5.24	6.57	5.72	7.31	6.31	5
Protein (%)*	37.37	37.90	38.59	38.46	38.56	40.51
Lipid (%)*	8.75	9.46	9.08	10.1	9.31	9.19
Ash (%)*	9.57	8.12	7.4	7.6	7.77	7.53
NFE (%)*[9]	39.07	37.95	39.21	36.53	38.05	37.77
Gross Energy(Mj kg^{-1})	19.69	18.55	20.22	18.93	19.26	20.23

*Dry matter basis.

[1] Scottish Fishmeal 70, United Fish Products Ltd, UK.

[2] Ewos-Bathgate Scotland, [3] AB Vista (AB-Agri Ltd). [4] Epanoil, Sevenseas Ltd, UK. [5] Corn oil.

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

[7] Roquette Frêres, France. [8] Holland and Barret. [9] Nitrogen-free extracts (NFE) = dry matter – (crude protein + crude lipid + ash).

ND – not determined. YPC_U = Yeast Protein Concentrate Unrefined, YPC_R = Yeast Protein Concentrate Refined, YPC_{PA} = Yeast Protein Concentrate Potable Alcohol, DDGS = Dried Distiller's Grain and Solubles.

Daily feed was corrected on a weekly basis following batch weighing after a 24 h starvation period as described in section 2.5. A 12 h light/12 h dark photoperiod was maintained throughout the trial duration.

4.2.3 Water quality

The water quality variables was measured as follows; water temperature, 24.42 ± 0.93 ^{o}C; dissolved oxygen, 7.07 ± 0.40 mg L^{-1}; total ammonia nitrogen (TAN), 0.06 ± 0.03 mg L^{-1}; nitrite, 0.07 ± 0.05 mg L^{-1}; nitrate, 32.96 ± 19.78 mg L^{-1}; and pH, 7.01 ± 0.30 (adjusted with $NaHCO_3$ as necessary) as described in section 2.6.

4.2.4 Growth parameters and chemical analysis

Specific growth rate (SGR), Final weight (FW), weight gain (WG), survival rate, feed conversion ratio (FCR), feed conversion efficiency (FCE), protein efficiency ratio (PER), apparent net protein utilisation (ANPU) and condition factor (K) were assessed as described in section 2.7.

Apparent net mineral utilisation was calculated by the following equation;

$$\text{Apparent net mineral utilisation (ANMU \%)} = \frac{(FBW \times \text{Conc.s.min.}) - (\text{IBW} \times \text{Conc.s.min.})}{Feed\ intake\ X\ Conc.s.min.} \times 100$$

Which;

IBW= Initial body weight, FBW= final body weight, Conc.s.min. = concentration of selected mineral.

4.2.5 Chemical composition analysis of the diets and fish carcasses

Diets and fish samples (initial and final) from the feeding trial were analysed according to AOAC (2002) standard methods for proximate composition. Fish sampled for whole body analysis (18 fish sampled at the start and 4 fish per tank at the end of trial) were ground and homogenized in a blender prior to chemical assays. Two samples per tank (2 fish per sample pooled together) were analysed to minimize the standard deviation between the samples.

Moisture content (dry matter) was determined using a drying oven (105 ºC for 24 h). Crude protein was calculated from sample nitrogen content and was determined using Kjeldahl apparatus (Gerhardt Kjeldatherm method, N% x 6.25) and crude lipid using ether extraction in multi-unit extraction Soxtec apparatus (dicloromethane extraction by Soxlhet method). Ash Content was analysed using a muffle furnace (incineration at 550ºC for 12 h). Gross energy analysed using (Parr bomb calorimeter). All protocols concerning analytical chemistry are described in section 2.8.

Amino acids analysed (except tryptophan) carried out by Sciantec Analytical Services Ltd. Laboratories (Yorkshire; UK) Table 4.2. Mineral compositions were conducted using spectrophotometer (ICP-MS) on a dry basis Table 4.3, as described in section 2.8.7.

Table 4.2 Amino acid profile for experimental diets.

	Control	YPC$_U$30	YPC$_R$30	YPC$_{PA}$30	DDGS15	DDGS30	Carp requirement **
Essential AA (%)							
Arginine	2.15	2.00	2.03	2.08	1.90	2.04	1.6
Histidine	0.95	0.97	0.99	0.99	1.06	0.94	0.8
Iso-Leucine	1.60	1.58	1.49	1.81	1.74	1.53	1.0
Leucine	2.74	2.68	2.74	2.86	2.66	2.70	1.30
Phenylanine	1.55	1.68	1.72	1.7	1.65	1.73	2.50[a]
Lysine	2.33	1.87	2.04	2.61	2.19	1.82	2.20
Threonine	1.47	1.34	1.59	1.72	1.44	1.18	1.50
Valine	2.03	1.92	1.84	2.23	2.21	1.67	1.40
Methionine	1.16	1.02	0.97	1.04	1.02	0.68	0.8
Tryptophan	ND	ND	ND	ND	ND	ND	0.30
Non-Essential AA (%)							
Alanine	2.10	1.85	1.84	2.30	1.90	1.55	ND
Aspartic acid	3.10	2.77	2.82	3.37	2.79	1.85	ND
Cysteine	0.53	0.58	0.67	0.62	0.71	0.65	ND
Glutamine	5.85	7.69	7.75	5.64	7.01	8.17	ND
Glycine	2.04	1.84	1.86	1.96	1.94	1.81	ND
Proline	1.57	2.16	2.25	1.47	1.41	2.46	ND
Serine	1.48	1.58	1.74	1.89	1.73	1.43	ND
Tyrosine	0.97	1.05	1.01	1.09	1.07	1.05	ND

*Not determined, **% values obtained from references as cited by NRC (2011),[a] with 1% Iso-leucine, YPC$_U$ = Yeast Protein Concentrate Unrefined, YPC$_R$ = Yeast Protein Concentrate Refined, YPC$_{PA}$ = Yeast Protein Concentrate Potable Alcohol, DDGS = Dried Distiller's Grain and Solubles.

Table 4.3 Mineral analysis for experimental diets. (n = 3)

Mineral	Control	YPC_U30	YPC_R30	$YPC_{PA}30$	DDGS15	DDGS30
g kg^{-1}						
Ca	12.06±3.18	8.33±0.14	8.75±0.50	8.22±0.53	9.11±0.16	8.28±0.21
K	8.26±1.98	9.70±0.59	7.39±0.13	6.26±0.08	7.65±0.06	7.61±0.09
Mg	2.55±0.59	2.65±0.16	2.22±0.08	1.94±0.04	2.33±0.04	2.36±0.02
Na	6.34±1.53	4.32±0.12	4.45±0.06	6.31±0.07	5.30±0.06	5.08±0.01
P	11.68±2.99	11.08±0.26	9.79±0.37	10.00±0.37	9.99±0.13	9.69±0.14
mg kg^{-1}						
Cr	2.57±0.66	0.92±0.19	0.76±0.56	1.22±0.43	1.33±1.08	0.82±0.43
Cu	17.22±3.73	13.00±1.73	15.53±1.00	33.08±0.50	16.58±0.53	16.10±0.79
Fe	215.75±59.94	197.90±18.94	202.08±6.49	172.68±36.38	198.11±3.80	200.89±20.87
Mn	61.99±15.20	78.77±0.41	62.53±2.53	63.04±1.58	63.47±2.17	63.98±0.20
Zn	106.47±30.22	95.64±4.91	88.39±3.47	83.76±0.95	94.03±1.40	114.93±29.74

Data are presented as mean ± S.D, YPC_U = Yeast Protein Concentrate Unrefined, YPC_R = Yeast Protein Concentrate Refined, YPC_{PA} = Yeast Protein Concentrate Potable Alcohol, DDGS = Dried Distiller's Grain and Solubles.

4.2.6 Histology

4.2.6.1 Light microscopy

Histological appraisal of the liver from 6 fish per experimental group was conducted at the end of the trial using light microscopy as described in section 2.9.1.

A photograph of sections of each fish's liver per treatment at 40X magnification was taken with an Olympus E-620 digital camera mounted on a Vanox Olympus research microscope model AHBT. Using the software Image J 1.43, numbers of nuclei were then counted in a standardized area per photograph. This was done by firstly calibrating exact sizes of photographs; then using a standardized 15x15 μm square located at the same coordinates in each image, all nuclei present inside the square, but not touching the square perimeter, were marked and a total number was calculated by the software.

4.2.6.2 Electron Microscopy

Samples for SEM were taken in the anterior and posterior region of gut from six separate fish per treatment as described in section 2.9.2. Microvilli density were assessed, sampling and processing protocols are described in section 2.9.2.1 as described in section 2.9.2. Samples from posterior region of the gut were observed from six fish per treatment for TEM and microvilli density were measured as described in section 2.9.2.2.

4.2.7 Haematological parameters

At the end of the trial, fish were scarified and blood collected from 10 fish per treatment as described in section 2.10.

4.2.7.1 Haemotocrit

Haemotocrit determination was assayed using heparinized capillary tubes as described in section 2.10.1.

4.2.7.2 Haemoglobin

Total blood haemoglobin concentration was determined using Drabkins spectrophotometric method as described in section 2.10.2.

4.2.7.3 Total erythrocyte counts

Erythrocyte counts were performed by diluting 20 µL of fresh blood with 1 mL of Dacies solution, and counts were performed with a Neubauer haemocytometer (Dacie and Lewis, 2001). A glass pipette were used and to ensure that the blood cells were re-suspended, a small quantity of the blood cell suspension were introduced on the platform of the haemocytometer at the edge of the cover slip to be drawn into counting area by capillary action. Then, total erythrocyte evaluations are carried out in the five small squares in the centre of the grid under a light microscope. The volume counted per square = $0.2 \times 0.2 \times 0.1 = 0.004\ mm^{-3}$.

4.2.7.4 Blood smears

Blood smears were prepared by adding a drop of blood onto a slide and allowed to air dry. Smears were fixed in 95% methanol and slides were stained using 6% Giemsa (BDH) for 20 min and washed twice for one min in distilled water. Slides were air – dried and mounted with cover slips using DPX (BDH). Images were taken with a DCMI30 digital camera (Brunel microscopes Ltd, Wiltshire, UK) using scopPhoto (ScopeTeck®, China) and a Medilux-12 light microscope (Kyowa).

4.2.7.5 Total leukocyte counts

Total leukocyte counts (neutrophil, monocyte, thrombocyte and lymphocyte) were performed with a digital imaging system scored blindly as total number of leukocytes per 1000 blood cells as described by (Merrifield, et al. 2010b).

4.2.7.6 Haematological indices

Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from RBC, Hct, and Hb according to the following formulae: MCV = (PCV x 1000)/RBC, MCH = Hb/RBC and MCHC = (Hb × 10)/Hct (Lee et al., 1998; Al-Dohail et al., 2011).

4.2.8 Liver enzyme assays

At the end of the experiment six fish per treatment were euthanized and livers were frozen as described in section 2.11. Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were assayed using the micro plate reader (Molecular Devices) as described in section 2.11.

The total protein content protein of the supernatant was determined as described in section 2.11.

4.2.9 Statistics

Statistical analysis (i.e. growth parameters, body composition, enzyme, histology) was carried out using One-Way ANOVA (SPSS 17.0). *Post hoc* LSD test was used to determine significant differences between means Percentage data was arcsine transformed prior to subsequent analysis and significant was accepted at $P < 0.05$ level.

4.3 Results

4.3.1 Growth parameters and feed efficiency

Growth performance and feed utilization data of common carp fed the six experimental diets are presented in Table 4.4. During the growth trial, all the fish readily accepted the experimental diets. The survival rate recorded during the experimental period was 100% for all experimental diets except DDGS30 which was 98%. There were significant differences between the final weights of the fish fed YPCU30 inclusion diet compared to fish fed control, YPCR30, DDGS15 and DDGS30 inclusion diets ($P<0.05$). Fish fed YPCPA30 inclusion diet exhibited significantly lower final weight compared to all other fish groups ($P<0.05$) (Table 4.4). Furthermore, the weight gain of fish fed YPCU30 was significantly ($P<0.05$) higher than fish fed the Control, YPCR30, DDGS15 and DDGS30 diets. Fish fed with YPCPA30 inclusion experienced significantly lower weight gain compared to control diet and replacement level diets (Table 4.4). The highest specific growth rate (SGR) was observed in the group YPCU30 (2.45) which had higher value than any other group, followed by groups DDGS15 (2.32), Control (2.31), DDGS30 (2.28) and YPCR30 (2.28). The lowest feed conversion efficiency was obtained in the group YPCPA30 (2.13±0.001) which was significantly lower than all other groups ($P<0.001$). Although trends towards elevated FCE were observed in all fish fed inclusion diets, significant elevations were observed in fish fed YPCU30.

Table 4.4 Growth performance and feed utilization of common carp fed the experimental diets for 8 weeks. (*n*=3)

	Control	YPCU30	YPCR30	YPCPA30	DDGS15	DDGS30
Initial weight (g)	15.26 ± 0.08	15.20 ± 0.00	15.14 ± 0.03	15.18 ± 0.03	15.18 ± 0.08	15.28 ± 0.06
Final Weight (g)	55.52 ± 0.34^{b}	60.00 ± 1.92^{a}	54.40 ± 0.34^{b}	49.92 ± 0.06^{c}	55.68 ± 0.85^{b}	55.57 ± 1.08^{b}
Weight Gain (g)	40.26 ± 0.25^{b}	44.80 ±1.92 ^{a}	39.26 ± 0.37^{b}	34.74 ± 03^{c}	40.50 ± 0.76^{b}	40.29 ± 1.14^{b}
SGR (% day^{-1})	2.31 ± 0.001^{b}	2.45 ± 0.057^{a}	2.28 ± 0.014^{b}	2.13 ± 0.001^{c}	2.32 ± 0.017^{b}	2.28 ± 0.002^{b}
FCE	87.22 ± 0.34^{ab}	91.34 ± 0.76^{a}	86.56 ± 1.24^{ab}	83.43 ± 0.07^{b}	87.52 ± 0.70^{ab}	89.26 ± 6.32^{ab}
FCR	1.33 ± 0.001^{b}	1.23 ± 0.027^{a}	1.35 ± 0.010^{bc}	1.43 ± 0.001^{d}	1.33 ± 0.000^{b}	1.37 ± 0.021^{c}
PER	2.33 ± 0.009^{ab}	2.41 ± 0.020^{b}	2.24 ± 0.032^{ab}	2.17 ± 0.002^{a}	2.27 ± 0.018^{ab}	2.20 ± 0.156^{a}
ANPU (%)	31.38 ± 0.50^{bc}	35.97 ± 0.93^{a}	30.49 ± 0.13^{cd}	29.54 ± 1.07^{d}	32.41 ± 0.42^{b}	32.37 ± 0.75^{b}
Condition factor (K)	1.41 ± 0.03^{ab}	1.48 ± 0.10^{a}	1.34 ± 0.07^{b}	1.39 ± 0.03^{ab}	1.46 ±0.12^{ab}	1.41 ± 0.09^{a}
Survival (%)	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	98 ± 2.00

Data are presented as mean ± S.D., Data in the same row with different superscript are significantly different (*P<0.05*), YPCU = Yeast Protein Concentrate Unrefined, YPCR = Yeast Protein Concentrate Refined, YPCPA = Yeast Protein Concentrate Potable Alcohol, DDGS = Dried Distiller's Grain and Solubles.

Table 4.5 Body proximate composition for common carp fed experimental diets for 8 weeks. *n* = 6 (n initial =3)

Whole Body composition	Initial	Control	YPCU30	YPCR30	YPCPA30	DDGS15	DDGS30
Moisture (%)	75.83 ± 0.43	74.46 ± 0.41^{ab}	76.18 ± 0.14 ^{c}	74.03 ± 0.01 ^{a}	75.90 ± 0.69^{bc}	74.85 ± 1.05^{abc}	75.34 ± 0.55^{abc}
Protein (%)*	56.00 ±1.82	57.37 ± 1.32^{a}	60.72 ±0.42^{b}	56.94 ± 0.59^{ac}	60.23 ± 2.26^{ab}	59.06 ±0.17^{abc}	60.04 ± 1.58^{abc}
Lipid (%)*	26.15 ± 0.59	28.87± 0.69^{c}	23.87 ±0.07^{a}	30.22 ± 0.18^{c}	23.91± 1.02^{a}	26.76 ± 0.04^{b}	25.62 ± 1.59^{ab}
Ash (%)*	11.39 ± 0.15	9.48 ± 0.44^{a}	11.80 ± 0.37^{d}	9.43 ± 0.57^{a}	11.07 ± 0.48^{cd}	9.99 ± 0.03^{ab}	10.66 ± 0.00b^{bc}
NFE (%)*	6.46±1.41	4.28± 2.45	3.61±0.72	3.41±0.16	4.79 ± 1.73	4.19 ± 0.16	3.68 ± 0.02
Gross energy (MJ kg^{-1})	24.71±0.20	25.51±0.01	24.04 ±0.52	25.26±0.13	24.46±0.24	25.08±0.08	24.76±0.31

* Dry matter basis, Data are presented as mean ± S.D., Data in the same row with different superscript are significantly different (*P<0.05*), Nitrogen-free extracts (NFE) = dry matter – (crude protein + crude lipid + ash), YPCU = Yeast Protein Concentrate Unref- ined, YPCR = Yeast Protein Concentrate Refined, YPCPA = Yeast Protein Concentrate Potable Alcohol, DDGS = Dried Distiller's Grain and Solubles.

While fish fed YPCPA30 FCE was significantly (*P<0.05*) decreased among the treatments (Table 4.4). Compared to the control group, the FCR was significantly improved with YPCU30 inclusion; however, YPCPA30 inclusion obtained worst (i.e. highest) FCR within the experimental group (*P<0.001*). PER for YPCU30 fed carp was improved significantly from those carp fed YPCPA30 and DDGS30 (*P<0.05*). On the other hand, protein efficiency ratio (PER) of fish fed diets Control, YPCR30 and DDGS15 did not differ significantly from YPCU30 fed fish (*P<0.05*). Apparent net protein utilisation (ANPU) was statistically highest for the YPCU30 and lowest for YPCPA30 inclusion diet. In addition, ANPU of the fish fed DDGS15, DDGS30 and Control differ significantly from the ANPU of fish fed YPCU30 and YPCPA30 (*P<0.05*). Furthermore, ANPU of fish fed YPCR30 was significantly lower from ANPU of fish fed YPCU30 DDGS15, DDGS30 and Control but is not differ significantly from the ANPU of fish fed YPCPA30 (*P<0.05*).The condition factor (K) of fish fed YPCR30 and YPCU30 are not statistically different from fish fed YPCPA30, DDGS15, DDGS30 and Control diets (*P<0.05*) but they are significantly different from each other (*P=0.039*).

