

UTILISATION OF *MORINGA OLEIFERA* (MORINGA) AND *PENNISETUM CLANDESTINUM* (KIKUYU) LEAF MEALS BY THREE COMMONLY CULTURED FISH SPECIES IN SOUTH AFRICA: *TILAPIA RENDALLI*, *OREOCHROMIS MOSSAMBICUS* AND *CLARIAS GARIEPINUS*

by

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DEDICATION

I dedicate this thesis to my husband Muzi, who has never left my side throughout the entire doctorate program and to my loving parents, whose words of encouragement and push for tenacity ring in my ears. You have been my best cheerleaders.

DECLARATION

I declare that the **UTILISATION OF *MORINGA OLEIFERA* (MORINGA) AND *PENNISETUM CLANDESTINUM* (KIKUYU GRASS) LEAF MEALS BY THREE COMMONLY CULTURED FISH SPECIES IN SOUTH AFRICA: *TILAPIA RENDALLI*, *OREOCHROMIS MOSSAMBICUS* AND *CLARIAS GARIEPINUS*** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references and that this work has not been submitted before for any other degree at any other institution.

Hlophe Samkelisiwe Nosipho

Date

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SAPSE ACCREDITED PAPERS PUBLISHED FROM THE THESIS

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2. Hlophe, S.N., Moyo, N.A.G. and Ncube, I. 2013. Postprandial changes in pH and enzyme activity from the stomach and intestines of *Tilapia rendalli* (Boulenger, 1897), *Oreochromis mossambicus* (Peters, 1852) and *Clarias gariepinus* (Burchell, 1822). *Journal of Applied Ichthyology*, 30: 35-41 **(Appendix B)**.
3. Hlophe, S.N. and Moyo, N.A.G. 2013. The aquaculture potential of *Tilapia rendalli* in relation to its feeding habits and digestive capabilities. *Physics and Chemistry of the Earth*, 66: 33-37 **(Appendix C)**.
4. Hlophe, S.N. and Moyo, N.A.G. 2014. Evaluation of kikuyu grass and moringa leaves as protein sources in *Oreochromis mossambicus* diet. *African Journal of Aquatic Sciences*, 39(3): 305–312 **(Appendix D)**.
5. Hlophe, S.N. and Moyo, N.A.G. 2014. Replacing Fishmeal with Kikuyu Grass and Moringa Leaves: Effects on Growth, Protein Digestibility, Histological and Haematological Parameters in *Clarias gariepinus*. *Turkish Journal of Fisheries and Aquatic Sciences*, 14: 795-806 **(Appendix E)**.
6. Hlophe, S.N., Moyo, N.A.G., Ngambi, J.W. and Ncube, I. 2015. Effect of exogenous enzyme supplementation on the growth performance and digestive enzyme activities in *Oreochromis mossambicus*. *Aquaculture Research* (accepted).

ABSTRACT

The ability to utilise dietary components differs between fish species. Digestive enzymes may be used to determine the efficiency of the digestive process. In this study, the activities of the digestive enzymes in *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* were explored. Protease, amylase, lipase and cellulase activities were measured in different parts of the digestive tract of the three fish species. The pH dynamics along the digestive tract were monitored. In all fish species, the presence of food led to a reduction in stomach pH. pH values of 1.54, 1.58 and 2.01 were recorded 12 hours after feeding in *Oreochromis mossambicus*, *Tilapia rendalli* and *Clarias gariepinus* respectively. Protease and amylase activities were significantly higher ($P < 0.05$, ANOVA) in the tilapias than in *Clarias gariepinus*. The tilapias may be pre-adapted to produce more protease and amylase to digest plant material which is more difficult to digest compared to animal matter. In all species amylase activity was significantly higher in the proximal intestine than in the other parts of the digestive tract ($P < 0.05$, ANOVA). The highest proteolytic activity was recorded in the distal intestines. This is because of the alkaline pH recorded in the proximal and distal intestines which favours for amylase and protease activity respectively. Lipase activities were not significantly different ($P > 0.05$) in all species. Marginal cellulase activities were recorded in all species. It is inferred here that phylogeny and not diet may be the main factor influencing enzyme activities as all fish were fed a similar diet.

Two locally available plant diets, kikuyu grass and moringa leaves, were tested as protein sources in the diet of a macrophagous fish, *Tilapia rendalli* (11.5±1 g). Nine diets (30% CP: 20 MJ/kg) were formulated by substituting fishmeal for kikuyu leaf meal (KLM) and moringa leaf meal (MLM). A control diet contained 10% fishmeal and no leaf meal. Fishmeal was replaced at 25, 50, 75 and 100% by KLM in diets: KLM 25, KLM 50, KLM 75 and KLM 100; then by MLM in diets MLM 25, MLM 50, MLM 75 and MLM 100. Each diet was fed to triplicate groups of fish for 60 days. The best growth (SGR, TGC) was in the control group. There was no significant ($P > 0.05$) decrease in SGR and TGC when KLM replaced up to 50% fishmeal. There was a significant ($P < 0.05$) decrease when MLM replaced >25% fishmeal. Kikuyu diets had no effect on villi height. A trend towards shorter villi was evident with increasing MLM. Digestive enzyme activities also decreased with increasing KLM and MLM

levels in the diet. Hepatocyte degradation was higher in fish fed moringa-based diets. Anti-nutrients (polyphenols, tannins, saponins and phytate) in moringa may have contributed to the poor growth, irritation of the enterocytes and hepatotoxic effects. These results show that replacing up to 25% fishmeal with KLM is effective in reducing the costs without negatively affecting the growth performance or health of *Tilapia rendalli*. Adding MLM, even at the lowest level (25%) was expensive and resulted in compromised growth and health.

The efficacy of KLM and MLM was also tested as alternative protein sources for *Oreochromis mossambicus* (12.5 ± 1 g) a microphagous herbivore. The same diets used for *Tilapia rendalli* were fed to triplicate groups of twenty fish for 60 days. Linear regressions of feed intake, SGR, PER and protein ADC with increasing levels of leaf meal were significant ($P < 0.05$). Superior growth performance, protein ADC and feed utilisation were also recorded in fish fed KLM-based diets than those fed MLM diets. When compared to *Tilapia rendalli*, *Oreochromis mossambicus* had superior growth performance and feed utilisation when fed the control diets and the lowest level of KLM. This was attributed to phylogeny. Protease, amylase and lipase decreased with increasing leaf meal levels and were higher in the intestine of fish fed KLM-based diets than those fed MLM-based diets. Fish fed MLM-based diets had higher number of goblet cells in the enterocytes, higher hepatocyte degradation and poor haematological parameters than those fed KLM diets. These adverse alterations were more pronounced in *Oreochromis mossambicus* compared to those observed in *Tilapia rendalli* feeding of the same diets. Cost benefit analysis also indicated that substitution fishmeal with KLM is a cheaper protein source in *Oreochromis mossambicus* diets. Kikuyu leaf meal may be used to replace up to 25% fishmeal without compromising the growth performance and health of *Oreochromis mossambicus*. Reduced growth and poor health was evident even at the lowest inclusion level of MLM.

The effects of replacing fishmeal with KLM and MLM in the diets of a predatory omnivore, *Clarias gariepinus* were also investigated. The same KLM and MLM-based diets used in the previous experiments were used. Each diet was randomly assigned to triplicate groups of *Clarias gariepinus* (30.5 ± 2 g) and fed to apparent satiation for 60 days. Significantly higher ($P < 0.05$) growth performance, feed and

protein utilisation was observed in *Clarias gariepinus* fed KLM diets compared to those fed MLM. Protein digestibility was higher in *Clarias gariepinus* fed the control diet than in both tilapias. However, in the treatment diets protein ADC was lower in *Clarias gariepinus* than in the tilapias. A decrease in the activity of digestive enzymes was also observed with increasing leaf meal level in the diet. This was attributed to the natural feeding habits and digestive adaptations of the different fish species. No histological alterations were found in liver of fish fed the control diet. Increased hepatocyte degradation was seen in fish fed higher levels of KLM and MLM in the diet. The enterocytes showed a significant increase in the number of goblet cells with increasing levels of MLM. Villi height decreased significantly ($P < 0.05$) when MLM replaced >75 fishmeal. The damage to the hepatocytes and enterocytes as well as the poor health condition shown by haematological parameters was more pronounced in *Clarias gariepinus* than in the tilapias. This suggests that the predatory fish is not equipped to utilise high levels of leaf meals in its diet. The results of this study indicate that KLM can replace up to 25% fishmeal and that adding MLM resulted in reduced performance. Higher profit index and lower incidence cost was observed KLM diets than in MLM diets.

Anti-nutrients in the leaf meals were the main factors leading to reduced feed intake and poor growth in fish fed the plant-based diet. Therefore, a subsequent study was undertaken to determine the efficacy of exogenous enzyme supplementation to reduce the negative effects of anti-nutrients and improve fish growth. A commercial multi-enzyme Natuzyme50[®] was supplemented at a rate of 0 (control), 0.25, 0.5, 0.75 and 1.00 g/kg DM feed in the best performing diet (KLM 25). These diets were tested in *Oreochromis mossambicus*. Natuzyme50[®] supplementation led to improved growth performance. Fish fed the diet containing 0.50 g/kg had the best growth performance and protein ADC and highest levels of digestive enzyme activities. At higher (>0.50 g/kg) enzyme supplementation levels, growth performance decreased. The improved growth performance with enzyme supplementation was attributed to the presence of enzymes such as cellulase and xylanase in the cocktail that are not naturally produced by fish. In addition, the activities of endogenous enzymes were enhanced. The optimal Natuzyme50[®] dietary level for optimal growth performance in *Oreochromis mossambicus* was 0.62 g/kg DM feed.

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CHAPTER 1:

GENERAL INTRODUCTION

1 INTRODUCTION

1.1 Aquaculture: Global overview

Aquaculture is the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants in a controlled environment (FAO, 2009). Aquaculture has been the fastest growing food sector for over 25 years and plays an important role in the global fight against hunger and malnutrition, particularly in developing countries. This sector has an average growth rate of 8.2% per year, compared with 1.3% for capture fisheries and 2.6% for total agricultural meat production (FAO, 2013). Currently, aquaculture accounts for nearly half (45.6%) of the world's food fish consumption, compared with 33.8% in 2000 (FAO, 2011). Estimates show that this growth will continue as demand increases with the growth in the human population (SOFIA, 2006).

Aquaculture has an approximately 4,000-year history, but it is only in the last 50 years that it has developed into an important worldwide industry. The importance of aquaculture production is set to increase substantially because of overfishing and an increasing demand for aquatic food. Overall capture fisheries production has remained stable at about 90 million tonnes per year from 2006 to 2011. The human population is expected to reach 8.5 billion by the year 2025, this implies that world fisheries will not be able to fulfil the demand for aquatic food. Evidently, only aquaculture has the potential to fill in the gap between supply and demand caused by the decline in wild catches and human population growth.

World fish-food supply has grown significantly at an average growth rate of 3.2% per year in the period 1961-2009, outpacing the increase of 1.7% per year in the world's population. World per capita food fish supply has also increased from an average of 9.9 kg in the 1960s to 18.4 kg in 2009 (FAO, 2011). Global production of fish from aquaculture has grown substantially in the past decade and hit a landmark in 2009, supplying half of the total fish and shellfish demand for human consumption (Brinker and Reiter, 2011). In 2010, aquaculture production reached 63.6 million tonnes, compared with 32.4 million tonnes in 2000 (FAO, 2012; Figure 1.1).

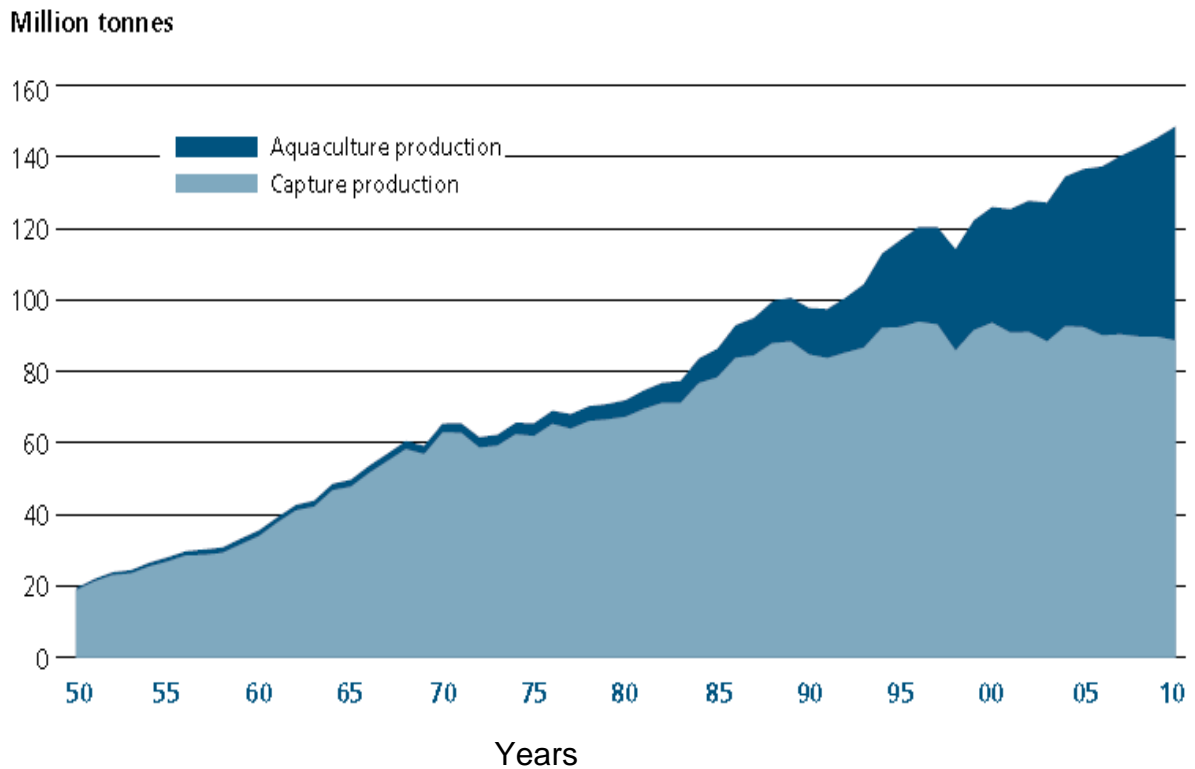


Figure 1.1: Total aquaculture production and global capture of fish from 1950-2010 (aquatic plants excluded). Data obtained from FAO, 2012

Aquaculture is practised in freshwater, brackish water and full-strength marine water. The percentage of production from freshwater aquaculture was 56% in 2010 (Figure 1.2). The culture of molluscs reached 24%; marine fish production on the other hand contributed only 3% of the world aquaculture production (Figure 1.2). Diadromos fish culture contributed 6% of the world production in 2010 (Figure 1.2). The average annual growth rate for freshwater aquaculture production from 2000 to 2010 was 7.2%, compared to 4.4% for marine aquaculture production (FAO, 2012).

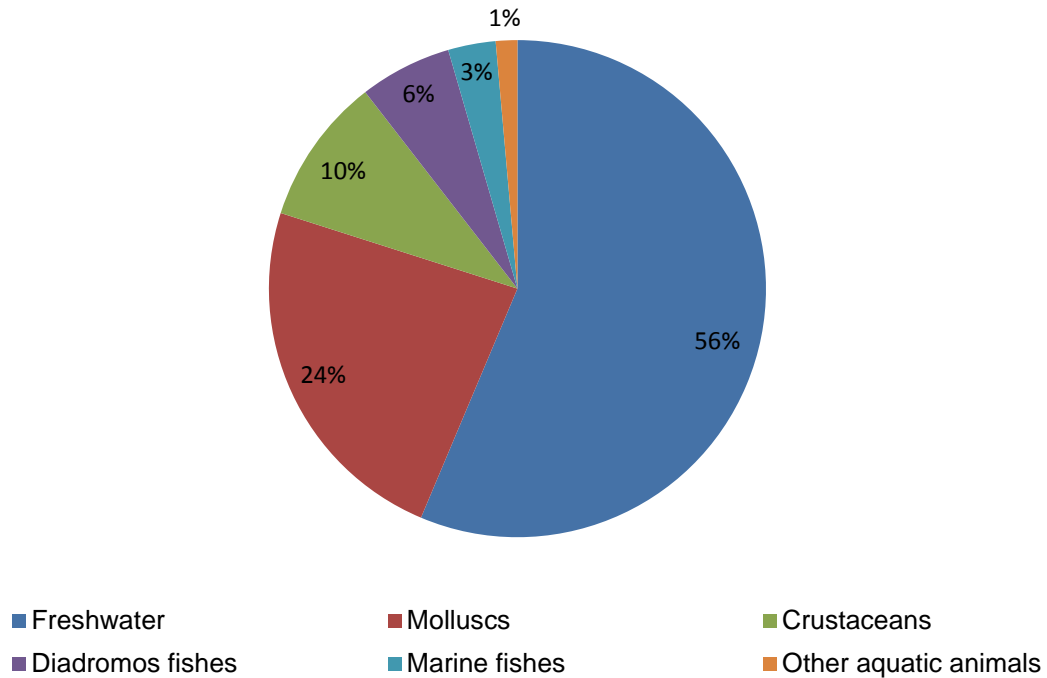


Figure 1.2: Composition of world aquaculture production in 2010 (data from FAO, 2012)

1.2 Leading aquaculture producers

Global finfish production amounted to approximately 83.67 million metric tonnes in 2011. China is the largest aquaculture producer accounting for about 60% of the total production (Figure 1.3). The global dominance of China in aquaculture production is due to its' over 2,000-year pro-active government policies in promoting aquaculture development within the country (Hishamunda and Subasinghe, 2003). At the continental level, Africa had the lowest aquaculture production accounting for 1.83% of global production in 2011 (FAO, 2013).

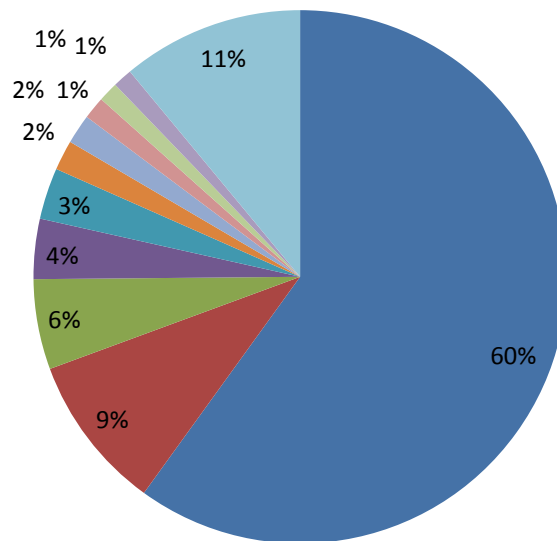


Figure 1.3: Global aquaculture production distribution in 2011 (Source: FAO, 2013)

Aquaculture production in Africa has increased by 60% since 1998 (FAO, 2012). Egypt is the leading aquaculture producer in Africa producing about 1 000 000 Metric tonnes in 2011. Nigeria and Zanzibar are also major aquaculture producers with 221 128 and 130 400 Metric tonnes respectively (Figure 1.4). Aquaculture production from South Africa for the same period was 6 457 Metric tonnes, contributing only 0.42% of the total production in Africa.

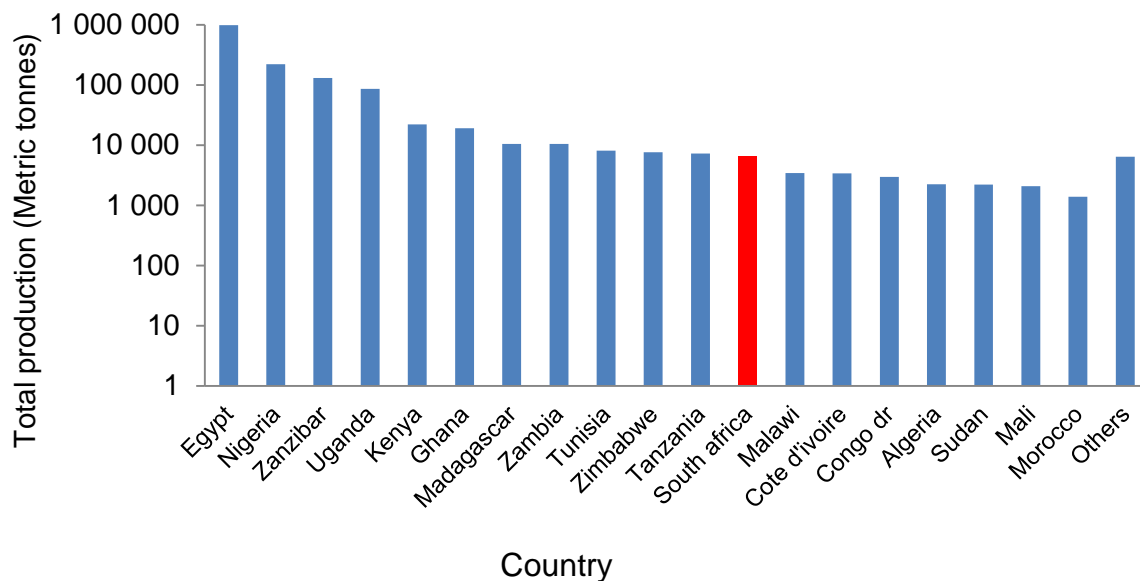


Figure 1.4: Estimated total aquaculture production in Africa by major producing country in 2011 (Source: FAO, 2013)

1.3 Aquaculture in Sub-Saharan Africa

Raising fish for sport purposes has a long history in sub-Saharan Africa. Trout was first introduced in South Africa between 1859 and 1896, in Kenya it was introduced in the late 1920s (Vincke, 1995) and the 1930s in Zimbabwe. Aquaculture on the other hand, is a relatively new phenomenon in this region (Satia and Bartley, 1997). In contrast to terrestrial agriculture, little or no traditional aquaculture knowledge exists among farmers in this region (Machena and Moehl, 2000). According to Maar *et al.*, (1979) the first African attempts to culture fish for human consumption were made in Kenya in 1924 using tilapia. This was followed by the Congo in 1937, Zambia in 1942 and Zimbabwe in 1952. Fish culture then spread to other countries and by the late 1950s, about 300,000 ponds had been constructed (Satia, 1989; Brummett and Williams, 2000). However, fish farming regressed sharply in the early 1960s as ponds were abandoned for several reasons including poor yields and shortage of skilled personnel or extension services and the lack of support from local governments (Machena and Moehl, 2000).

There was renewed interest in aquaculture in the late 1970s and 80s. In spite of this new interest, the development of this industry still faces major challenges. These include lack of access to quality fish feed, lack of suitable species, lack of quantity

and quality fingerlings, insufficient research and extension services, limited coordination between research and development sectors poor development of aquaculture policies, and inaccessibility of capital (Machena and Moehl, 2000; Hishamunda, 2007).

Aquaculture in Africa has great potential, about 43% of continental Africa has been identified as suitable for both small-scale and commercial farming of tilapia, African catfish and carp (Aguilar-Manjarrez and Nath, 1998). About 15% of this is classified as “most suitable” with a possibility of getting almost two crops per year of both tilapia and African catfish. This classification for the suitability of aquaculture is based on a multi-criteria evaluation that takes into account several factors including water requirements, soil and terrain suitability, inputs availability, farm-gate sales and infrastructure. In southern Africa, about 23% was ranked as suitable for commercial aquaculture but less than 5% is utilised (Kapetsky, 1994; Aguilar-Manjarrez and Nath, 1998). Aquaculture in this region has remained mostly rural, a secondary and part-time activity which takes place in small freshwater ponds within small farm holdings. Over 80% of fish farmers in sub-Saharan Africa are small scale, practicing extensive aquaculture on a non-commercial basis mainly to improve household food security (Hecht, 2007).

1.4 Aquaculture in South Africa

The South African aquaculture industry was established in the late 1960s and 1970s. During this period, several government agencies promoted freshwater aquaculture by constructing up to 13 well-equipped hatcheries across the country to supply fingerlings to both private and government projects. At present only 3 of the 13 government hatcheries are operational, albeit, at a much lower capacity and efficiency (DAFF, 2010). The main reasons for the reduced activity in aquaculture include high production costs (60% of which is feed related costs). Secondly, there was little planning and support available to the farmers or farmers associations. Basic training in fish biology, husbandry skills and marketing is lacking. Additionally, the brood stock used in these projects was randomly selected from locally available fish with no attention to improved strains or selection for favourable traits such as fast growth or temperature tolerance.

1.4.1 Marine aquaculture

Marine aquaculture production in South Africa is focussed on abalone, mussels (Spanish and brown), oysters, seaweeds, prawns, dusky and silver kob, yellow tail, Atlantic salmon, clownfish, white margined sole, west and east coast rock lobster, scallop and blood worm. Abalone culture is a well-established sector and contributes up to 62% of the marine aquaculture production in South Africa (Figure 1.5). Abalone is the big success story of South African aquaculture. This species is highly priced in southeastern Asia. Recently there has been an upsurge in interest of abalone culture because the wild population has been radically reduced through poaching. Marine aquaculture production increased substantially between 2007 and 2008 (Figure 1.6).

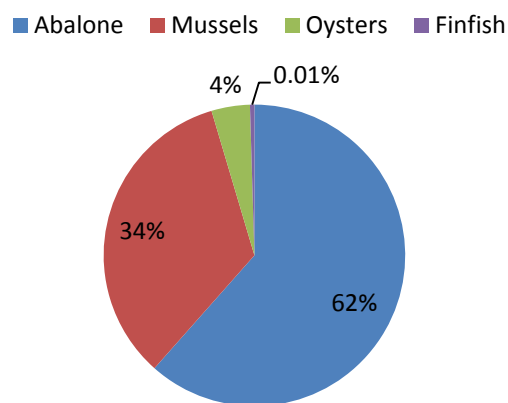


Figure 1.5: Main species contributing to marine aquaculture in South Africa in 2011 (Data from DAFF, 2013)

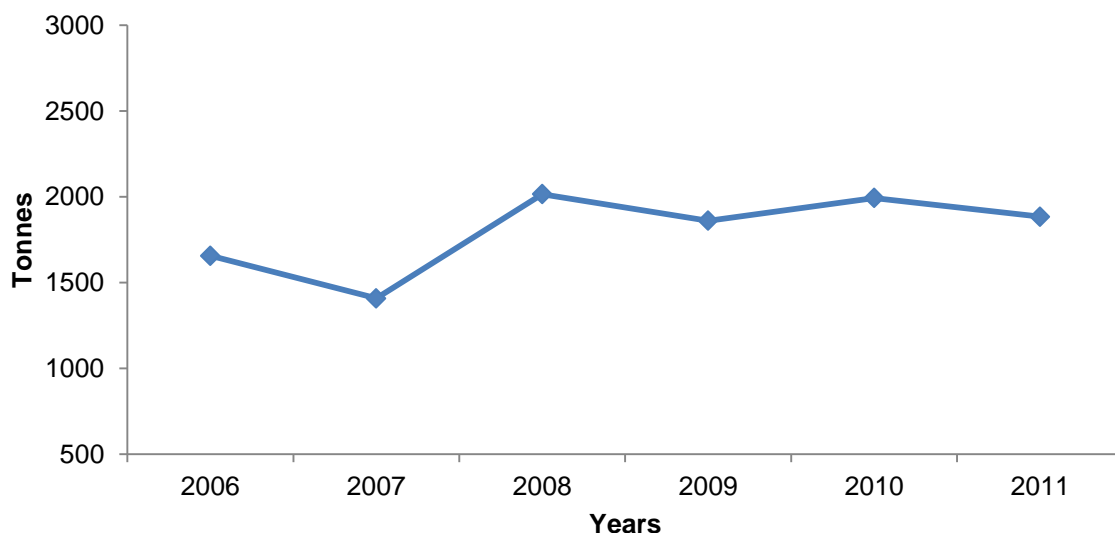


Figure 1.6: South African marine aquaculture production (DAFF, 2013)

1.4.2 Freshwater aquaculture

Freshwater fish culture is limited by the supply of suitable water. Trout is the most cultured freshwater species and its distribution expands across all provinces in South Africa. Tilapias are the second largest cultured fish species in the country with farms located across the country. Ornamental fish farming also plays an important role in freshwater culture after tilapias. Other freshwater species cultivated on a small scale include the African catfish and freshwater crayfish (Figure 1.7). Freshwater culture in South Africa has also increased from 2 000 tonnes in 2006 to almost 3 000 tons in 2011 (Figure 1.8).

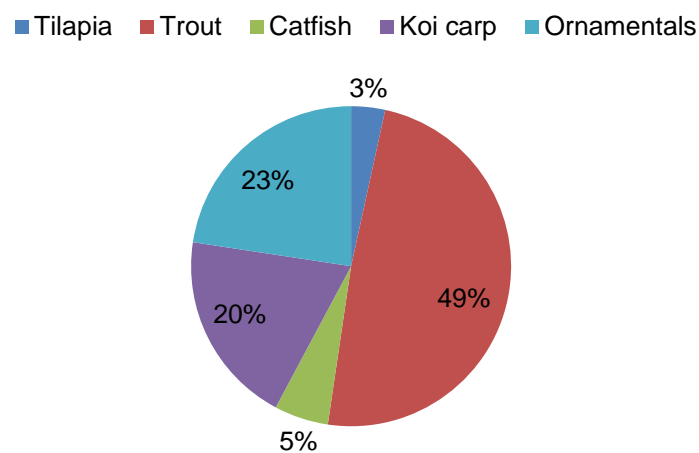


Figure 1.7: Main species contributing to freshwater aquaculture in South Africa in 2011 (Data from DAFF, 2013)

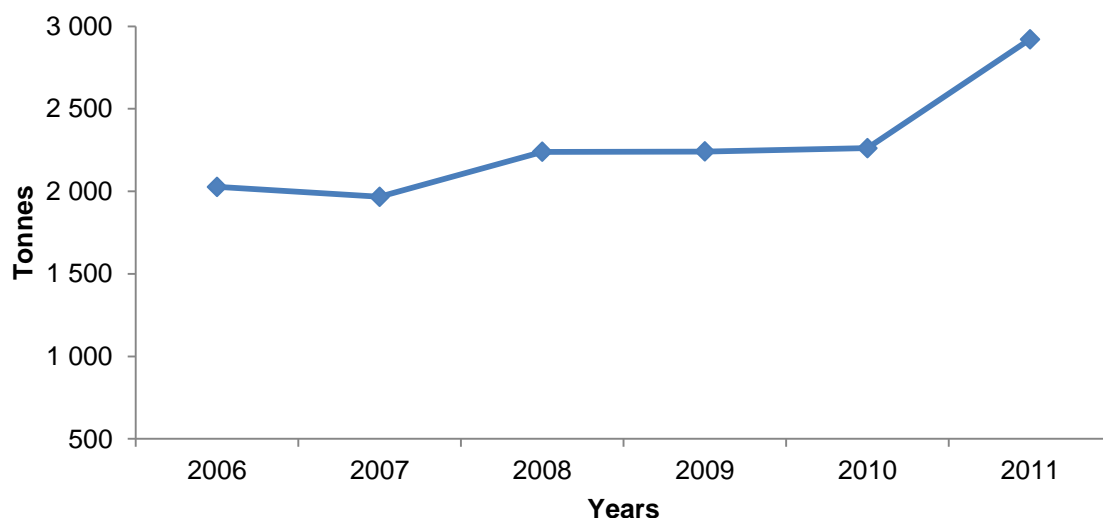


Figure 1.8: South African freshwater aquaculture production (DAFF, 2013)

Trout, the main freshwater fish species produced in South Africa, is an exotic species with high environmental restrictions and requires high water volume and management. This causes limitations in the development of the freshwater sector. Therefore, for this sector to improve, the culture of endemic fish species should be encouraged. South Africa has several freshwater species, however, very little is known about their potential for aquaculture. These species have contributed significantly to fish catches from South African rivers and dams. *Tilapia sp.* is the most important and abundant freshwater fish, that is relatively easy to breed. Another well-accepted and fast growing fish species whose production should be explored is the African catfish (*Clarias gariepinus*). However, these species have no formal market and therefore their market value is currently low.

1.4.1 Efforts to increase freshwater aquaculture

The Department of Agriculture Forestry and Fisheries (DAFF) in South Africa has recently finalised the Aquaculture Research and Technology Development Programme (ARTDP). This programme was developed to provide a strategic framework for aquaculture research and technology development in South Africa. The programme has two divisions (industry diversification and sustainable production) and focusses on creation and development of new knowledge and technology. The industry diversification division comprises of six programmes, namely; nutrition and feed development, new species, genetics, production systems, technology transfer and pilot/demonstration projects as well as marketing and post-harvest technology. The sustainable production division focusses on environmental interactions, food safety and animal health and welfare. According to DAFF report (2012), the ARTDP will fast-track development of an economically and environmentally sustainable aquaculture industry in South Africa.

Recent research efforts to increase freshwater production in South Africa include the use of an old age farming tradition to increase natural feed production in ponds through the addition of manures (Rapatsa and Moyo, 2013). These authors reported that chicken manure increases primary production in ponds and this could be an important diet for the filter feeding *Oreochromis mossambicus*. In another study, these authors also determined the effect of effective microbes (EMs) on primary production and fish health and reported that the mechanism of EMs was

unpredictable and at high doses, stress related histological alterations were observed in *Oreochromis mossambicus* (Rapatsa and Moyo, 2013). These authors however, only investigated ways of increasing primary production and this cannot provide adequate nutritional support especially in semi-intensive or intensive culture as maximum growth is restricted by insufficiency of nutrients from primary production (Edwards *et al.*, 2000). Improved production is only possible through provision of supplementary feed to sustain the increased demand for nutrients. It is therefore important to find cheaper sources of supplementary feeding to promote growth.

1.5 Challenges facing the aquaculture industry

A review conducted by the FAO (2012) shows that Sub-Saharan countries are lagging behind in aquaculture development, mainly due to the lack of a well-developed aquaculture fish feed production sector. In addition, the aquaculture industry in South Africa has limited technical expertise, unlike terrestrial livestock, fish are difficult to culture because knowledge of water chemistry is required and diseases and stress are only noticed once the fish start to die and spread quickly in the aquatic environment. Physical limitations including water scarcity, temperature required for optimal growth also contribute to the poor development of this sector. Furthermore, there is need for accurate information regarding markets so that realistic business models can be developed. At present, little is known about the regional or local market in most Southern African countries.

Feed costs represent one of the largest operating cost of most aquaculture operations (El-Sayed, 2006). Aquaculture is no different from any other terrestrial farming activity in that production is totally reliant upon the provision and supply of nutrient inputs (Tacon and Metian, 2008). Therefore, the supply of feed inputs will have to increase for this sector to maintain its average growth of 8-10 percent per year. Feed inputs may include the use of industrially compounded aquafeeds, farm-made aquafeeds, or the use of natural food organisms of high nutrient value. Diets in aquaculture are principally based on conventional feed-stuffs such as fish oils and fishmeal (Goddard, 1996). Traditionally, fishmeal has been used as the main protein source in fish diets because of its well-balanced amino acid profile, adequate fatty acids, high levels of essential vitamin and mineral composition, high palatability and high digestibility (Nguyen *et al.*, 2009). Fishmeal has been reported to offer major

benefits to fish health, including improved immunity against disease, high survival, increased growth performance and reduced incidences of deformities (FAO, 1986). As a result, aquaculture has been utilising most of the global fishmeal produced. The amount of fishmeal used in aqua feeds has grown, rising from 1.87 to 3.73 million tonnes between 1995 and 2008. This increase has not been supported by the increase in the production of fishmeal from capture fisheries. On the contrary, this growth was sustained by a decrease in the use of fishmeal in other sectors, such as poultry and pig feeds. By the year 2010 aquaculture was using 63 percent of the global fishmeal produced (Figure 1.9). The current global production of fishmeal, however, is decreasing at an average of 1.7 percent (Tacon *et al.*, 2011).

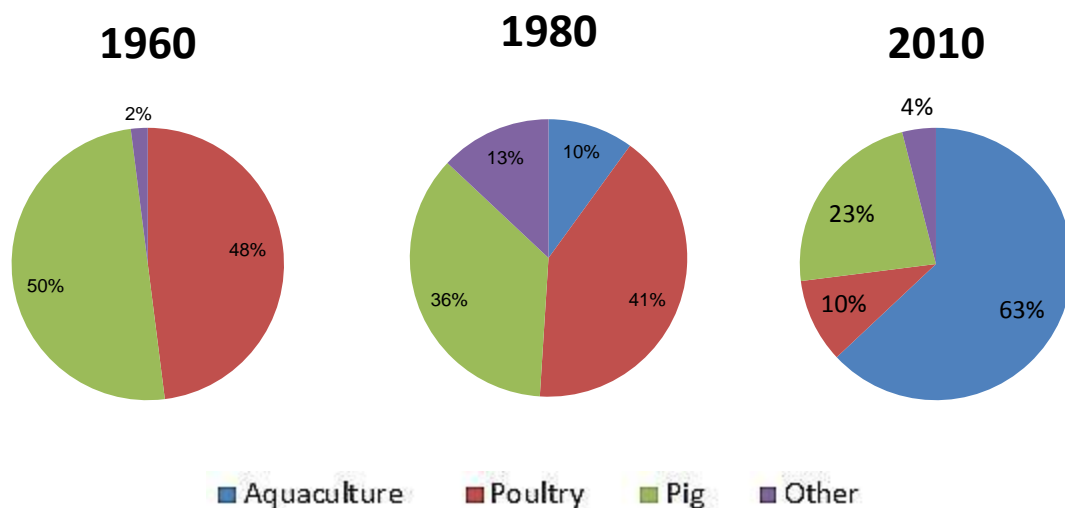


Figure 1.9: World fishmeal consumption between 1960 and 2010. Data obtained from IFFO (2011)

The aquaculture feed production sector is also the largest consumer of global fish oil. By the year 2010, this sector was using 88 percent of fish oil (Figure 1.10). This dependence upon fish oil produced from wild fish as the main lipid source in aquafeeds is also a major concern faced by the aquaculture industry. Thus, fish oil replacement is critical for a sustainable aquaculture production (SOFIA, 2006; Tacon, 2004).

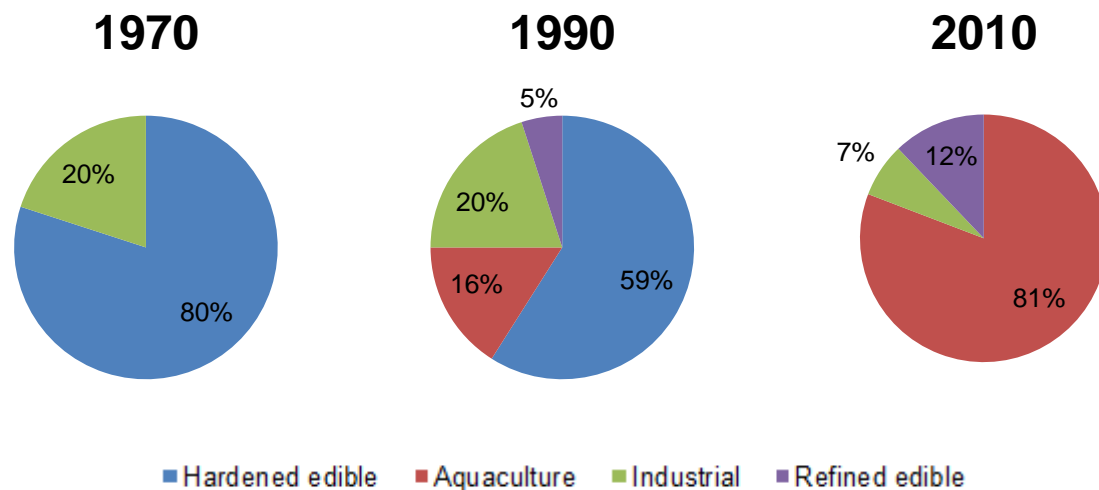


Figure 1.10: World fish oil consumption between 1970 and 2010. Data obtained from IFFO (2011)

Fluctuations in the availability of fishmeal are linked to variations in capture landings of fish used for fishmeal production. Overfishing and unsustainable fishery management have caused some of the variation, but the large deviations are mainly due to the El Niño phenomena (Sintayehu *et al.*, 1996). This has resulted in continuous increases in the price of fishmeal, negatively affecting the profitability of aquaculture enterprises (Sintayehu *et al.*, 1996). The limitations in the availability of feed resources have forced the aquaculture industry to search for alternative sources of protein to use as fishmeal substitutes in aquafeeds.

Efforts to find sustainable protein sources for aquafeeds

For this sector to maintain its current average growth rate of 8-10% per year, the supply of feed inputs (especially protein sources) will have to grow at a similar rate. The decrease in global production of fishmeal illustrates that the sustainability of the aquaculture sector will depend on sustained supply of suitable proteins for aquafeeds. The irrefutable fact is that the wild feed resource is finite. If aquaculture production is to continue to grow, the industry needs to focus on the use sustainable alternatives and supplements to fishmeal and fish oil. It is important for fish nutritionists to identify alternative dietary ingredients that will reduce the amount of fishmeal and fish oil contained in aquaculture feeds while maintaining the important human health benefits of farmed fish.

Finding alternatives to fishmeal is important not only in sustaining aquaculture production but also for reducing aquaculture operation costs. Alternatives to fishmeal are available from plant and animal protein sources as well as single-celled proteins such as microalgae, bacteria and yeast. Some alternative animal protein sources that have shown potential as fishmeal substitutes include meat and bone meal (El-Saidy and Gaber (2003), poultry by-product meal (primarily from the chicken industry) and feather meal (Stickney, 2009). In spite of the promising results obtained, there is public concern with the use of animal by-products in fish feeds. This is based on evidence that diseases such as Bovine Spongiform Encephalopathy (BSE) commonly known as a mad-cow disease and prion may be introduced in the consumer food chain (Naylor *et al.*, 2009; Stickney, 2009; Davies and Gouveia, 2010). Even though microbial and algal species are considered innovative protein sources for aquafeeds, high production costs limit their use (FAO, 2012). Therefore, research on aquafeed development has mainly focused on plant protein sources in the last two decades.

Plant proteins are a good alternative source for fishmeal because they are readily available worldwide at low cost (Dersjant-Li, 2002 and Naylor *et al.*, 2009). Plant proteins represent the major dietary protein source used within feeds for lower trophic level fish species such as tilapias, carps and catfish. They are the second major source of dietary protein and lipid sources after fishmeal and fish oil for shrimps and European higher trophic level fish species (Tacon *et al.*, 2011).

Many studies have been conducted to evaluate the replacement of fishmeal in practical fish diets with cheap, locally available plant sources. These include; terrestrial plant seeds such as soybean (Lin and Luo, 2011), a combination of soybean and cottonseed (El-Saidy and Saad, 2011), a combination of soybean, sunflower, and cottonseed, (Chebbaki *et al.*, 2010), rapeseed meal (Davies *et al.*, 1990). As well as aquatic plants such as duckweed (Chowdhury *et al.*, 2008) and *Azolla spp* (Fiogbe, *et al.*, 2004), plant leaves; leucaena leaf meal (Osman, *et al.*, 1996), cassava leaf meal (Ng and Wee, 1989) and leaf protein concentrate such as Rye grass and alfalfa leaf protein concentrate (Olvera-Novoa *et al.*, 1990). The replacement of fishmeal in practical diets without reducing the performance would result in more profitable production. Therefore, plants with high protein levels are

preferred because dietary protein affects the growth performance in fish (Musuka *et al.*, 2009).

The search for alternatives to fishmeal has largely been focused on conventional sources such as oil seed cakes and meals due to their higher protein content compared to leaf meals (Table 1.1). Some plant protein sources such as soybean and sunflower have been commercialised and have multiple uses in both human consumption and other terrestrial animal feeds. Their use may not be sustainable in some countries where it would have to be imported to meet demand and this increases production costs. Thus, there is a need to evaluate more readily available and locally produced sources of protein. Exploring a wider variety of plant protein resources to be used in aquafeeds will provide the basis for a more economic feed.

Table 1.1: Gross nutritional composition of some ingredients used in fish feeds (%)

Ingredients	Nutritional composition				
	Protein	Lipid	Carbohydrates	Fibre	Ash
Fishmeal	60-75	5-20	1-4	1-3	10-25
Oil seed meal and cakes	20-60	1-15	20-55	4-20	6-15
Leaf meals	11-30	2-7	38-65	10-20	4-19
Cereals	5-14	1-9	75-80	2-10	2-5
Cereals by products	6-17	4-15	34-65	10-30	4-18
Root crops	2-10	1-3	70-90	1-4	2-6

Source: Hossain (1996)

It is clear from the preceding sections that the lack of affordable fish feeds is one of the major constraints in the development of the aquaculture industry in South Africa and the rest of the world. Accordingly, in the current study two plant protein resources were tested as possible replacements for fishmeal in aquafeeds. *Pennisetum clandestinum* (kikuyu grass) and *Moringa oleifera* (moringa) leaves have been selected because of the high protein content in the leaves and wide availability in South Africa. Previous studies on the use of kikuyu grass as a protein source in *Tilapia rendalli* diets indicated that fresh kikuyu grass was preferred over other plant sources and readily digested by *Tilapia rendalli* (Hlophe and Moyo, 2011). The grass

was also explored as a fishmeal substitute in *Tilapia rendalli* diets. Hlophe *et al.* (2011) reported that this grass (26% protein) can be used to replace up to 20% of fishmeal without any significant effect on growth performance of *Tilapia rendalli*. Higher inclusion levels of kikuyu (above 20%) resulted in reduced performance. This was attributed to the presence of anti-nutritional factors in the grass. However, Hlophe *et al.* (2011) did not identify or destroy the anti-nutritional factors that may be present in the grass. There is need therefore, to identify the anti-nutritional factors present in kikuyu grass and to explore its possible use in other commonly cultured fish species in South Africa.

Moringa, also known as the miracle plant, is a member of the family Moringaceae. It is fast growing and widely available in the tropics and subtropics with great economic importance for the food and medical industry (Foidl *et al.*, 2001). Moringa has been used traditionally to cure a number of diseases and it is a source of many vitamins, minerals and antioxidants (Foidl *et al.*, 2001). The leaves are rich in carotenoids, ascorbic acid and iron (Makkar and Becker, 1997). Moringa is widely grown in many parts of South Africa. The leaves and pods are recognised as a food source for humans and a dry season feed for animals (Makkar and Becker, 1997). In recent years, there has been increased hype in the use of moringa in South Africa and the government has encouraged rural farmers to grow moringa in a bid to combat malnutrition.

Freshwater fish culture in South Africa is dominated by three endemic species: *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus*. These fish species have different natural feeding habits that may affect their ability to utilise plant protein resources in their practical diets. *Tilapia rendalli* adults generally feeds on macrophytes (Meschiatti and Arcifa, 2002; Weljange *et al.*, 2006; Hlophe and Moyo, 2011). *Oreochromis mossambicus* is a microphagous effective algal feeder (Doupé *et al.*, 2010; El-Sayed, 2006). *Clarias gariepinus* is a benthopelagic, predatory omnivore, feeding on a wide variety of items from small fish to detritus (Groenewald, 1964; Dadebo, 2000; Skelton, 2001; Rad *et al.*, 2004; Kadye and Booth, 2012). Improving the culture of these well-accepted fish species is the key to the development of the freshwater aquaculture industry in South Africa. Efforts to

improve fish culture should focus on the development of good quality, low cost feeds to meet the nutritional requirements of the each fish species.

For a plant protein source to be included in aquafeeds, its utilisation should be tested in different fish species because fish species differ in their sensitivity and response to anti-nutrients found in plant protein sources (Francis *et al.*, 2001; Chong *et al.*, 2002; Gatlin *et al.*, 2007). It is important that plant ingredients be tested for utilisation by each fish species before inclusion in its diet. Several studies have shown that the ability of fish to utilise plant diets differs from species to species (Collins *et al.*, 2012; Chaudhuri *et al.*, 2012). The utilisation of dietary nutrients is dependent upon efficient digestion especially the production of relevant digestive enzymes.

The central objective of this research was to determine effect of replacing fishmeal with kikuyu and moringa leaf meals in the diets of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus*. The specific objectives were to determine:

- The digestive capabilities of these fish species.
- To determine the inclusion levels of the leaf meals for optimal productivity
- The effect of exogenous enzyme supplementation on productivity as well as determine the supplementation level for optimum productivity.

1.6 Thesis layout

The efficacy of kikuyu grass and moringa leaves to replace fishmeal in the diet of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* was assessed. This thesis is divided into eight Chapters, each addressing a step in the utilisation of leaf meal-based diet by these fish species.

Chapter 2

In this chapter, relevant literature on morphological adaptations to feeding on plant diets reviewed. Furthermore, literature on the use of different plant protein sources in replacing fishmeal and advances made to improve their utilisation in aquafeeds is explored.

Chapter 3

In this chapter, the digestive capabilities of all the fish species are explored. The type and quantity of digestive enzymes produced are measured and related to each species' ability to effectively utilise plant-based diets.

Chapter 4

This chapter focuses on the utilisation of kikuyu and moringa-based diets by *Tilapia rendalli*. Utilisation is measured by growth performance indices, protein digestibility and the activity of digestive enzymes. Moreover, the effect of the plant diets on histological and haematological parameters is determined.

Chapter 5

Oreochromis mossambicus's ability to utilise kikuyu and moringa-based diets is compared to that of the closely related *Tilapia rendalli*. The differences are highlighted.

Chapter 6

In this chapter, commonly cultured a predatory omnivore in South Africa (*Clarias gariepinus*) is fed the kikuyu and moringa-based diets. Its ability to utilise these plant diets is compared to that of the tilapias.

Chapter 7

In this chapter, the effectiveness of exogenous enzyme supplementation to improve the utilisation of kikuyu-based diets by *Oreochromis mossambicus* was investigated.

Chapter 8

This chapter focuses on the general discussion, summing the findings from the previous chapters and comparing these to other published works. Recommendations on future studies are given and an overall conclusion to the study is presented.

Chapter 9

A complete reference list of all the sources cited in the thesis is given in this chapter.

CHAPTER 2:

LITERATURE REVIEW

2 LITERATURE REVIEW

2.1 The origin and distribution of the Tilapine fish

Tilapia is a common name given to three genus (*Oreochromis*, *Sarotherodon* and *Tilapia*). The name tilapia was an effort by A. Smith (its author), to spell the bushman word for 'fish' which began with a click, rendered 'Til' (Trewavas, 1982). Tilapias are euphemistically referred to as 'aquatic chicken' due to their high growth rates, adaptability to a wide range of conditions, ability to reproduce in captivity and feeding at low trophic levels (El-Sayed, 2006). They are a fresh water group of fish originating exclusively from Africa (excluding Madagascar), the Jordan Valley and the surrounding coastal rivers (Philippart and Ruwet, 1982; Trewavas, 1983). Outside Africa, they are widely distributed in South and Central America, Southern India, Sri Lanka and Lake Kinneret, Israel (Philippart and Ruwet, 1982). Tilapias are currently found in various ecological water systems that vary in depth, alkalinity or salinity, including slow-moving rivers and flood plain pools and swamps, small shallow lakes, large deep lakes, impounded water bodies, isolated crater lakes, sodalakes, thermal springs, volcanic craters, open and closed estuaries and lagoons (Philippart and Ruwet, 1980; Lowe-McConnell, 2000). Although tilapias inhabit a wide range of ecosystems, it is generally assumed that tilapias evolved from a marine ancestor which penetrated fresh water and that this accounts for the large number of euryhaline species (Philippart and Ruwet, 1982).

2.2 Feeding biology of tilapias

The natural geographic range of tilapine fish includes large habitat diversity. This attribute enables effective utilisation of a wide variety of food items, ranging from insect larvae to algae, weeds and macrophytes (Skelton, 2001; El-Sayed, 2006). Young tilapia fry like that of most fish species are largely carnivorous, feeding on zooplankton as well as aquatic larvae and terrestrial insects that fall into the water (Moriarty, 1973; Lowe-McConnell, 1975; Brown and Gratzek, 1980; Trewavas, 1983). As they grow, tilapia fingerlings gradually begin taking more plant foods, including phytoplankton and macrophytes (Moriarty, 1973; Lowe-McConnell, 1975; Bowen, 1982; Trewavas, 1983; Lim, 1989).

Generally, all tilapias are herbivorous. Herbivorous fish are defined as those whose food constitutes more than 50% plant material by weight or volume, at least in some period of their life (Opuszynski and Shireman, 1995). Most tilapia species are opportunistic, changing their diet to whatever food items are abundant or available.

The digestive system of tilapias is relatively simple and unspecialised. Tilapias are equipped with a pharyngeal mill that has bi- and tricuspid teeth situated in the throat, which aid in cutting, tearing and macerating of fibrous plant material (De Silva and Anderson, 1995). This process allows for easier peristaltic mixing and increased exposure to digestive enzymes. Tilapias have a very short oesophagus connected to a thin-walled small sac-like stomach. In the stomach, hydrochloric acid (HCL) is secreted to create an unusually low pH, frequently <1.5 (Bowen, 1976; Perschbacher *et al*, 2010) which facilitates the lyses of prokaryotic and eukaryotic cell walls to expose the cytoplasm to digestive enzymes. Tilapias have long intestines (seven to thirteen times the total fish length) which reflect their herbivorous feeding nature because vegetable foods are digested less readily than animal sources (Caulton, 1976; Balarin and Hatton, 1979; El-Sayed, 2006). Furthermore, herbivorous fish compensate for the shortage of protein in plants by ingesting higher quantities of food.

2.3 The red-breast tilapia: *Tilapia rendalli* (Boulenger, 1897)

Tilapia rendalli has a typically deep body that is laterally compressed, with a convex head (Figure 2.1). It has a protruding mouth with prominent bicuspid teeth. Its body and head are covered with relatively large cycloid scales that are not easily dislodged (Ross, 2000; Skelton, 2001). Mature fish are olive green to brown in colour, and have a bright red chest and throat; hence, it is commonly known as Redbreast tilapia (Skelton, 2001). *Tilapia rendalli* has dark vertical bars. Its caudal fin has a spotted upper half and a red or yellow half. This red or yellow pigment is also present on the anal fin. It has a carmine flush on the lower flanks, behind the pectoral fin and this is not confined to one sex or to mature fish. The long dorsal fin is spiny, the rear is soft rayed, and the anal fins have hard spines and soft rays. The pectoral and pelvic fins are large and anterior in an advanced configuration (Ross, 2000). It attains a total length of 400 mm and weight of up to 2 kg (Skelton, 2001) and may live up to 7 years. *Tilapia rendalli* have well developed sense organs

represented by prominent snares and a clearly visible and interrupted lateral line. The eyes are relatively large, providing the fish with an excellent visual capability (El-Sayed, 2006). Figure 2.2 represents a global distribution of *Tilapia rendalli*.