4.3.2 Carcass composition and mineral analysis

Body composition data of fish fed various types of dietary YPC and DDGS are summarized in Table 4.5 Moisture content was increased significantly (*P<0.05*) only with the YPCU30 and YPCPA30 dietary inclusion compared to the control diet. Significant differences were apparent for whole-body protein which tended to increase significantly (*P<0.05*) in fish fed YPCU30 dietary inclusion compared to the fish fed fishmeal diet. Fish fed other experimental diets did not display any differences in body protein content. A similar tendency was found for PER which was found to be maximum at YPCU30 inclusion in the diet. Compared to the control, lipid contents

decreased significantly (*P<0.05*) in fish fed on experimental diets, except for fish fed YPCR30 which was not significantly different (*P<0.05*). Whole-body ash was significantly higher in fish fed YPCU30 (11.83±0.373), YPCPA30 (11.10±0.492) and DDGS30 (10.68±0.004) compared with fish fed control diet (9.50±0.439) (*P=0.005*). However, there was no significant differences in the fish fed YPCR (9.44±0.576) and DDGS15 (10.00±0.025) compared with fish fed the control diet (9.50±0.439) (*P< 0.05*). Body gross energy level was comparable in fish fed all diets. No significant (*P<0.05*) differences were apparent with regards to nitrogen free extracts (NFE) contents.

Carcass mineral levels can be seen in Table 4.6. A clear trend of elevated carcass calcium levels was observed with dietary inclusions of various types of YPC leading to significant increases (*P<0.05*) with YPCU30 and YPCPA30. Similar results were observed with respect to phosphorous whereby significantly higher levels were observed in all YPC fed groups, except the DDGS15 fed group. Additionally, higher levels of carcass sodium and magnesium were observed in fish fed YPCU30 and YPCPA30 diets. Potassium levels were significantly (*P<0.05*) higher only in fish fed YPCU30, and iron levels were significantly (*P<0.05*) lower only in fish fed YPCPA30. Copper levels were significantly (*P<0.05*) higher in carp fed YPCPA30 than fish fed other dietary groups. Zinc levels were significantly (*P<0.05*) lower in fish fed YPCU30, DDGS15 and DDGS30 diets and no significant differences were apparent in fish fed DDGS30 and YPCPA30. Chromium and manganese levels were not affected by dietary inclusion of YPCU, YPCR, YPCPA and DDGS.

The Apparent Net Mineral Utilisation (ANMU) is presented in Table 4.7.The ANMU of phosphorus, sodium, magnesium and chromium for all diets tested showed significant differences of retention efficiencies between treatments. The control fed carp had (*P<0.05*) significantly lower phosphorus and magnesium efficiency than the carp fed other diets. Also the retention of sodium and chromium in fish fed various types of YPC

and DDGS were significantly higher than fish fed control diet except YPCPA30 dietary inclusion which was significantly (*P<0.05*) lower than carp fed the control diet. The highest value for ANMU of copper was obtained in fish fed YPCU30 inclusion and YPCPA30 fed fish lowest, significant differences were found between treatments (*P<0.05*). YPCPA30 had a significantly higher potassium value of ANMU in comparison to other dietary treatments (*P<0.05*). Fluctuating values for ANMU of iron were observed in fish fed all dietary treatments compared to the iron value in fish fed the control diet, which were significantly different (*P<0.05*). Control fed fish had significantly higher zinc ANMU (*P<0.001*) compared to all other dietary groups.

Table 4.6 Mineral analyses for common carp (whole body) fed on experimental diets for 8 weeks. (*n*=6)

Mineral	Initial	Control	YPC$_U$30	YPC$_R$30	YPC$_{PA}$30	DDGS15	DDGS30
g kg^{-1}							
Ca	24.10±1.82	17.89±0.51^{a}	25.15±1.34^{c}	17.82±0.38^{a}	21.71±0.58^{b}	18.02±0.02^{a}	19.54±0.54^{a}
K	7.81±0.24	8.94±0.24^{ab}	9.90±0.03^{c}	8.93 ± 0.21^{ab}	9.42±0.31^{bc}	8.80±0.26^{a}	8.98±0.11^{ab}
Mg	1.08±0.06	0.88±0.01^{a}	1.11±0.02^{d}	0.89±0.01^{a}	1.02±0.01^{c}	0.88±0.18^{a}	0.96±0.00^{b}
Na	3.11±0.14	2.99±0.05^{ab}	3.35±0.08^{c}	2.82±0.01 ^{a}	3.13±0.19 ^{b}	2.86±0.03 ^{a}	2.90±0.00 ^{a}
P	16.86±0.96	14.11±0.52^{a}	18.46±0.50^{d}	14.28±0.05a^{b}	16.46±0.25^{c}	14.16±0.37^{a}	15.19±0.49^{b}
mg kg^{-1}							
Cr	0.38±0.13	0.72±0.17	0.44±0.03	0.49±0.30	0.38±0.18	0.47±0.13	0.40±0.04
Cu	9.41±0.45	7.92±0.90^{ab}	8.57±0.07^{abc}	7.25±0.69 ^{a}	9.93±0.56 ^{c}	9.36±0.45^{bc}	8.45±1.16^{abc}
Fe	90.70±1.18	73.34±1.09	75.97±2.08	72.89±0.55	71.03±0.62	75.30±7.05	71.19±1.56
Mn	7.22±0.43	2.79±0.25	3.08±0.30	2.73±0.12	2.88±0.15	2.61±0.15	2.96±0.26
Zn	282.97±0.43	202.96±11.85^{c}	172.46±7.37^{a}	195.95±1.47^{bc}	224.53±0.29^{d}	179.43±3.16^{ab}	188.08±0.52^{abc}

Data are presented as mean ± S.D., Data in the same row with different superscript are significantly different (*P<0.05*).

Table 4.7 Mineral Retention for common carp fed experimental diets for 8 weeks. (*n*=6)

Mineral	Control	YPC$_U$30	YPC$_R$30	YPC$_{PA}$30	DDGS15	DDGS30
Macro Mineral %						
Ca	94.89±0.40^{d}	95.72±1.68^{d}	88.44±0.51^{b}	84.80±0.04^{a}	91.39±0.14^{c}	85.96±2.07^{ab}
K	23.32±0.0^{b}	22.54±0.42^{a}	26.34 ± 0.17^{d}	29.24±0.02^{e}	24.54±0.02^{c}	23.66±0.57^{b}
Mg	6.37±0.03^{a}	8.54±0.18^{d}	7.69±0.06^{c}	9.52±0.01^{e}	7.06±0.01^{b}	7.60±0.18^{c}
Na	9.57±0.039^{b}	16.41±0.339^{e}	12.72±0.092^{d}	8.94±0.005^{a}	10.75±0.003^{c}	10.75±0.260^{c}
P	23.24±0.09^{a}	35.34±0.73^{d}	28.45±0.22^{c}	29.48±0.02^{c}	27.11±0.02^{b}	28.86±0.70^{c}
Micro Mineral %						
Cr	6.74±0.029^{b}	10.23±0.203^{e}	14.82±0.089^{f}	5.74±0.003^{a}	7.87±0.008^{c}	9.46±0.229^{d}
Cu	8.88±0.036^{b}	13.12±0.305^{e}	8.75±0.071^{b}	5.56±0.003^{a}	11.57±0.002^{d}	9.63±0.233^{c}
Fe	6.45±0.026^{b}	7.37±0.183^{d}	6.89±0.054^{c}	6.55±0.005^{b}	7.24±0.006^{d}	6.09±0.148^{a}
Mn	0.41±0.001^{cd}	0.43±0.026^{d}	0.39±0.008^{c}	0.28±0.001^{a}	0.31±0.007^{b}	0.40±0.010^{cd}
Zn	33.96±0.14^{d}	28.88±0.98^{b}	39.14±0.35^{e}	43.01±0.03^{f}	31.16±0.10^{c}	25.41±0.62^{a}

Data are presented as mean ± S.D., Data in the same row with different superscript are significantly different (*P<0.05*), YPC$_U$ = Yeast Protein Concentrate Unrefined, YPC$_R$ = Yeast Protein Concentrate Refined, YPC$_{PA}$ = Yeast Protein Concentrate Potable Alcohol, DDGS = Dried Distiller's Grain and Solubles.

4.3.3 Histological analyses

The results of the histological examinations of the microvilli density and microvilli lengths are presented in Table 4.8. The density of microvilli in the posterior region of fish fed YPC$_U$30 (4.35±0.82 AU), YPC$_{PA}$30 (4.13±0.53 AU), DDGS15 (3.62±0.95 AU), DDGS30 (3.45±0.66 AU) was decreased compared with the control fed fish (4.34±0.42 AU) with no significant differences were found among the treatments (*P<0.05*), However, there was a significant difference in the fish fed YPC$_R$30 (2.44±1.15 AU) compared to the control fish (4. 34±0.42 AU) (Figure 4.2, Table 4.8). There were no significant (*P<0.05*) differences in the anterior region (Figure 4.3, table 4.8).

The number of hepatocytes (field of view 225 μm^2) in the liver of fish fed YPC$_{PA}$30 were not significantly different to those fish fed the control diet (*P<0.05*). Significant differences in the number of hepatocytes were observed between fish fed with YPC$_R$30 or YPC$_{PA}$30 and DDGS30, which they had a fewer number of hepatocyte compared to the fish fed with the control diet (*P<0.05*). Furthermore, no significant (*P<0.05*) differences were obtained in the number of hepatocytes in the fish fed YPC$_U$30 and DDGS15 diets with fish fed the control diet.

Hepatic structure showed that the liver of the fish fed the control, YPC$_U$ and YPC$_R$ diets appeared to be healthy with no signs of pathological change. The liver from fish fed the YPC$_{PA}$ appeared to be abnormal as vacuolisation and relatively disorganised hepatocyte structures were observed. The hepatic structure of this fish appears to be subject to some form of pathological change with areas of vacuolization, comparatively disorganized hepatic ultrastructure and distinct hepatocytes absent. Vacuolization was also noticed, but not as prominently, fish fed the DDGS15 and DDGS30 diets (Figure 4.1; Table 4.8).

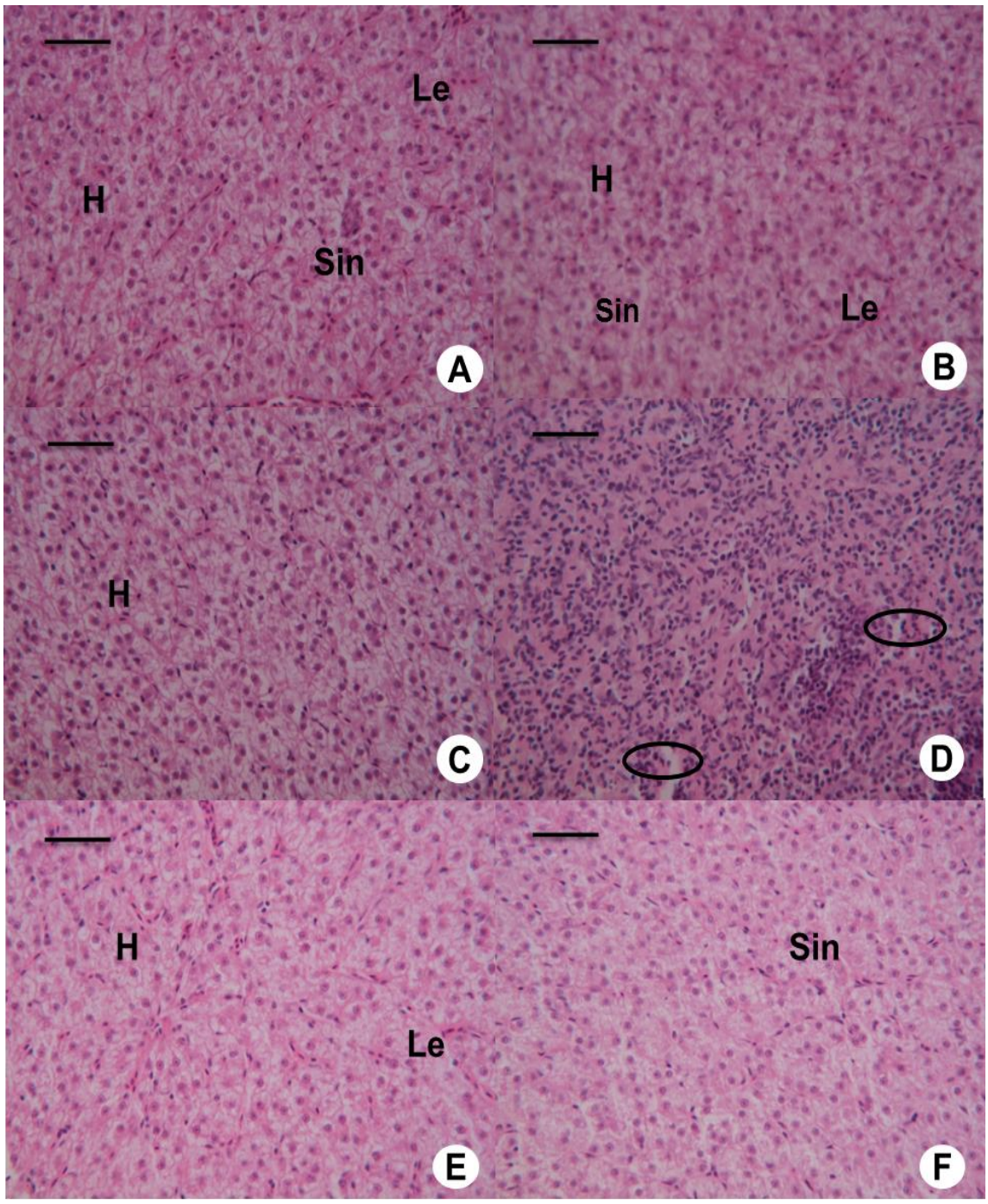


Figure 4.1 Photomicrograph of liver section of carp stained with haematoxylin and eosin. Fish were fed (A) fishmeal (B) YPC$_{U}$30, (C) YPC$_{R}$30, (D) YPC$_{PA}$30, (E) DDGS15 and (F) DDGS30. (Scale bar = 50 µm). Hepatocytes, Le: Leukocytes, Sin: Sinusoid and Circle areas the slight necrosis or hypertrophy of liver cells (hepatocytes).

Table 4.8 Microvilli morphology and liver structure of common carp fed on experimental diets. (*n* =6)

Variable	Intestine	Control	YPCU30	YPCR30	YPCPA30	DDGS15	DDGS30
Hepatocytes		182.3±20.32^{ab}	175.2±10.80^{ab}	158.7±14.02^{a}	230.2 ±109.03^{b}	173.2±27.32^{ab}	151.8±28.59^{a}
Microvilli density*	Anterior	1.75±0.45	1.76±0.37	1.90±0.12	1.91±0.45	1.75±0.34	1.51±0.19
	Posterior	4.34±0.42^{b}	4.35±0.82^{b}	2.4±1.15^{a}	4.13±0.53^{b}	3.62±0.95^{ab}	3.45±0.66^{ab}
Microvilli length (μm)	Posterior	0.98±0.32^{a}	1.45±0.13^{b}	1.2±0.03^{ab}	0.94±0.05^{a}	-	1.31±0.38^{ab}

* Arbitrary unit.

Data are presented as mean ± S.D.

Data in the same row with different superscript are significantly different (*P<0.05*).

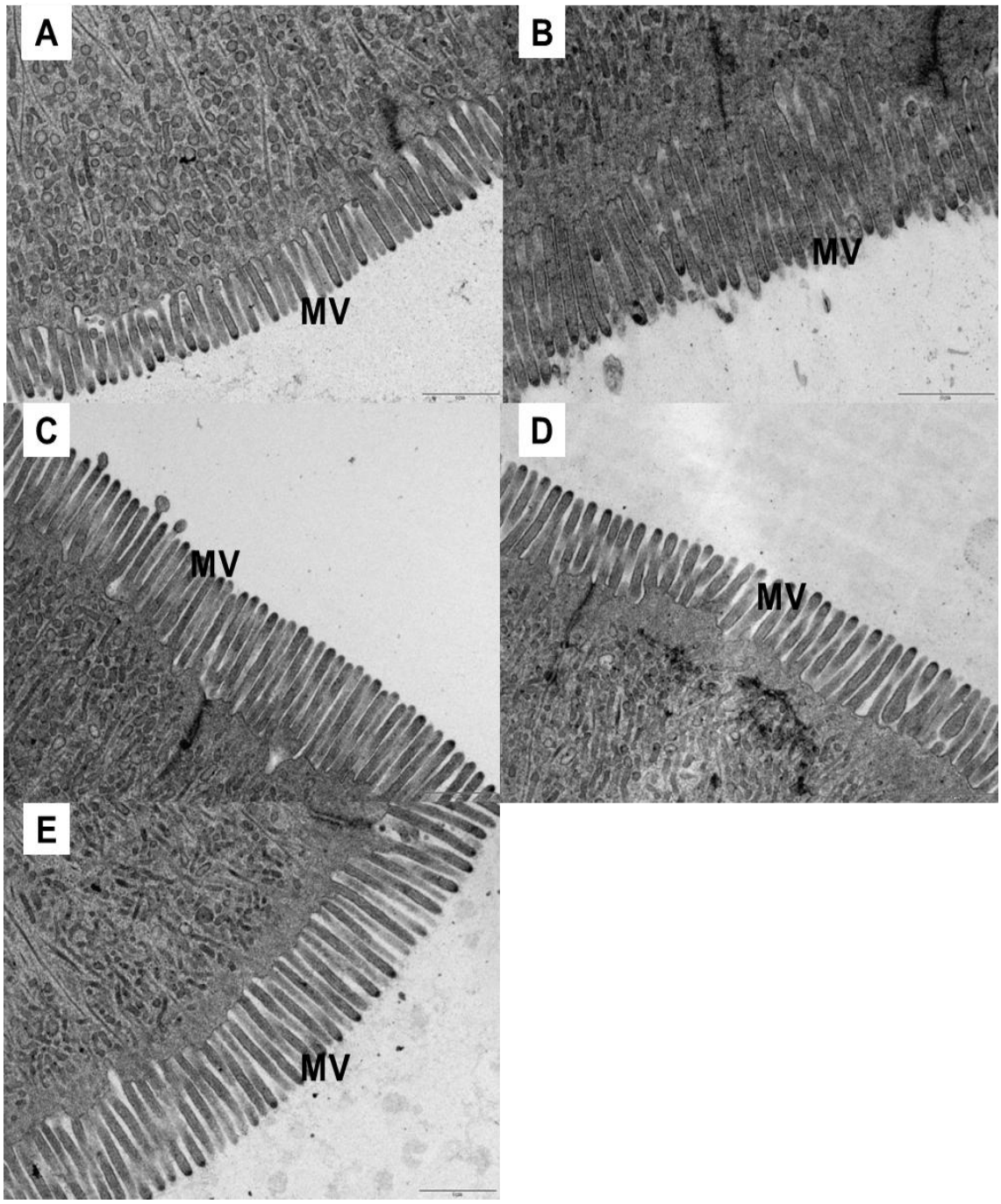


Figure 4.2 Comparative TEM micrographs of the posterior intestine of (A) control fed fish, (B) YPCU30 fed fish, (C) YPCR30 fed fish, (D) YPCPA30 fed fish and (E) DDGS30 fed fish. Microvilli length appear longer and healthier significantly in the group YPCU compare to the other treatments. (Scale bar = 1 µm). MV: Microvilli.