Figure 2.1: Adult *Tilapia rendalli*

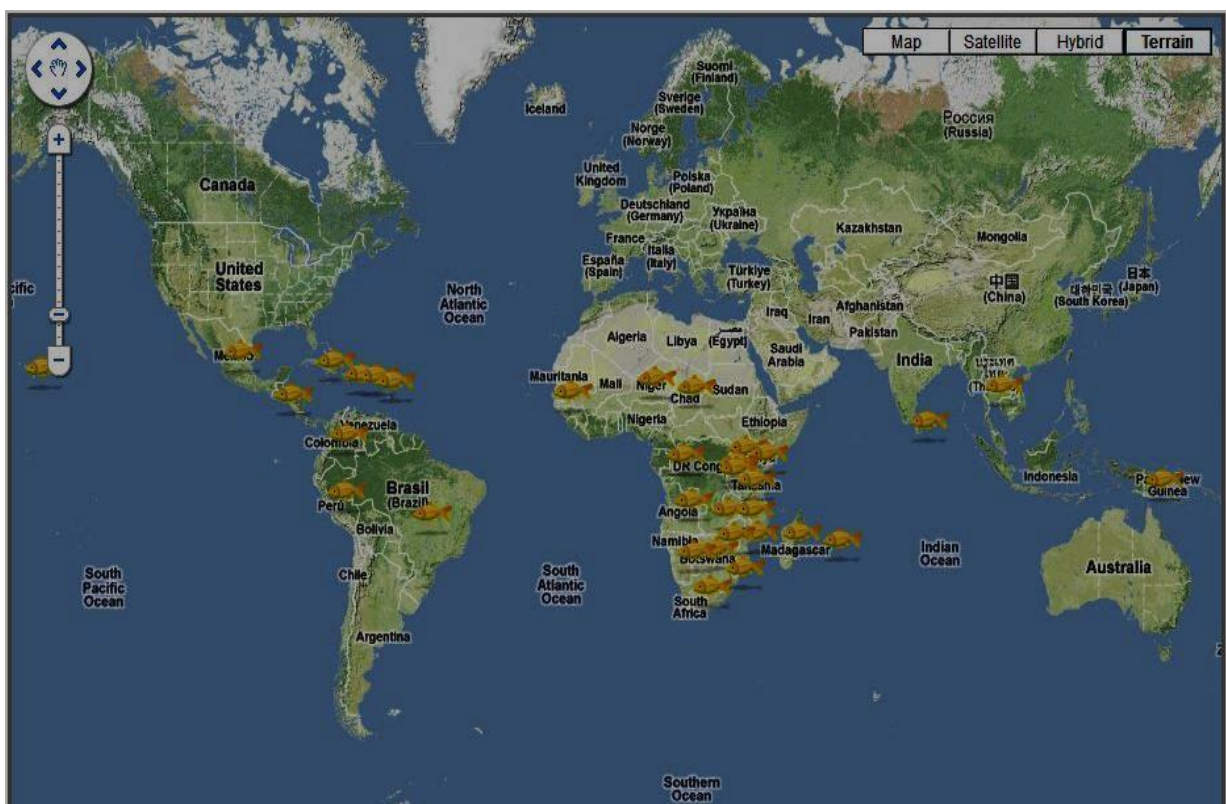


Figure 2.2: The distribution of *Tilapia rendalli*

2.3.1 Adaptation to feeding on plant diets

Tilapias of the genus *Tilapia* tend to take coarser food including macrophytes and are often used to control weed growth in irrigation channels, ponds and dams (Abdel-Malek, 1972; Kenmuir, 1973; Caulton, 1976; Lowe-McConnell, 1982). The juveniles generally feed on plankton and adults on macrophytes (Bowen, 1982). *Tilapia rendalli* has been described as an opportunistic feeder because of its ability to change its feeding habits in the absence of a desired diet. *Tilapia rendalli* feeds on submerged marginal vegetation when macrophytes are not present. Studies by Batchelor (1978) indicate that *Tilapia rendalli* feed almost exclusively on inundated marginal vegetation when present.

The ability of *Tilapia rendalli* to digest plant protein has not been fully exploited, as tilapia culture is often focussed on the faster growing *Oreochromis* species. The few studies conducted on *Tilapia rendalli* are often carried out in semi-intensive low cost systems. Mataka and Kang'ombe (2007) conducted an experiment to determine the effect of substituting maize bran with chicken manure on the production of *Tilapia rendalli* juveniles in semi-intensive pond culture. They reported a higher specific growth rate (SGR) in ponds where 75% maize bran and 25% chicken manure was applied than in ponds where only maize bran was fed. In another study, Soko and Likongwe (2002) also reported that the addition of chicken manure increased the specific growth rate (SGR) in *Tilapia rendalli* fed maize bran in ponds. Experiments have been done with success in feeding *Tilapia rendalli* with Napier grass (*Pennisetum purpureum*). Chikafumbwa (1996) concluded that feeding *Tilapia rendalli* with Napier grass was an effective low cost feed for African small holder farmers and that supplementary feeding was not necessary when *Tilapia rendalli* was fed Napier grass in pond culture. In a recent study, *Tilapia rendalli*'s ability to utilise plant protein was evident when fed kikuyu grass, cabbage, duckweed and vallisneria, *Tilapia rendalli* was able to breakdown some of the cell wall and attain high digestibility values (Hlophe and Moyo, 2011). In a study conducted by Micha *et al.* (1988), *Tilapia rendalli* outperformed *Oreochromis niloticus* when fed *Azolla microphylla* only. Although there was 10% mortality in *Oreochromis niloticus*, all the *Tilapia rendalli* survived. This suggests that even though *Tilapia rendalli* does not grow as fast as tilapias of the *Oreochromis* genus, it may be the solution to the

current problem of high feed costs in aquaculture because of its ability to utilise higher plants.

From the above studies, *Tilapia rendalli's* ability to utilise plant diets in nature is irrefutable. However, no work has been done on the use of prepared leaf meal-based diets in the culture of *Tilapia rendalli*. The ability of *Tilapia rendalli* to feed on plant-based diets makes it a good candidate for aquaculture because they can be fed on plant diets that are cheaper and readily available compared to fishmeal. Thus, one of the key objectives of this study was to determine the ability of this fish species to utilise kikuyu and moringa leaf meal-based protein.

2.4 The Mozambique tilapia: *Oreochromis mossambicus* (Peters, 1852)

Oreochromis mossambicus (Figure 2.3) is characterised by a moderately deep body with a straight head profile in juveniles and females. Mature males develop a concave head profile with enlarged jaws and teeth that are projected forward. A single dorsal fin is present and the anal fin has only three spines (Van der Elst, 1999; Skelton, 2001). Juveniles have a silvery colour with six to seven vertical bars and three spots along the flanks. Adults have a silvery olive to deep blue-grey colour with red margins along the dorsal and caudal fins. Breeding males turn deep greyish black with a distinct white colour around the lower head and throat region and flame red dorsal and caudal fin fringes (Van der Elst, 1999; Skelton, 2001). *Oreochromis mossambicus* has a wide distribution (Figure 2.4).



Figure 2.3: Adult *Oreochromis mossambicus*

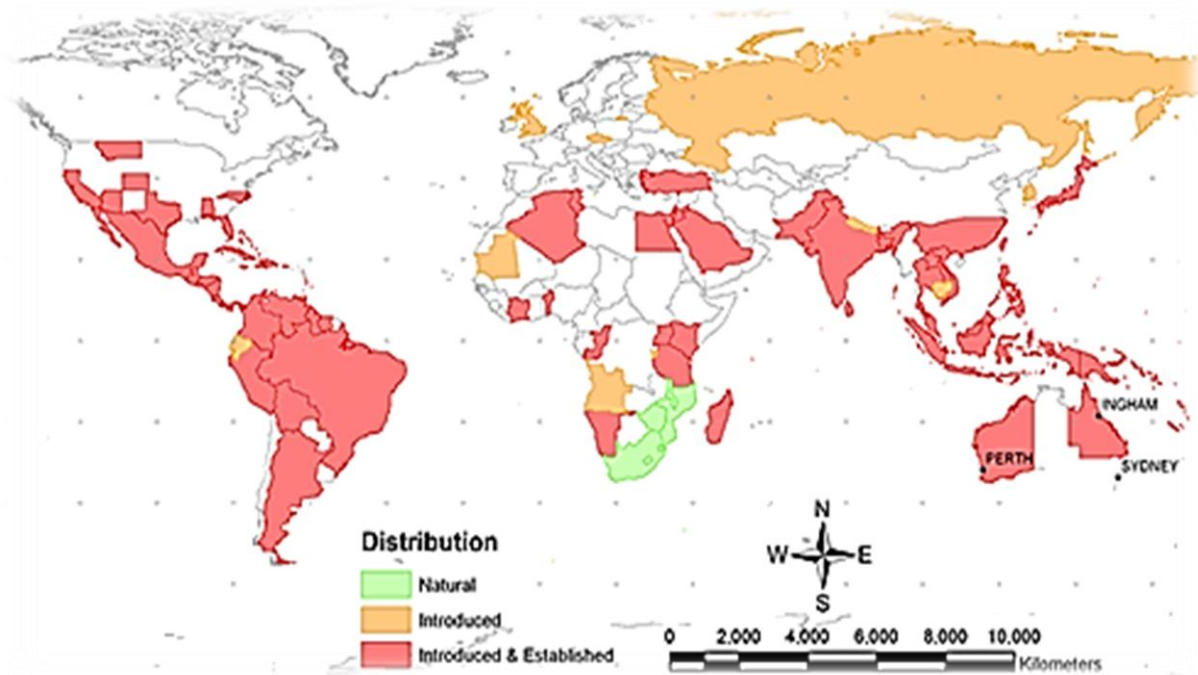


Figure 2.4: *Oreochromis mossambicus* distribution

2.4.1 Adaptation to feeding on plant diets

Oreochromis mossambicus is a microphagous feeder, feeding predominantly on phytoplankton; particularly diatoms (Skelton, 2001), adults also eat aquatic insects (Kotze *et al.*, 1999; Van der Elst, 1999; Skelton, 2001). Several authors have described *Oreochromis mossambicus* as an algal feeder (El-Sayed, 2006; Doupé *et al.*, 2010). Earthworms, small fish, even bottom sledge rich in organic matter all form important dietary components for *Oreochromis mossambicus* (Kotze *et al.*, 1999).

As previously stated, *Oreochromis mossambicus* dominates tilapia production in South Africa. It has a high growth rate after *Oreochromis niloticus* whose production in South Africa is prohibited. *Oreochromis mossambicus* being a herbivore is also pre-adapted to utilising plant-based diet and has evolved both morphological and physiological adaptations to aid in ingestion and mastication of plant material. Its culture however is mainly based on fishmeal as the main protein source. Its ability to utilise plant diets is not fully exploited, despite reports of low stomach pH values (Perschbacher *et al.*, 2010) enabling it to breakdown cell wall in blue-green algae. It was therefore necessary to determine the ability of this microphagous herbivore to utilise kikuyu and moringa leaf meal-based protein in its diet.

2.5 The origin and distribution of the clariid catfish

Catfish of the genus *Clarias* are broadly distributed in Africa and Asia. A number of species of the genus *Clarias* and their hybrids are cultured, mainly because of their fast growth rate, disease resistance and amenability to high-density culture (Haylor, 1993; Huisman and Richter, 1987). The main cultured species in this group are *Clarias gariepinus*, *Clarias batrachus*, *Clarias macrocephalus*, and *Clarias agullaris*.

A characteristic feature of clariid catfish is their ability to breathe atmospheric air and tolerate low dissolved oxygen levels (Bruton, 1979). This is an important factor in aquaculture as atmospheric air contains approximately 30 times more oxygen per unit volume than water. *Clarias gariepinus* can reach over 130 cm in length and 12.8kg in weight (Skelton, 2001). *Clarias gariepinus* is a good candidate for aquaculture because of its ability to feed on a variety of food items, its fast growth rate, a high degree of hardiness and survival in poorly oxygenated waters. *Clarias gariepinus* has a sedentary life style and inhabits a variety of habitats from temperate to tropical streams, rivers, pans, underground sinkholes, swamps, shallow and deep lakes (Uys, 1989), submerged rice-fields, ponds and impoundments (Potts *et al.* 2008).

2.5.1 The African catfish: *Clarias gariepinus* (Burchell, 1822)

Clarias gariepinus (Figure 2.5) has a bony elongated body with no scales. It is recognised by long dorsal and anal fins, which give it a rather eel-like appearance. *Clarias gariepinus* is equipped with an air-breathing labyrinthic organ arising from gill arches. The gills have wide openings and the first gill arch has 24 to 110 gill rakers (Skelton 2001). Its large helmet-like head is depressed and has small eyes, a broad terminal mouth with four pairs of prominent barbels (Figure 2.5). It has long spineless dorsal and anal fins and an adipose fin. Its caudal fin is rounded, and its colour varies dorsally from dark to light brown and is often mottled with shades of olive and grey while the underside is a pale cream to white (Skelton 2001). *Clarias gariepinus* can grow to a very large size with a maximum reported length of 170 cm (IGFA, 2001) and weight of 60 kg (Robbins *et al.*, 1991).



Figure 2.5: *Clarias gariepinus*

Clarias gariepinus occurs naturally in Israel, Syria and southern Turkey and Africa. It is possibly the most widely distributed fish in Africa (Daget *et al.*, 1984; Skelton, 2001). According to Picker and Griffiths (2011), the native range of *Clarias gariepinus* covers most of the African continent (Figure 2.6), making it an important aquaculture fish species in South Africa.

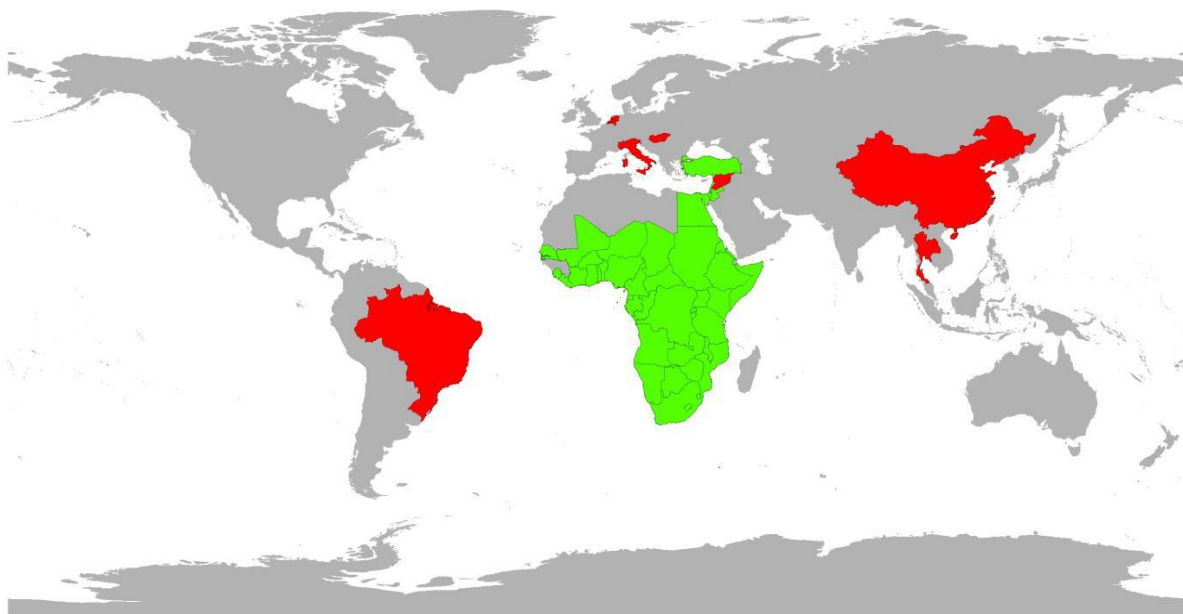


Figure 2.6: Native (green) and introduced (red) ranges of *Clarias gariepinus* globally (Data source: GISD, 2012)

2.5.1.1 Digestive morphology and feeding biology of *Clarias gariepinus*

Clarias gariepinus is equipped with bands of fine, sharp teeth on the pre-maxillary and dentary bones that enable it to grasp its prey and prevent its escape. The vomerine and pharyngeal tooth bands also perform this function but also serve to

incapacitate the prey through crushing (Skelton, 2001). The teeth on the vomerine band may perform some mastication, but large prey is swallowed whole. The oesophagus is short and dilatable serving as a passage to the highly expandable stomach. The stomach is differentiated into corpus and pyloric regions. The stomach is muscular and can crush and tumble the food to facilitate digestion (Bruton, 1979). The intestine is simple, fairly short and thin walled. The walls of the terminal portion of the intestine (rectum) are slightly thicker and more muscular than the rest of the intestine. These morphological adaptations indicate a dependency on animal diets.

In its natural range, *Clarias gariepinus* is a predatory omnivore that feeds on fish, invertebrates, plant material, plankton, reptiles, and amphibians (Munro 1967; Bruton 1979; Merron 1993; Winemiller and Kelso-Winemiller 1996). Larvae are almost exclusively dependent on zooplankton for the first week of exogenous feeding. *Clarias gariepinus* is nonetheless regarded primarily as a piscivore. Several authors reported that juvenile *Clarias gariepinus* feed in decreasing order of preference on insects, crustacea, molluscs, detritus and plankton. Sub-adults and adults feed predominantly on fish (Bruton, 1979; Mbewaza-Ndawula, 1984; Uys, 1989). Predation is most efficient on relatively slow moving bottom-living organisms but fast prey, such as fish, can be caught using pack-hunting tactics. *Clarias gariepinus* can vary its food according to availability (Clay, 1979) and is considered an opportunistic omnivore (Uys, 1989). This makes *Clarias gariepinus* a good candidate for aquaculture as it can be fed on available feed resources. Furthermore, it is the most widely cultured warm water fish in South Africa (DAFF, 2012).

2.6 The use of plant protein sources in replacing fishmeal in aquafeeds

The rapid development of the aquaculture industry has been accompanied by rapid growth of aquafeed production. Fishmeal-based diets are particularly important in the early stages of development because of the high-protein content, excellent amino acid profile, high-nutrient digestibility and no anti-nutrients (Rumsey, 1993; Glencross *et al.*, 2007; Barrows *et al.*, 2008). The decline in global fishmeal production necessitates a growing and urgent need to find substitutes to fishmeal in aquafeeds.

Plant proteins are a good alternative source for fishmeal because they are readily available worldwide at low cost (Dersjant-Li, 2002 and Naylor *et al.*, 2009). Plant proteins are the second major source of dietary protein and lipid sources after fishmeal and fish oil in aquafeeds (Tacon *et al.*, 2011). Among the most promising alternative protein sources are varieties of grain legumes, pulses and cereals as reviewed by Gatlin *et al.* (2007); Hardy (2010); Enami (2011). Several studies have shown that plant proteins (soybean meal, sunflower, corn gluten-meal, wheat, maize, yeast, maize gluten, detoxified jatropha kernel meal and wheat gluten meals) could be incorporated up to 50% in diet of omnivorous fish species without affecting fish performance (Pongmaneerat *et al.*, 1993; Escaffre *et al.*, 1997; Mazurkiewicz, 2009; Kumar *et al.*, 2011; Marković *et al.*, 2012).

Soybean meal is the most commonly used plant protein source to partially or completely replace fishmeal in diets in fish feeds because of its high protein content and digestibility, relatively well-balanced amino acid profile and steady supply. However, increasing demand for soybean meal for both human consumption and animal feed has resulted in the increase in the global price of soybean meal (Kumar *et al.*, 2011). In addition, the production of soybeans in some countries such as South Africa is low and importing it, is increasingly expensive. There is a need to explore the use of non-conventional feed sources as protein sources in aquaculture diets that have the capacity to yield the same output as conventional feeds, and perhaps at cheaper cost. One possible source of cheap protein is the leaf meals. Leaf meals are not only a good protein source but also provide some essential vitamins such as vitamins A and C, minerals and oxycarotenoids.

2.7 Utilisation of leaf meals in fish diets

Several leaf meals have been studied with respect to their suitability as protein sources in fish diets. These include; leucaena, *Leucaena leucocephala* (Pantastico and Baldia, 1980; Ferraris *et al.*, 1986; Santiago *et al.*, 1988), sesbania, *Sesbania sesban* (Hafez *et al.*, 2000), mulberry, *Morus spp* (Vijayakumar Swamy and Devaraj, 1995), alfalfa, *Medicago sativa* (Yousif *et al.*, 1994), acacia, *Acacia auriculiformis* (Mondal and Ray, 1999), papaya, *Carica papaya* (Eusebio and Coloso, 2000), water hyacinth, *Eichornia crassipes* (Hasan and Roy, 1994), duckweed, *Lemna polyrhiza* (Bairagi *et al.*, 2002), duck lettuce, *Ottelia alismoides*, water snowflake, *Nymphoides*

indicum (Patnaik *et al.*, 1991) and peanut *Arachis hypogaea* (Garduno-Lugo and Olvera-Novoa, 2008). In most of these studies, the leaf meals could only replace \leq 25% of fishmeal protein. Higher replacement levels led to poor palatability (Hassan *et al.*, 1997), poor nutrient utilisation (Eusebio *et al.*, 2004), poor growth (Eusebio and Coloso, 2000), pathological lesions (Hafez *et al.*, 2000) and/or poor reproductive performance (Santiago *et al.*, 1988). These studies show that the use of leaf meals in fish feeds is possible, but limited by the presence of anti-nutritional factors and low protein levels. It is against this background that two plant protein resources (kikuyu grass and moringa leaves) were selected in this study.

2.7.1 Kikuyu grass (*Pennisetum clandestinum*)

Kikuyu grass (*Pennisetum clandestinum*) is a creeping perennial grass that possesses strongly noded stolons above ground and rhizomes below ground (Quinlan *et al.*, 1975). Kikuyu grass originates from East Africa (Zaire and Kenya) from a region that is home to the Kikuyu tribe and from the Ngorongoro Crater, which has natural grasslands of *Pennisetum clandestinum* (Skerman and Riveros, 1990). It was introduced in South Africa in 1909 (Stapf, 1921). Its ease of cultivation, high growth rate, drought resistance and thickly matting habit, make this an excellent lawn grass. Kikuyu is significantly more frost tolerant than other commonly grown tropical grasses in the sub-tropics (Quinlan *et al.*, 1975). The advantage of kikuyu grass over most other tropical grasses is its ability to grow well in autumn with minimum deterioration in forage quality and retains its nutritive levels in the dry season (Mears and Humphreys, 1974). Kikuyu grass is highly productive, yielding up to 30 000 kg/ha of dry matter (Cross, 1978). This grass has relatively high protein content and a good amino acid profile (Marais, 2001; Flukerson *et al.*, 2007; Hlophe and Moyo, 2011).

Kikuyu is one of the most important summer pasture species in South Africa (Miles *et al.*, 1995). It is recognised as an important pasture for increased milk yields in dairy cattle (Malleon *et al.*, 2009), increased growth rates in beef cattle (Louw and Bartholomew (1998) and in lambs De Villiers (1998). However, there is paucity of information on the utilisation of kikuyu grass meal as a protein source in formulated diets for fish.

2.7.2 Moringa (*Moringa oleifera* Lam)

Moringa (Moringa oleifera Lam) belongs to the moringaceae family and is indigenous to Northern India and Pakistan (Bosch, 2004; Makkar and Becker 1997). *Moringa* is now widely cultivated in the tropics (Fahey *et al.*, 2001). There are thirteen species of moringa trees in the family moringaceae and *Moringa oleifera* is the most widely cultivated species (Kristin, 2000). *Moringa* is also known as the miracle tree. It has been used traditionally to cure a number of diseases (Foidl *et al.*, 2001). *Moringa* leaves have quality attributes that make it a potential replacement for fishmeal in aquafeeds. The leaves have high protein content (28-36%), are rich in carotenoids, ascorbic acid and iron (Makkar and Becker, 1997). In recent years, this tree has been promoted as an outstanding indigenous source of highly digestible protein, calcium, iron and vitamin C for humans, suitable for utilisation in many developing countries including South Africa where undernourishment is a major concern (Fahey *et al.* (2001). As a result, there has been an upsurge of interest in moringa cultivation and consumption in South Africa and many health claims have been reported. The government has encouraged farmers to grow moringa trees in a drive to decrease malnutrition in many rural areas of South Africa.

An appreciable amount of work has been done on the use of moringa leaf meal as a replacement for fishmeal in fish diets (Madalla, 2008; Afuang *et al.*, 2003; Richter *et al.*, 2003). Another advantage of using moringa as a protein resource in fish feeds is that it is a perennial plant that can be harvested several times in one growing season.

Efficient utilisation of these plant diets depends on the fish's ability to breakdown, digest and absorb dietary nutrients. To ensure effective utilisation, it is important to identify anti-nutrients in both kikuyu and moringa leaves and develop strategies to alleviate their effect. Previously, most researchers concentrated on ways to destroy anti-nutrients in plant diets. However, this usually resulted in substantial loss of essential nutrients (Madalla, 2008). Therefore, it is important to find ways of improving the utilisation of plant-based diets by enhancing the digestive capability in fish. Digestion and the subsequent utilisation of food in fish depends on the following three main factors (1) the ingested food and the extent to which it is susceptible to

the effect of the digestive enzymes (2) the activity of the digestive enzymes (3) the length of time the food is exposed to the action of digestive enzymes (Hepher, 1988).

2.8 Enzyme activity

Digestive enzymes are one of the most important factors that influence the efficiency of feed utilisation in fish and characterisation of these enzymes provides some information regarding the digestive capacity of fish to hydrolyse carbohydrate, protein and lipid of feed ingredients (Essa *et al.*, 2010; Lemieux *et al.*, 1999). An investigation of the digestive secretions in fish may clarify some aspects of their nutritive physiology and help optimise the utilisation of dietary nutrients by different fish species.

Studies on the relationship between feeding habits and enzyme activities in fish are fragmentary and contradictory. There are two schools of thought on the factors affecting enzyme activity; some authors argue that enzyme activity is principally a function of diet (Saha and Ray, 1998) while others maintain that it is largely affected by phylogeny (Chan *et al.*, 2004; German *et al.*, 2004; Chaudhuri *et al.*, 2012). Most of these studies, however, looked at enzyme activities of wild-caught fish feeding on a wide range of uncontrolled food items, thus making it difficult to compare between different species or conclude if enzyme activities are controlled by phylogeny or by diet.

Alpha amylase is produced in the pancreas and has been identified in pancreatic juice, the stomach and intestines. In general, the activity of amylase differs from species to species and appears to be related to their feeding habits. Amylase, which is needed for the hydrolysis of carbohydrates, responds to the level of dietary carbohydrate. De Silva and Anderson (1995) reported an increase in amylase activity in *Oreochromis mossambicus* when the fish was changed to a starch-rich diet. Hidalgo *et al.* (1999) and Hofer *et al.* (1982) postulated that amylase activity depends on the natural diet of each species, herbivorous and omnivorous fish having more activity than carnivores.