Microvilli length from the posterior region increased from 0.98±0.32 µm in carp fed control diet to 1.45±0.13 µm in carp fed YPCU30 diet. Although, the microvilli length in the posterior region of carp fed YPCR30 (1.19.± 0.03 µm) and DDGS30 (1.19±0.28 µm) was longer than microvilli length of carp fed control diet (0.98±0.32) but no

significant (*P<0.05*) differences were observed (Figure 4.4, Table 4.8). The microvilli length of fish fed YPCPA30 diet (0.94±0.38 µm) was comparable with carp fed control diet (0.98±0.32) (*P<0.05*).

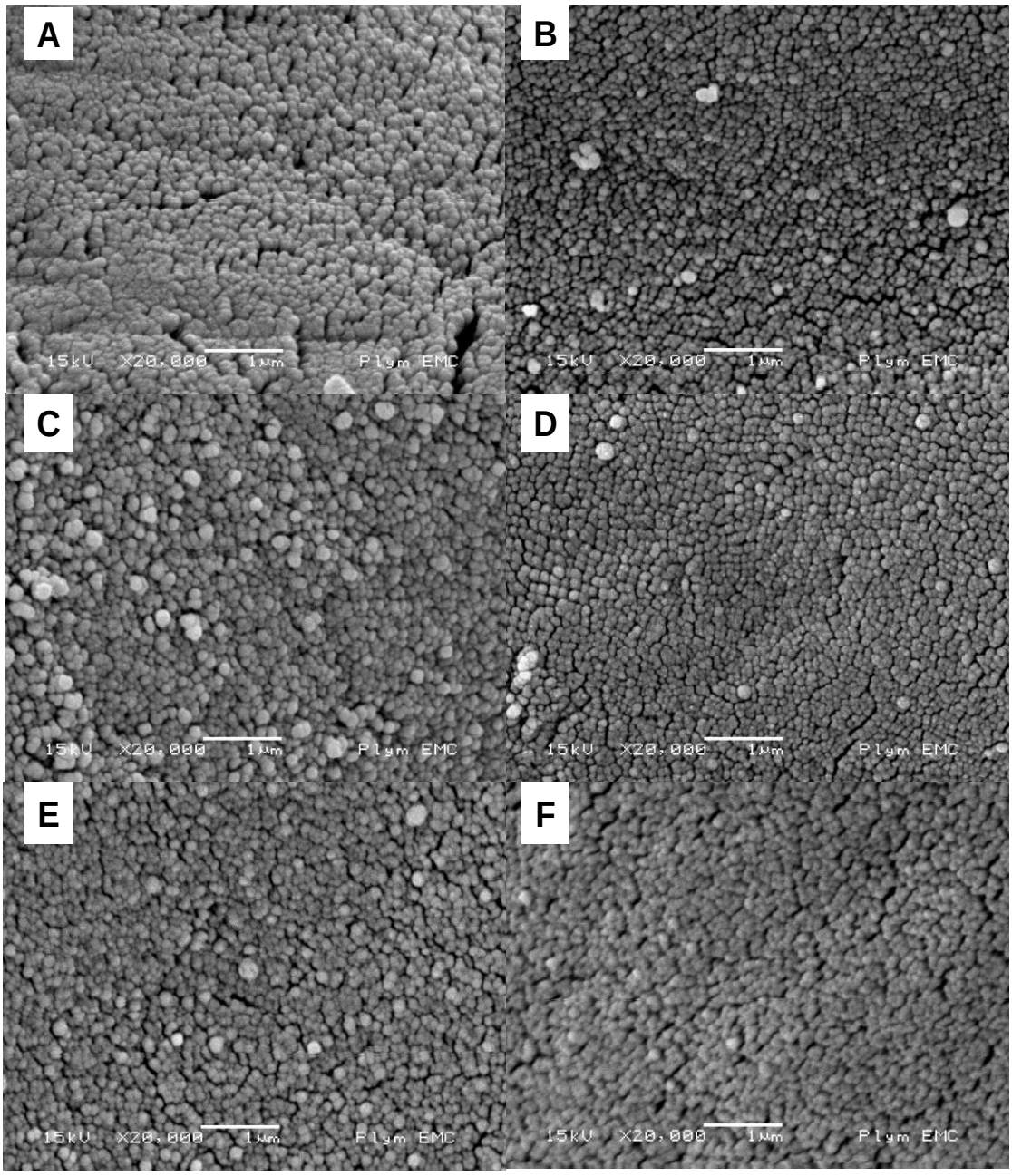


Figure 4.3 Comparative SEM micrographs of anterior intestine of carp fed (A) control fed fish, (B) YPCU fed fish (C) YPCR fed fish (D) YPCPA fed fish, (E) DDGS15 fed fish and (F) DDGS30 fed fish. There are no distinctive differences of the microvilli density between the treatments. (Scale bar = 1 µm)

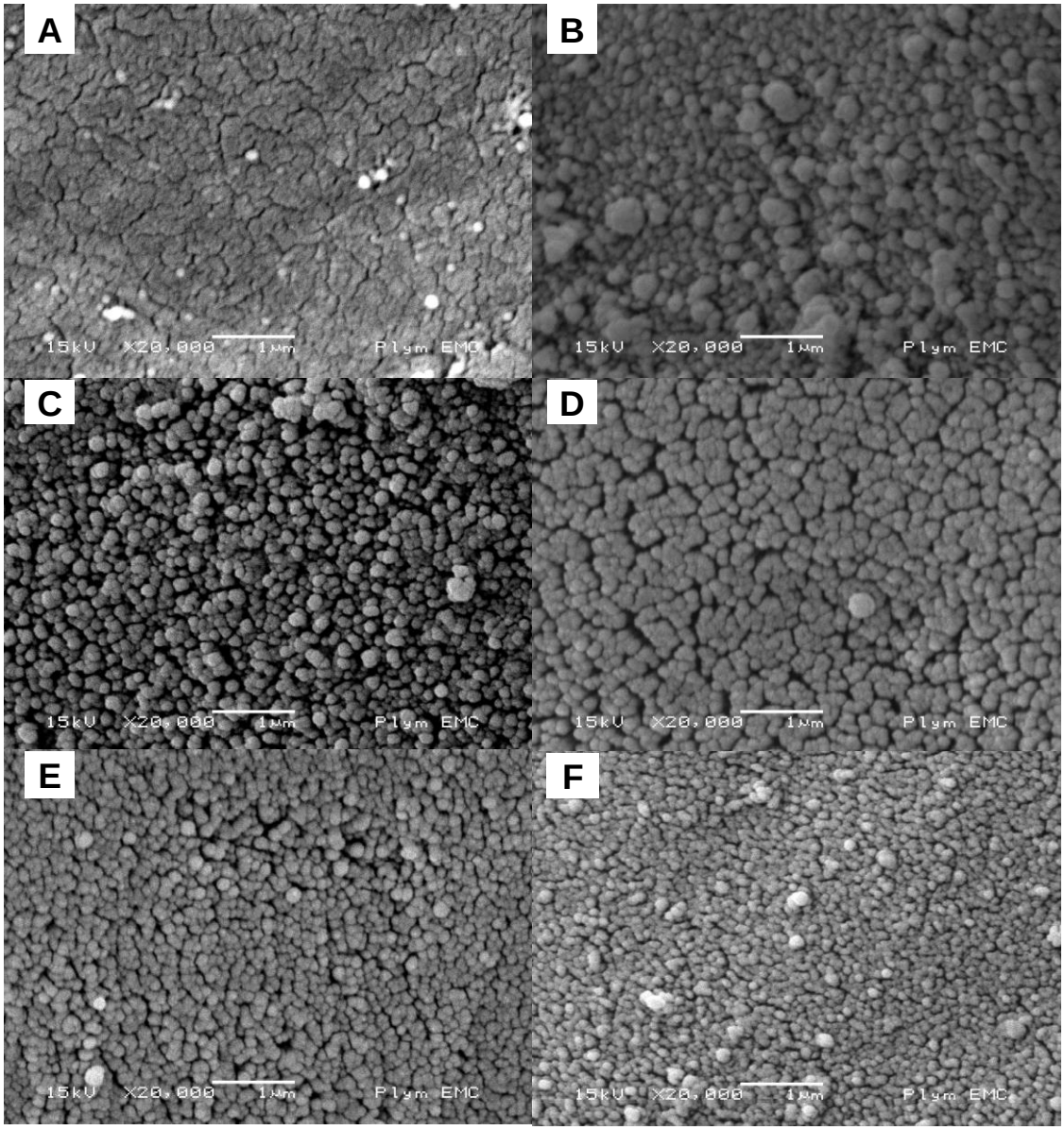


Figure 4.4 Comparative SEM micrographs of posterior intestine of carp fed (A) control fed fish, (B) YPC$_U$ fed fish, (C) YPC$_R$ fed fish (D) YPC$_{PA}$ fed fish, (E) DDGS15 fed fish and (F) DDGS30 fed fish. There are significant differences of the microvilli density in YPC$_R$ fed fish compared to other treatment groups. (Scale bar = 1 µm)

4.3.4 Blood parameters

Haematological measurements for the different groups of fish are shown in Table 4.9. No significant ($P<0.05$) differences were observed in the haematocrit (Hct), haemoglobin (Hb), mean corpuscular haemoglobin concentration (MCHC) or mean corpuscular haemoglobin (MCH) from fish fed the different experimental diets. The

erythrocyte levels (RBCC) of fish fed DDGS30 and YPCU30 diets were significantly higher than of fish fed the YPCPA30 diet. There were no statistically significant differences in RBCC of fish fed control, DDGS15 and YPCR30 inclusion diets compared to the other groups (*P<0.05*) (Table 4.8). The total number of leucocytes (WBC) was significantly higher (*P<0.001*) in fish fed the YPCU30 diet (88.25 ± 7.30 per 1000 blood cells) than in the fish fed the DDGS15 (61.38± 13.29) and YPCPA (61.38±11.27) diets; fish fed control and YPCR30 (79.00± 9.20) diets were not different to fish fed the YPCU30 (88.25± 7.30) diet. While, fish fed DDGS30 (68.50± 8.99) were significantly lower than those fish fed YPCU30 (88.25± 7.30) and control (81.00) ± 9.58) diets (*P<0.05*). The mean corpuscular volume (MCV) of fish fed the control and YPCPA30 diets were significantly (*P<0.05*) higher than fish fed YPCU30 diet (*P*=0.012). Furthermore, there were no significant (*P<0.05*) differences between fish fed YPCR30, DDGS15 and DDGS30 compared to the fish fed the control and YPCPA30 or YPCU30 diets (*P<0.05*).

4.3.5 Enzymatic analyses

Hepatopancreas ALAT activities of fish fed experimental diets (various types of YPC and DDGS inclusion) had no significant differences between dietary treatments (*P<0.05*) (Table 4.9). ASAT activities decreased with dietary inclusion of various types of YPC and DDGS and compared to the control and were statistically (*P<0.05*) lower in all inclusion dietary treatments (YPCU30, YPCR30, YPCPA30, DDGS15 and DDGS30) (Table 4.9).

Table 4.9 Haematological parameters and liver enzymes of common carp after 8 weeks of feeding on experimental diets. (*n*=10)

	Control	YPC$_U$30	Y$_{PCR}$30	YPC$_{PA}$30	DDGS15	DDGS30
Haematocrit (%)	44.8 ± 2.62	41.60 ± 3.73	43.40 ± 3.95	43.50 ± 4.88	44.30 ± 5.01	44.90 ± 3.45
Haemoglobin (g dL^{-1})	6.93± 0.70	6.78 ± 0.44	7.13 ± 0.44	6.61 ± 0.32	6.68 ± 0.96	7.14 ± 0.75
RBC (10^6 µL)	1.27 ± 0.04^{ab}	1.36 ± 0.04^{b}	1.29 ± 0.17^{ab}	1.23 ± 00.19^{a}	1.31 ± 0.12^{ab}	1.39 ± 0.14^{b}
Leukocytes counts*	81.00 ± 9.58^{cd}	88.25 ± 7.30^{d}	79.00 ± 9.20^{bcd}	71.88 ± 11.27^{abc}	61.38 ± 13.29^{a}	68.50 ± 8.99^{ab}
MCV (fl)	381.22 ± 29.42^{b}	327.10 ± 28.99^{a}	368.51 ± 72.87^{ab}	389.29 ± 70.00^{b}	366.45 ± 47.48^{ab}	350.18 ± 33.24^{ab}
MCH (pg)	54.59 ± 5.72	49.93 ± 3.03	56.08 ± 8.16	55.05 ± 8.30	51.21 ± 8.07	51.84 ± 7.77
MCHC (g dL^{-1})	14.36 ± 1.47	15.39 ± 1.77	15.44 ± 1.87	14.31 ± 1.77	14.12 ± 2.38	14.82 ± 1.88
ALAT (U mg^{-1} protein)	0.95 ± 0.27	0.90 ± 0.50	1.02 ± 0.76	0.79 ± 0.34	0.84 ± 0.16	1.15 ± 0.68
ASAT (U mg $^{-1}$ protein)	3.84 ± 1.21^{b}	2.17 ± 1.17^{a}	1.76 ± 0.76^{a}	1.68 ± 0.97^{a}	2.17 ± 1.40^{a}	2.35 ± 0.88^{a}

Data are presented as mean ± S.D, Data in the same row with different superscript are significantly different (*P<0.05*), * Number of leukocytes per 1000 blood cells, RBC-Red Blood cells, MCV-Mean Corpuscular Volume, MCH-Mean Corpuscular Haemoglobin. MCHC-Mean Corpuscular Haemoglobin Concentration, YPC$_U$ = Yeast Protein Concentrate Unrefined, YPC$_R$ = Yeast Protein Concentrate Refined, YPC$_{PA}$ = Yeast Protein Concentrate Potable Alcohol, DDGS = Dried Distiller's Grain and Solubles.

4.4 Discussion

This study showed that the growth performance and feed utilization of fingerling common carp improved with the dietary YPCU30 inclusion diet, compared to those fed the FM based diet alone. However, in the case of the fish fed YPCPA30 inclusion, growth and feed utilization were reduced compared to control fed fish. There were no negative effects on growth performance and feed utilization in the case of the fish fed YPCR30, DDGS15 and DDGS30 inclusion diets compared with the control diet group. These results indicate that substitution of YPCU, YPCR and DDGS could enhance growth performance and feed conversion of fingerling common carp, and the result is consistent with previous results observed on Lake trout (Rumsey et al., 1990), sea bass (Oliva-Teles and Gonçalves 2001), Nile tilapia (Lim et al., 2005; Shelby et al., 2008), common carp (Kukačka and Mareš 2008), and sunshine bass (Gause and Trushenski 2011a, b). Some researchers consider that high levels of yeast may invoke negative effects (Davies and Wareham 1988; Rumsey et al., 1991). Attack and Matty (1979) found a reduction of feed intake in rainbow trout fed a 40% brewer's yeast diet compared to a control diet. In contrast, Rumsey et al. (1992) observed that there was no negative effect on feed intake when rainbow trout were fed a diet where 50% of FM was replaced with brewer's yeast. Kukačka and Mareš (2008) showed that the substitution with 15% corn DDGS generated better growth than 30% corn DDGS in the diets of common carp but no adverse effects were found in the fish fed 30% DDGS compared to the control fish fed diet. Nile tilapia has shown no reduction in growth and feed utilization effects when fed up to a 50% substitution of FM with brewers waste (Zerai et al., 2008). However, Nile tilapia fed biofuel DDGS (more similar to that used in this study) produced significantly lower weight gain than those fed FM, although a 17.5% DDGS diet gave similar feed conversion ratio and protein efficiency ratio to FM fed fish (Schaeffer et al., 2010).

Apparent net protein utilization (ANPU) had significantly a better effect on growth when fish fed the YPCU30 diet rate than controlfish fed, YPCPA30, DDGS15 and DDGS30 diets. The ANPU for the YPCR30 diet had significantly lower effect on growth rate compared to all other dietary treatments. The results from this trial supported a previous study undertaken by Oliva-Teles and Gonçalves (2001), who indicated that ANPU of juvenile sea bass fed diets containing yeast was superior to bass fed a fishmeal based diet. On the contrary, in rainbow trout the inclusion of mixed single cell protein (including yeasts) for casein in semi-purified diets, though not affecting growth negatively did affect apparent net protein utilisation ANPU (Murray and Marchant 1986). However, as in the current study, these workers recorded that the ANPU increased in trout with the increasing the levels of yeast extract (Rumsey et al., 1992). Rumsey et al., 1991 stated that some of the non-protein nitrogen (NPN) may be used as a source of non-essential amino acids. This may support the good performance in terms of protein assimilation for tilapia if this species can reflect the excellent profile protein and amino acid profile of the formulated diets for Nile tilapia. Also, the high rate of FCR for fish fed YPCU30 could account for the best growth rate and feed performance.

It was interesting that the moisture was found to increase significantly in fish fed the YPCU30 and YPCPA30 compared with different fish fed groups. The protein content also increased in carp fed YPCU30 compared with other dietary treatments. These results for whole body composition are in general agreement with the findings in tilapia galilee reported by Abdel-Tawwab et al. (2010). In contrast, the lipid content of the carcass was lower in fish fed the experimental diets in comparison to the control diet, except YPCR30. Similar findings were reported by Abdel-Tawwab et al. (2010). An interesting result was the statistically greater ash content of carp fed the YPCU30, YPCPA30 and DDGS30 compared to those fish fed with control, YPCR30 and DDGS15

diets and this complies with the results of tilapia with data recorded by Abdel-Tawwab et al., (2010). These results are also supported by the results in Chapter 3 where carp fed YPCU diets had significantly higher lipid content in whole body fish compared to carp fed control diet.

More detailed research has been conducted to investigate the mineral requirements of cultured fish of a number of species (Watanabi et al., 1997; Papatryphon et al., 1999; Roy and Lall 2006). It is well known that, fish may derive minerals either from diet or the surrounding water. The characteristic concentration and functional forms of minerals and trace elements need to be maintained within narrow ranges for essential metabolic activities in cells and tissues and organs. The biochemical, physiological and functional pathologies which occur as a result of mineral deficiencies depend on several factors, such as the duration and degree of mineral deprivation. In the present investigation, it appears that increased inclusion of different YPC sources and DDGS can elevate the tissue retention levels of specific minerals due to the increased dietary contribution of phosphorus, magnesium, potassium, sodium, chromium, copper, iron, manganese and zinc from this source and reduction in the overall ash content as fish meal is reduced. This is particularly evident for the macro-elements phosphorus, sodium and magnesium and for the trace element chromium (Roy and Lall 2006). An improved retention of phosphorus has environmental consequences and may lead to the reduced need to supplement diets with inorganic phosphorus sources (Nwanna et al., 2010). In general, the outcome of the second trial with carp confirms the results which were observed in Chapter 3 for mineral balance and retention.