With respect to proteolytic activity, there are some disagreements, with some authors (Hidalgo *et al.*, 1999; Kuz'mina 1990) stating that proteolytic activity is less

dependent on the nutritional habits (carnivorous / omnivorous) than amylase activity. Kapoor *et al.* (1975); Fange and Grove (1979) and Ugolev *et al.* (1983) on the other hand, maintain that the ratio amylase: protease activity in omnivorous and herbivorous fish was higher than in carnivorous fish.

Lipase activity has been found in extracts of the pancreas, pyloric caeca and upper intestine, but was almost non-existent in the stomach and in the lower intestine. The principal site for lipase activity is the mucosal layer but in some species, like the *Siberian sturgeon*, there is hydrolysis in the stomach by gastric lipase (Gisbert *et al.*, 1999). However, the primary site of lipid hydrolysis for most species appears to be in the pyloric caeca and anterior intestines (Halver and Hardy, 2002), and all fat-digestive enzymes are known to act in alkaline media. Klahan *et al.* (2009) working on Nile tilapia, reported that digestive enzyme activities also depended on the organs studied. The protease and lipase activities appeared to be high in the intestines while amylase was more active in the liver.

It is evident that the feeding habits (herbivorous, omnivorous or carnivorous) of different fish species and the type of diet may affect the activity of the different enzymes produced during the digestive process. An understanding of the digestive capabilities of the three important warm water aquaculture fish species (*Clarias gariepinus*, *Oreochromis mossambicus* and *Tilapia rendalli*) in South Africa is fundamental to enhance their exploitation. Therefore, a comparative study on the activities of digestive proteolytic, amylase and lipase activity may reveal the capacity of these species to utilise protein, carbohydrates or lipids and consequently their ability to utilise plant-based diets.

To date there is no animal or plant protein available with an essential amino acid profile that can satisfy the dietary requirements of fish apart from fishmeal (Tacon and Jackson, 1985). This is attributed mainly to the presence of anti-nutritional factors, low protein content and poor amino acid profile in plant diets (Ogunji, 2004; Francis *et al.*, 2001; Francis *et al.*, 2002). As a result, studies on total or partial replacement of fishmeal in fish diets have resulted in reduced growth performance.

2.9. Anti-nutritional factors and their effects on utilisation of nutrients

Anti-nutritional factors are compounds mainly organic, defined as substances that interfere with food utilisation negatively affecting palatability, growth performance and health of animals (Makkar *et al.*, 1993). These are naturally occurring chemical substances in plants. They are possibly the main factors limiting inclusion of plant ingredients in fish diets (Krogdahl *et al.*, 2010). Their presence may result in poor palatability, chronic intoxication, poor feed intake, alteration of gut morphology, interference with the digestion and utilisation of dietary nutrients (Francis *et al.*, 2001).

Increasing levels of plant ingredients in fish diets often leads to decreased digestibility of the diet and nutrients (El-Sayed, 1999). Anti-nutritional factors have been reported to play a role in reducing the digestibility of plant-based diets. Saponins (Ikedo *et al.*, 1996), tannins (Guillaume and Métailler, 1999) and fibre (Gaber, 2006) are reported to form poorly digestible complexes with nutrients. Saponins are also known to inflict damage on the intestinal mucosa thus interfering with the digestion process (Bureau *et al.*, 1998). The chemical and physical structure of fibre creates a barrier between nutrients and digestive enzymes hence lowering digestion. Fibre may also disrupt enzyme activities through adsorption or immobilization (Gaber, 2006).

Fishmeal replacement with leaf meal protein sources has been adopted in the past, but little is known about their effects on the digestive organs and possible repercussions on fish health. Numerous papers regarding the effect of dietary soybean products on the gut histology of carnivorous fish have been published (van den Ingh *et al.*, 1991; 1996; Baeverfjord and Krogdahl, 1996; Aslaksen *et al.*, 2007; Merrifield *et al.* 2009). However, very few studies have been undertaken on the effect of leaf meal-based diets on the histology of the digestive organs in omnivorous and herbivorous fish. Uran *et al.* (2008) reported intestinal inflammation caused by soybean meal in *Cyprinus carpio* L. Recently, Mahmoud *et al.* (2014) observed degeneration of intestinal mucosa, enteritis characterised by aggregation of lymphocytes and focal detachment of epithelial lining in *Oreochromis niloticus* fed soybean-based diets. With continued efforts of finding more alternatives to fishmeal, it is important to also determine the effects these 'new protein' sources on intestine

histology. The effects of common leaf meals such as kikuyu grass and moringa on the digestive morphology of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* remain unknown.

The nutritional state of fish can also be determined by monitoring changes in blood parameters (Zhou *et al.*, 2012; Eslamoo *et al.*, 2012). The quantity and quality of feed (especially protein) and the presence of anti-nutritional factors in the feed (Lindsay, 1977; Akinola and Abiola, 1991; Esonu *et al.*, 2001; Akinmutimi, 2004) influence haematological components. This important indicator of fish health has often been overlooked in the selection of plant-based fishmeal replacers. Thus, one of the objectives of this study was to determine the effect of feeding kikuyu and moringa leaf meal-based diets on the haematological parameters in *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus*.

2.10 The use of exogenous enzymes in fish diets

Exogenous enzymes may be used to inactivate anti-nutritional factors and enhance the nutritional value of plant-based protein in feeds (Dalsgaard *et al.*, 2012). The use of exogenous enzymes in fish diets may be effective in reducing the negative effects of plant-based diets by denaturing the anti-nutrients and enhancing digestion (Krogdahl *et al.*, 2010; Santigoa *et al.*, 2010; Denstadli *et al.*, 2011; Dalsgaard *et al.*, 2012).

Exogenous enzymes are widely applied in feed for monogastric animals including pigs and poultry as a means to increase the nutritional value of plant-based protein sources by reducing the anti-nutritional effects (Cowieson *et al.*, 2006). In comparison, there is limited information on the effects of enzymes in fish feed. Enzymatic supplementation main goals are: 1) improve nutrient digestibility and utilisation, 2) reduce the effect of anti-nutritional factors, 3) improve the utilization of non-starch polysaccharides (NSP), 4) improve endogenous enzymes activity, 5) minimize environmental pollution caused by residuals and 6) spare the use of amino acids on enzyme synthesis (Carter *et al.*, 1994; Soltan, 2009; Lin *et al.* 2007). Enzymes provide a natural way to transform complex feed components into absorbable nutrients. The increased use of plant-based proteins sources in fish feeds necessitates the use of enzymatic supplements to improve their utilisation.

Several authors have reported an increase in growth performance in fish whose diets were supplemented with exogenous enzymes (Carter *et al.*, 1994; Farhangi and Carter, 2007). Lin *et al.* (2007) reported that supplementing 0.1% of a commercially prepared exogenous enzyme (neutral protease, α -glucanase and xylanase) into plant-based diets for juvenile hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) significantly increased specific growth rate, feed efficiency ratio and apparent protein retention. On the contrary, Ogunkoya *et al.* (2006) did not find any effect on the growth and feed efficiency after adding graded levels of a commercial enzyme cocktail to rainbow trout diets containing up to 200 g/kg of soybean meal (SBM). Similarly, Farhangi and Carter (2007) reported that adding commercial enzymes: Energex™, Bio-Feed™ Pro and Alpha galactosidase™ separately or in combination to a diet containing 50% of de-hulled blue lupin for rainbow trout was ineffective.

Currently, results on the effect of exogenous enzyme supplementation in fish diets are inconclusive. There is limited research on the effect of exogenous enzyme supplementation on the enzyme activities in omnivorous and herbivorous fish. Additionally, most of the work done on enzyme supplementation has been done in fish fed seed/legume-based diets. There is paucity of information on the effect of adding exogenous enzymes in leaf meal protein resources. Thus, one of the objectives of the present study was to evaluate the effects of a commercial multi-enzyme cocktail on the growth performance as well as on the activities of digestive enzymes in *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* fed kikuyu and moringa-based diets.

The general objective of this study was to assess the digestive capabilities of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus*, determine their ability to utilise kikuyu and moringa-based diets and develop strategies to enhance the utilisation of these plant-based diets.

CHAPTER 3:

**POSTPRANDIAL CHANGES IN PH AND
ENZYME ACTIVITY FROM THE STOMACH
AND INTESTINES OF *TILAPIA RENDALLI*,
OREOCHROMIS MOSSAMBICUS AND
*CLARIAS GARIEPINUS***

3.1 INTRODUCTION

Intensive fish culture has become essential to meet the increased demand for farmed fish. Therefore, fish nutritionist must develop diets that are nutritionally suited and can be effectively utilised by different fish species. In the development of fish feed, it is important to understand the nutritional physiology of the fish species. Furthermore, the conditions (i.e. high stocking rates) in these systems accentuate the need for a proficient enzyme system (Shiau, 1990).

Warm water aquaculture in South Africa is dominated by three fish species; *Clarias gariepinus* with well documented predatory/omnivore feeding habits (Dadebo, 2000; Skelton, 2001; Rad *et al.*, 2004). *Oreochromis mossambicus* is an effective algal feeder (El-Sayed, 2006; Doupé *et al.*, 2010) and *Tilapia rendalli* that predominately feeds on macrophytes (Weliange *et al.*, 2006; Hlophe and Moyo, 2011). The digestive tract of *Tilapia rendalli* and *Oreochromis mossambicus* are largely similar, being characterised by a small sac-like stomach and long coiled intestines (El-Sayed, 2006). On the other hand, *Clarias gariepinus* has a masculine, distensible stomach and relatively short intestines indicating a dependency on animal-based diets (Hecht *et al.*, 1988). These observations were based on stomach content analysis. However, stomach content analysis does not provide information on the suitability of the diet for optimal growth performance. Digestive enzymes are a useful tool in determining the dietary components most effectively utilised.

Digestive enzymes are a good indicator of digestive capabilities of fish; their activities determine the successful breakdown and utilisation of nutrients in a diet, optimising growth and productivity (Debnath *et al.*, 2005; Mohanta *et al.*, 2008). Accordingly, characterisation of digestive enzymes provides some information regarding the digestive competence of fish to hydrolyse protein, carbohydrate and lipid of feed ingredients (Essa *et al.*, 2010).

There are few inconclusive studies on the relationship between feeding habits and enzyme activities in fish. Some authors report that the activity of digestive enzymes is dependent on the diet (Saha and Ray, 1998), while others maintain that it is largely affected by phylogeny (Chaudhuri *et al.*, 2012; Chan *et al.*, 2004; German *et*

al., 2004). Most of these studies however, were based on wild caught fish with no control of the diet or other environmental factors such as temperature.

Despite the widespread culture of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus*, very little work has been done on the enzyme dynamics of these species when fed formulated diets. De Silva and Anderson (1995) indicated that *Oreochromis mossambicus* displayed a higher level of amylase activity when fed on a high starch diet. The enzyme activities of *Tilapia rendalli* have not been investigated before. Uys and Hecht (1987) reported high activities of pancreatic amylase in *Clarias gariepinus*.

Proteins are one of the most expensive fractions of aquaculture feeds, therefore it is important that they be fully utilised by the fish. Protein digestion is dependent not only on the source of protein but also on the fish's capability to produce enough proteolytic enzymes. Hidalgo *et al.* (1999) reported that natural nutritional habits do not determine the concentration of the proteolytic enzymes. Several authors have reported that protease activity in omnivorous and herbivorous fish is lower than in carnivorous fish (Kapoor *et al.*, 1975; Fange and Grove, 1979; Ugolev *et al.*, 1983). Some authors however have reported a high proteolytic potential in non-carnivorous fish (Hidalgo *et al.*, 1999; Kuz'mina, 1990), stating that vegetable proteins are difficult to digest and require high activity to be well utilised. On the contrary, De Silva and Anderson (1995) reported a fall in protease activity with a decrease in the proportion of fishmeal in the diet. This may indicate a correlation between the activity of protease and the amount of fishmeal in the diet.

Enzyme activity is dependent on pH; therefore, some authors determine digestive enzyme activity at a particular pH (Chaudhuri *et al.*, 2012; Essa *et al.*, 2010) or over a range of pH values (Klahan *et al.*, 2009). The pH in the digestive tract however changes with the presence or absence of food. Thus for accurate enzyme activity, it is important to determine activity in a buffer with pH corresponding to the pH in the particular organ at the specified time after feeding. This is one of the few studies in which enzyme activity in the different organs is measured in the actual pH recorded of the organ at collection.

The lack of information on the protease, amylase, lipase and cellulase activity in fish species of aquaculture importance may hinder the development of suitable feeds. This chapter therefore focusses on the changes in pH and enzyme activities in the digestive tract of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* in response to one meal of formulated feed in order to distinguish species-specific differences from diet-elicited effects.

3.2 OBJECTIVES

The specific objectives of this chapter were to determine:

- i. the effect of time after feeding on protease, amylase, lipase and cellulase enzyme activities in the stomach and intestines of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus*.
- ii. the effect of time after feeding on pH in the stomach and intestines of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus*.

3.3 MATERIALS AND METHODS

3.3.1 Experimental system

The experiments were carried out in a recirculating water system (Figure 3.1) in a greenhouse. The system consisted of 64 self-cleaning rectangular fibreglass tanks (1.5 m³). Water was pumped to each tank at a rate of 1 L per minute from an 8000 litre biofilter. In each tank (experimental unit), aged water was maintained at the 1 m³ mark with the use of drain pipes fitted at the bottom of the tank. Water was drained from bottom of the tanks through standpipes into a series of biofiltration tanks (Figure 3.1). An Elektror side channel blower Model: D7300 (Karl W. Miller) and an air stone were used to blow and diffuse air in each tank respectively. Twenty percent of the water in the system was replaced biweekly with aged water.

One hundred and twenty sub-adult fish of each fish species [*Tilapia rendalli* (25 ± 2 g), *Oreochromis mossambicus* (30 ± 2 g) and *Clarias gariepinus* (42 ± 2 g)] were stocked in triplicate tanks at 40 fish per tank.

3.3.2 Water quality management

Daily measurements were taken for temperature, dissolved oxygen, ammonia and pH using a handheld YSI (556 MPS) multi-meter as described in Chapter 3 section 3.4.3.

3.3.3 Fish and sample preparation

Fish were acclimatised to the experimental environment for 2 weeks. During this period and prior to stocking, all fish were offered commercial tilapia pellets (Table 3.1) at 4% body weight. All fish were fed on the same diet in order to distinguish species-specific differences from diet-elicited effects. Fish were starved for 24 hours before sampling. The starvation period of 24 hours was considered sufficient for complete gastric evacuation based on an earlier study (Hlophe *et al.*, 2011). Fifteen fish from each species (5 fish per tank) were sampled before feeding (0 hours) for enzyme and pH determination. The remaining fish were fed 4% of their body weight. Thereafter, 15 fish from each species (5 fish per tank) were sampled at four hour intervals (4, 8, 12, 16, 20 and 31 hours). The fish were killed with a blow on the head and dissected on ice.

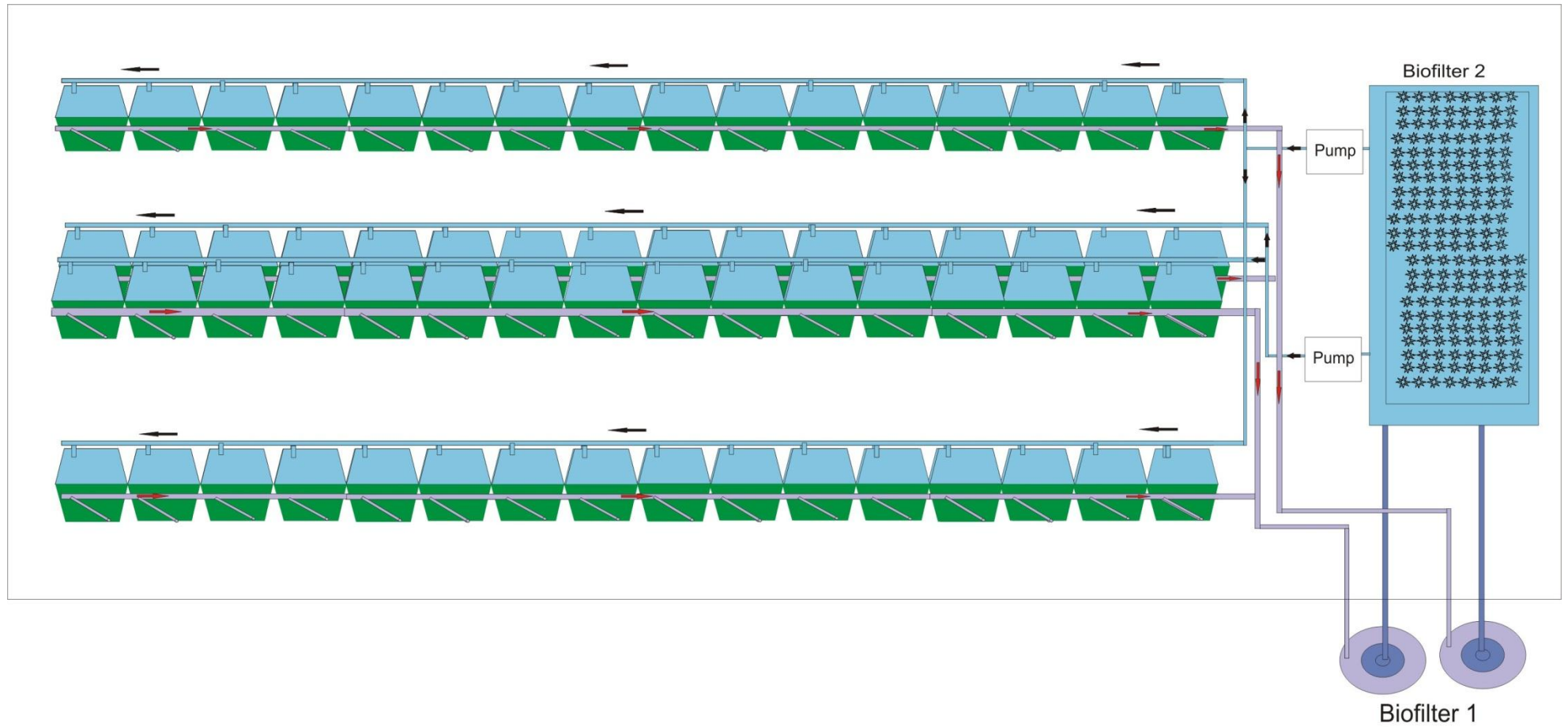


Figure 3.1: Recirculating system used for the experiments with fibre glass tanks

Table 3.1: Proximate composition of the commercial tilapia diet used in the study

Parameter	Amount
Protein	34.48 %
Carbohydrates	17.20 %
Gross energy	17 MJ/kg
Ash	10.78 %
Moisture	7.80 %
Fat	2.97 %
Crude fibre	2.30%

3.3.4 Gastrointestinal tract pH measurements

The digestive tract of each fish was divided into three parts; the stomach, the proximal intestine (PI) and the distal intestine (DI). The intestine of each fish was divided into two equal sections: the first half was the PI and the second half was the DI. The pH measurements were taken using a Crison Basic 02 pH meter (Lasec-SA). At each sampling time, the tip of the microelectrode (diameter 4 mm) was inserted into small slits made in the stomach, the PI and the DI. For measuring pH in the PI and DI, three slits were made (at 15 cm intervals for the tilapias and 3 cm for *Clarias gariepinus*) along the length of each and the tip of the microelectrode inserted into the openings. Accordingly, the pH recorded for the PI and DI at any sampling time was a mean of these 3 measurements. At each sampling time, the stomach, proximal and distal intestines of all sampled fish were pooled (per species) and stored in marked plastic sample bags at -86°C until used for the analysis of enzyme activity.

3.3.5 Enzyme activity and characterization of digestive enzymes

Crude enzyme preparations

For the different fish species, stomach, proximal and distal intestines were separately homogenised (1:2 w/v) with 50 mM Tris-HCl buffer (pH 7.5) in an ice water bath, using a tissue homogeniser. The preparation was centrifuged at 4 200 × g for 60 minutes at 4°C. The floating lipid fraction was discarded and the aqueous supernatant recovered and kept at -20°C until used.

Enzyme activity was measured for samples collected only at 0, 12 and 31 hours after feeding. The activity was measured at these times because the stomach pH was significantly different at these times. The pH recorded in the specific part of the digestive tract at collection, was used to determine the pH of the buffer at which enzyme activity was measured. All enzyme readings of the pooled samples were done in triplicate.

3.3.5.1 Characterization of protease

Protease activity was measured by the increase in cleavage of short chain polypeptides (Bezerra *et al.*, 2005). All samples were analysed in triplicate. Total protease activity was determined using 1% (w/v) azocasein, prepared in a 0.1 M phosphate buffer pH 7. One twenty five μl of the substrate (azocasein) was incubated with 5 μl of the crude enzyme extract and 40 μl of the buffer solution for 30 minutes at 30°C. Then, 100 μl of 20% (w/v) trichloroacetic acid (TCA) was added to stop the reaction. After 15 minutes, centrifugation was carried out at 2 800 x g for 10 minutes. One hundred μl of the supernatant was added to 1 M NaOH (100 μl) in a flat bottom 96 well micro titre plate and the absorbance was measured at 440 nm against a blank (prepared in the same way without the crude enzyme extract). The protease-specific activity was expressed by the change in absorbance per minute per mg protein of the enzyme extract ($\Delta\text{Abs min/mg protein}$).

3.3.5.2 Characterisation of amylase

Amylase activity was determined in triplicate using the 3, 5-dinitrosalicylic acid (DNS) method (Bernfeld, 2006). Starch substrate (1% w/v) was prepared in 0.1 M phosphate buffer pH 7. Thirty three μL of the substrate was incubated with 3 μL crude enzyme extract and 27 μL buffer solution for 10 minutes at 30°C. Thereafter, 100 μL of 1% of the DNS solution was added and the solution was boiled for 5 minutes. After boiling, the solution was cooled to 4°C and then read at 540 nm using a Multimode Analysis Software version 3.3.0.9 (DTX 880). Blanks were similarly prepared, without the crude enzyme extracts. Maltose (10–100 mM) was used for the preparation of the calibration curve. The amylase-specific activity was defined by the μmol of maltose produced per minute per mg protein at the specified condition.

3.3.5.3 Characterisation of lipase

The lipase activity was also evaluated in triplicate using the protocol from Markweg *et al.* (1995) where the substrate, 0.01 M para-nitrophenylpalmitate (pNNP) is dissolved in iso-propanol. The pNNP substrate (10 µl) was added to 12.5 µl of the crude enzyme extract and 215 µl of the 0.1 M phosphate buffer (pH 7). The absorbance was measured at 410 nm against a blank similarly prepared, without the crude enzyme extract. Para-nitrophenol (pNP) at a concentration of 0.1 – 10 µg/ml was used for the preparation of the calibration curve. The lipase-specific activity was defined by the µmol of p-nitrophenol produced per minute per mg protein at the specified condition.

3.3.5.4 Characterisation of cellulase

For the characterisation of cellulase activity, the same protocol used for amylase was used, substituting the starch substrate with carboxyl-methyl-cellulose (1% w/v). Glucose (0.5-10 mM) was used for the preparation of the calibration curve. The cellulose-specific activity was defined by the µmol of glucose produced per min per mg protein at the specified condition. Enzyme (amylase/cellulase) activity for each organ is reported as the mean of activities determined at zero, twelve, and thirty one hours after feeding.

3.3.5.5 Protein concentration

The protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

3.3.6 Statistical analysis

Data on pH and enzyme activity was tested for normality using the Shapiro-Wilk normality test. One way analysis of variance was used to determine the effect of time on the pH of the stomach, PI and DI in each species. A three-way analysis of variance (ANOVA) was used to determine the effects of fish species, part of the digestive tract and time after feeding on the activity of different enzymes. Tukey's post hoc analysis was used to separate significant means. All statistical analysis was done on IBM SPSS statistics 21 software.

3.4 RESULTS

3.4.1 Postprandial changes in pH

The pH in the stomach was more than 4 in all fish species before feeding (Figure 3.2). The presence of food resulted in a significant ($P < 0.05$) decrease in pH 12 hours after feeding for all species. The lowest stomach pH was 1.54 in *Oreochromis mossambicus*, 1.58 in *Tilapia rendalli* and 2.01 in *Clarias gariepinus*. Initial pH values were restored 31 hours after feeding (Figure 3.2). Stomach pH was significantly higher ($P < 0.05$) in *Clarias gariepinus* than in *Oreochromis mossambicus* at feeding and 31 hours after feeding. However, 12 hours after feeding, no significant differences were observed in all three fish. On the other hand, time after feeding had a significant effect ($P < 0.05$) on the stomach pH for all fish.

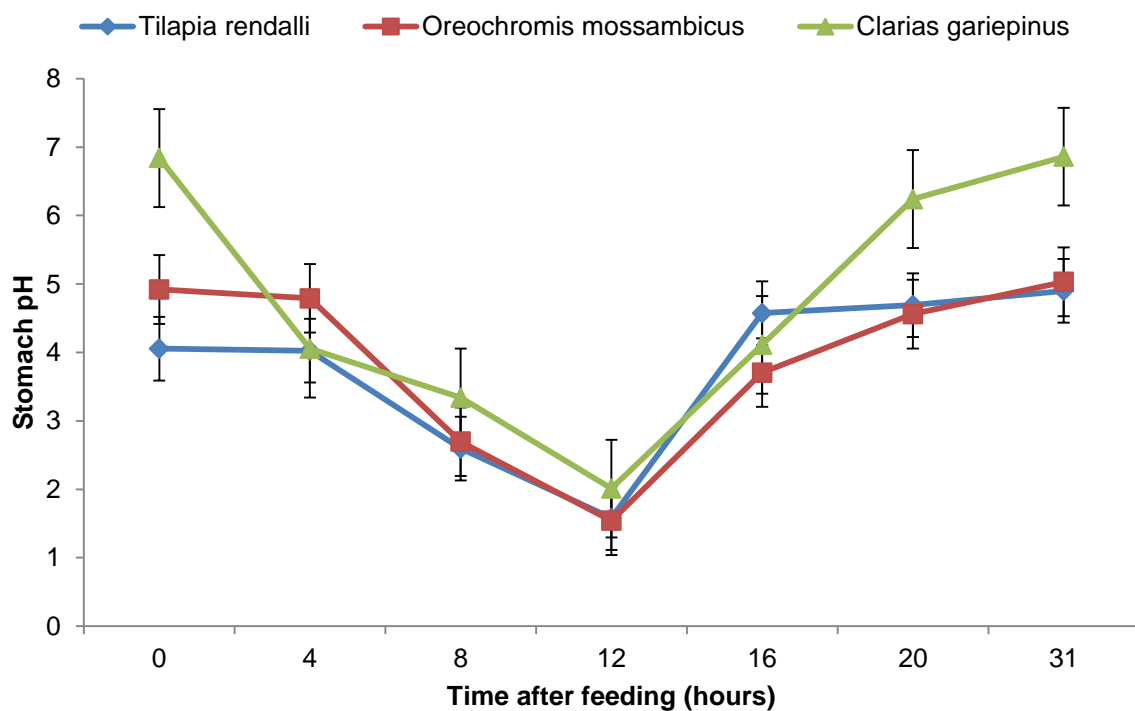


Figure 3.2: Postprandial changes in the pH of the stomach in *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* ($n = 15$ fish / species / sampling time). Values are mean of 3 replicates \pm standard error

The pH in the proximal intestines, was significantly higher ($P < 0.05$) than that of the stomach in the three fish species. The proximal intestines had alkaline pH values (7-8) throughout the experimental period and showed no significant differences

($P > 0.05$) with time after feeding for all species (Figure 3.3). In the distal intestine (Figure 3.4), the pH values were slightly higher but not significantly different ($P > 0.05$) from those of the proximal intestine. No significant differences were observed with time after feeding or between the three fish species.

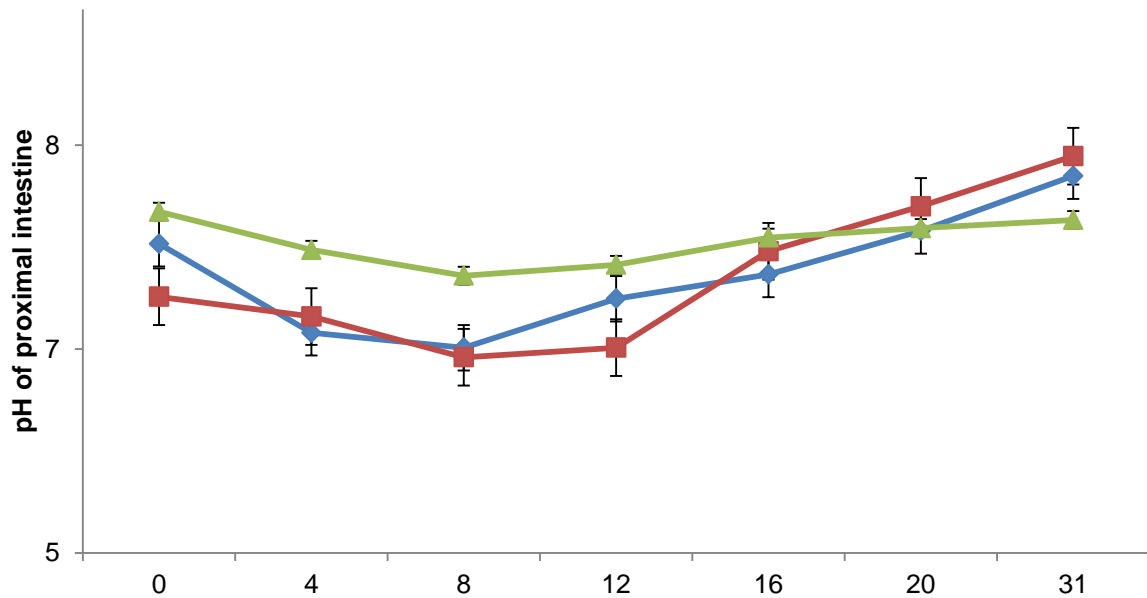


Figure 3.3: Postprandial changes in the pH of the proximal intestine in *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* ($n = 15$ fish / species / sampling time). Values are mean of 3 replicates \pm standard error

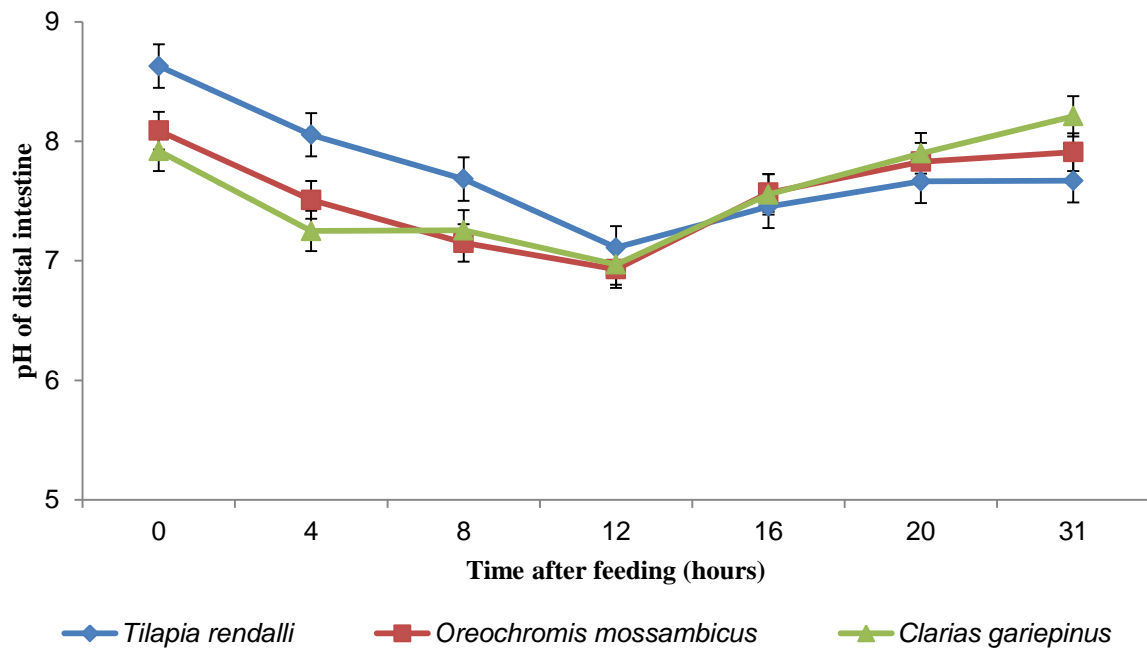


Figure 3.4: Postprandial changes in the pH in the distal intestine of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* (n = 15 fish / species / sampling time). Values are mean of 3 replicates ± standard error

3.4.2 Enzyme activities

3.4.2.1 Protease specific activity

Effect of fish species: Protease activity was significantly lower ($P < 0.05$) in *Clarias gariepinus*, than in the tilapias. No significant differences ($P > 0.05$) were observed between *Oreochromis mossambicus* and *Tilapia rendalli* (Table 3.2).

Effect of part of digestive tract: Protease activity was significantly different ($P < 0.05$) between the stomach, proximal and distal intestines in all species. The highest activity was recorded in the distal intestine in all fish species. In the stomach, *Clarias gariepinus* had significantly higher ($P < 0.05$) protease activity (0.95 $\mu\text{mol}/\text{min}/\text{mg}$ protein) than the tilapias. In the DI however, *Clarias gariepinus* had significantly lower ($P < 0.05$) protease activity (16.22 $\mu\text{mol}/\text{min}/\text{mg}$ protein) than *Tilapia rendalli* and *Oreochromis mossambicus* (18.91 and 18.09 $\mu\text{mol}/\text{min}/\text{mg}$ protein respectively; Table 3.2). Protease activity was not significantly different ($P > 0.05$) in the stomach, PI and DI of *Tilapia rendalli* and *Oreochromis mossambicus* (Table 3.2).

Effect of time: Time after feeding had a significant effect ($P < 0.05$) on protease activity in all three fish species ($p > 0.05$, ANOVA: Table 3.2). In the stomach, protease activity was highest at 31 hours after feeding and lowest just before feeding (0 hours). In the PI and DI, protease activity peaked 12 hours after feeding in all fish species. There was a significant interaction between the fish species and time after feeding in the stomach, PI and DI.

3.4.2.2 Amylase activity

Effect of fish species: Amylase activity was significantly lower ($P < 0.05$) in *Clarias gariepinus* compared to that of *Tilapia rendalli* and *Oreochromis mossambicus* (Table 3.3). *Tilapia rendalli* had higher amylase activities than *Oreochromis mossambicus*, although these differences were not significantly different ($P > 0.05$).

Effect of part of digestive tract: Amylase activity was highest in the proximal intestine of all fish species compared to stomach and DI (Table 3.3). *Clarias gariepinus* had significantly lower ($P < 0.05$) amylase activity in the stomach and DI than the tilapias.

Effect of time: Amylase activity differed significantly ($P < 0.05$) with time after feeding and was highest 12 hours after feeding for all fish species (Table 3.3). A significant ($P < 0.05$) interaction between the fish species and time after feeding in the stomach, PI and DI was observed.

Table 3.2: Protease activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein) in the stomach, proximal and distal intestines of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* (n = 15 fish / species / sampling time). Values are mean of 3 replicates \pm standard deviation

Time (hours)	Stomach				PI				DI			
	0	12	31	Mean	0	12	31	Mean	0	12	31	Mean
<i>Tilapia rendalli</i>	0.26 \pm 0.02	0.96 \pm 0.31	0.79 \pm 0.11	0.66 \pm 0.31 ^b	7.53 \pm 0.62	20.41 \pm 0.41	13.53 \pm 0.98	13.82 \pm 5.61 ^a	10.16 \pm 0.16	25.14 \pm 0.87	21.44 \pm 1.21	18.91 \pm 6.79 ^a
<i>Oreochromis mossambicus</i>	0.37 \pm 0.01	0.52 \pm 0.01	0.88 \pm 0.04	0.59 \pm 0.23 ^b	8.25 \pm 1.05	21.86 \pm 1.33	12.11 \pm 0.54	14.07 \pm 6.12 ^a	9.18 \pm 1.11	26.64 \pm 1.09	18.48 \pm 1.04	18.09 \pm 7.61 ^a
<i>Clarias gariepinus</i>	0.84 \pm 0.17	1.32 \pm 0.12	0.68 \pm 0.09	0.95 \pm 0.33 ^a	7.14 \pm 1.47	17.74 \pm 1.73	11.01 \pm 1.24	11.96 \pm 4.71 ^b	9.71 \pm 0.94	19.53 \pm 1.71	19.43 \pm 1.08	16.22 \pm 4.90 ^b
Effects	Probability (P = 0.05)											
Species	*				*				*			
Time	*				*				*			
Species*time	*				*				*			

Means within a column and main effect not sharing a common superscript differ (P<0.05); * = P <0.05; NS = not significantly different.

Table 3.3: Amylase activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein) in the stomach, proximal and distal intestines of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* (n = 15 fish / species / sampling time). Values are mean of 3 replicates \pm standard deviation

Time (hours)	Stomach				PI				DI			
	0	12	31	Mean	0	12	31	Mean	0	12	31	Mean
<i>Tilapia rendalli</i>	6.61 \pm 0.01	7.30 \pm 0.00	5.90 \pm 1.24	6.54 \pm 0.93 ^a	11.12 \pm 0.65	26.34 \pm 2.93	11.13 \pm 0.18	16.12 \pm 7.69 ^b	8.34 \pm 2.11	9.00 \pm 1.37	6.01 \pm 0.02	7.79 \pm 1.87 ^a
<i>Oreochromis mossambicus</i>	7.31 \pm 4.01	6.25 \pm 0.00	5.10 \pm 0.55	6.15 \pm 2.22 ^a	13.11 \pm 0.44	22.00 \pm 2.05	10.50 \pm 0.41	15.21 \pm 5.42 ^{ab}	10.20 \pm 2.15	8.01 \pm 0.15	4.95 \pm 0.37	7.90 \pm 2.75 ^a
<i>Clarias gariepinus</i>	0.21 \pm 1.23	2.05 \pm 0.17	1.31 \pm 0.55	1.55 \pm 0.45 ^b	10.20 \pm 0.75	14.02 \pm 6.17	9.32 \pm 0.81	11.45 \pm 4.01 ^b	4.62 \pm 0.58	5.53 \pm 0.45	1.01 \pm 0.07	3.73 \pm 2.11 ^b
Effects	Probability (P = 0.05)											
Species	*				*				*			
Time	NS				*				*			
Species*time	NS				*				*			

Means within a column and main effect not sharing a common superscript differ (P<0.05); * = P <0.05; NS = not significantly different.

3.4.2.3 Lipase specific activity

Effect of fish species: *Clarias gariepinus* had significantly higher ($P < 0.05$) lipase activity than *Oreochromis mossambicus* and *Tilapia rendalli* (Table 3.4).

Effect of part of digestive tract: Lipase activity was significantly different ($P < 0.05$) in the parts of the digestive tract examined. The highest activity was recorded in the proximal intestine, followed by the distal intestine and lowest lipase activity was recorded in the stomach for all three fish species.

Effect of time: Time after feeding had a significant effect on lipase activity in all fish species. Lipase activity was highest 12 hours after feeding in all fish species (Table 3.4).

3.4.2.4 Cellulase activity

Effect of fish species: Low cellulase activities were observed in all species. Cellulase activity differed significantly ($P < 0.05$) between the three fish species (Table 3.5). *Oreochromis mossambicus* had the highest activity.

Effect of part of digestive tract: The part of the digestive tract also significantly affected cellulase activity. No cellulase activity was found in the proximal intestine of all fish species.

Effect of time: Cellulase activity was significantly lower 31 hours after feeding. No significant differences were observed in cellulase activity between 0 and 12 hours after feeding (Table 3.5).

3.4.3 Water quality parameters

Water temperature ranged from 25.0°C to 28.4°C (average 26.1±2.2°C). The pH values in all the experimental tanks varied within a range of 7.2-7.7. Dissolved oxygen ranged between 6.24-7.10 mg/l. Total ammonia ranged between 0.62-0.87 ppm.

Table 3.4: Lipase activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein) in the stomach, proximal and distal intestines of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* (n = 15 fish / species / sampling time). Values are mean of 3 replicates \pm standard deviation

	Stomach				PI				DI			
Time (hours)	0	12	31	Mean	0	12	31	Mean	0	12	31	Mean
<i>Tilapia rendalli</i>	4.50 \pm 0.35	2.13 \pm 0.14	8.81 \pm 0.12	5.12 \pm 2.93 ^c	15.26 \pm 0.84	23.36 \pm 1.68	19.37 \pm 1.21	19.32 \pm 3.68 ^b	18.57 \pm 1.01	15.20 \pm 1.06	21.58 \pm 1.23	18.66 \pm 2.77 ^c
<i>Oreochromis mossambicus</i>	5.32 \pm 0.98	4.54 \pm 0.75	9.12 \pm 0.52	6.36 \pm 2.23 ^b	13.57 \pm 1.02	25.23 \pm 1.47	22.79 \pm 1.05	20.51 \pm 5.42 ^b	21.61 \pm 1.14	19.00 \pm 1.07	23.21 \pm 1.24	21.12 \pm 2.21 ^b
<i>Clarias gariepinus</i>	9.25 \pm 0.12	6.12 \pm 0.34	12.73 \pm 0.18	9.36 \pm 2.85 ^a	32.46 \pm 1.58	36.92 \pm 1.73	33.21 \pm 1.55	34.19 \pm 2.53 ^a	29.21 \pm 1.87	31.00 \pm 1.47	28.56 \pm 1.65	29.61 \pm 1.98 ^a
Effects	Probability (P = 0.05)											
Species	*				*				*			
Time	*				*				*			
Species*time	*				*				*			

Means within a column and main effect not sharing a common superscript differ (P<0.05); * = P <0.05; NS = not significantly different.

Table 3.5: Cellulase activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein) in the stomach, proximal and distal intestines of *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus* (n = 15 fish / species / sampling time). Values are mean of 3 replicates \pm standard deviation

	Stomach				PI				DI			
Time (hours)	0	12	31	Mean	0	12	31	Mean	0	12	31	Mean
<i>Tilapia rendalli</i>	0.24 \pm 0.09	0.01 \pm 0.00	0.02 \pm 0.00	0.09 \pm 0.12 ^b	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.0 ^a	0.04 \pm 0.23	0.07 \pm 0.01	0.00 \pm 0.00	0.096 \pm 0.10 ^a
<i>Oreochromis mossambicus</i>	0.00 \pm 0.00	0.70 \pm 0.43	0.10 \pm 0.07	0.29 \pm 0.41 ^a	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.0 ^a	0.00 \pm 0.00	0.01 \pm 0.00	0.00 \pm 0.00	0.003 \pm 0.01 ^b
<i>Clarias gariepinus</i>	0.00 \pm 0.00	0.02 \pm 0.54	0.08 \pm 0.21	0.035 \pm 0.38 ^b	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.0 ^a	0.00 \pm 0.00	0.20 \pm 0.61	0.00 \pm 0.00	0.077 \pm 0.14 ^a
Effects	Probability (P = 0.05)											
Species	*				NS				*			
Time	*				NS				*			
Species*time	*				NS				*			

Means within a column and main effect not sharing a common superscript differ (P<0.05); * = P <0.05; NS = not significantly different.

3.5 DISCUSSION

The decrease in pH observed in the stomach after feeding, indicates that all three fish species secrete hydrochloric acid (HCl) in the stomach (Klahan *et al.*, 2009) and that secretion is stimulated by the presence of food. The decrease of stomach pH after feeding has been reported in the Nile tilapia (Getachew, 1989) and in other fish species such as the White Sea bream (Yufera *et al.*, 2012) and the gilthead sea bream (Nikolopoulou *et al.*, 2011). Hydrochloric acid produced in the stomach functions mainly to convert the inactive zymogen pepsinogen into the active protease enzyme pepsin, as well as assisting in the physical breakdown of prey skeletal elements by softening and initiating the breakdown of food (Yufera *et al.*, 2012). In tilapias, it is also important for the rupturing of cell walls of macrophytes and algae, especially the thick walled gelatinous coated blue-green algae.

Digestion in stomach-less fish does not involve acid digestion as these fish either fail or do not need to produce hydrochloric acid (Yufera *et al.*, 2012). *Clarias gariepinus* has a well- developed masculine stomach that is capable of acid secretion by day 4 of exogenous feeding (Verreth *et al.*, 1992). *Tilapia rendalli* and *Oreochromis mossambicus* on the other hand lack a true stomach (El-Sayed, 2006), possessing a modified pocket-like structure. The stomach pH however, was lower in the tilapias (1.54 in *Oreochromis mossambicus*) than in *Clarias gariepinus*. This may imply that tilapias are genetically predisposed to produce more hydrochloric acid, which is crucial in the lysis of cells of the blue-green algae, an important food item for tilapias. However, more studies still need to be done using different diets to conclude if the lower pH in the stomach of the tilapias was not dietary related. In the proximal intestine, the pH was around neutral for all fish species and did not differ with time after feeding. Secretions from the liver (bile) and pancreas contain bicarbonate that helps to neutralise the acidic chyme, creating a neutral environment, suitable for enzyme digestion (Nikolopoulou *et al.*, 2011). In the DI, although the pH values remained above neutral and higher than the values in the PI, these differences were not significant in all fish species.

Tilapia rendalli had the highest total protease activity closely followed by *Oreochromis mossambicus* and *Clarias gariepinus* had the lowest activity. In the stomach, *Clarias gariepinus* had higher (although not significant) protease activity

than the tilapias. Protein digestion is initiated in the stomach of fish by pepsin and hydrochloric acid (Xiong *et al.*, 2011). According to Uys and Hecht (1987), catfish rely on their thick walled stomach for mechanical breakdown of food (thus secrete less pepsin) unlike the tilapias which require a highly acidic medium to enable biochemical breakdown of food. The very acid environment in the stomach of *Tilapia rendalli* and *Oreochromis mossambicus* may have resulted in low protease activity, as most proteolytic enzymes are active in an alkaline environment (De Silva and Anderson, 1995). In the proximal intestine, the pH was markedly increased in all species probably because of slightly alkaline pH recorded in this site. The protease activity in the proximal intestine was highest in *Tilapia rendalli* followed by *Oreochromis mossambicus* and was lowest in *Clarias gariepinus*. As reported in other studies (Chaudhuri *et al.*, 2012; Klahan *et al.*, 2009), the principal site for protease activity in this study was the distal intestine in all fish species. The distal intestine is also the site where the highest pH values were recorded in all species.

Total proteolytic activity was higher in the tilapias (*Tilapia rendalli* and *Oreochromis mossambicus*) than in the catfish. This is despite that catfish being a predatory omnivore, naturally feeds on a higher protein diet than tilapias. The higher proteolytic activities recorded in the tilapias concur with results reported by Chaudhuri *et al.* (2012) who found higher protease activities in *Terapon jarbua* whose diet was mainly of plant origin than in fish which fed predominantly on animal diets. The higher protease activity recorded in the tilapias may be an adaptive approach they assumed to efficiently utilise the low protein content in their natural diets. Hofer (1982) also advanced this argument and suggested that in order to make up for the lower protein in their diets, herbivorous fish increased consumption rate and enzyme production. Hidalgo *et al.* (1999) on the other hand, could not find any differences in proteolytic activities in fish classified as herbivorous, omnivorous or carnivorous. All these authors however, worked with wild caught fish whose diet was not controlled. In the present study, all fish were fed the same diet; the differences observed in protease activity therefore, were probably due to species differences. Although it would have been ideal to investigate the effect of the different natural diets on protease activity, the major challenge is that there is considerable diet overlap between the three fish species. The role played by proteolytic enzymes cannot be overlooked in the selection of an aquaculture species as they are important in the utilisation of the

most limiting dietary component (protein) and consequently affect growth and production. The highest protease activity was recorded in the intestines. This is where the pH was around neutral in all fish species. Several authors have reported higher protease activities in neutral or slightly alkaline pHs (Klahan *et al.*, 2009; Hidalgo *et al.*, 1999).

The total amylase activity was also significantly higher in the tilapias. Both tilapias are herbivorous in nature and feed mainly on plant diets higher in carbohydrates than those of the catfish. The tilapias may be physiologically adapted to utilise carbohydrates. This may explain the high amylase activities observed in the tilapias even when all species were fed the same formulated commercial fish pellets. This implies that both tilapias have a higher potential for carbohydrate digestion and therefore, could be fed high carbohydrate based diets. This is very important in aquaculture species as the prices of fishmeal continue to increase and more plant diets are used in aquafeeds. The low amylase activities obtained for *Clarias gariepinus*, in conjunction with the anatomy of its digestive tract (a large distensible muscular stomach and a short intestine) reflect its principal dependency on protein digestion for its growth. These results are in agreement with Caruso *et al.* (2009) who reported that α -amylase activity was lower in carnivorous fish, stating that carnivorous fish may not be equipped to produce high level of the amylase enzyme. The source of amylase in the proximal intestine may be the intestinal glands and the pancreas (Papoutsoglou and Lyndon, 2003; De Silva and Anderson, 1995).

The relatively high lipase activities recorded in all the fish species suggests that these fish are all capable of utilising lipids in their diets. Lipase activity however, was significantly lower in the tilapias than in the catfish in all parts of the digestive tract. These results concur with Opuszynski and Shireman (1995) who reported that lipase activity was lower in herbivorous fish. The low lipase activity may be related to the feeding habits of tilapias whose herbivorous diets typically have low fat content. The low lipase activity observed in this study and previous studies may suggest that the diet of these tilapias should have lower lipid content compared to that of *Clarias gariepinus*. Lipase is produced by the pancreas into the proximal intestine. This explains the higher lipase activities recorded in this region. Klahan *et al.* (2009) working on Nile tilapia also reported that lipase was more active in the proximal

intestines. The proximal intestine is also an ideal site for lipid digestion, as fat digesting enzymes act in an alkaline media (Klahan *et al.*, 2009).

Cellulase activity is generally lacking in the gastrointestinal tract of tilapias (El-Sayed, 2006). However, in this study cellulase activity was detected, albeit, with low activity in all fish species. The low cellulase activity observed indicates that the digestibility of plant-based diets may be enhanced by exogenous cellulase supplementation. These results confirm earlier studies that *Tilapia rendalli* was capable of breaking down cellulose in plant diets (Hlophe and Moyo, 2011). Saha *et al.* (2006) also reported low cellulase values in *Oreochromis mossambicus*. Marginal levels of cellulase activity in the distal intestine of these fish species may be a combination of endogenous cellulase and that from the microflora of the digestive tract. However, in this study no measures were taken to identify the source of cellulase.

Time after feeding is important in studies of enzyme activities as fish are able to anticipate feeding times and therefore maximise food utilisation through increased enzyme secretion (Montoya *et al.*, 2010). In the current study, the highest enzyme activity was recorded 12 hours after feeding and lowest just before feeding (0 hours) in all fish species. This implies that these fish increased enzyme secretion in response to the presence of food in the stomach. Results by Vera *et al.*, 2007 and Montoya *et al.*, 2010 indicate that enzyme activity increases in fish fed at regular intervals and that postprandial increase in enzyme activity was evident. It can be inferred here that for maximum utilisation of dietary components, feed should not be withdrawn for intervals greater than 12 hours as enzyme production decreases after this time. All three fish species in this study were fed the same diet; therefore, diet may not be an overriding factor in the differences observed in protease and amylase enzyme activities.

It has now been established that the digestive enzyme profile of the three fish species are different even when fed on the same diet. Thus, a subsequent study was undertaken, to determine the effect of plant diets (kikuyu and moringa) on the macrophyte feeding *Tilapia rendalli*, which had the highest levels of protease and amylase activities.

CHAPTER 4:

**THE EFFECT OF REPLACING FISHMEAL
WITH *PENNISETUM CLANDESTINUM* AND
MORINGA OLEIFERA IN THE DIET OF
*TILAPIA RENDALLI***

4.1 INTRODUCTION

Tilapias feed low on the trophic level in nature and are accustomed to satisfying much of their nutritional needs from plant sources (Fitzsimmons, 1997). Despite the ability of wild tilapia to utilise plant proteins efficiently, commercial tilapia diets have mainly focused on using fishmeal as the main source of protein. Fishmeal is a preferred protein source in aquaculture diets because of its high nutritional value (essential amino acid profile, essential fatty acids, digestible energy, vitamins and minerals) and high palatability (Tacon, 1993).

In recent years, researchers have focussed on searching for alternative protein ingredients for use in aquaculture feeds. Plants with high protein levels are preferred because dietary protein enhances growth performance in fish (Musuka *et al.*, 2009). Research efforts have focused on the replacement of fishmeal with less expensive locally available sources (Enami, 2011) especially meals from processed seedcakes. Most studies carried out to determine the effects of replacing fishmeal with plant proteins in tilapia diets have focussed mainly on soyabean meal (Wee and Shu, 1989, Shiau *et al.*, 1989, Webster *et al.*, 1992). Other plant-based fishmeal replacers explored include maize gluten meal (Wu *et al.*, 1995); lupins (Fontainhas- Fernandes *et al.*, 1999); rapeseed (Davies *et al.*, 1990) and distillers dried grains (Coyle *et al.*, 2004).

There is need to continually search for new sources of protein in fish diets because competition from other sectors has led to the commercialisation of some of the widely used plant protein resources (soybean meal, rapeseed meal and sunflower meal). Leaf meals are potentially good sources of protein because of their rapid growth enabling several harvest times per growing season compared to seed meals. An appreciable amount of work has been done on the use of different leaf meals as inexpensive sources of protein in fish feeds [cassava (Ng and Wee, 1989); sweet potatoe (Adewolo, 2008); alfalfa (Yousif, *et al.*, 1994); moringa (Richter *et al.*, 2003); *Leucaena leucocephala* (Bairagi *et al.*, 2004)].

In this chapter, the effect of replacing fishmeal with two locally available plant leaf meals in the diet of a macrophagous fish, *Tilapia rendalli* is explored. *Tilapia rendalli*

is one of the cultured warm-water, freshwater fish species in South Africa and has wide market acceptability. It is pre-adapted to utilising plant-based diets and is widely known for its voracious appetite for macrophytes (Munro, 1967) and ability to change its diet to whatever resources are available (Meschiatti and Arcifa, 2002; Weliang, *et al.*, 2006). *Tilapia rendalli* is able to efficiently utilise plant protein with its adults feeding almost exclusively on a plants in nature (Hlophe, 2012).