As stated previously, biochemical markers relating to metabolism are useful indicators of general health and function in the animals. This is also true for animals to assess their nutritional status.

ALAT and ASAT have an important role in amino acid synthesis in higher vertebrates including fish. Excess deposition of energy as glycogen or lipid can enhance the activities of several key enzymes involved in glycolysis, lipogenesis as well as protein and amino acid synthesis and degradation. Also ALAT and ASAT has been a useful indicator of tissue injury or hepatotoxicity in human and animals and as bio-marker of adaptive reactions (Samsonva et al., 2003.). Gaye-Siessegger et al. (2007) found that three purified diets differing only in their non-essential amino acid composition had observed the higher affect on the ASAT activity but the ALAT activity remained unaffected of Nile tilapia. Sugita et al. (2001) tested the response of enzyme activities linked with metabolic regulation in hepatopancreas and muscle of carp. In this study, ALAT activity was unaffected by the substitution of fishmeal in the diets with various types of YPC and DDGS. In contrast however, ASAT activity was significantly decreased in carp fed diets with YPC and DDG at all levels (YPCU30%, YPCR30%, YPCPA30%, DDGS15%and DDGS30% of inclusion, respectively) compared to carp fed the control diet. In contrast to this finding, Abdel Tawwab et al., (2010) found that a diet containing live baker's yeast did not affect either ALAT or ASAT of Galilee tilapia *Sarotherodon galilaeus* (L.) while, the ALAT and ASAT were elevated with copper treated groups. Also they found that the results of ASAT activity with copper exposure supported our previous findings in Chapter 3. Also Carver and Walker (1995) and Sato et al. (1995) recorded that different dietary yeasts had no significant effects on ALAT and ASAT activities in the human and rat. The findings in the current trial indicate that dietary yeast inclusion may influence the liver metabolism of carp fed dietary yeast compared to fish fed a fishmeal based control diet but not adversely. Deteriorations in ASAT activity could have been associated with a decreased need for pyrimidine biosynthesis which involves aspartate as a substrate. Various types of YPC and DDGE are rich sources of purines and pyrimidine bases within nucleic acids and may have

reduced demand within the hepatocytes of carp fed different types of YPC and DDGE thus sparing the need for *de novo* synthesis.

Haematological analysis often provides valuable information for health assessment and subsequent management of cultured fish (Cnanni et al., 2004; Hoseinifar et al., 2011). In the present study haemotocrit, haemoglobin, MCH and MCHC levels were all not affected by any of the experimental diets. These results are in agreement with the results found in Chapter 3 for haematocrit and haemoglobin. Welker et al. (2007) observed no negative effects on the haematological parameters Haemotocrit (Htc) and Haemoglobin (Hb) when Channel catfish (*Ictalurus punctatus)* fed on 0.2% dietary whole cell brewer's yeast **(***S. cerevisiae*) Compared to control fed fish, total leukocyte counts were significantly reduced in fish fed DDGS (15-30%) dietary inclusion, and in the fish fed YPCR30. However carp receiving YPCPA30 remained unaffected, whilst fish fed YPCU30 had elevated levels. Reque et al. (2010) observed that the total leukocyte count for Nile tilapia was not affected by 2% dietary yeast inclusion. The levels of red blood cells counts (RBCC) in the fish fed YPCU30 and DDGS30 were significantly elevated compared to fish fed YPCPA30 whereas no significant differences were obtained in the fish fed diets of YPCR30 and DDG15 in comparison to those fish fed FM based diet. This result is consistent with the findings of Abdel-Tawwab et al. (2008) who reported that the supplementation of the diets with 0.1–0.5 % commercial baker's yeast (*S. cerevisiae*) significantly increased RBCC in Nile tilapia. The results from the current study may indicate a general improvement of fish health when fed an YPC and/or DDGS dietary inclusion over a period of growth and development in young fish.

Morphological examination of the liver and gastro intestinal tract gastrointestinal tract was also undertaken in this trial. Histological analysis of the liver showed a significant difference in the number of nuclei between carp fed YPCR30 and DDGS30 with those fed fishmeal only**.** The number of nuclei was lower in fish fed the YPCR30 and

DDGS30 diets than fish fed control diet but this difference was not significant. This is possibly due to the size or number of the cells. In this study it is uncertain why nuclei numbers were affected. Evidence indicated that DDGS30 samples had disruptions in hepatic cell layers which may have affected hepatocyte number. However this was not found in the YPCR30 fish although these did have similar overall nuclei counts. The observed reduction in the hepatocyte number in fish fed YPCR may possibly be due to removal of the valuable nutritional components during the washing process (AB-Agri Patent # 109203A1, 2010). This result is supported by a study conducted by Rumsey et al. (1990) that observed lower growth performance and nutritional status in lake trout (*Salvelinus namaycush*) fed washed brewer's yeast compared to fish fed unwashed yeast. Unfortunately there is not enough information available on washed yeasts as novel proteins, particularly in terms of their effects on histology. Further study needs to be done in the future to indicate health implications and morphology and structure of liver of fish fed novel proteins such as yeasts and single cell protein (SCPs). With the known limitations of substitution of FM with DDGS it can be proposed that DDGS at 30% inclusion may result in affects due to higher fibre levels in the DDGS.

The liver is a useful indicator of health and nutrition status (Blanchard et al., 2008; Marchand et al., 2008; Wold et al., 2009; Berntssen et al., 2010), and it is suggested that fish fed YPCR30 and DDGS30 had fewer hepatocytes compared to fish fed other experimental diets but these were not significant different due to some sort of nutritional stress being indicated by differences in hepatic morphology. It is possible that YPCR30 and DDGS30 are less but still suitable diets for carp in comparison to control, YPCU30, YPCPA30 and DDGS15 diets.

It seems apparent the only information on livers in experimental feeding studies of this type is general information on hepato-somatic index (HSI). In a study conducted by Li et al. (2005) the inclusion of brewer's yeast at level of 2% fed red drum (*Sciaenops*

ocellatus) was shown to have no negative affect on the HIS. Furthermore, Thompson et al. (2001) showed that brewer's yeast had no adverse effects on the HSI whether at 10, 20, 30 or 50% inclusion into a FM diet for European seabass. This would indicate that inclusion of brewing yeasts into diets, can not only benefit fish as previously discussed but also poses no apparent threat to energy reserves of fish. However, it is possible that pathological changes may still occur and determining this requires more extensive of liver histology investigations.

The gastrointestinal tract of carp was also studied in this trial to evaluate the effect of diets on morphology and ultrastructural changes. SEM (scanning electron microscopy) analyses in the present study revealed that various types of yeasts and distillers dried grain with solubles (DDGS) inclusion exerted no major change on microvilli density in the anterior intestinal region. Microvilli density in the posterior intestinal region was however affected, fish fed YPC had a higher dense microvilli structure which can be a possible explanation for better growth performance and food utilisation of carp in general receiving yeast protein concentrated (YPC). TEM (transmission electron microscopy) analyses of the intestine revealed that various types of yeasts and DDGS30 can increase the microvilli length, especially in the case of fish fed YPCu30 diets. Similar increases in microvilli density have been observed with 2% mannan oligosaccharide (MOS) dietary supplementation have been reported for cobia larvae (Slaze et al., 2008), and also Dimitroglou et al. (2009) who observed the same results with gilthead sea bream fed mannan oligosaccharide (MOS).

4.5 Conclusions

Using ethanol production co-products and potable alcohol co- products in aquaculture is an area of growing current interest. DDGS has often been seen to be a viable partial replacement for fishmeal at inclusion levels of approximately 20 to 50%. There are a few studies on use of yeast as a protein replacement for FM in diets for fish which can be used up to 50% inclusion with no impact on growth and health of the animal. Results of this trial indicated no significant difference in weight gain of carp fed DDGS up to at least 30% compared to that of fish fed the FM diet. YPCU appeared to be the most successful ingredient within the different yeast protein concentrates tested against FM replacement. However, carp fed YPCPA dietary inclusion had the lower growth rate and feed utilization of all source tested. Through histological analysis of the liver, this study indicated that 30% DDGS and refined yeast protein concentrate (YPCR) fed carp had reduced numbers of nuclei although this was not significantly different from the control, thus they may not be as suitable for long term studies (more than 6 months) due to dietary imbalances of some form being the likely cause of observed results. This is a possible detection of future health implications in fish should they be fed the level of ingredients in question for an extended period of time, for example in a situation of commercial production. Further research is therefore needed to confirm this statement. In addition, liver enzyme analysis suggested that the different yeast protein concentrates and DDGS may offer better performance in carp perhaps due to their additional micro-nutrients functional properties as dietary ingredient. Furthermore, the haematological index for the current study seems to support the use of yeast protein concentrate and DDGS. Using YPC and DDGS will help to reduce the cost of cultured finfish via increased productivity, profitability whilst meeting sustainability criteria.

Chapter 5

Utilisation of refined bio-fuel derived yeast protein concentrate (YPC$_R$) in tilapia diet

5.1 Introduction

In aquaculture production, fishmeal is an expensive ingredient and the main source of dietary protein in the diets (Carter and Hauler 2000; Naylor et al., 2000; Edwards et al., 2004; Gaber 2006; Tacon and Metian 2008a) and some researchers have explored the potential of alternative protein sources in tilapia diets including plant protein (soybean meal (SBM), canola meal ,corn gluten meal, sunflower meal, cotton seed, peanut meal), animal protein by-products (meat meal, meat and bone meal, blood meal, poultry by-product meal, hydrolysed poultry feather meal, offal and other derivatives from poultry, swine and cattle) (El-Sayed and Tacon 1997; Li et al., 2006; Meeker 2006; Gatlin et al., 2007). However, there are some restrictions in using proteins from animal by-products or plant by-product sources including deficiency of one or more EAAs (lysine, isoleucine and methionine), palatability, availability and cost (Hasan 2000; Gatlin et al., 2007; Zerai et al., 2008; FAO 2009). Therefore, a novel protein with high nutritional content and low cost will be advantageous as a new alternative protein in specific aquafeeds for tilapia. In this respect, dried distiller's grains with solubles (DDGS) and single cell proteins (SCPs) are the most viable candidates to be employed as protein sources to these species. In this respect a partial replacement of FM by maize distiller's grains with soluble (MDGS), gluten meal and gluten feed in diets for Nile tilapia fingerlings yielded better growth than fish fed a control diet (Wu et al., 1994, 1995; Tudor et al., 1996).

Single cell proteins include live or dead micro-organisms such as algae, bacteria and yeasts. Nutritionists have already conducted much research with single cell proteins as alternative protein of fishmeal in fish diets. For example; Chow and Woo (1990) investigated that the filamentous alga *Spirulina* sp. has successfully been substituted up to 20% of commercial eel diet without any effect on growth, appetite, and amylase/protease activities of Mozambican tilapia.

Moreover, Viola and Zohar (1984) stated that bacterial SCPs (Pruteen, 70% crude protein) could replace up to 50% of the FM within a 30% crude protein diet with no negative effect in the growth of caged-reared tilapia hybrids *(O. niloticus* X *O. aureus),* whereas fish performance was reduced when Pruteen (SCP) fully replaced FM in the diets. In addition, results indictated that there was no reduction in fish growth, but at higher substitution levels, growth performance and feed efficiency was decreased due to either EAA deficiency or low feed intake (Davies and Wareham 1988) when rainbow trout fry were fed on the diets containing 40% of SCPs (Eurolysine Fodder Protein - EFP, 64% crude protein) in replacement of fishmeal in the diet.

Among SCPs the yeast has received most attention because yeast has a very good nutritive profile (rich in protein and B-complex vitamins and B-glucans), ease for handling, widely available with low cost (Olvera-Novoa et al., 2002; Zerai et al., 2008). Yeast has considerable potential to be used as a partial replacement for fishmeal. Previous research with tilapia indicated that the inclusion of fishmeal with yeast improved growth rate and feed utilisation (Lara-Flores et al., 2003). The use of yeast and yeast derivatives resulted in better growth performance when Nile tilapia were fed at level of 20% dietary yeast (Hisano et al., 2007). Furthermore, Oduro-Boateng and Bart-Plange (1988) documented that Pito brewery waste could be used as a full dietary substitute for FM for *T. busumana* without adversely affecting the growth, and there were significant economic savings. However, brewery draffs have been recommended at up to 30% replacement for FM for Nile tilapia with no significant reduction on performance (Pouomogne 1995). Moreover, in a study done by Barnes (2006a, b) there was a significant improvement in rainbow trout survival and growth with the inclusion of fermented yeast in the diet during the first feeding period and efficiency in nutrient utilisation was equal to fish fed control diets (Oliva-Teles and Gonçalves 2001). Yeast has been used successfully as a partial substitution of fishmeal in commercial aquafeeds

(Rumsey et al., 1990, 1991; Oliva-Teles and Gonçalves 2001; Olvera-Novoa et al., 2002; Lunger et al., 2006; Zerai et al.. 2008; Gause and Trushenski 2011a, b) as well as to improve fish growth and immune response (Ortuño et al., 2002; Li and Gatlin 2003, 2004, 2005; Li et al., 2005; Ghosh et al., 2005).

The current study investigated the new novel protein (yeast protein concentrate refined) for replacing fishmeal in tilapia diets. The yeast protein concentrate refined is a by-product from the bioethanol production process (when cereal is fermented to produce ethanol the resulting residue is converted into dried distiller's grains with solubles (DDGS). Approximately half of the protein within the DDGS is a yeast component. Recently with the help of new technology ethanol yeast has been refined (separated) from DDG and washed out by a special method and air dried. Subsequently, the new product YPCR has a protein content of 56% (AB-Agri Patent # 109203A1, 2010).

The aims of this study was to evaluate the effect of washed ethanol yeast protein concentrate refined (YPCR) levels on growth performance, feed utilization, body composition, mineral analyses, key liver enzyme activities and histological changes in both the liver and gut of Nile tilapia.

5.2 Materials and Methods

5.2.1 Dietary preparation

Four isonitrogenous (38%) and isolipidic (8%) diets were formulated, to replace fish meal protein by three levels of refined yeast protein concentrates YPCR (*S. cerevivisiae*) which were substituted at 10%, 20% and 30% g kg^{-1}. Dietary ingredients were mixed in a Hobart food mixer (Hobart Food Equipment, Australia, model no: HL1400 – 10STDA) with warm water until a soft slightly moist consistency was achieved. This

was then cold press extruded (La Monferrina P6, La Monferrina, Asti, Italy) to produce a 2 mm pellet see section 2.3. Dietary chemical composition is shown in Table 5.1.

5.2.2 Fish rearing and system design

The experiments were conducted at the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth, UK. Red tilapia (*Oreochromis niloticus*) was obtained from Fishgen, Swansea, UK. All fish were Genetically Male Tilapia (GMT®) as described in section 1.6. After 6 weeks acclimation and on-growing, 29 fish (12.39 ±0.08 g) were randomly distributed into 12 x 80 L fibreglass tanks, each provided with 99% re-circulated aerated freshwater at a rate of 300 L h^{-1}. Each treatment was conducted in triplicate. Fish were fed either the experimental diets at 4% biomass per day (equal rations at 09.00, 13.00 and 17.00 H) for 8 weeks. The system was a 2,500 Litre re-circulating system with 12x80 L tanks. The flow rate in the tanks was approximately 100 L h^{-1} see section 2.4. Illumination of the aquariums was achieved with fluorescent tubes set to a 12 h light 12 h dark photoperiod. Daily feed was corrected on a weekly basis following batch weighing after a 24 h starvation period as described in section 2.5.

5.2.3 Water quality

Water quality was recorded daily using a HQ40d Dual-Input pH and DO meter. Water temperature was maintained at an average of 24.44 ± 1.02 ºC throughout the trial with an immersed heater. Dissolved oxygen was maintained at an average of 7.60 ± 1.00 mg L^{-1} by an air stone in each tank. The pH was maintained at an average 6.44 ± 0.45 and adjusted with sodium bicarbonate as necessary. The total ammonia nitrogen was maintained at 0.06 ± 0.06 mg L^{-1}. The nitrite was maintained at 0.03 ± 0.03 mg L^{-1}. The

nitrate was maintained at 35.73 ± 30.30 mg L^{-1}. Water changes were conducted twice a week to maintain safe water quality parameters, as described in details in section 2.6.

Table 5.1 Dietary formulations, proximate composition and energy content of the experimental diets.

Ingredient g kg^{-1}	Control	YPC$_R$ 10	YPC$_R$ 20	YPC$_R$ 30
Herring Meal LT92[1]	419.5	384.2	261.4	182.3
Yeast protein concentrate refined (YPC$_R$)[2]	0	100	200	300
Wheat Carrier Flour[3]	508	440.5	460.5	436.7
Fish Oil[4]	0	0	5	15
Vegetable Oil[5]	27.5	30.3	28.1	21
Viten[6]	20	20	20	20
Vitamin and mineral Premix[7]	20	20	20	20
Molasses[8]	5	5	5	5
Proximate composition (%)				
Moisture	5.68	6.34	4.41	4.46
Protein*	38.74	37.56	38.02	38.04
Lipid*	9.26	9.88	8.33	8.91
Ash*	9.29	8.60	7.79	6.88
NFE*[9]	37.03	37.62	41.45	41.71
Gross Energy (MJ kg^{-1})	19.17	19.37	19.59	19.74

*Dry matter basis

[1] Scottish fish meal 70, United Fish Products Ltd, UK.
Sigma-Aldrich Ltd, UK.

[2] AB Vista (AB-Agri Ltd)

[3] Ewos-Bathgate Scotland

[4] Epanoil, Sevenseas Ltd, UK.

[5] Corn oil.

[6] Roquette Frêres, France.

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

[8] Holland and Barret

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

ND – not determined

Table 5.2 Amino acid profile for experimental diets.

Amino Acid	Control	YPCR10	YPCR20	YPCR30	Tilapia requirement[1]
Essential AA (%)					
Arginine	2.27	2.26	2.99	1.88	1.20
Histidine	1.06	1.02	0.94	1.07	1.00
Iso-Leucine	1.66	1.73	1.55	1.66	1.80
Leucine	2.8	2.79	2.73	2.72	1.90
Phenylanine	1.67	1.71	1.66	1.73	1.10
Lysine	2.56	2.23	2.00	1.77	1.30-1.40
Threonine	1.65	1.61	1.45	1.45	1.10
Valine	2.12	2.23	1.89	2.00	1.60
Methionine	1.3	1.17	1.02	0.93	0.80-1.10
Tryptophan*	ND	ND	ND	ND	0.28
Non-Essential AA (%)					
Alanine	2.01	2.08	1.84	1.92	ND
Aspartic acid	3.41	3.15	2.85	2.62	ND
Cysteine	0.69	0.73	0.61	0.72	ND
Glutamine	5.82	6.76	7.26	8.24	ND
Glycine	2.04	2.18	1.8	2.06	ND
Proline	1.58	1.29	2.36	2.2	ND
Serine	1.84	1.92	1.71	2.03	ND
Tyrosine	1.08	1.06	1.03	1.11	ND

*Not determined.