Replacement of fishmeal in fish diets without reducing the growth performance would result in more profitable aquaculture enterprises. In this chapter, the potential for kikuyu grass (*Pennisetum clandestinum*) as a protein source in *Tilapia rendalli* diets is compared to that of moringa (*Moringa oleifera*) leaves. The negative effects caused by plant-based diets on the digestive morphology and growth performance have been extensively studied in carnivorous fish (Collins *et al.*, 2012; Caballero *et al.*, 2003; Krogh *et al.*, 2000). However, the adverse effects of plant-based diets on the digestive physiology of herbivorous fish have been overlooked. Previous research done on fishmeal replacement diets fed to herbivorous fish has focussed on growth performance. Understanding the effect of plant-based diets on the digestive enzyme activities, morphology of the digestive organs and health of fish is pivotal in finding ways to reduce their adverse effects. The main objective of this chapter was to investigate the effect of replacing fishmeal with different levels of kikuyu and moringa leaf meals on the growth performance, digestive enzyme activities, morphology of digestive organs (liver and intestine histology) and haematological parameters in *Tilapia rendalli*.

4.2 OBJECTIVES

The specific objectives of this study were to determine:

- i. the effect of partially and totally replacing fishmeal with different levels of kikuyu and moringa leaf meals on the growth performance, liver histology, intestine histology, digestive enzyme activities and haematological parameters in *Tilapia rendalli*.
- ii. incidence cost and profit index of partially and totally replacing fishmeal with different levels of kikuyu and moringa leaf meals in the diet of *Tilapia rendalli*.

4.3 MATERIALS AND METHODS

4.3.1 Fish acclimatisation

A total of 405 *Tilapia rendalli* fingerlings (12 ± 2.0 g) obtained from Flag Boshielo Dam, South Africa were transported to the University of Limpopo, Aquaculture Research Unit and kept in the experimental tanks where they were acclimatised for one month. The fish were fed a maintenance ration of standard commercial tilapia diet containing 34% protein, 3% lipid and a gross energy content of 17 MJ/kg dry matter.

4.3.2 Feed preparation

Fresh kikuyu grass (lawn) was harvested from the Aquaculture Research Unit, University of Limpopo. Moringa leaves were purchased from the Patient Wellness Centre, Lebowakgomo, Limpopo Province, South Africa. Kikuyu grass and moringa leaves were dried under a shade and milled using a hammer mill. The proximate and amino acid composition of kikuyu leaf meal (KLM), moringa leaf meal (MLM) and fishmeal used is given in Table 4.1.

Nine isonitrogenous (crude protein 30%) and isoenergetic (gross energy 20 MJ/kg) diets were formulated by substituting fishmeal for KLM and MLM. Ingredients were purchased from a local feed manufacturer (Wille Pille, Mokopane, South Africa). The control diet did not contain any plant leaf meal but 10% fishmeal (Table 4.2). In the KLM diets, fishmeal was substituted at 25, 50, 75 and 100% with KLM in diets designated as KLM 25, KLM 50, KLM 75 and KLM 100 respectively. Similarly, in diets designated as MLM 25, MLM 50, MLM 75 and MLM 100 fishmeal was replaced with MLM at 25, 50, 75 and 100% respectively. Maize meal and maize gluten meal were adjusted to regulate protein and energy levels. Chromic oxide (Cr_2O_3) was added in each diet (0.5%) as an inert marker. The diets were formulated using the Winfeed 3, EFG Software (Natal).

Dry ingredients were mixed for 30 minutes in a Hobart mixer (Belle, Mini 150; England) to ensure that the mixture was well homogenized. Oil was added and blending continued for 15 minutes. Water was added at 10-20% v/w to provide a pelletable mixture. This mixture was passed through a Hobart pelletiser fitted with a

1.0 mm die to attain pellets of desired size. Pellets were dried in the sun, after drying the pellets were packed in labelled polythene bags, sealed and stored at -20°C until used.

Table 4.1: Proximate and amino acid composition of kikuyu, moringa leaf meals and fishmeal used

Components	KLM	MLM	Fishmeal	Dietary amino acid requirements for tilapia
Dry matter (%)	93.63	92.00	90.50	
Protein (%)	26.84	32.09	64.80	
Energy (MJ kg ⁻¹)	17.99	18.30	12.90	
Ash (%)	9.50	7.17	21.10	
Fat (%)	4.38	1.99	5.60	
Fibre (%)	18.58	10.10	0.00	
Methionine (%)	0.45	0.33	2.95	0.40
Arginine (%)	1.44	1.20	5.82	1.13
Tyrosine (%)	0.80	0.99	3.4	1.79
Histidine (%)	0.51	0.58	2.43	0.42
Threonine (%)	0.93	0.80	4.31	1.17
Isoleucine (%)	1.12	0.87	4.68	0.80
Leucine (%)	2.33	2.05	7.60	1.35
Phenylalanine (%)	1.34	1.39	4.21	1.00
Lysine (%)	1.42	1.10	7.57	1.51
Valine (%)	1.56	1.10	5.25	0.88
Cysteine (%)	0.43	0.25	0.91	0.53

Amino acid requirements for tilapia adopted from Jauncey *et al.* (1983)

Table 4.2: Ingredients (%) and proximate composition of experimental diets

	Control	KLM 25	KLM 50	KLM 75	KLM 100	MLM 25	MLM 50	MLM 75	MLM 100
Fishmeal replaced	0	25	50	75	100	25	50	75	100
KLM	0.00	7.50	15.00	22.50	30.00	0.00	0.00	0.00	0.00
MLM	0.00	0.00	0.00	0.00	0.00	7.27	14.72	21.70	29.00
Fishmeal	10.62	7.73	5.25	2.77	0.00	8.00	5.31	2.70	0.00
Soybean meal	7.32	7.32	7.32	7.32	7.32	7.00	7.00	7.00	7.00
Canola meal	18.00	18.00	18.00	18.00	18.00	18.71	18.71	18.71	18.71
Sunflower meal	16.80	16.80	16.80	16.80	16.80	16.80	16.80	16.80	16.80
Maize gluten	11.60	12.00	12.00	12.00	12.00	11.60	11.60	11.60	11.60
Wheat middlings	2.17	2.17	2.17	2.17	2.17	5.00	5.00	5.00	5.00
Maize meal	28.56	23.45	18.37	13.30	8.25	18.08	13.25	8.73	4.39
Canola oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix ¹	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin premix ²	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Binder	2.00	2.00	2.00	2.00	2.00	4.00	4.00	4.00	4.00
Chromic oxide	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Dry matter (%)	94.69	92.40	92.32	91.32	91.40	94.65	95.22	95.47	94.84
Crude protein (%)	30.03	29.87	29.96	29.71	30.07	29.84	29.98	30.48	30.38
Energy (MJ kg ⁻¹)	20.21	20.34	20.48	20.53	20.47	20.58	20.42	20.26	20.36
Fat (%)	3.94	4.65	4.52	4.31	3.93	3.60	3.78	3.84	3.35
Crude fibre (%)	7.04	8.07	8.88	10.31	12.91	7.03	7.59	7.88	8.54

¹ Mineral premix (g kg⁻¹): KH₂PO₄, 502; MgSO₄ · 7H₂O, 162; NaCl, 49.8; CaCO₃, 336; Fe(II) gluconate, 10.9; MnSO₄ · H₂O, 3.12; ZnSO₄ · 7H₂O, 4.67; CuSO₄ · 5H₂O, 0.62; KI, 0.16; CoCl₂ · 6H₂O, 0.08; ammonium molybdate, 0.06; NaSeO₃, 0.02

² Vitamin premix (g or IU kg⁻¹ premix); thiamine, 5; riboflavin, 5; niacin, 25; folic acid, retinol palmitate, 500,000 IU;1; pyridoxine, 5; cyanocobalamine, 5; cholecalciferol; 50,000 IU; a-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; ascorbic acid, 10; choline chloride, 100; biotin, 0.25

4.3.3 Experimental system

The experiments were carried out in a recirculating water system (Figure 3.1) in a greenhouse described in Chapter 3, Section 3.3.1.

4.3.4 Water quality management

Dissolved oxygen, water temperature and ammonia were monitored every two days using a hand held YSI (556 MPS) water quality multi parameter instrument. pH was monitored biweekly using a pH meter (CG 840, Schott). Photoperiod was natural.

4.3.5 Experimental design, diet allocation and feeding

A completely randomised design was used in this experiment. At the beginning of the experiment, fish were individually weighed on a Mettler PM 6000 balance to the nearest 0.1 g. Each diet was randomly allocated to triplicate groups of *Tilapia rendalli* (12.00 ± 2 g) stocked at 15 fish per tank. All fish were hand-fed their allocated diet to apparent satiation (until one or two pellets remain uneaten for 2 minutes). Feed was offered three times daily at 0900, 1300 and 1700 hours. The amount of feed consumed in each tank was recorded daily. The feeding experiment was undertaken in 60 days.

Faeces collection

Faecal samples were siphoned from each tank three times per day (2 hours after each feeding). Faecal samples from the same dietary treatments were pooled, excess water was drained and the samples stored in a freezer (-20°C). Faecal collection started from the third week of the experiment and continued until sufficient faecal matter was collected for analysis.

4.3.6 Proximate composition of leaf meals, experimental diets and faeces

All the diets and ingredients were analysed for dry matter, crude protein, crude lipid, crude fibre and gross energy following the procedures stipulated by the Association of Official Analytical Chemists (AOAC International, 2012). All samples were analysed in triplicate.

4.3.6.1 Dry matter

A sample of the feed ingredients and diets was dried to constant weight for 24 hours in an oven maintained at 105°C. Dry matter was expressed as a percentage of the initial sample weight.

4.3.6.2 Crude Protein

Crude protein (CP) was calculated from the nitrogen content of each sample. Nitrogen content of the dry matter was determined on the LECO FP2000 Nitrogen Analyser, which quantifies the nitrogen fraction using the Dumas combustion method. Protein content was then calculated as percent (%) nitrogen x 6.25.

4.3.6.3 Crude Lipid

Lipid content was assessed using the solvent extraction method that uses a Soxhlet extraction unit (Tecator Soxtech HT 1043 Extraction unit). Crude lipid in diets and ingredients was determined with petroleum ether at 50°C. This method depends upon the heating of solvent, which is allowed to pass through the sample to extract the lipid. The extract is collected and when the process is completed the solvent is evaporated and the remaining crude lipid is dried and weighed. Crude lipid was then calculated as: $\text{Crude lipid (\%)} = (\text{extracted lipid} / \text{sample weight}) \times 100$.

4.3.6.4 Crude fibre

Crude fibre was determined as loss in weight after ignition of dried lipid-free residues after digestion with 1.25% sulphuric acid solution and 1.25% sodium hydroxide solution. This method depends upon digestion of moisture free and solvent extracted sample with weak acid solution and then with weak base solution. The remaining residue is then ashed for 2 hours at 550°C in a muffle furnace and the difference in weight on ashing was regarded as the crude fibre (hydrolysis resistant organic matter). The extracted fibre was then expressed as a percentage of the original un-

defatted sample and calculated as: Fibre (%) = [(digested sample – ashed sample) / sample weight] x 100.

4.3.6.5 Gross energy

Gross energy was determined using an automatic adiabatic bomb calorimeter (Gallenkamp and Co Ltd, England). The method is based on combustion in a 'bomb' chamber; once the sample is burned, the resulting heat is measured by a rise in temperature of water surrounding the bomb. Benzoic acid was used as a standard. The energy value obtained from the standard of known energy was used to calibrate the system. The energy content in each sample was calculated according to the formula: Gross Energy (kJ/g) = [(final temperature-initial temperature) x 10.82]-0.0896 / sample weight (g).

where: 10.82 = Heat capacity of the calorimeter (kJ/K)

0.0896 = Combined energy value of nickel wire and cotton (kJ/g).

4.3.6.6 Chromic Oxide

Chromic oxide in diets and faeces was analysed using Furukawa and Tsukahara (1966)'s method. This method involves the digestion of the sample by concentrated nitric acid and subsequent oxidation of chromic oxide with 70% perchloric acid. An orange colour is formed by the oxidation of chromium III to chromium VI. This was then read on a spectrophotometer (Uvikon 810) at 350 nm against distilled water. Chromic oxide was then calculated as: Weight of chromic oxide in sample = (Absorbance – 0.0032 / 0.2089)

Chromic oxide (%) = weight of chromic oxide/ sample weight (mg)] x 100

4.3.6.7 Amino acids

To estimate the amino acid concentrations in fishmeal, KLM and MLM, samples were hydrolysed with 6N HCl at 110°C for 24 hours. For the determination of methionine and cysteine, samples were treated with performic acid to oxidise methionine to methionine sulfone and cystine to cysteic acid (Kasproicz-Potocka *et al.*, 2013). All samples were then hydrolysed at 1108°C in vacuo in 6 M-HCl for 22 hours and analysed in an automatic AA analyser (4151 Alpha Plus Amino Acid Analyser; Pharmacia LKB Biochrom Ltd, Cambridge, UK).

4.3.6.8 Anti-nutrients

4.3.6.8.1 Tannins and phenols

Total tannins and condensed tannins in KLM and MLM were determined using the spectrophotometric methods described by Makkar *et al.* (1993). Tannins were calculated as the difference between phenolics before and after tannin removal from the extract with the use of insoluble polyvinyl pyrrolidone. Phenolic content was measured with Folin-Ciocalteu reagent. Condensed tannins were measured with butanol-HCl-Fe³⁺ reagent (Porter *et al.*, 1986). Total phenols and tannins were then expressed as tannic acid equivalent and condensed tannins as leucocyanidin equivalent.

4.3.6.8.2 Phytate

Phytate content was determined using the colorimetric method described by Vaintraub and Lapteva (1988). Ground samples (0.5 g for each leaf meal) were stirred in 10 ml of 3.5% hydrochloric acid for 1 hour. The contents were centrifuged at 3500 x g for 10 min to obtain the supernatants. Suitable aliquots of the supernatants were then diluted with distilled water to make up 3 ml, which was used for the assay.

4.3.6.8.3 Saponins

Total saponin content was determined using the spectrophotometric method, as used by Makkar *et al.* (1993). Ground samples (0±5 g of beach leaf meal) were mixed with 10 ml of 80% aqueous methanol and stirred overnight using a magnetic stirrer. The mixture was then centrifuged at 3500 x g for 10 min and the supernatants were collected. The residues were subsequently washed three times with 5 ml of 80% aqueous methanol then centrifuged and the supernatants were collected in volumetric flasks. Aliquot samples from the flasks were then used for saponin determination. The results were expressed as diosgenin equivalent from a standard curve plotted using different concentrations of diosgenin in 80% aqueous methanol.

4.3.7 Growth performance parameters

All fish from each tank were bulk weighed once every two weeks. At the end of the experimental period, fish were individually weighed and the following growth performance indices were calculated:

Specific growth rate (SGR) is the average percentage weight gained per day between any two weighings.

(SGR was calculated according to Winberg (1956) as:

$$\text{SGR (g/day)} = \left[\frac{\ln W_t - \ln W_0}{t} \right] \times 100\%$$

where: W_t = final body weight (g)

W_0 = initial body weight (g)

t = time feeding period (days)

\ln = natural Logarithm (log)⁻¹⁰

Thermal-unit growth coefficient (TGC) offers a simple mode of growth rate comparison in fish as it is a standardised measure of growth that is unaffected by live weight, time interval and water temperature. TGC was calculated according to Iwama and Tautz (1981) as:

$$\text{TGC} = 100 \times \frac{\text{FBW}^{0.333} - \text{IBW}^{0.333}}{\sum T^{\circ}\text{C} \times \text{days}}$$

where: FBW = final body weight (g/fish)

IBW = initial body weight (g/fish)

T = temperature.

Feed Conversion Ratio (FCR) is the amount of body weight gained for every kilogram of feed consumed.

$$\text{Feed conversion ratio (FCR)} = \frac{\text{food consumed (g)}}{\text{mass gained (g)}}$$

Protein efficiency ratio (PER) is defined as fish weight gained per gram of crude protein fed. PER indicates the efficiency of protein utilisation and is calculated as:

$$\text{PER} = \frac{\text{Increase in body mass (g)}}{\text{Protein consumed (g)}}$$

Digestibility Determination

Chromic oxide (Cr_2O_3) was used as an inert indicator in the experimental diets. It passes unaffected by digestion through the alimentary tract of fish, providing a

convenient method of measuring digestibility without the need of quantitative collection of faeces (Furukawa and Tsukahara, 1966).

Apparent digestibility coefficient for protein

Apparent digestibility coefficient (ADC) for protein was calculated as:

$$\text{Protein ADC (\%)} = 100 \left(1 - \left(\frac{\% \text{ Cr}_2\text{O}_3 \text{ diet}}{\% \text{ Cr}_2\text{O}_3 \text{ in faeces}} \right) \times \left(\frac{\% \text{ protein in faeces}}{\% \text{ protein in diet}} \right) \right).$$

4.3.8 Histological analysis

Liver samples and 1 cm segments from the small intestine were obtained from the mid intestine of fifteen fish per dietary treatment. The samples were submerged in 10% neutral buffered formalin for 24 hours. Thereafter, the tissues were dehydrated in 70% ethanol until embedded in paraffin, sectioned and stained with hematoxylin and eosin according to standard histological techniques in graded ethanol series, and embedded in paraffin wax for histological examination. Short ribbons of sections (3-5 μm) were cut and placed into a heated water bath to flatten the sections. They were then mounted on glass slides and dried before staining with haematoxylin and eosin. Slides were examined and photographed under light trinocular microscopy at 400X (Leica Microsystems model DM750, Leica, Bannockburn, IL, USA). Images were captured with a DVC digital camera (Digital Video Camera Company, Austin, TX) mounted on a BH-2.

McFaden *et al.* (1997)'s criteria was used to categorise the differences in (liver) hepatocyte condition. Each specimen was assigned one of 3 grades (1-3), a healthy specimen scoring 1 and a degraded liver scoring 3 as outlined in Table 4.3. The hepatocyte degradation mean value of liver samples from each treatment was the mean score of all samples in that treatment.

Table 4.3: Histological criteria used for scoring of *Tilapia rendalli* hepatocytes

Tissue	Grade		
	1 (Healthy)	2 (Intermediate)	3 (Degraded)
Liver nuclei	Lightly granular, small and distinct	Abundant granules, enlarged or indistinct nucleoli	Small dark and pyknotic
Cytoplasm	Structured: Varied texture, scattered granules with eosin positive patches	Homogenous: Granular slight variability in staining property.	Hyaline: Lacking texture, dark small and often separated the cell boundary
Vacuolation	Vacuoles follow cell boundary and encroach on the nucleus.	Small occasional vacuoles.	Poor vacuolation, cells shrink leading to large sinusoidal spaces.

Adapted from McFadzen *et al* (1997)

Villi heights and widths (μm) from the intestine samples were measured on Image J (1.46) software. Twenty measurements were taken from each slide. The number of goblet cells per segment was counted according to Baeverfjord and Krogdahl (1996). The number of goblet cells was expressed as the total number of phenotypically mature goblet cells (based on the intensity of staining, the size of the apical region and morphology) per 100 epithelial cells.

4.3.9 Digestive enzyme analysis

At the end of the feeding period, whole intestines from five fish in each dietary treatment were pooled and stored in marked plastic sample bags at -86°C until used for analysis of enzyme activity. The intestines were homogenised (1:2 w/v) with 50 mM Tris-HCl buffer (pH 7.5) in an ice water bath, using a tissue homogeniser. The preparation was centrifuged at $4\ 200 \times g$ for 60 minutes at 4°C . The floating lipid fraction was discarded and the aqueous supernatant recovered and kept at -20°C until used. Protease, amylase and lipase activities were determined as described in Chapter 3, Section 3.3.5.

4.3.10 Haematological analysis

On termination of the experiment, blood samples were taken from five fish per tank (15 fish per dietary treatment) for haematological analysis. Blood was drawn through caudal venous puncture into lavender top EDTA tubes (with anticoagulant) for total blood count. Total blood count was done on a Systemex, XT-1800i blood analyser. For glucose determination, blood was drawn into glucose tubes with sodium fluoride and for protein and blood urea nitrogen (BUN) determination; blood was drawn into serum tubes with acid citrate dextrose. Analysis for plasma glucose was done on a 600: UniCel DxC General Chemistry Analyzer.

4.3.11 Cost benefit analysis

Assuming that all other operating costs remained constant and the cost of ingredients was the only variable cost, the following economic indicators as used by Bahnasawy *et al.* (2003) were calculated:

$$\text{Incidence cost} = \frac{\text{cost of feed}}{\text{quantity of fish produced (kg)}}$$

$$\text{Profit index} = \frac{\text{local market value of fish}}{\text{cost of feed}}$$

4.3.12 Statistical analysis

Each tank was considered an experimental unit. Percentage data was arcsine transformed prior to carrying out regression analysis. Linear regression was performed on feed intake, growth performance parameters (TCG, SGR), feed utilisation parameters (FCR, PER) and apparent digestibility of protein for each diet. The regressions were significant when $P < 0.05$. Analysis of covariance (ANCOVA) was used to test if there were significant differences between the KLM and the MLM regressions for each parameter. Normality and homogeneity of variance was confirmed using the Shapiro-Wilk normality test and Levene test respectively (SAS, 2008). Kruska Wallis test was used to determine if there are any significant differences in the histological scores. Data on the haematological parameters were subjected to a one way analysis of variance (ANOVA). Significance was accepted at probabilities < 0.05 . All statistical analysis was carried out on SAS (SAS, 2008).

4.4 RESULTS

4.4.1 Anti-nutrients in kikuyu grass and moringa leaves

Both KLM and MLM contain polyphenols, tannins, phytate and saponins (Table 4.4). The levels of both tannins and total polyphenols were significantly higher ($P<0.05$) in MLM than in KLM. Phytate levels were significantly higher in MLM than in KLM. Saponin levels in MLM were also significantly higher (more than three times) than those in KLM ($P<0.05$) (Table 4.4).

Table 4.4: Anti-nutritional factors (g/kg) in KLM and MLM used in the experimental diets

	KLM	MLM
Total polyphenols	14.82±4.7 ^a	43.02±6.1 ^b
Tannins	8.40±0.5 ^a	12.10±1.1 ^b
Phytate	6.42±1.3 ^a	25.41±3.7 ^b
Saponins	20.31±4.2 ^a	79.32±8.5 ^b

4.4.2 Growth performance and protein apparent digestibility

4.4.2.1 Weight gain

No mortality was observed for the duration of the experiment (60 days). Fish fed the fishmeal-based control had the highest ($P<0.05$) body weight gain and this was evident from the second week of the experiment (Figure 4.1). The inclusion of KLM led to reduced weight gain. *Tilapia rendalli* fed KLM 100 had the lowest weight gain (Figure 4.1). Similarly, replacing fishmeal with increasing levels of MLM in the diet of *Tilapia rendalli* resulted in reduced weight gain (Figure 4.2).

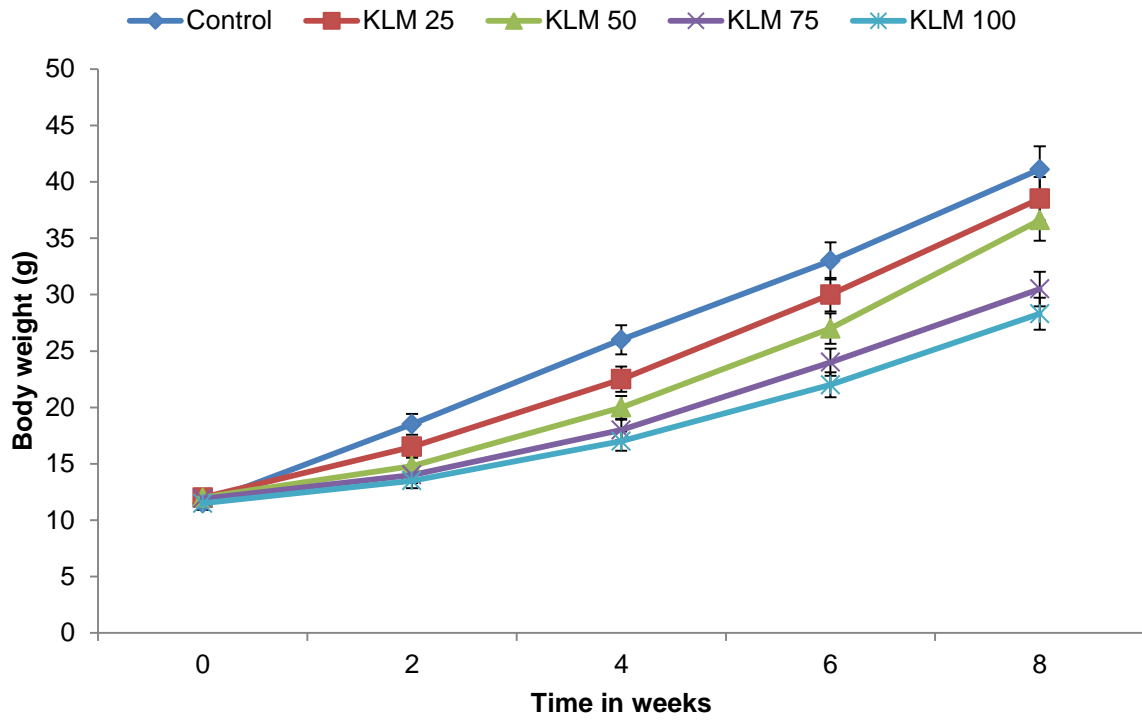


Figure 4.1: Change in body weight of *Tilapia rendalli* fed KLM diets

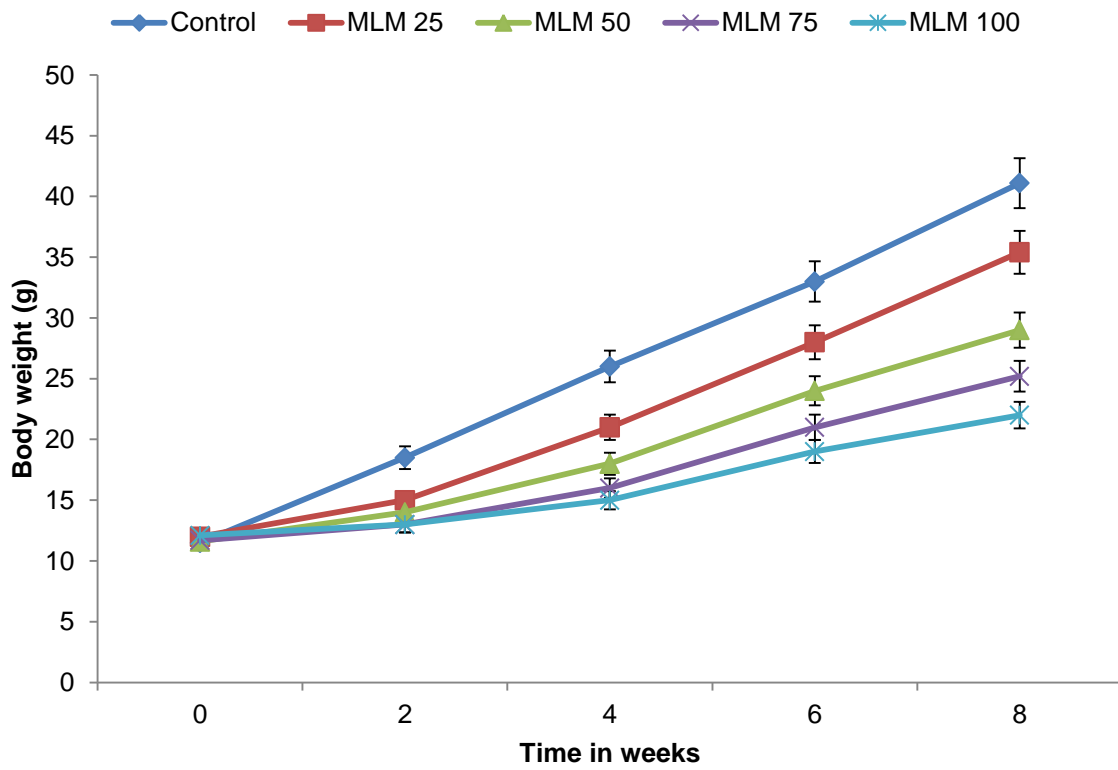


Figure 4.2: Change in body weight of *Tilapia rendalli* fed MLM diets

4.4.2.2 Feed intake

The control diet was readily consumed and fish in this group had the highest feed intake (Figure 4.3A; Figure 4.3B). Inclusion of KLM (Figure 4.3A) in the diet had a significantly ($P < 0.05$) negative linear relationship with feed intake. Fish showed reluctance to consume diets containing higher levels of MLM and were observed to spit out pellets a few times before actual ingestion. Thus, increasing MLM in the diet also had a significant ($P < 0.05$) negative linear relationship with feed intake (Figure 4.3B). Feed intake in kikuyu treatment groups was significantly higher than in the moringa treatment groups ($P < 0.05$, ANCOVA).