[1]values obtained from references as cited by the Committee on Nutrient Requirements of Fish and Shrimp (NRC) (2011)

Table 5.3 Mineral analysis for experimental diets. (*n*=3)

Mineral	Control	YPCR 10	YPCR 20	YPCR C30
g kg^{-1}				
Ca	9.96 ± 0.36	10.06 ± 0.75	9.02 ± 0.77	7.63 ± 0.99
K	7.91 ± 0.24	7.83 ± 0.21	7.03 ± 0.09	6.76 ± 0.04
Mg	2.40 ± 0.07	2.45 ± 0.10	2.21 ± 0.02	2.19 ± 0.10
Na	6.10 ± 0.18	5.67 ± 0.18	4.44 ± 0.05	3.64 ± 0.07
P	10.81 ± 0.39	10.90 ± 0.59	9.67 ± 0.09	9.26 ± 0.38
mg kg^{-1}				
Cr	1.25 ± 1.14	1.22 ± 0.86	1.05 ± 0.48	1.20 ± 0.74
Cu	16.38 ± 0.80	15.92 ± 0.87	17.39 ± 2.44	17.93 ± 1.60
Fe	192.68 ± 8.00	227.83 ± 26.29	214.14 ± 35.51	213.39 ±15.30
Mn	55.64 ± 1.35	69.76 ± 3.02	62.13 ± 1.32	64.51 ± 2.05
Zn	93.34 ± 3.21	104.86 ± 3.28	86.66 ± 0.45	98.32 ± 15.18

Data are presented as mean ± S.D.

Data in the same row with different superscript are significantly different ($P<0.05$).

5.2.4 Growth parameters and feed utilisation

Specific growth rate (SGR), Final weight (FW), weight gain (WG), survival rate, feed conversion ratio (FCR), feed conversion efficiency (FCE), protein efficiency ratio (PER), apparent net protein utilisation (ANPU) and condition factor (K) were assessed as described in section 2.7.

Also Hepatosomatic index was calculated by the following equation;

$$\text{Hepatosomatic Index HSI (\%)} = \frac{\text{Liver weight}}{\text{Fish weight}} \times 100$$

5.2.5 Chemical composition analysis of the diets and fish carcasses

Diets and fish samples (initial and final) from the feeding trial were analysed according to AOAC (2002) standard methods for proximate composition. Fish sampled for whole body analysis (18 fish sampled at the start and 4 fish per tank at the end of trial) latter each two fish pooled separately (2 sample per tank were analysed to minimize the standard deviation between the samples), grounded and homogenized in a blender prior to chemical assays for nutritional values available in each replication.

Moisture content (dry matter) was determined using drying oven (105 ºC for 24 h). Crude protein was calculated from sample nitrogen content was determined using Kjeldahl apparatus (Gerhardt Kjeldatherm method, N% x 6.25) and crude lipid using ether extraction in multi-unit extraction Soxhlet apparatus (dicloromethane extraction by Soxhlet method). Ash Content was analysed using a muffle furnace (incineration at 550 ºC for 12 h). Gross energy analysed using (Parr bomb calorimeter).

All protocols concerning analytical chemistry are described in section 2.8. Amino acids analysed (except tryptophan) carried out by Sciantec Analytical Services Ltd. Laboratories (Yorkshire; UK) Table 5.2.). Mineral compositions were conducted using spectrophotometer (ICP-MS) on a dry basis Table 5.3 as described in section 2.8.7.

5.2.6 Histology

5.2.6.1 Light microscopy

A histological appraisal under the light microscope six fish per experimental group at the termination of the trial was used. Liver dissected for histological examination as described in section 2.9.1.

5.2.6.2 Electron microscopy

Posterior intestinal samples from five fish per experimental group were obtained for microvilli density by Scanning electron microscopy SEM as described in section 2.9.2.1. Transmission electron microscopy TEM of microvilli length and five fish were retained per experimental group as described in section 2.9.2.2.

5.2.7 Enzymatic assays

At the end of experimental six per treatment were euthanized and liver were freezed as described in section 2.11. Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were assayed using the micro plate reader (Molecular Devices) as described in section 2.11.

The total protein content protein of the supernatant was determined as described in section 2.11.

5.2.8 Intestinal microbiological analysis

At the end of the trial three fish per tank were sampled to isolate intestinal material from fish fed the experimental diets. After dissecting aseptically, samples were pooled by tank resulting in three replicates per treatment (200 mg) each constituting material from three fish. DNA extraction, PCR amplification of the 16S rRNA V3 region, DGGE and subsequent phylotype isolation and sequencing were conducted as described in section 2.12.

5.2.9 Statistical analysis

Statistical analysis was performed using One-Way ANOVA (SPSS 17.0). *Post hoc* LSD test was used to determine significant differences between means Percentage data was arcsine transformed prior to subsequent analysis and significant was accepted at $P< 0.05$ level.

5.3 Results

5.3.1 Growth performance

After eight weeks of culture, growth performance (final weight, weight gain and specific growth rate (SGR)) and feed utilisation (FCE, FCR) of tilapia were not impaired by dietary inclusion of the refined yeast protein concentrate (YPC_R) However, 30% of YPC_R dietary inclusion resulted in significantly lower weight gain (*P=0.025*) and SGR (*P=0.011*) compared with the control group (*P<0.05*). Apparent net protein utilization (ANPU) was not significantly different in any treatment (Table 5.4). A significant decrease of hepatosomatic index (HSI) (Figure 5.1) was found in the fish fed YPC_R 30% (1.97 ± 0.17) compared to the control fish fed (2.48 ± 0.17) (Figure 5.1).

The condition factor (K) was not statistically different between the groups. Compared to the control group, survival rate for all levels of YPCR inclusion were higher but not significantly (*P<0.05*).

Table 5.4 growth performance, food utilization and survivals of tilapia fed the test diets for 8 weeks. (*n* = 3)

Variable	Control	YPCR10	YPCR20	YPCR30
Initial Weight (g)	12.42 ± 0.12	12.45 ± 0.04	12.34 ± 0.06	12.36 ± 0.08
Final Weight (g)	59.02 ± 3.58	58.65 ± 1.28	56.72 ± 4.18	55.34 ± 2.12
Weight Gain (g)	41.73 ± 0.39^{a}	42.15 ± 1.99^{a}	40.28 ± 0.39^{ab}	38.23 ± 2.32^{b}
SGR (% day^{-1})	2.70 ± 0.03^{a}	2.70 ± 0.02^{a}	2.65 ± 0.06^{ab}	2.58 ± 0.05^{b}
FCE	93.02 ± 1.47	92.89 ± 2.49	91.41 ± 0.79	92.03 ± 0.25
FCR	1.075 ± 0.017	1.077 ± 0.029	1.094 ± 0.009	1.087 ± 0.003
PER	2.40 ± 0.04	2.47 ± 0.07	2.40 ± 0.02	2.42 ± 0.01
ANPU (%)	30.24 ± 1.79	29.65 ± 0.39	29.32 ± 2.45	28.07 ± 1.52
Condition factor (K)	1.75 ± 0.07	1.69 ± 0.15	1.77 ± 0.05	1.71 ± 0.08
Survival (%)	91.95 ± 5.27	93.10 ± 3.45	93.10 ± 6.90	93.10 ± 5.90

Data are presented as mean ± S.D.
Data in the same row with different superscript are significantly different (*P<0.05*).

5.3.2 Whole body composition

The whole-body chemical composition of fish is presented in Table 5.5. No significant differences were found in whole-body moisture, protein, lipid, ash or gross energy content (*P<0.05*).

Whole-body mineral levels are shown in Table 5.6. A clear trend of elevated carcass iron levels was observed with increasing YPCR inclusion leading to significant differences at YPCR inclusion levels of ≥ 20% *(P<0.001).* Additionally, chromium levels were higher in all YPCR groups but not significantly compared to the control group (*P=0.613*). Furthermore, zinc levels were higher in fish fed control diet in comparison with fish fed dietary YPCR inclusion, while no significant differences were reported within dietary treatments (*P=0.156*). Calcium, potassium, magnesium, sodium,

phosphorus, copper and manganese were not affected by dietary inclusion of YPCR ($P<0.05$).

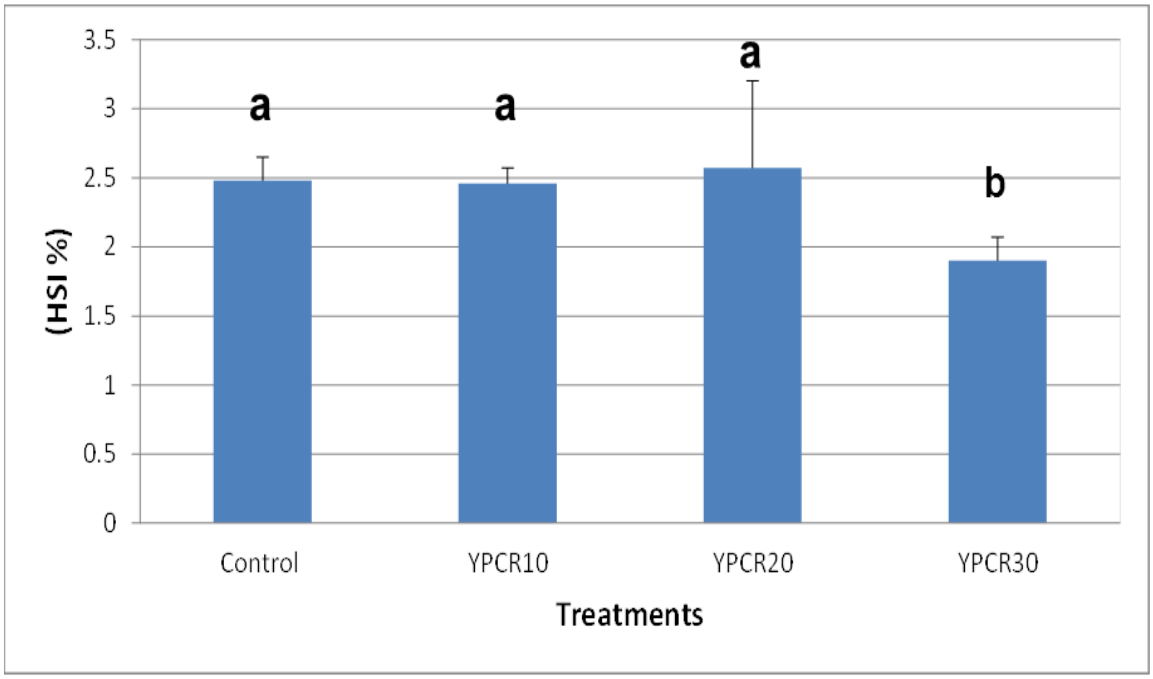


Figure 5.1 Hepato-somatic index (HSI) of tilapia fed on experimental diets for duration of 8 weeks trials. Different superscripts are significantly different (*P<0.05*).

Table 5.5 whole-body proximate composition (%) of the initial fish and fish fed the experiment diets. (*n*=6)

Whole Body composition	Initial*	Control	YPCR10	YPCR20	YPCR30
Moisture (%)	71.97 ± 0.31	71.43 ± 0.47	71.32 ± 0.19	72.12 ± 0.84	73.60 ± 4.46
Protein (%)	52.24 ± 1.27	53.43 ± 0.62	52.76± 0.89	54.01 ± 0.37	53.20 ± 0.91
Lipid (%)	27.53 ± 0.86	25.72 ± 0.69	26.51 ± 2.33	25.51 ± 2.00	24.45 ± 3.22
Ash (%)	15.28 ± 0.95	14.32 ± 0.21	14.56 ± 0.25	14.27 ± 0.52	14.34 ± 0.18
NFE (%)	4.96 ± 1.65	6.53 ± 0.19	6.17 ± 2.23	6.21 ± 2.04	8.01 ± 3.10
Gross Energy (MJ kg^{-1})	23.34 ± 0.41	23.65 ± 0.69	23.91 ± 0.22	24.09 ± 0.10	24.24 ± 0.17

*n=3. Nitrogen-free extracts (NFE) = dry matter – (crude protein + crude lipid + ash). Data are presented as mean ± S.D. Data in the same row with different superscript are significantly different ($P<0.05$).

Table 5.6 Whole-body mineral profile for tilapia fed on experimental diets for 8 weeks. (*n*=6)

Mineral	Initial*	Control	YPCR10	YPCR20	YPCR30
g kg^{-1}					
Ca	32.18 ± 5.92	33.05 ± 1.50	34.35 ± 0.95	33.79 ± 0.38	33.15 ± 0.91
K	7.15 ± 1.71	8.63 ± 0.04	8.40 ± 0.11	7.98 ± 0.82	8.40 ± 0.18
Mg	0.97 ± 4.12	1.08 ± 0.03	1.08 ± 0.01	1.04 ± 0.01	1.03 ± 0.02
Na	2.72 ± 0.63	3.23 ± 0.27	3.19 ± 0.08	3.04 ± 0.33	3.31 ± 0.03
P	20.18 ± 3.73	20.39 ± 1.17	21.28 ± 0.45	21.45±0.41	21.16 ± 0.48
mg kg^{-1}					
Cr	1.22 ± 0.79	0.60 ± 0.16	0.61 ± 0.10	0.67 ± 0.12	0.75 ± 0.18
Cu	13.70 ± 4.12	14.17 ± 1.00	12.68 ± 0.98	13.39 ± 0.98	13.76 ± 0.56
Fe	71.04 ± 26.66	56.16 ± 4.03[a]	57.01 ± 3.66 [a]	66.98 ± 6.15 [b]	94.18 ± 2.76[c]
Mn	4.19 ± 1.25	9.68 ± 1.28	8.40 ± 2.30	7.37 ± 2.41	7.70 ± 1.58
Zn	57.12 ± 13.63	73.57 ± 19.13	56.22 ± 1.00	56.47 ± 1.98	57.47 ± 11.24

*n=3. Data are presented as mean ± S.D. Data in the same row with different superscript are significantly different ($P<0.05$).

5.3.3 Light microscopy

Histological examinations of the intestine found that all groups showed no signs of tissue damage or necrosis (Figure 5.2). No statistical differences in the villi length was observed in the posterior intestine (Table 5.7). However, the number of goblet cells in the posterior intestine increased with the increasing dietary levels of YPC_R inclusion; the highest improvement was observed in the fish fed YPC_R30 diet (12.35±1.99 µm), followed by fish fed YPC_R 20 diet (10.00±0.89 µm) which were both significantly ($P<0.05$) higher than fish fed the control (6.72±1.01 µm) or YPC_R10 (7.90±1.04 µm) diets.

However, an increase of lipid accumulation in the livers was noticed with the increasing dietary levels of YPC_R and in turn general decrease in hepatocyte density was observed with more clustering of nuclei and more diffuse cytoplasmic organisation (Figure 5.3).

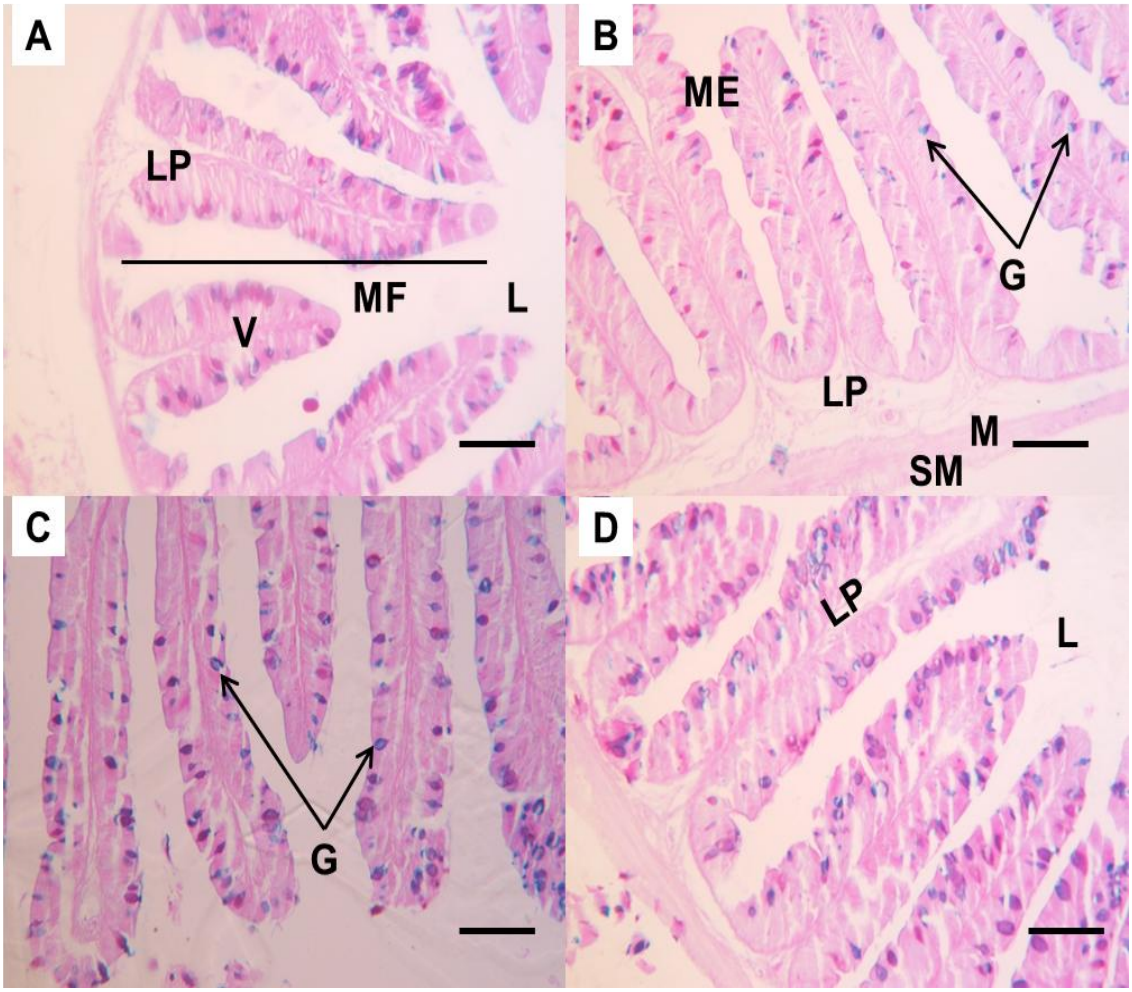


Figure 5.2 Light micrograph (alcian blue and PAS staining) of posterior intestine from fish fed, (A) control diet, (B) YPC$_R$10diet, (C) YPC$_R$20 diet and (D) YPC$_R$30 diet showing a significant improvement in the number of goblet cells with increasing YPC$_R$ inclusion level in the dietary treatment. (Scale bar = 100 µm). L: Lumina, LP: Lamina properia, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells, V: Villi.