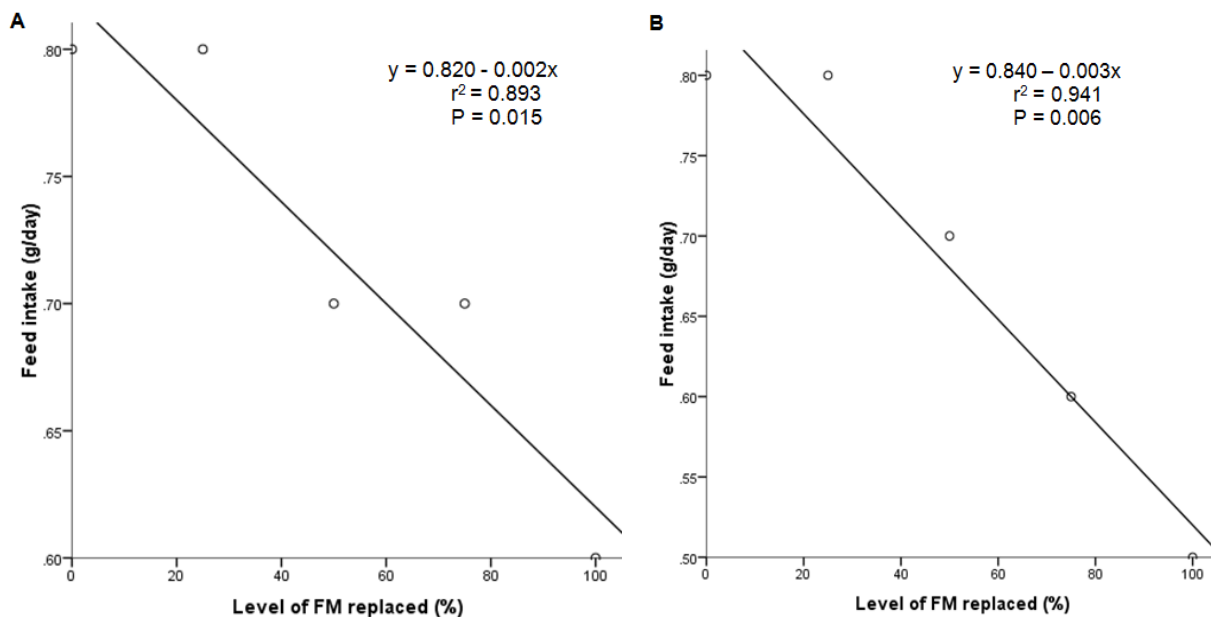


Figure 4.3: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on feed intake in *Tilapia rendalli*

4.4.2.3 Thermal growth coefficient

The decline in feed intake was accompanied by a decline in growth performance. *Tilapia rendalli* fed the control diet had the highest thermal growth coefficient (Figure 3.4A; Figure 4.4B). Replacing fishmeal with KLM (Figure 4.4A) and MLM (Figure 4.4B) in the diet had a negative linear relationship with TGC. Thermal growth coefficient was significantly ($P < 0.05$) reduced when KLM replaced more than 50% fishmeal. On the other hand, MLM could only replace 25% fishmeal in the diet without a significant decrease in TGC. Fish fed MLM 100 had the lowest growth

TGC. Fish fed kikuyu diets showed significantly higher TGC ($P < 0.05$; ANCOVA) than those fed moringa diets.

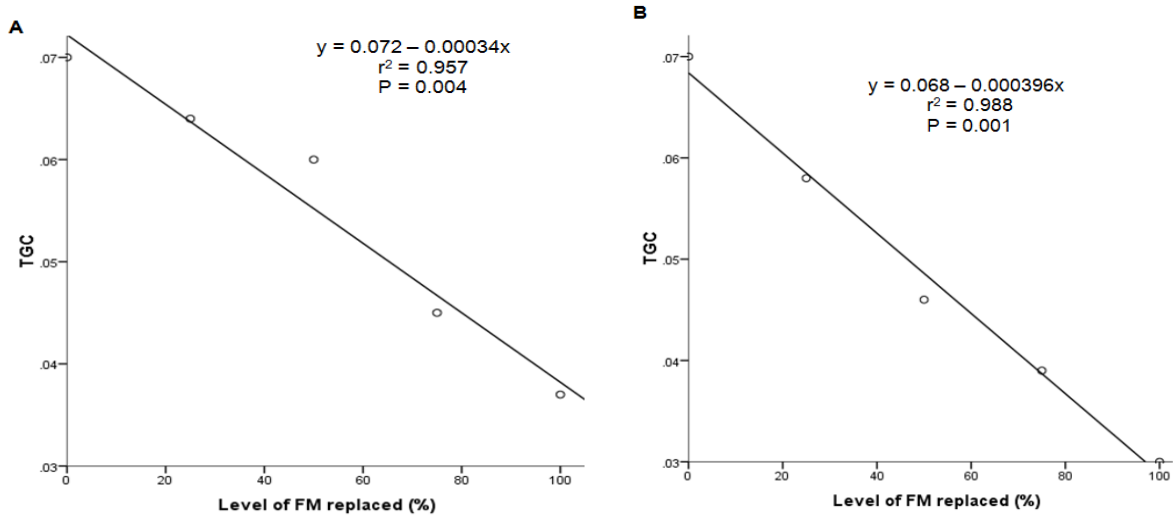


Figure 4.4: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on thermal growth coefficient in *Tilapia rendalli*

4.4.2.4 Specific growth rate

Specific growth rate was highest in *Tilapia rendalli* fed the control diet. SGR was higher in fish fed KLM diets than in those fed MLM diets. Increasing levels of both KLM (Figure 4.5A) and MLM (Figure 4.5B) had negative linear relationships ($P < 0.05$) with SGR. The negative linear relationship between SGR and level of fishmeal replaced in the diet for KLM diets and MLM diets was significantly different ($P < 0.05$, ANCOVA).

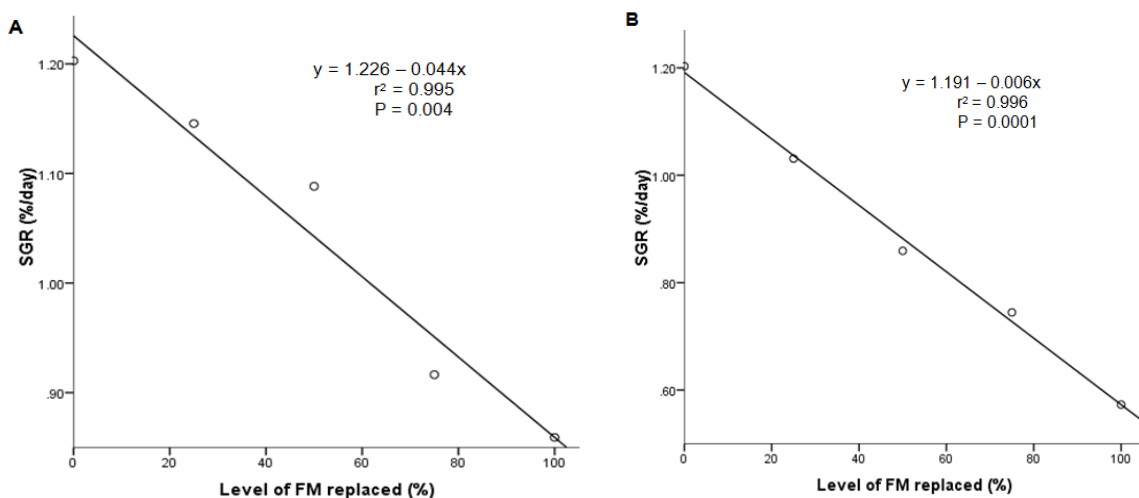


Figure 4.5: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on specific growth rate in *Tilapia rendalli*

4.4.2.5 Feed conversion ratio

The best FCR was recorded in fish fed the control diet (Figure 4.6A; Figure 4.6B). Replacing fishmeal with increasing levels of both leaf meals had a significant ($P < 0.05$) positive linear relationship with FCR. Increasing KLM to replace more than 25% of fishmeal led to FCR values that were significantly higher ($P < 0.05$) than the control (Figure 4.6A). When MLM replaced fishmeal (Figure 4.6B), the FCR values were higher than those of KLM-based diets. FCR in the KLM treatment groups was significantly lower than in the moringa treatment groups ($P < 0.05$, ANCOVA).

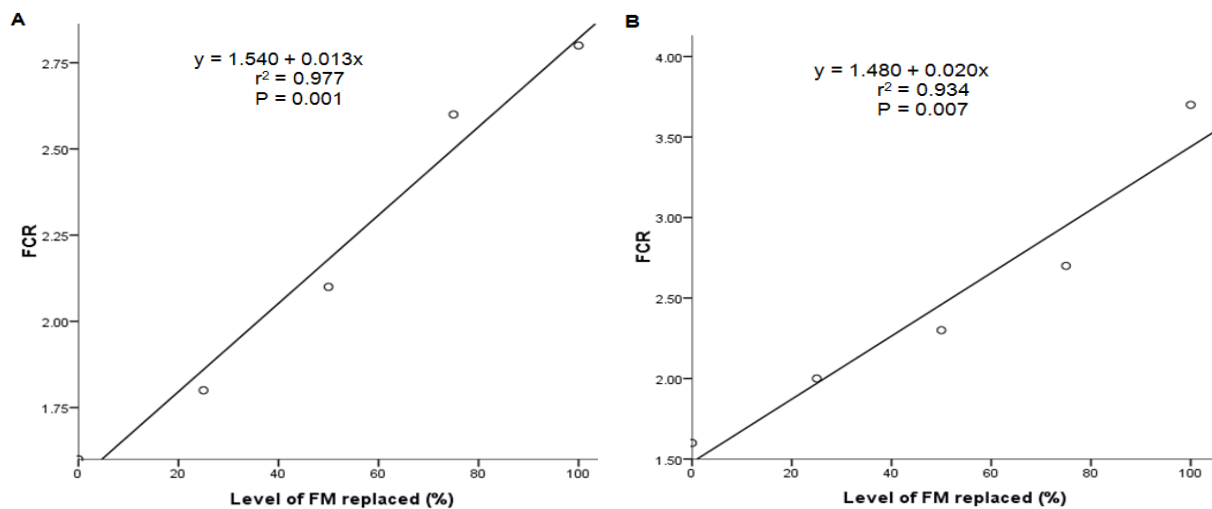


Figure 4.6: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on feed conversion ratio in *Tilapia rendalli*

4.4.2.6 Protein efficiency ratio

Protein efficiency ratios obtained show that protein in KLM is utilised better than that in MLM. In the KLM-based diets, the PER of fish fed KLM 25 (1.88 ± 0.91) was not significantly different ($P > 0.05$) from that of fish fed the control diet (2.10 ± 0.94) (Figure 4.7A). On the contrary, PER of fish fed MLM-based diets were significantly lower ($P < 0.05$) than the control even at the lowest inclusion level (MLM 25: 1.69 ± 1.05). Significant negative linear regressions were obtained for PER in both KLM (Figure 4.7A) and MLM diets (Figure 4.7B).

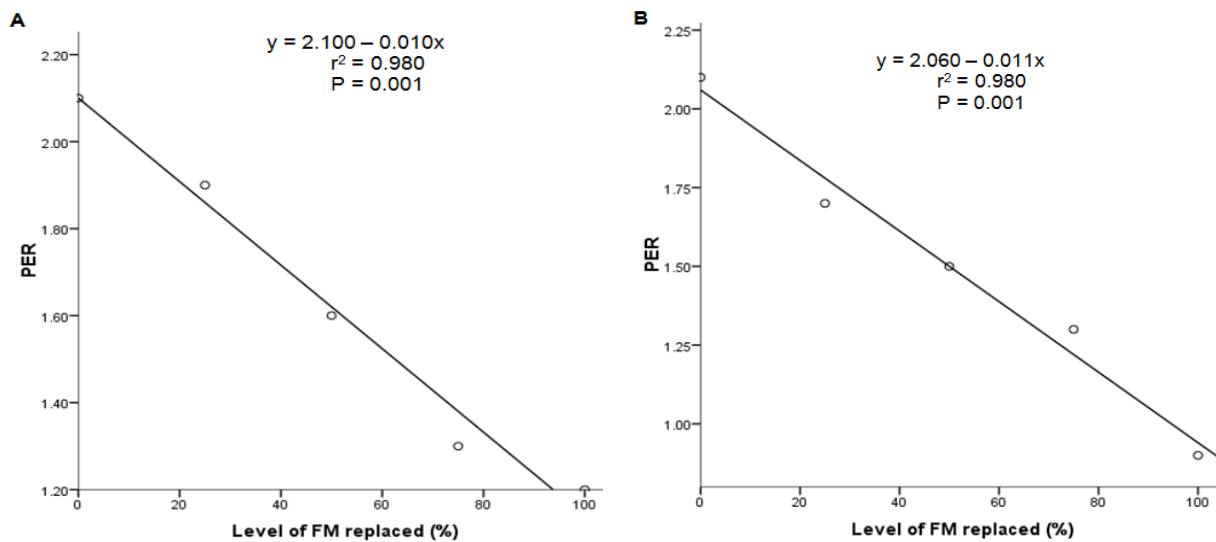


Figure 4.7: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on protein efficiency ratio in *Tilapia rendalli*

4.4.2.7 Apparent digestibility coefficient

The values recorded for ADC (protein) also show that KLM was digested better than MLM. ADC for protein was highest (82.09%) in fish fed the control diet (Figure 4.8A; Figure 4.8B). ADC decreased when fishmeal was replaced with either KLM or MLM. The relationship for protein ADC with KLM and MLM were both linear and significant ($P < 0.05$). The ADC for protein was significantly higher ($P < 0.05$, ANCOVA) in KLM-based diets (Figure 4.8A) compared to the MLM-based diets (Figure 4.8B).

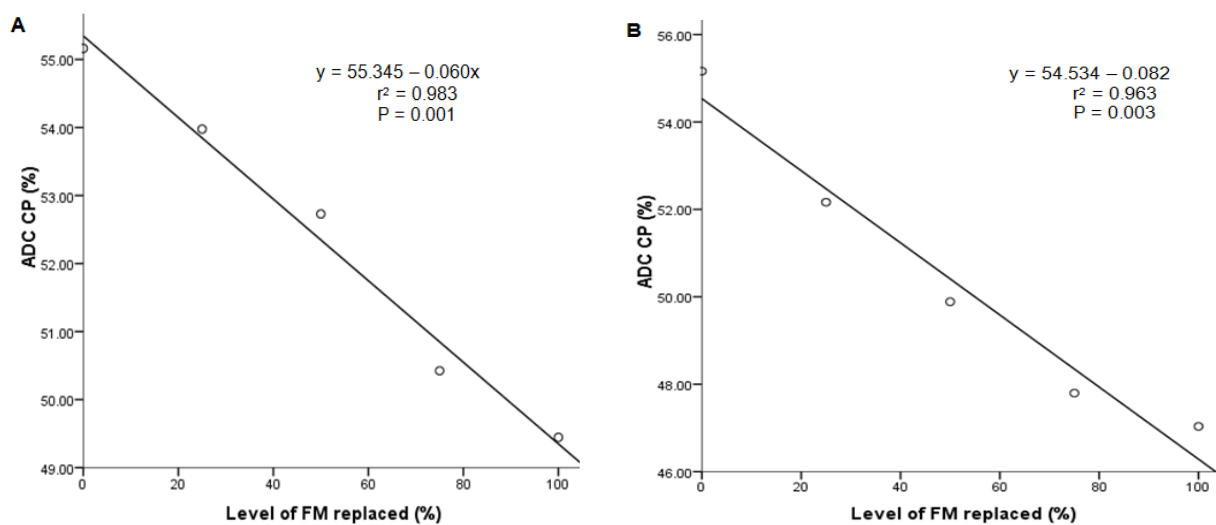


Figure 4.8: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on apparent digestibility coefficient for protein in *Tilapia rendalli*

4.4.3 Effect of plant diets on intestine histology

There was no apparent effect of KLM-based diets on the villi length in *Tilapia rendalli* (Table 4.5). In fish fed MLM diets however, decreasing villi length was evident as the level of MLM in the diet increased. This decrease in villi length however, was not significant ($P>0.05$).

An increase in the number of goblet cells in *Tilapia rendalli* enterocytes with increasing leaf meal inclusion in the diet was observed. The goblet cell number increased with increasing levels of both KLM and MLM. The increase in goblet cell number was not significant ($P>0.05$) in both leaf meals. Fish fed MLM-based diets had higher goblet cell numbers than those fed KLM diets (Table 4.5).

Table 4.5: Histological values of the mid intestine of *Tilapia rendalli* fed different inclusion levels of kikuyu and moringa leaf meals

		% of fishmeal replaced by the plant meals in the diet				
		0	25	50	75	100
<i>Villi length (μm)</i>						
KLM	488.6 \pm 12 ^a	481.3 \pm 15 ^a	476.8 \pm 10 ^a	484.2 \pm 18 ^a	479.9 \pm 11 ^a	
MLM		479.8 \pm 20 ^a	472.0 \pm 15 ^a	469.7 \pm 10 ^a	461.0 \pm 18 ^a	
<i>Villi width (μm)</i>						
KLM	68.2 \pm 11 ^a	72.7 \pm 21 ^a	66.9 \pm 23 ^a	65.4 \pm 21 ^a	69.3 \pm 17 ^a	
MLM		69.7 \pm 16 ^a	71.9 \pm 19 ^a	68.5 \pm 10 ^a	71.8 \pm 14 ^a	
<i>Goblet cells (per 100 epithelial cells)</i>						
KLM	397 \pm 18 ^a	381 \pm 12 ^a	402 \pm 22 ^a	400 \pm 13 ^a	423 \pm 24 ^a	
MLM		386 \pm 16 ^a	416 \pm 17 ^a	429 \pm 20 ^a	447 \pm 18 ^a	

KLM-kikuyu leaf meal; MLM-moringa leaf meal

There were no degenerative changes observed in the intestine of *Tilapia rendalli* fed lower levels of KLM (KLM 25 and KLM 50). However, when the level of KLM was increased to replace more than 50%, an increase in the number of goblet cells was evident (Figure 4.9). In fish fed the MLM-based diets on the other hand, higher incidence of goblet cells was recorded when MLM replaced more than 25% fishmeal (Figure 4.10).

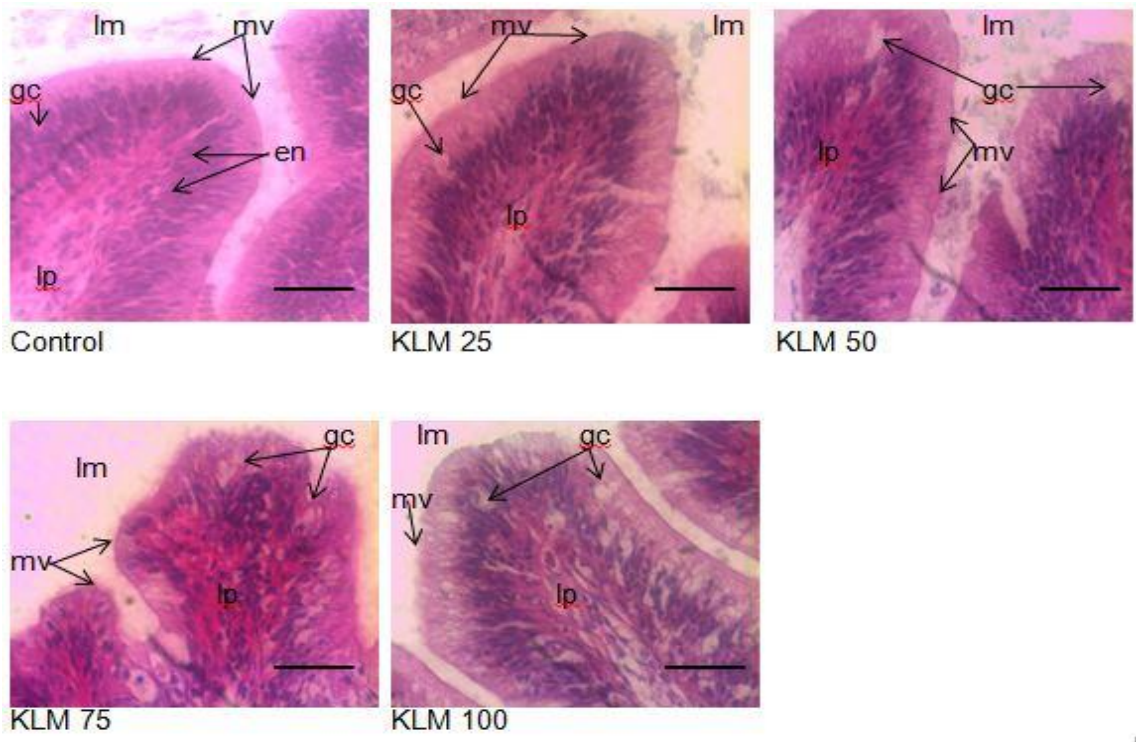


Figure 4.9: Histological sections (H and E-stained) of the intestine from the mid gut of *Tilapia rendalli* fed kikuyu-based diets. lm-lumen; lp-lamina propria; arrows point to-mv, microvilli; gc, goblet cells. Scale bar 10 μ m

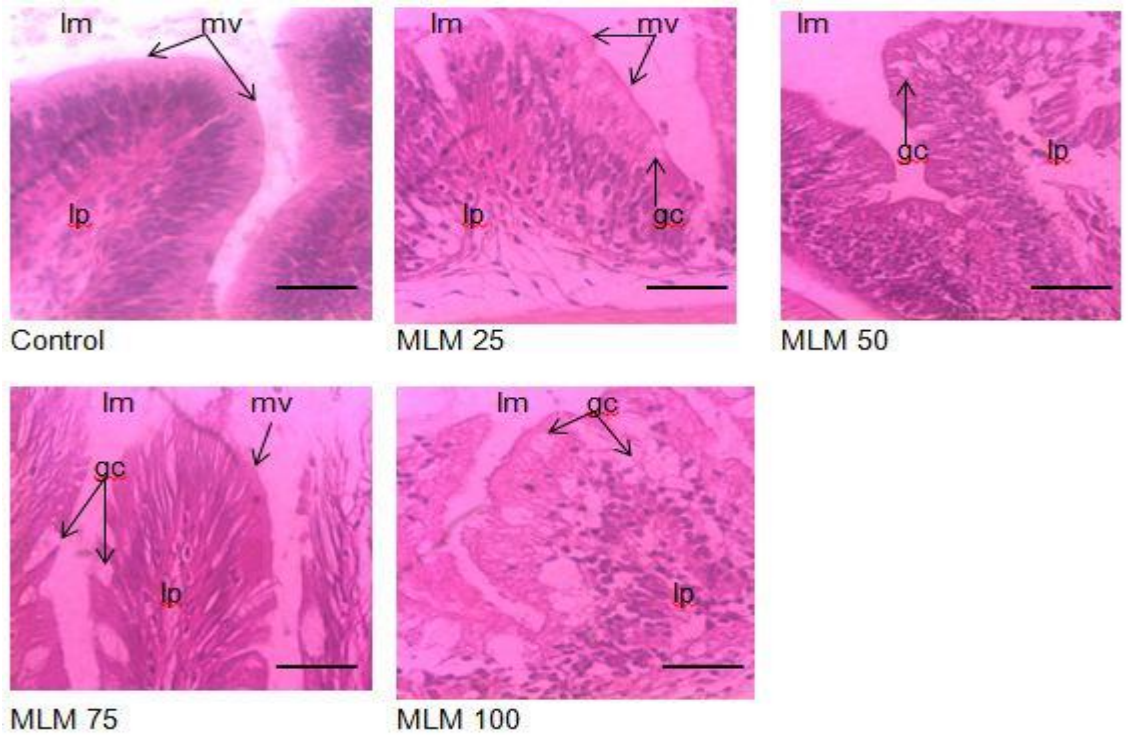


Figure 4.10: Histological sections (H and E-stained) of the intestine from the mid gut of *Tilapia rendalli* fed moringa-based diets. lm – lumen; lp, lamina propria; arrows point to-mv, microvilli; gc, goblet cells. Scale bar 10 μ m

4.4.4 Effect of plant diets on liver histology

Liver histology showed centrally located lightly granular nuclei with distinct boundaries of the hepatocytes and normal vacuolation in *Tilapia rendalli* fed the control diet (Figure 4.11). This group was assigned a hepatocyte degradation mean value of 1.33 (Table 4.6). Fish fed KLM 25 showed a slight decrease in vacuolisation (Figure 4.11). The alterations were however not significantly different ($P>0.05$) from the control (Table 4.6). Increasing the level of KLM in the diet resulted in increased degradation of the hepatocytes. Fish fed KLM 75 and KLM 100 showed an increased number of pyknotic nuclei, reduced vacuolation and increased sinusoidal space (Figure 4.11). The hepatocyte degradation mean values (1.90 and 2.02 respectively) were significantly higher ($P<0.05$) than the control (1.33; Table 4.6).

Hepatocyte histology in *Tilapia rendalli* fed the moringa-based diets displayed greater degradation when compared to the kikuyu diets at the same inclusion levels. Fish fed MLM 25 showed granular cytoplasm and increased sinusoids (Figure 4.12). The hepatocyte degradation score (1.80) was significantly greater ($P<0.05$) than that of the control (1.33: Table 4.6). When the level of MLM in the diet increased, the degree of hepatocyte degradation also increased. In MLM 75 and MLM 100 treatments, the hepatocyte degradation values were 2.43 and 2.63 respectively (Table 4.6). These hepatocytes were irregularly shaped with the nucleus pushed to the periphery of the cells; increased sinusoidal space and fatty degeneration were also observed (Figure 4.12).