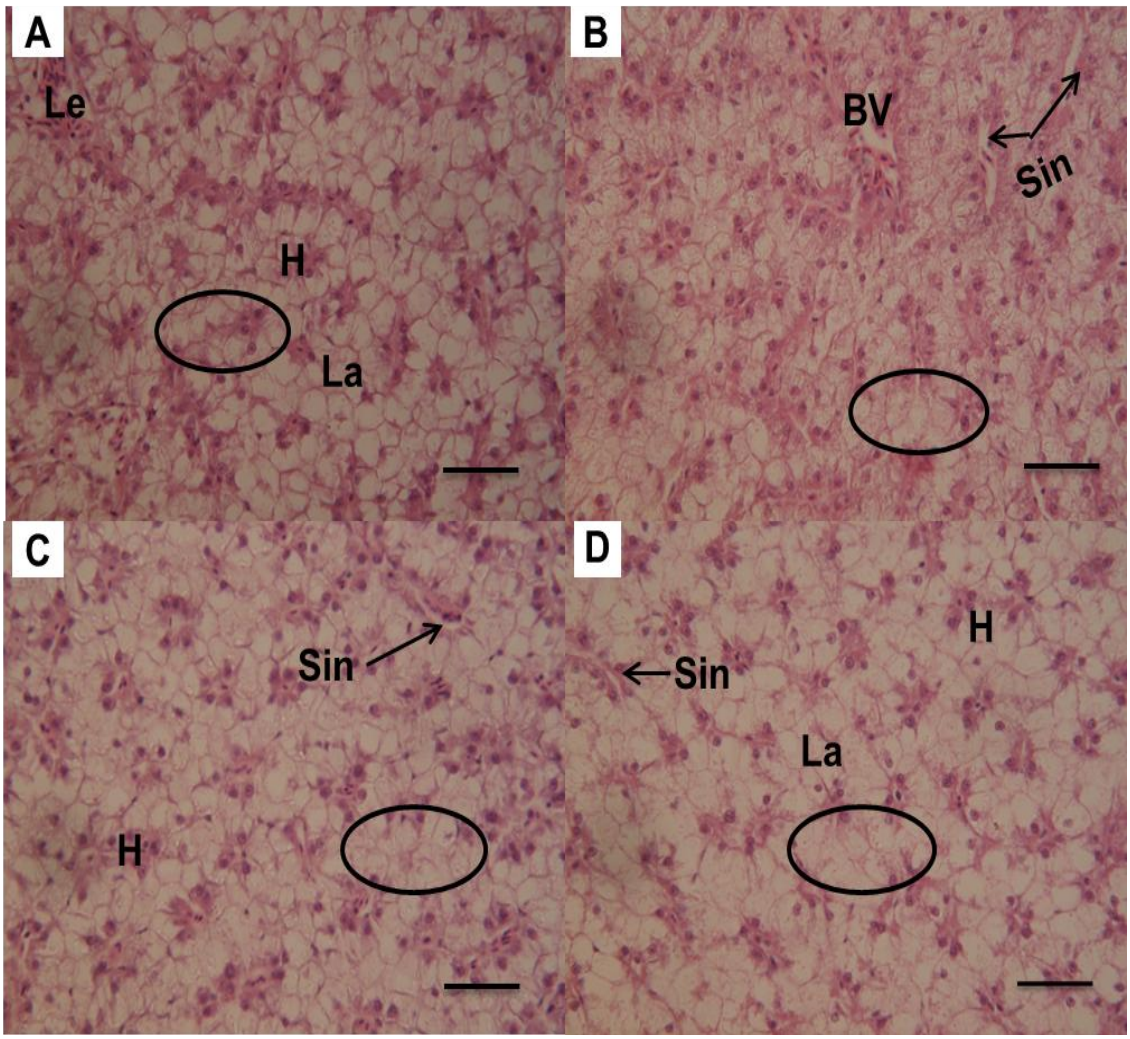


Figure 5.3 Light microscopy of representative tilapia liver sections obtained on termination of the study showing A) control fed group and B, C and D (10, 20 and 30% inclusion of YPC$_R$ respectively). Higher YPC$_R$ inclusion (≥ 20%) appeared to decrease general hepatocyte density with increasing lipid accumulation causing more clustering of nuclei and more diffuse cytoplasmic organisation. (Scale bar = 50 µm). BV: Blood vessel, H: Hepatocytes, Le: Leukocytes, Sin: Sinusoid, La: Lipid accumulation, Circle (areas indicates) clustered hepatocytes that contain large amount of lipid.

Table 5.7 Intestinal morphology of tilapia fed on experimental diets. (*n*=6)

Parameter	Control	YPCR10	YPCR20	YPCR30
Microvilli density (μm)	1.23 ± 0.11^{a}	1.59 ± 0.23^{b}	1.31 ± 0.03^{ab}	1.33 ± 0.19^{ab}
Microvilli length (μm)	1.28 ± 0.03^{a}	1.61 ± 0.20^{ab}	1.65 ± 0.11^{ab}	1.87 ± 0.35^{b}
Mucosal fold length (μm)	387.56 ± 22.26	412.91 ± 45.86	422.01 ± 70.12	408. 10± 93.90
Goblet cells (per 100 μm)	6.72 ± 1.01^{a}	7.90 ± 1.04^{a}	10.00 ± 0.89^{b}	12.35 ± 1.99^{c}

Data are presented as mean ± S.D.

Data in the same row with different superscript are significantly different ($P<0.05$).

5.3.4 Electron microscopy

5.3.4.1 Scanning electron microscopy SEM

The results of the histological examinations of the microvilli density and microvilli length are presented in Table 5.7. The density of microvilli in the posterior region of fish fed YPC$_R$ 10% (1.59±0.23 AU), YPC$_R$ 20% (1.31±0.03AU), YPC$_R$ 30% (1.33±0.19 AU) were enhanced compared with the control fed fish (1.23±0.11AU), while significant ($P<0.05$) differences were only observed in the fish fed YPC$_R$ 10% ($P=0.025$) (Figure 5.4).

5.3.4.2 Transmission electron microscopy TEM

The transmission Electron microscopy TEM observed that dietary inclusion levels of YPC$_R$ increased the microvilli length in fish for posterior region. Microvilli length for the posterior region of control fed fish was shortest (1.28±0.03 µm) and longest at higher inclusion levels of YPC$_R$30% fish fed (1.87±0.35 µm). However, there were significant differences in microvilli length in the fish fed YPC$_R$ 30% diet (P=0.009), compared to the microvilli length in fish fed control diet ($P<0.05$) (Figure 5.5).

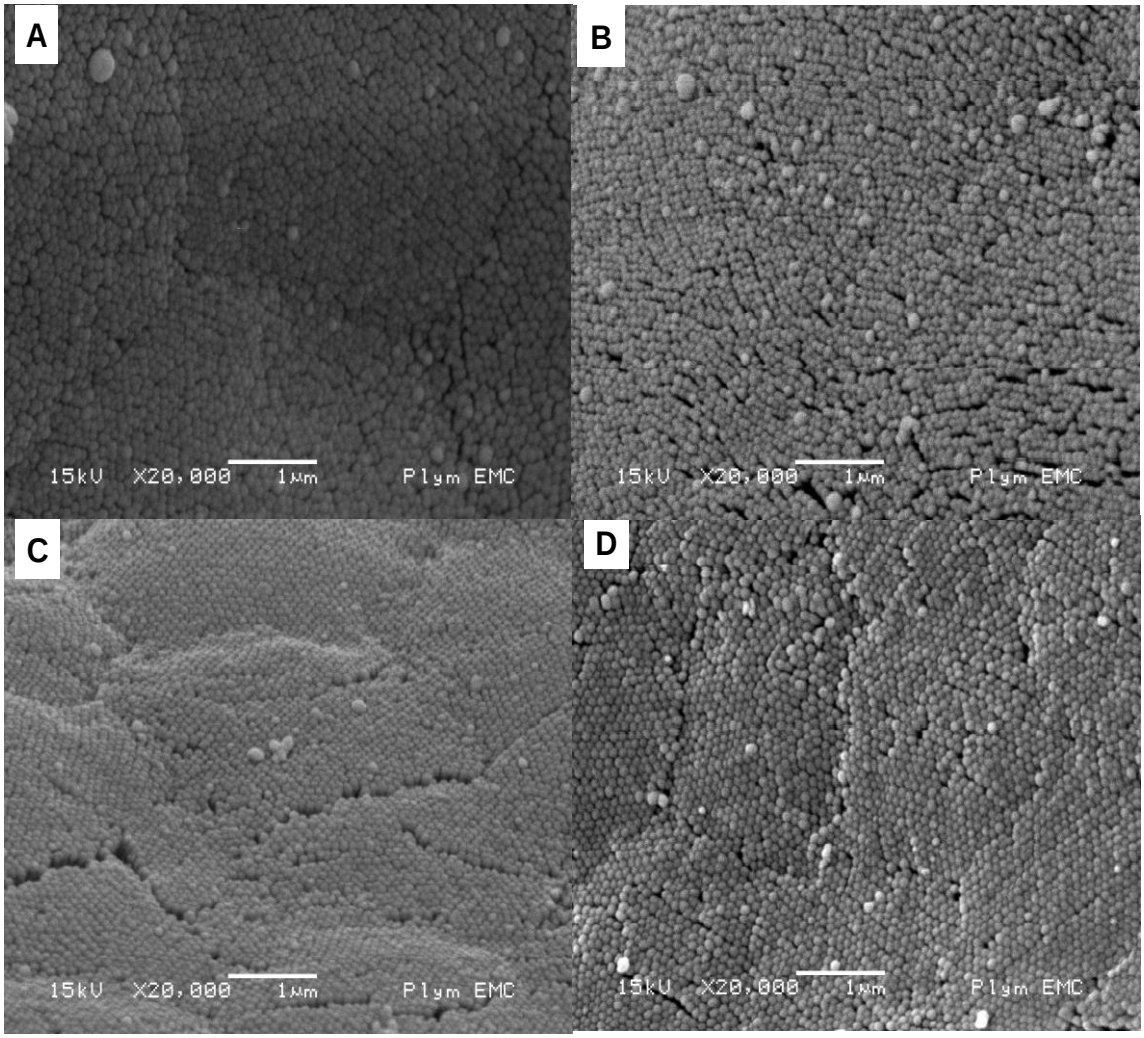


Figure 5.4 SEM micrographs of posterior intestine of tilapia fed the (A) control (B) YPCR10 (C) YPCR20 and (D) YPCR30, the YPCR inclusion diets. Microvilli are equally regular and similar density among all treatments. (Scale bar = 1µm)

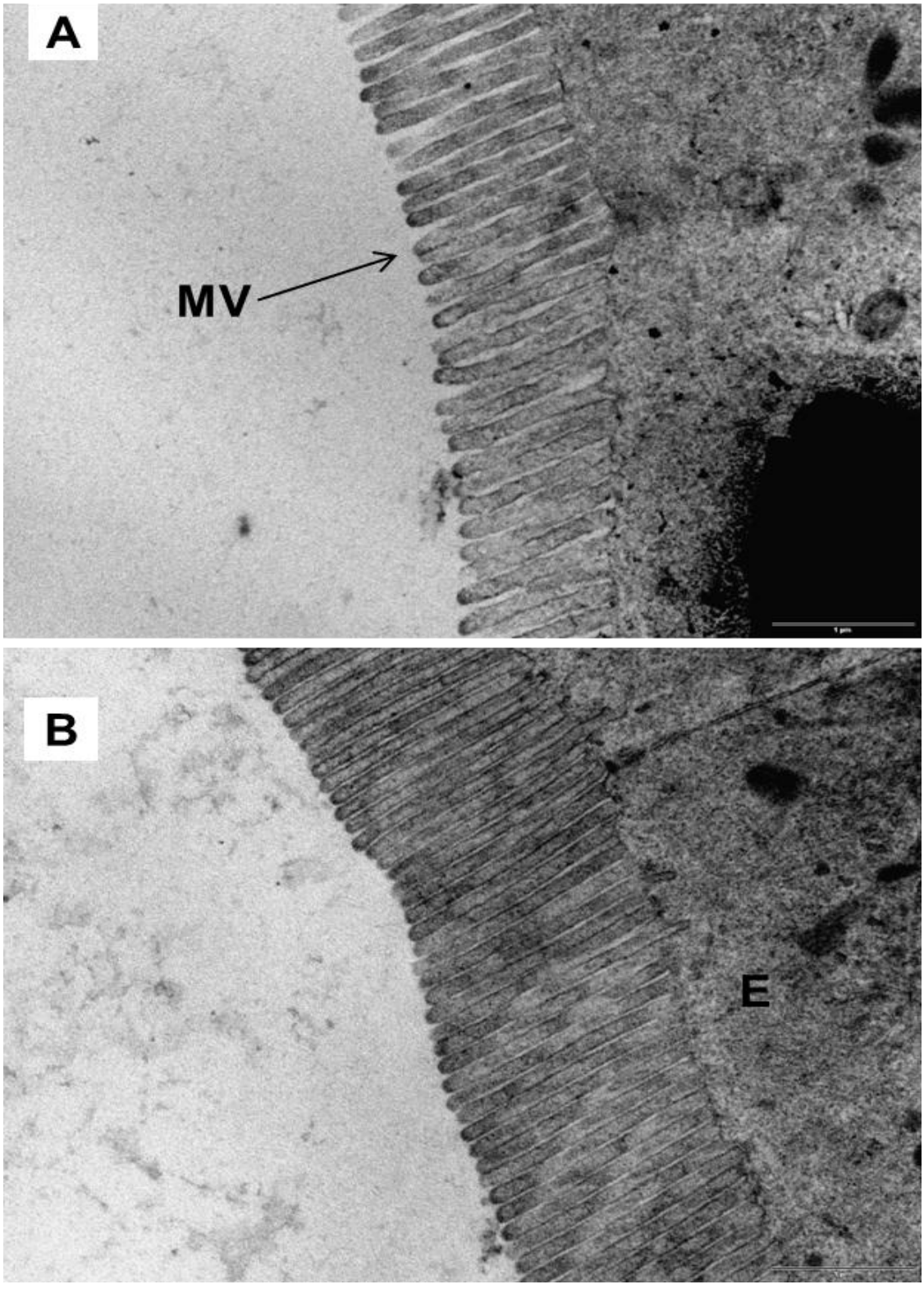


Figure 5.5 Comparative TEM micrographs of the posterior intestine of tilapia fed either (A) control, (B) YPC$_R$ substitute diets, Microvilli are longer and denser in YPC$_R$ fed fish. (Scale bar = 1µm). MV: Microvilli, E: Enterocyte.

5.3.5 Enzyme activity

Hepatopancreas alanine amino transferase (ALAT) activity in liver of the juvenile tilapia fed the control diet was high but no significant differences were observed in all treatment groups ($P<0.05$). Similarly, aspartate amino transferase (ASAT) activity in fish fed the control diet was high and no significant differences were found among the dietary groups ($P<0.05$) (Figure 5.6).

5.3.6 Intestinal microbiological analysis

PCR-DGGE fingerprints of the microbial profiles reveal clear differences in similarity, as depicted in Tables 5.8 and 5.9. The average numbers of phylotypes between treatments were: control = 7.33 ± 0.58, YPCR10 = 11.67 ± 0.58, YPCR = 22.00 ± 0.00 and YPCR30 = 13.33 ± 0.58. The number of phylotypes were significantly higher in YPCR20 than all other treatments and the control values were significantly lower than all other treatments ($P<0.01$). Tables 5.8 and 5.9 further illustrates the dissimilarity between treatments, whereby each set of replicates within treatments showed high similarity to one another (>0.91) but all YPC treatments were highly dissimilar to the control (similarity for control vs YPCR10 = 0.39 ± 0.04, control vs YPCR20 = 0.33 ± 0.02 and control vs YPCR30 = 0.35 ± 0.04).

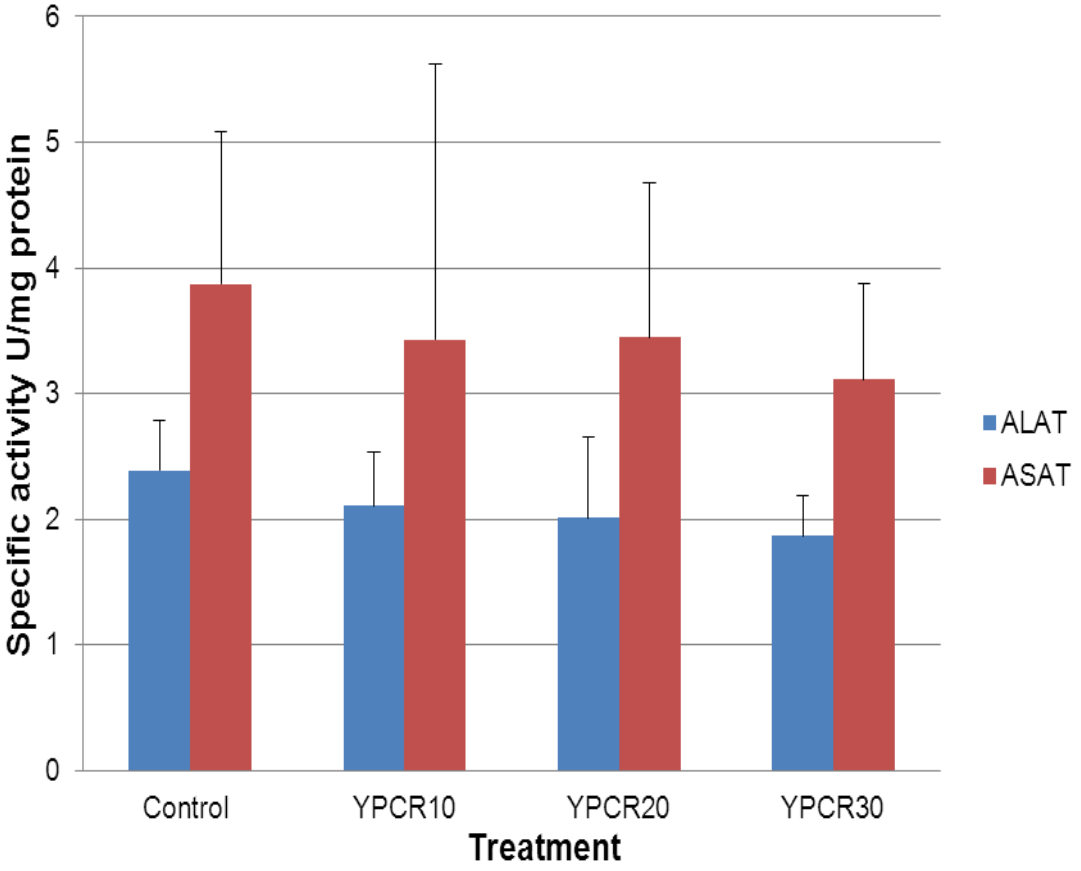


Figure 5.6 Specific activities of ALAT and ASAT enzymes in the liver of tilapia fed on experimental diets for 8 weeks.

Table 5.8 Similarity half matrix between presence/absence of DDGE bands of tilapia intestinal microbial populations fed the experimental diets (Jaccard calculation).

		Control			YPCR10			YPCR20			YPCR30		
		1	2	3	4	5	6	7	8	9	10	11	12
	1	1.00											
	2	1.00	1.00										
Control	3	0.88	0.88	1.00									
	4	0.36	0.36	0.43	1.00								
	5	0.36	0.36	0.43	1.00	1.00							
YPCR10	6	0.38	0.38	0.46	0.92	0.92	1.00						
	7	0.32	0.32	0.36	0.55	0.55	0.50	1.00					
	8	0.32	0.32	0.36	0.55	0.55	0.50	1.00	1.00				
YPCR20	9	0.32	0.32	0.36	0.55	0.55	0.50	1.00	1.00	1.00			
	10	0.33	0.33	0.40	0.67	0.67	0.71	0.59	0.59	0.59	1.00		
	11	0.31	0.31	0.38	0.63	0.63	0.67	0.57	0.57	0.57	0.93	1.00	
YPCR30	12	0.33	0.33	0.40	0.76	0.76	0.71	0.52	0.52	0.52	0.86	0.93	1.00

Table 5.9 Similarity coefficients, by treatment (mean ± SD), of the bacterial DGGE profiles from intestinal communities of tilapia after feeding on the experimental diets.

	Mean
Control vs Control	0.92 ± 0.07
Control vs YPCR10	0.39 ± 0.04
Control vs YPCR20	0.33 ± 0.02
Control vs YPCR30	0.35 ± 0.04
YPC_R10 vs YPCR10	0.95 ± 0.05
YPC_R10 vs YPCR20	0.53 ± 0.03
YPC_R10 vs YPCR30	0.69 ± 0.05
YPC_R20 vs YPCR20	1.0 ± 0.00
YPC_R20 vs YPCR30	0.56 ± 0.19
YPC_R30 vs YPCR30	0.91 ± 0.04

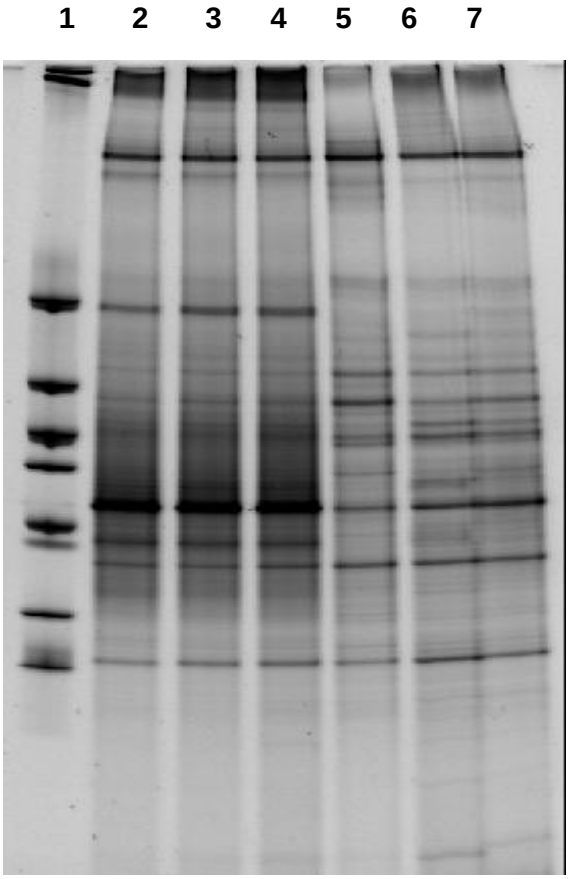


Figure 5.7 Example of DGGE profiles obtained for the bacterial communities associated with the GI tract of tilapia fed control (lanes 2 to 4) and $YPC_R30\%$ (lanes 5 to 7) lane 1 is 100 bp ladder loaded as a standard to assess staining.

5.4 Discussion

The use of ethanol yeast has been evaluated by several researchers as a novel ingredient to FM substitution in the diet for some species notably carp (Chapter 3 and 4) and sunshine bass *Morone chrysops* x *M. Saxatalis* (Gause and Trushenski 2011a, b). In this study, weight gain and SGR in Nile tilapia fed replacement diets which contained 10 and 20% YPC$_R$ were equal to those fed the control FM based diet. However, tilapia fed a diet with 30% inclusion of YPC$_R$ resulted in a significant decrease in weight gain and SGR compared with those fish fed the control diet. The findings from this study are not consistent with the results for Chapters 3 and 4 with carp which gave better growth and feed utilization results for all YPC. The low SGR and weight gain are possibly due to the palatability issue or nutrient utilisation problems in the diet such as a content of lysine and methionine levels in the experimental diets, but NRC requirements for tilapia were met (2011). Both methionine and lysine levels decreased with increasing amount of YPC$_R$ in the test diet. The essential amino acid (EAA) requirement of tilapia has been reported by different workers and these appear to be similar to other species when expressed as percent of protein in the diet or as a percent of whole protein fish carcass protein (NRC 2011). These are represented in Chapter 1 and 2 based mainly on experimental diets for tilapia using semi-purified ingredients. In this study, gross dietary methionine and lysine was reduced from 1.3 to 0.93 and 2.56 to 1.77 respectively although the actual digestibility of these amino acids was not determined. Therefore, at 30% inclusion of YPC$_R$ lowered DCP (Digestible Protein) and hence reduced EAA availability would have possibly contributed to an unbalanced EAA profile for juvenile tilapia thus impairing growth and feed efficiency.

In the investigation similar final body weights, SGR, FCR, PER and carcass composition was observed for tilapia fed all treatments except the 30% YPC$_R$ inclusion

producing a significant reduction in these parameters. Perhaps indicating greater sensitivity for tilapia fed higher levels of YPCR.

However other researchers have reported comparable growth performance with tilapia fed diets containing a range of plant based protein and energy rich ingredients. El-Saidy and Gaber (2003) found that SGR of Nile tilapia were comparable when FM was replaced by 25, 50, 75 and 100% a mixture of plant protein. Indeed the SGR of Nile tilapia was not affected when up to 50% dietary FM was replaced with cottonseed meal (Mbahinzireki et al., 2001) which obviously did not cause any apparent nutrient deficiency or imbalance.

The apparent net protein utilisation (ANPU) in this study was affected only in tilapia fed 30% inclusion of YPCR diet. In contrast to this, different results were found by Oliva-Teles and Gonçalves (2001) and Ozório et al. (2010) who reported a positive effect on growth and apparent retention when supplementing sea bass feed with 30% and pacu (*Piaractus mesopotamicus*, Holmberg 1887) feed with up to 50% brewer's yeast respectively. This reduction in the ANPU may be because of the deficiency and imbalance of the amino acid profile Table (5.2). Probably another possible reason is the tilapia could not utilise the non-protein nitrogen properly as a consequence of reduced protein efficiency for utilisation (ANPU). The result from current study is not confirming the ANPU results from carp study in Chapter 4.

Whole body carcass composition was slightly altered by the level of YPCR in the diet. However, these differences were not statistically significant. In addition, there was a trend towards a decrease in the lipid content with increasing the level of YPCR in the diet but not significant. Similar results were reported by Gause and Trushenski (2011a) and this complies with the results presented in Chapter 3 in terms of moisture, protein and lipid. Fish fed on 30% YPCR inclusion had a lower content of crude lipid and fish fed on control diet had higher content of lipid. This suggests that may there were

protein/energy imbalances in the diets as presented to tilapia when fed higher amounts of YPCR.

In the 30% YPCR treatment group there was a significantly reduced hepato-somatic index HSI (Figure 5.1) compared to other treatment groups, this perhaps being due to excess energy in the dietary treatment or imbalance in the lipid or protein in the dietary inclusion of fishmeal as another explanation for the lower HSI. As in this study, Gause and Trushenski (2011b) found that diets containing different level of ethanol yeast were observed a significant effect on HSI of sunshine bass. Contrary to this result, Gause and Trushenski (2011a) used ethanol yeast as a protein source in the diets and no impact on HSI were recorded during the course of their investigation. Furthermore, Li et al. (2005) found that when red drum (*Sciaenops ocellatus*) were fed on 2% of brewer's yeast diet as an immunological supplement, there was no observed affect on HIS. Moreover, when evaluated as a protein source, brewer's yeast did not show any significant differences in HIS whether at 10, 20, 30 or 50% inclusion into a FM diet for European sea bass (Thompson et al., 2001).

All animals, including fish require minerals for their normal life proceses. Fish have a limited capability to absorb minerals from both diets and water (Lim et al., 2001). In this study there was a trend towards an increase in iron levels in the whole fish carcass with higher inclusion levels of YPCR in the dietary treatments, this is possibly due to higher iron availability in the YPCR compared to fishmeal. Furthermore, there were no negative effects on other mineral retention in whole body content, although there were some variations of chromium and zinc levels in the fish. On the contrary, overall mineral composition of the carcass was more pronounced for carp (Chapters 3 and 4) It should be noted that Li et al. (1996) Eya and Lovell (1997) observed that in Channel catfish, a reduction of bone mineralization, decreased bone strength and reduced weight

gain occurred due to phosphorus deficiency. This was not an issue in the present study with tilapia as phosphorus levels were uniform in diets and resulting fish carcass.

It is well known that enzyme activity levels associated with intermediately metabolism are affected by nutritional status and diet composition. Sugita et al. (2001) tested the response of enzyme activities linked with metabolic regulation in hepatopancreas and muscle of carp. In this study, ALAT activity was fluctuated with substitution of fishmeal in the diets, but was not significantly different among the dietary treatment.

ALAT and ASAT activities have an important role in amino acid synthesis in higher vertebrates including fish. Excess deposition of energy as glycogen or lipid can modulate the activities of several key enzymes involved in glycolysis, lipogenesis as well as protein and amino acid synthesis and degradation. Also ALAT and ASAT has been a useful indicator of tissue injury or hepatotoxicity in human and animals and as bio-marker of adaptive reactions (Samsonva et al., 2003; Lindblom et al., 2007). Gaye-Siesseger et al. (2007) found that diets containing varying protein to carbohydrate ratio affected the specific hepatic enzymes of amino acid metabolism in tilapia including ALAT and ASAT.

In the current study, ALAT activity in tilapia was decreased slightly with increasing YPC$_R$ inclusion in the diets but was not statistically significant among the dietary treatments. However, ASAT activity was marginally increased with increasing YPC$_R$ inclusion respectively compared to carp fed a similar control diet. In contrast to this result, Abdel Tawwab et al. (2010) concluded that a diet containing live baker's yeast had no negative effect on the ALAT and ASAT activities of Galilee tilapia *Sarotherodon galilaeus* (L.). The results from Chapters 3 and 4 indicate that the inclusion of YPC$_U$ and various types of YPC and various types of YPC and DDGS in the experimental diets significantly decreased the specific activity of ASAT in the carp liver. Previously, Carver and Walker (1995) and Sato et al. (1995) determined that

there were no significant differences in ALAT and ASAT activities in both the human and rats fed different dietary yeasts. The results of this study suggest that there are no negative impact on liver metabolism of tilapia fed dietary YPCR yeast compared to fish fed a fishmeal based diet at the substitution levels tested.

Scanning electron microscopy (SEM) data in the present study revealed that refined yeast protein concentrate (YPCR) inclusion had some positive changes on microvilli density in the posterior intestinal region. Significantly higher density of microvilli was observed in fish fed 10% YPCR diet. This may be a possible explanation for similar growth performance and feed utilisation with the control diet in the current study due to better gut absorptive capacity. However this result does not agree with the results obtained for the posterior region of carp intestine in Chapter 4. Transmission electron microscopy (TEM) analyses of the intestine revealed that YPCR has the capability to increase the microvilli length; the better improvements of microvilli length was observed in the fish fed 30% YPCR diets. Similar results were reported only when carp fed YPCU diet in Chapter 4. Also, this result could be supported by the finding of Salze et al. (2008) which was found that when cobia larvae fed on 2% mannan oligosaccharide (MOS) dietary supplementation enhanced the microvilli length. In addition, Dimitroglou et al. (2009) obtained the same results with experiments conducted on gilthead sea bream.

Villi length improved with the increasing YPCR inclusion in the diet indicating that the elevated substitution of fish meal with a yeast concentrate altered intestine morphology; possibly due to the crude fibre component within the nitrogen free extract (NFE) and reduction in available (starch) carbohydrate in the higher inclusion diet. Similar findings have been reported by Aslaksen et al. (2007) and Merida et al. (2010) for sea bass and Atlantic salmon. Moreover, goblet cells were increased with the higher substitution

levels of YPC$_R$, possibly due to fibre content in nitrogen free extract of the diet. The similar tendency has been confirmed with carp in Chapter 3.

PCR-DGGE fingerprints of the microbial profiles reveal clear differences in the microbial communities, as revealed by significantly lower number of phylotypes in the control group than all other treatments and the high dissimilarity (ca. 0.70) between the microbial profiles of the control fed fish to that of the yeast fed fish. These findings are contradictory to the data obtained in the carp study of Chapter 3, whereby no significant impacts on carp gut microbiota structure was observed with the inclusion of dietary yeast. However, several studies have reported that the gut microbiota of fish may be sensitive to dietary inclusion of yeast; examples include beluga sturgeon (Hoseinifar et al., 2011) and red drum (Burr et al., 2008) and a further study has demonstrated that the gut microbiota of tilapia is sensitive to a *S. cerevisiae* fermentation product (DVAQUA®) (He et al., 2009). When comparing the findings of chapters 3 and 5 it is evident that the gut microbiotas of the two different fish species have different degrees of sensitivity to yeast. Indeed, the gut microbiota of the two species is reported to be composed of different microbial species. For instance the carp gut microbiota is reported to consist of *Weissella* spp., *Halomonas* spp., *Cobetia* spp., *Streptococcus* spp., *Cetobacterium* spp., *Vibrionaceae* spp., *Flavobacteruim* spp., *Lactococcus* spp., *Pediococcus spp.*, *Enterococcus* spp., *Lactobacillus* spp, *Bacillus* spp., *Acinetobacter* spp., *Aeromonas* spp., *Moraxella* spp. and *Pseudomonas* spp., (Sugita et al., 1997; Cai et al., 1999; Mahmoud et al., 2004; Chapter 3). Whereas, *Weissella* spp., *Staphylococcus* spp., *Lactobacillus* spp., *Agrobacterium* spp., *Cyanobacterium* spp., *Enterobacter* spp., *Thermus* spp., *Fusobacterium* spp., *Clostridium* spp., *Mycobacterium* spp., *Flavobacterium* spp., *Anoxybacillus* spp., *Cetobacterium somerae*, *Rhizobium* spp., *Brevundimonas* spp., *Acinetobacter* spp., *Pseudomonas* spp.,

Shewanella spp., *Bacillus* spp. *and Enterobacter* spp. are common gut microbes in tilapia (He et al., 2009; Merrifield et al., 2010c, 2011; Zhou et al., 2010).

5.5 Conclusion

The results from this trial with tilapia showed that a YPCR protein ingredient can be used to considerably substitute FM in the diets of Nile tilapia without adversely affecting growth performance and feed utilization upto (30%) inclusion levels. Higher levels will lead to lower SGR and weight gains compared to tilapia fed moderate levels of yeast. The partial YPCR inclusion in tilapia diets also has no adverse effects on carcass composition and mineral profile. Iron level in tilapia carcass has been increased with the increasing of YPCR dietary levels. Furthermore, the new product has been shown to enhance intestinal morphology in the tilapia fed substitution diets.

In general, therefore, it seems that the YPCR could be used as a safe and effective primary or secondary protein to partially replace fishmeal in the diets of tilapia. Optimal replacement of FM with YPCR in diet for Nile tilapia needs further investigation. Additionally, inclusion of YPCR protein ingredients in Nile tilapia commercial diets will offer a significant reduction in feed costs and lead to move sustainable feed formulation for this species.

However, this ingredient should be tested in large scale growth trials conducted under commercial conditions to ensure transferability of the present findings to commercial production.

Chapter 6

General discussion and conclusion

In order to reduce the cost of warm water fish diets, nutritional research is now heavily focused on developing novel sustainable protein sources e.g. from plant and animal by-products / single cell proteins (SCP's) such as bacteria, yeasts and algae, as viable alternatives to FM without imparting any negative impact on fish production and health. Novel technologies will be the key factor in producing these alternatives to generate an increased range of options and ingredients suited to a wider spectrum of species. An example of such novel technology is the production of yeast protein concentrate (YPC) from the biofuel production processes and similar products from potable alcohol fermentation processes. Which are traditional routes such as the use of Distillers Dried Grains (DDG) from mainly corn based alcohol generating industries in the USA, Canada and Asia-Pacific region.

The use of yeast as an alternative protein source for different fish species (e.g. rainbow trout, sunshine bass) diets has been demonstrated by several studies to date (Rumsey et al., 1990, 1991; Gause and Trushenski 2011a, b). This has been primarily in order to reduce the inclusion for FM in aquafeeds where this commodity has been a basic component of the diet for many scenarios. FM is an excellent protein source of high biological value but is now seen as potentially unsustainable if aquaculture production of high value species continues to expand as predicted. This is obviously a serious constraint and as stated in Chapter 1 must be addressed to underpin the expansion of aquaculture. Although the problem is more acute in high value carnivorous species such as salmon, sea bass, sea bream and turbot for example, it is still an issue for other fish such as carp, tilapia and catfish. Carp production world wide requires 17 % of the total global production of FM and therefore attempts to reduce this burden are to be welcomed.

The aim of this research programme was to investigate the viability of partial substitution of fishmeal with bioethanol-/brewing-derived by-products (YPC_U, YPC_R and DDGS YPC_{PA}) from a defined industrial source namely AB-Agri within the UK. This company has a requirement to make assessments of its patented technology using a sequence of trials with fish. In the programme of investigation we tested these products on formulated feeds for warm water species only, i.e. carp and tilapia as model fish for future applications where the potential could be applied for other higher value species as well. Growth performance and feed utilization of carp and red tilapia fed diets with YPC inclusion achieved the same overall performance as carp or Nile tilapia fed basal control diets with FM as the primary protein source. In some cases, the growth performance and feed utilization was significantly improved (refer to Chapters 3 and 4), thus confirming the findings of Rumsey et al. (1990, 1991) Olvera-Novoa et al. (2002) and Gause and Trushenski (2011a, b) who also reported positive outcomes in fish fed various yeast sources.

The study conducted in Chapter 3 showed that their inclusion in warm water species (carp) fed diets with different levels of YPC_U were either comparable or considerably superior than those fed FM diets, in terms of both growth performance and feed utilization as well as other nutritional parameters employed in routine fish nutrition investigations. The final weight gain and PER were significantly improved when fish were fed (YPC_U) substitution diets. However, SGR was significantly higher in carp fed 15% and 20% YPC_U inclusion diets compared to FM fed carp. FCR and FCE were considerably enhanced in carp fed all dietary inclusion levels of YPC_U except at the 50% inclusion level. This indicates a very good performance for yeast derived protein for warm water fishes in general but with some restrictions at higher incorporation levels in diets.

As well as growth, body composition was somewhat affected to varying degrees for carp and tilapia but this was not deemed to be of any significance with respect to the relatively short duration of trials where fish only reached sub harvest sizes.

Whole body ash significantly increased with increasing inclusion levels of YPCU but in the case of whole body gross energy content, this decreased with increasing inclusion level of YPCU and was significantly reduced at $\geq$20% which indicated that some specific prior treatments of yeast could affect nutrient retention and especially tissue mineral profile and level. More specifically, the inclusion of yeast protein concentrate seems to affect mineral content in fish as a result of different relative bioavailability characteristics resulting from different processing techniques employed. Mineral content of the carcass (calcium, potasium, magnesium, phosphorus, manganese, and zinc) increased with the inclusion level of YPCU and at all levels of inclusion was significantly higher than the FM reference diet. All other mineral content (sodium, chromium, copper and iron) in the carcass of carp were unaffected by the inclusion of YPCU in the diet. Since mineral status is important in fish with respect to health and their nutritional quality for human consumption, importance should be given to this finding since mineral premixes are costly and any natural sources with higher bioavailability should be examined further.

It was important in all studies to evaluate the effects of the YPC products on the major organs of the digestive system and therefore both the liver and gut were examined for any adverse changes in ultra-structure and physiological as well as metabolic function.

Light microscopy of carp liver showed that the level of YPCU inclusion did not influence its morphology and structure at the end of the feeding experiments. The intestinal tract were compared by examination of a specific region (distal) which is deemed to be more sensitive to diet effects in fish, especially trout and salmon where

some diets such as those very high in plant ingredients especially soya bean meal can cause enteritis and other lesions. The light microscopy evaluation of carp intestine revealed that YPCu inclusion did not influence villi length and epithelial leucocytes of the anterior and posterior intestinal region respectively. However, the number of goblet cells in the posterior region was significantly elevated with the increasing YPCu inclusion level of $\geq$15%. In contrast, no significant differences were reported in the anterior region in terms of the number of goblet cells with varying YPCu inclusion level.

Intestinal microbiota profiles for carp fed the experimental diets reported in Chapter 3 were similar in all samples regardless of YPCu inclusion level with no significant differences observed in the microbial ecological diversity or relative abundance.

Metabolic assessment of fish may have shown the effects of YPC on liver function if higher dietary levels were implicated in elevated glycogen and fat deposition or the possibility of increased nucleic acid intake and associated metabolic processes for protein synthesis. Assays of key metabolic enzymes associated with amino acid metabolism were therefore determined for groups of fish fed the respective diets. These were aspartate amino transferase and alanine amino transferase respectively. Any increases might have signified excessive levels of amino acids indicating greater biosynthesis of dietary protein from amino acids, and a decrease in activity possibly a sign of liver damage and impaired metabolic function. It was found that hepato-pancreas ASAT activity was influenced by YPCu level, with a significant decrease occurring at 20% and 50% inclusion. ALAT activity decreased with increasing YPCu inclusion levels but without any significant differences. This might indicate evidence of metabolic constraints associated with higher incorporation of yeast into carp diets.

ASAT activity was affected by various types of YPC and DDGS inclusion, with a significant decrease occurring at all levels of the various types of YPC and DDGS

substitution level. ALAT activity also decreased with various types of YPC and DDGS levels but no significant differences were found that indicated serious metabolic constraints to their use at a set level of 30% incorporation.

The study described in Chapter 4 revealed the potential of using various processing technologies of YPC (YPC_U, YPC_R and YPC_{PA}) at a set inclusion level of 30%, and DDGS at inclusion levels of 15% and 30% with 10% YPC_R as FM replacement in the diet of carp. The overall growth performance and feed utilization when substituting FM with YPC_U and YPC_R at the levels of 30% and DDGS at the levels of 15% and 30% (with 10% YPC_R for both) was comparable to or better than was observed with the reference diet. However, the substitution of FM with YPC_{PA} resulted in significantly lower in terms of weight gain, SGR, FCR and ANPU compared to the reference diet. Similarly, dietary inclusion of 7.5, 15, 20 and 50% of YPC_U resulted in equal or improved growth performance and feed utilization of carp (Chapter 3) and this is true for sunshine bass fed with 27-41% ethanol yeast (Gause and Trushenski 2011a, b). Body composition of carp was significantly elevated by various types of YPC and DDGS dietary inclusion in terms of protein and ash. The gross energy was equal in fish fed various inclusion level of YPC and DDGS inclusion compared to a control group of fish receiving primarily FM.

Mineral retention of carp was improved with dietary inclusion of various types of YPC and DDGS in comparison to carp fed with control diet (i.e. phosphorus, sodium, magnesium and chromium) as found in the previous study outlined in Chapter 3, and is consistent with work reported on sunshine bass by Gause and Trushenski (2011a, b).

Haematological parameters indicated that neither YPC nor DDGS inclusion levels affected haematocrit, haemoglobin and mean corpuscular haemoglobin concentration. Red blood cells counts and mean corpuscular volume were slightly altered by various

types of yeast and DDGS. Total leukocytes counts were also assessed in carp fed various types of YPC and DDGS and a reduction was only recorded in carp fed with DDGS at 15% and 30% inclusion.

The gastrointestinal tract and liver histology of carp were examined for dietary treatments containing different YPC sources and DDGS. Indeed, TEM of intestinal tract of YPC and DDGS fed carp revealed significantly elevated microvilli length inthe anterior and posterior intestinal region compared with carp fed the control diet. However, SEM showed that the microvilli density remained unaffected by the dietary treatment in the anterior intestinal region.

The liver showed a normal histological structure, and no obvious changes were found in terms of the pathology of fish fed different levels of YPC and DDGS. The number of hepatocytes was significantly elevated in the carp fed YPC_{PA} inclusion level compared to other dietary groups.

In Chapter 5, partial substitution of FM with YPC_R demonstrated that there was a comparable result with tilapia fed incremental YPC_R inclusion levels in terms of FCR, FCE, PER, ANPU and K-value, with tilapia fed on a high quality FM based diet. However, at the highest inclusion level (30%), the weight gain, SGR and HSI were significantly lower than the control group fish. Contrary to the results of Chapter 3 with carp there was no negative impact of growth performance with YPC_U at even a 50% inclusion level. Also in comparison to the Chapter 4 studies with carp there was no indication of any improvements of weight gain and SGR with a 30% dietary inclusion YPC_R for carp.

For tilapia, no significant differences were found in carcass composition with respect to moisture, protein, lipid, ash, NFE and gross energy.

It was found that iron levels in the carcass of YPCR fed tilapia were significantly elevated compared with FM fed tilapia. However, chromium and zinc levels were slightly increased in the carcass of YPCR fed tilapia in comparison to tilapia fed FM diet. The levels of calcium, potassium, magnesium, sodium, phosphorus, copper and manganese remained unaffected regardless of the level of YPCR. These findings support the results from Chapter 4.

In Chapter 5, the activities of key liver metabolic enzymes were reported for tilapia fed the yeast source. YPCR- fed Nile tilapia did not exhibit any differences in terms of ALAT and ASAT activity, when compared to FM-fed fish. These findings are in agreement with those of carp fed YPC or various types of YPC and DDGS dietary inclusion (Chapters 3, 4).

Likewise the histological evaluation of the gastrointestinal tract and liver of tilapia were undertaken at the end of the trial period to examine the effects of diet on general health.

Evaluation of the posterior region of the intestine by SEM in Chapter 5 indicated that the microvillus density was significantly increased with YPCR inclusion level. This finding was not in evidence for carp from the investigations reported in Chapter 4. Further, TEM evaluation of microvilli length in the posterior intestine of tilapia showed a significant increase compared with FM fed tilapia. These results were also observed in carp (Chapter 4). A significant increase in the number of goblet cells in the posterior region was also confirmed by light microscopy which was linked to increasing levels of YPCR inclusion in the diet. This result was consistent with the findings reported in Chapter 3 for the same region of carp intestine. However, no significant differences were found in terms of the mucosal fold length in this region, and this was also similar to the finding in the same region of carp (Chapter 3). Histological examination of the liver of tilapia revealed that YPCR inclusion could have a negative effect on hepatocyte

density, whilst elevating lipid accumulation, indicating some potential for limiting yeast protein for tilapia in dietary formulations.

In contrast to the findings presented in Chapter 3, whereby it was observed that the gut microbiota of carp where not significantly affected by dietary YPCU, Chapter 5 shows that the gut microbiota of tilapia are affected by dietary YPCR inclusion. Future studies are required to elucidate the identity of the microbes affected in order to ascertain if these changes have any relevance to the host.

Experimental design is fundamental in the interpretation of fish nutrition investigations and there are many approaches that can be undertaken when we examine the scientific literature. This programme of work followed the orthodox conventions where a standard diet is formulated to meet the known nutritional requirements for fish species based on NRC data and specific known references.

These experiments focused on a reference FM based diet with a high quality Low Temperature (LT) FM within a semi-purified formulation. The value of the yeast protein concentrate is assessed within the context of a 100% FM protein level meeting the target protein requirements for either species of around 38% of the diet. In more practical formulations with reduced FM content and combinations of animal and plant by-products, then YPC may be a complete replacement of FM providing that the amino acid balance of the diet is met by a complimentary array of ingredients and the possible supplementation of the yeast with synthetic L-methionine or DL-methionine or hydroxyl- methionine analogues to effect a balanced essential amino acid mixture.

One of the most important aspects of the consideration of any novel feed ingredient is its digestibility coefficients for respective nutrients especially protein, essential amino acids (EAA's) and energy. YPC's will be particularly variable in terms of digestibility and this is primarily due to the cell wall structure of yeast leading to reduced availability

of cellular content. Therefore yeast needs to be effectively processed by hydrolysis and /or mechanical fractionation to elevate nutritional value. Another consideration is the high nucleic acid content of yeast which could be detrimental but more important is the contribution of nucleic acid to Non- Protein Nitrogen (NPN) leading to erroneous formulations in respect of the 'true protein' content of the diet. In these studies, diets were formulated to be iso-nitrogenous to provide diets of similar crude protein level as undertaken by many workers in this field. It could be argued that a higher substitution of FM protein by YPC would have been feasible if the exact digestibility as well as the true protein level were determined accurately. However, digestibility was not measured for either carp or tilapia and diets were not strictly formulated on a digestible amino acid basis or supplemented by crystalline amino acids. These areas remain to be examined in more comprehensive studies and using practical diets generated by computer software such as linear least cost formulation approaches.

In summary, this programme of research has validated a novel feed ingredient with potential use in the aqua feed sector. Although limited in duration, feeding trials with juvenile carp and tilapia demonstrate the considerable potential for using a yeast protein concentrate from the production of both potable alcohol and biofuel derived sources using wheat based substrates in this case. Both juvenile carp and tilapia responded well to the yeast and DDGS by-products in terms of palatability, acceptance, nutritional value and ability to partially replace a significant amount of fish meal as the primary protein within balanced diets for warm water fish species.

It should be emphasised that trials with fast growing juvenile fish are an excellent preliminary approach for evaluating feed ingredients in experimental diets. However this represents only a partial stage in the production of fish leading to large market weight fish at harvest. In carp and tilapia production for instance, fish are typically 300-1000 g at market or even larger in some cases. The longer term significance of

incorporating yeast concentrates and DDGS for carp and tilapia through to harvestable weight was not addressed in the present programme of work but is full appreciated to be necessary and desirable in the future for a complete understanding of the available options. Apart from finding whether additional dietary constraints or even higher potential inclusion of these products is feasible, there are implications to fish composition and quality. Appearance, texture and taste are all vital for consumer acceptance of the 'finished' product. It is therefore advocated that specialised 'taste panel' assessors be linked to future experimental studies to judge the quality of farmed fish fed yeast and DDGS for their market value and consumer preferences.

By proportion, carp is the most dominant freshwater fish being raised in the world with tilapia fast approaching record levels of production globally. The salmon and trout industry is of obvious relevance in the high value end of aquaculture but more expensive fish such as sea bream, sea bass, turbot and species like cobia and barramundi are representative of an expanding marine based aquaculture sector. The strategic use of fish meal in diets for carnivorous fish are a serious issue and we need to urgently extend our range of alternative feed ingredients to these species if we are to provide a viable and sustainable industry. YPC and DDGS are now high on the agenda for extended feasibility studies similar to the experiments reported here due to the increased potential of bio-ethanol derived co-products in this decade and beyond. Therefore the application of such ingredients for other fish species must be undertaken and such studies are now in hand at Plymouth University and other institutions to offer a new generation of acceptable alternatives to fish meal for wider applications in the aqua feed sector. It should be finally appreciated that YPC and DDGS are only a partial solution to providing an alternative feed ingredient source to reduce or replace fish meal. It is most likely that they will be used together with more traditional materials and inter-

changed according to availability and price. Nonetheless, they will be a valuable additional source in the list of feed ingredients at our disposal.

As an illustration of the potential inclusion of these ingredients into warm water diet formulations, a cost benefit exercise was performed using Linear Least Cost Formulation by applying a commercial software programme (FeedSoft ^{TM}). The data provided realistic market prices for September 2011 for major commodities such as fish meal, soya bean meal, wheat feed, fish oil and distillers dried grains and solubles (DDGS). An average commercial price was used for yeast protein concentrate to simulate the expected value of yeast within the context of its nutritional value with particular attention to protein level and amino acid profile.

Tables 6.1,2,3 shows a typical practical diet formulation where fish meal level is held at 15% with soya bean meal as the secondary protein ingredient. A realistic 610 € per tonne cost is encountered for Diet A. Diet B allows for the inclusion of YPC which must fall below the price of soya bean meal (550 €) to allow inclusion at its shadow price setting of 510 € per tonne. The resulting feed is projected to be 600 € per tonne which is only a marginal saving.

Diet C was designed to allow DDGS and YPC to be included to partially replace soya bean meal, wheat and with a lower fish meal restriction at 5% of the diet. This diet satisfied the nutritional constraints of carp and tilapia and is based on the dietary restrictions resulting from the work in this research programme. Such practical diets must of course be tested in a series of feeding trials with fish. There are numerous theoretical scenarios where YPC and DDGS can be offered in combination with other ingredients for efficient diets for fish species. Such formulations are beyond the scope of this chapter but will need much more reliable data on digestibility of nutrients,

restriction levels, availability and cost of raw materials for accurate formulations for fish production.

Wheat production is a vital prerequisite for the biofuel and potable alcohol industry to generate the resulting co-products discussed and evaluated in this thesis. This is of significant value in the European context and also in Central Asia and the Russian Federation where wheat is a major commodity. Wheat production is also of considerable importance in North America (USA and Canada) but in this region, corn has been the traditional material for DDGS and by-products of fermentation. Therefore it will be of economic importance to undertake more studies in this area to make this process more efficient. Increased competition for the use of cereals for the production of

Table 6.1 Standard diet (diet A) for warm water fish (carp and tilapia) with cost of 0.61 € kg^{-1}; 610€ $Tonnes^{-1}$.
Ingredient restrictions

Ingredient	Price (EUR)	Min (%)	Max (%)	Usage (%)	Batch (kg/t)
Wheat feed	0.30			45.37	453.70
Soybean (Hamlet HP100)	0.55			23.06	230.60
Herring Meal LT92	1.50	15.00		15.00	150.00
Glutalys	0.58	10.00		10.00	100.00
Corn oil	0.50	1.00		3.07	30.70
Vitamin Premix	1.60	2.00	2.00	1.00	20.00
Fish oil	1.00	1.50	1.50	1.50	15.00

Nutrient restrictions

Nutrient	Units	Min Limit	Max Limit	Actual	Shadow
Dry Matter (DM)	%		100.00	86.38	0.00
Crude Protein	%	38.00	38.00	38.00	0.01
Digestible crude protein	%			9.24	
Crude lipid	%	8.00	8.00	8.00	0.00
Crude Fibre	%			1.68	

Table 6.2 Standard diet and YPC (diet B) for warm water fish (carp and tilapia) with cost of 0.60 € kg^{-1}; 600 € Tonnes^{-1}.
Ingredient restrictions

Ingredient	Price (EUR)	Min (%)	Max (%)	Usage (%)	Batch (kg/t)
Wheat feed	0.30			43.43	434.30
Yeast AB-Agri Scotland	0.51			19.62	196.20
Herring Meal LT92	1.50	10.00		15.00	150.00
Soybean (Hamlet HP100)	0.55	10.00		10.00	100.00
Glutalys	0.58	5.00		5.00	50.00
Corn oil	0.50	1.00		3.45	34.50
Vitamin Premix	1.60	2.00	2.00	2.00	20.00
Fish oil	0.50	1.50	1.50	1.50	15.00

Nutrient restrictions

Nutrient	Units	Min Limit	Max Limit	Actual	Shadow
Dry Matter (DM)	%		100.00	86.65	0.00
Crude Protein	%	38.00	38.00	38.00	0.01
Digestible crude protein	%			9.24	
Crude lipid	%	8.00	8.00	8.00	0.00
Crude Fibre	%			1.31	0.00

Table 6.3 Standard diet with YPC and DDGS (diet C) for warm water fish (carp and tilapia) with cost of 0.50 € kg^{-1}; 500 € Tonnes^{-1}.

Ingredient restrictions

Ingredient	Price (EUR)	Min (%)	Max (%)	Usage (%)	Batch (kg/t)
Yeast AB-Agri Scotland	0.51			29.11	291.10
Wheat feed	0.30			28.36	283.60
DDGS AB-Agri	0.20	10.00	15.00	15.00	150.00
Soybean (Hamlet HP100)	0.55	10.00	10.00	10.00	100.00
Herring Meal LT92	1.50	5.00	5.00	5.00	50.00
Glutalys	0.58	5.00		5.00	50.00
Fish oil	1.00	3.00		3.00	30.00
Corn oil	0.50	1.00		2.53	25.30
Vitamin Premix	1.60	2.00	2.00	2.00	20.00

Nutrient restrictions

Nutrient	Units	Min Limit	Max Limit	Actual	Shadow
Dry Matter (DM)	%		100.00	89.44	0.00
Crude Protein	%	38.00	38.00	38.00	0.01
Digestible crude protein	%			3.08	
Crude lipid	%	8.00	8.00	8.00	0.00
Crude Fibre	%			0.91	0.00

‘first generation’ biofuels compete with the food chain either directly for use of cereals in human nutrition or as components for domestic ‘farm’ animals. Ruminant and mono-gastric animals including mainly poultry and swine require substantial amounts of grains in their complete feeds. This is true also of aquaculture which is making significant demands on global feed resources.

The development of solid state fermentation systems and ligno-cellulosic technologies to utilise plant fibre to create ‘second generation’ based biofuels will lead to more flexibility in the production of bio-ethanol to release cereals for other uses. The resulting separation of yeast from the resulting co-products will warrant more studies with terrestrial animals and fish species in the years to come. There will also be further competition with the emerging ‘third generation’ biofuel industries with much being discussed about the additional potential of algae as a feed source for fish.

The significance of these technologies for application in aquaculture is enormous and we are on the threshold of a new era in applied fish nutrition where the nutritional value of these by-products is enhanced by their possible functional properties as immune-modulators, prebiotic or even probiotic bio-active feed additives or supplements in aqua feeds. There is clearly scope for an inter-disciplinary approach to their evaluation and the fish nutrition sciences are being directed towards these objectives.

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