Table 4.6: Hepatocyte degradation scores of *Tilapia rendalli* fed different inclusion levels of kikuyu and moringa leaf meals (100 hepatocytes per treatment)

	% of fishmeal replaced by the plant meals in the diet				
	0	25	50	75	100
<i>Hepatocyte degradation score</i>					
KLM	1.33±0.6 ^a	1.63±0.5 ^a	1.87±0.3 ^{ab}	1.90±0.5 ^{bc}	2.20±0.4 ^c
MLM	1.33±0.6 ^a	1.80±0.2 ^{ab}	2.27±0.7 ^b	2.43±0.4 ^c	2.63±0.6 ^d

Values in the same row with the same superscripts are not significantly different ($P>0.05$)

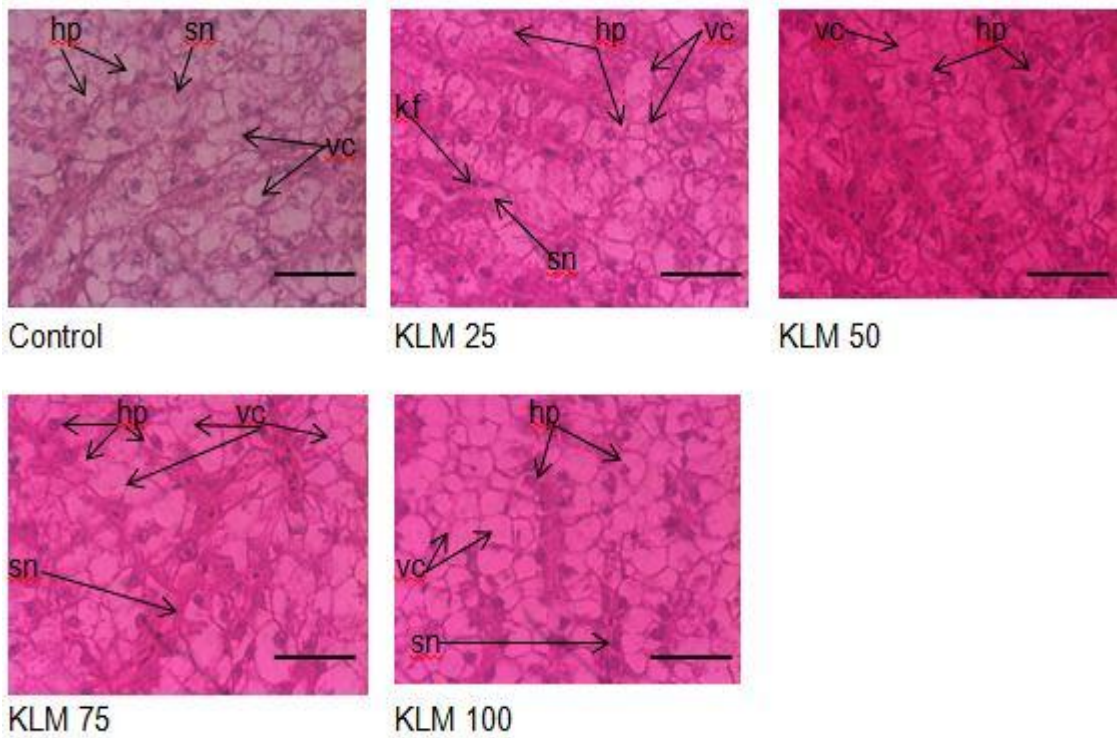


Figure 4.11: Histological sections (H and E stained) of the liver of *Tilapia rendalli* fed kikuyu-based diets. Arrows point to: hp, hepatocytes; vc, vacuoles; sn, sinusoids; kc, kupffer cells. Scale bar 10 μm

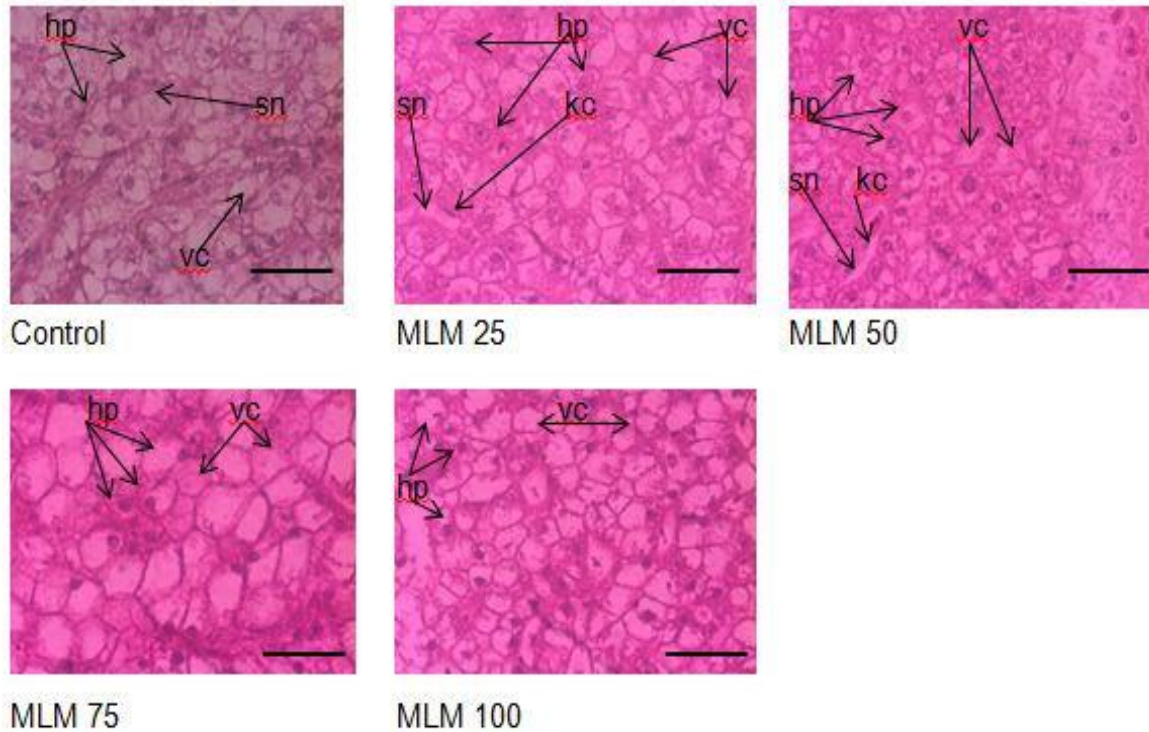


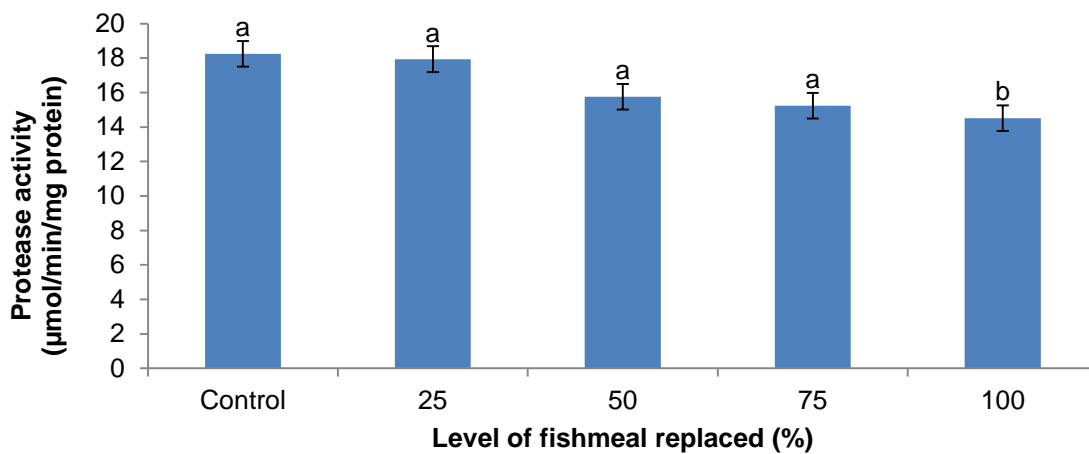
Figure 4.12: Histological sections (H and E stained) of the liver of *Tilapia rendalli* fed moringa-based diets. Arrows point to: hp, hepatocytes; vc, vacuoles; sn, sinusoids; kc, kupffer cells. Scale bar 10 μm

4.4.5 Effect of plant diets on the activities of digestive enzymes

4.4.5.1 Protease activity

Protease activity was highest (18.24 $\mu\text{mol} / \text{min} / \text{mg}$ protein) in fish fed the control diet and decreased with increasing levels of plant meal in the diet (Figure 4.13). In the KLM diets, the decrease in protease activity was only significant ($P < 0.05$) when more than 75% of dietary fishmeal was replaced (Figure 4.13A). Protease activity in fish fed the MLM-based diets decreased significantly when more than 25% fishmeal was replaced (Figure 4.13B).

A



B

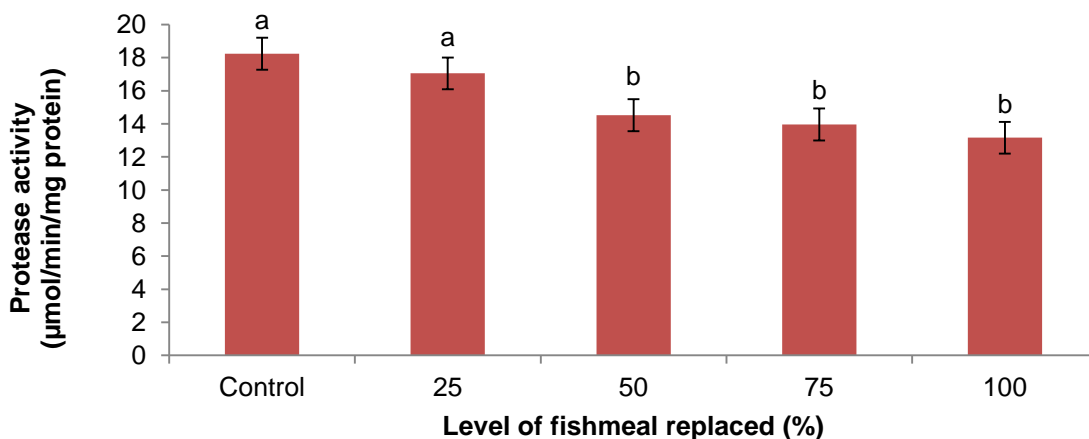


Figure 4.13: Effect of replacing fishmeal with KLM (A) and MLM (B) on the protease activities in *Tilapia rendalli* intestine. Bars with different letters are significantly different ($P < 0.05$)

4.4.5.2 Amylase activity

Tilapia rendalli fed the control diet also had the highest level of amylase activity in the intestine (24.32 $\mu\text{mol}/\text{min}/\text{mg}$ protein) (Figure 4.14A and Figure 4.14B). Amylase activity decreased significantly ($P < 0.05$) as the level of KLM replaced more than 50% fishmeal in the diet (Figure 4.14A). In the MLM diets, a significant decrease ($P < 0.05$) in amylase activity was observed when MLM replaced more than 25% fishmeal (Figure 4.14B).

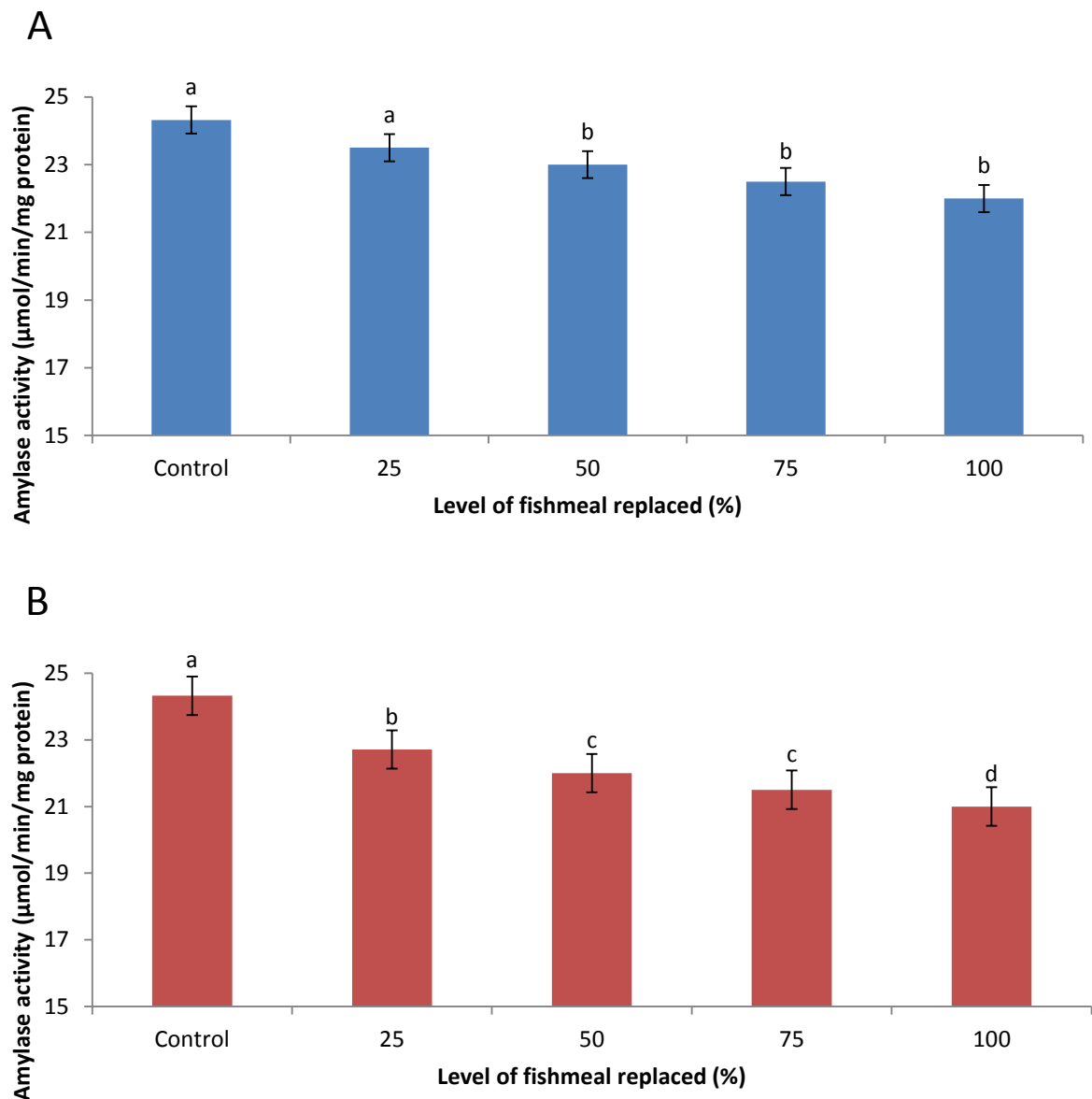


Figure 4.14: Effect of replacing fishmeal with KLM (A) and MLM (B) on the amylase activities in *Tilapia rendalli* intestine. Bars with different letters are significantly different ($P < 0.05$)

4.4.5.2 Lipase activity

Replacing dietary fishmeal with KLM did not have any significant effect ($P>0.05$) on the lipase activity in *Tilapia rendalli* intestine (Figure 4.15A). The addition of MLM on the other hand, led to significantly lower ($P<0.05$) lipase activity in fish fed diets MLM 75 and MLM 100 (Figure 4.15B).

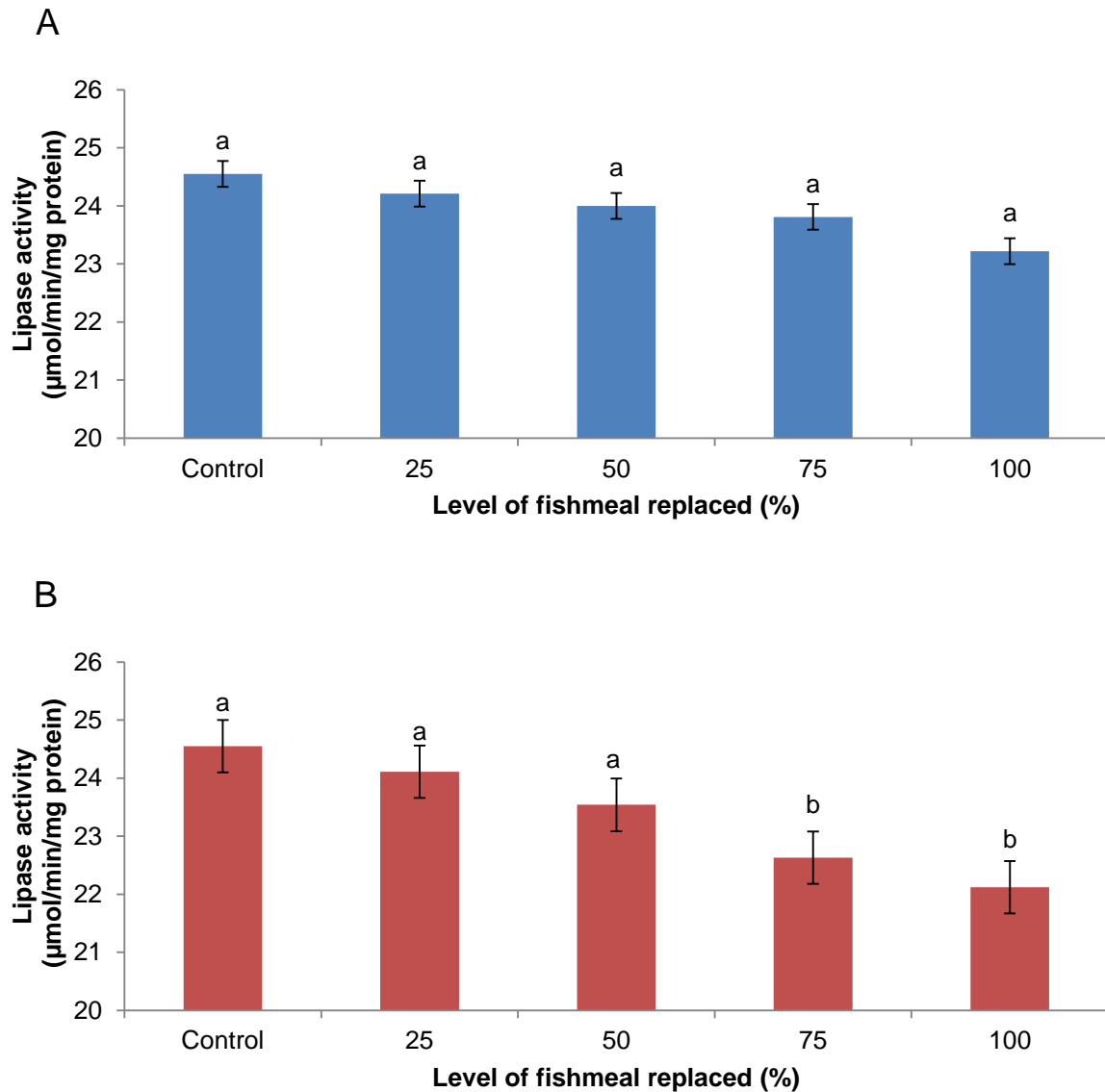


Figure 4.15: Effect of replacing fishmeal with KLM (A) and MLM (B) on the lipase activities in *Tilapia rendalli* intestine. Bars with different letters are significantly different ($P<0.05$)

4.4.6 Effect of plant diets on haematological parameters

In fish fed the KLM diets, white blood cells (WBC) were not significantly different ($P>0.05$) to those of fish fed the control diet (Table 4.7). The red blood cells (RBC)

and hematocrit (HCT) decreased with increasing KLM and these were not significantly different ($P>0.05$) to the control. There were no significant differences ($P>0.05$) in plasma glucose of *Tilapia rendalli* fed all KLM diets when compared to the control. Plasma protein and blood urea nitrogen (BUN) were also not significantly ($P>0.05$) different from the control diet at all KLM inclusion levels in diet (Table 4.7).

In the moringa-based diets, WBC increased with increasing MLM inclusion and were significantly higher ($P<0.05$) than the control in *Tilapia rendalli* fed diets where MLM replaced more than 25% fishmeal (Table 4.8). The RBCs and HCT were significantly lower ($P<0.05$) in MLM100 than in fish fed the control diet. Plasma glucose in all treatments was not significantly different ($P>0.05$) from the control. Plasma protein and blood urea nitrogen (BUN) were also not significantly ($P>0.05$) different from the control (Table 4.8).

Table 4.7: The Effect of kikuyu-based diets on haematological parameters of *Tilapia rendalli* (n = 15)

	Control	KLM 25	KLM 50	KLM 100
WBC ($10^3\mu\text{l}$)	264.41 \pm 7.5 ^a	255.11 \pm 8.2 ^a	244.19 \pm 5.9 ^a	260.03 \pm 4.5 ^a
RBC ($10^6\mu\text{l}$)	1.49 \pm 0.24 ^a	1.37 \pm 0.20 ^a	1.23 \pm 0.32 ^a	1.16 \pm 0.15 ^a
HCT (l/l)	0.26 \pm 0.14 ^a	0.25 \pm 0.21 ^a	0.24 \pm 0.13 ^a	0.16 \pm 0.11 ^a
Hb (g/dl)	6.00 \pm 1.1 ^a	6.60 \pm 2.1 ^a	5.20 \pm 1.3 ^a	6.60 \pm 1.4 ^a
MCV (fL)	181.81 \pm 3.4 ^a	182.48 \pm 5.2 ^a	195.12 \pm 6.1 ^a	139.70 \pm 7.2 ^a
MCH (pg)	42.00 \pm 2.1 ^a	48.18 \pm 3.5 ^a	42.30 \pm 2.4 ^a	56.90 \pm 2.2 ^a
MCHC (g/dL)	23.08 \pm 4.1 ^a	26.40 \pm 5.7 ^a	21.67 \pm 2.6 ^a	41.25 \pm 8.1 ^a
Glucose (g/dl)	10.20 \pm 2.1 ^a	13.40 \pm 2.3 ^a	12.40 \pm 1.5 ^a	8.00 \pm 1.4 ^a
Total protein (g/l)	32.00 \pm 2.5 ^a	28.00 \pm 2.1 ^a	27.00 \pm 1.0 ^a	26.00 \pm 2.2 ^a
BUN	1.00 \pm 0.11 ^a	1.20 \pm 0.12 ^a	1.50 \pm 0.21 ^a	1.10 \pm 0.13 ^a

Values are mean of 15 determinations \pm standard deviations. Values in the same rows with the same superscripts are not significantly different ($P>0.05$). WBC- white blood cells; RBC- red blood cells; HCT- hematocrit; Hb- haemoglobin; MCV- mean corpuscular volume; MCH- Mean corpuscular haemoglobin; MCHC- mean corpuscular haemoglobin concentration; Glucose- plasma glucose; BUN- Blood urea nitrogen

Table 4.8: The Effect of moringa-based diets on haematological parameters of *Tilapia rendalli* (n = 15)

	Control	MLM25	MLM50	MLM100
WBC ($10^3\mu\text{l}$)	264.41 \pm 7.5 ^a	268.75 \pm 9.2 ^a	271.14 \pm 4.2 ^b	298.91 \pm 7.3 ^b
RBC ($10^6\mu\text{l}$)	1.49 \pm 0.24 ^a	1.44 \pm 0.11 ^a	1.43 \pm 0.25 ^a	1.27 \pm 0.18 ^b
HTC (l/l)	0.26 \pm 0.14 ^a	0.27 \pm 0.21 ^a	0.27 \pm 0.15 ^a	0.12 \pm 0.05 ^b
Hb (g/dl)	6.00 \pm 1.1 ^a	5.60 \pm 1.2 ^a	6.80 \pm 1.5 ^a	4.70 \pm 1.2 ^a
MCV (fL)	181.81 \pm 3.4 ^a	187.50 \pm 3.8 ^a	181.21 \pm 4.5 ^a	169.06 \pm 6.1 ^a
MCH (pg)	42.00 \pm 2.1 ^a	38.90 \pm 3.1 ^a	45.64 \pm 2.4 ^a	56.20 \pm 3.5 ^a
MCHC (g/dL)	23.08 \pm 4.1 ^a	20.74 \pm 1.3 ^a	25.19 \pm 2.5 ^a	39.17 \pm 3.4 ^a
Glucose (g/dl)	10.20 \pm 2.1 ^a	9.80 \pm 1.9 ^a	8.50 \pm 1.3 ^a	8.00 \pm 1.2 ^a
Total protein (g/l)	30.00 \pm 2.5 ^a	27.00 \pm 1.5 ^a	26.00 \pm 2.0 ^a	26.00 \pm 1.5 ^a
BUN	1.00 \pm 0.11 ^a	1.60 \pm 0.21 ^a	1.00 \pm 0.14 ^a	1.20 \pm 0.18 ^a

Values are mean of 15 determinations \pm standard deviations. Values in the same rows with the same superscripts are not significantly different ($P>0.05$). WBC- white blood cells; RBC- red blood cells; HCT- hematocrit; Hb- haemoglobin; MCV- mean corpuscular volume; MCH- Mean corpuscular haemoglobin; MCHC- mean corpuscular haemoglobin concentration; Glucose- plasma glucose; BUN- Blood urea nitrogen

4.4.7 Water quality parameters

Water temperature ranged between 25.0-28.0°C (average 26.1 \pm 2.2°C). The pH values in all the experimental tanks were within a range of 7.2-7.7. Dissolved oxygen ranged between 6.24-7.10 mg/l. Total ammonia ranged between 0.62-0.87 ppm. All the water quality parameters were within the acceptable limits for *Tilapia rendalli*.

4.4.8 Cost benefit analysis

The economic analysis indicated lower incidence cost when KLM was added to the diet. The inclusion of MLM on the other hand, resulted in higher incidence costs. Furthermore, the profit index was increased with increasing levels of KLM in the diet. Adding MLM in *Tilapia rendalli* diets however, reduced the profit index (Table 4.9).

Table 4.9: Cost benefit analysis feeding *Tilapia rendalli* on kikuyu and moringa-based diets

	% of fishmeal replaced by the plant meals in the diet				
	0	25	50	75	100
<i>Kikuyu-based diets</i>					
Incidence cost	0.02	0.02	0.02	0.02	0.01
Profit index	1.27	1.32	1.37	1.42	1.48
<i>Moringa-based diets</i>					
Incidence cost	0.02	0.02	0.03	0.03	0.03
Profit index	1.27	1.12	1.04	0.97	0.91

4.5 DISCUSSION

The growth performance of *Tilapia rendalli* decreased as the level of both plant meals in the diet increased. These results are consistent with several studies on the replacement of fishmeal with plant-based protein (Garduno-Lugo and Olvera-Nova, 2008; Sheeno and Sahu, 2006; Luo *et al.*, 2012). At all inclusion levels, the growth parameters measured were poorer in the moringa-based diets than the kikuyu-based diets. MLM had higher protein content than KLM. Conventionally, plants with high protein content result in superior growth performance and are preferred as fishmeal replacers in fish diets. The merit of a protein source however, is not only dependent on the amount of protein but also on the amino acid composition and the biological availability thereof (El-Sayed, 2006). All diets in this study had the same protein content (30%). The essential amino acid composition of the two leaf meals was largely similar. Lysine and methionine are the most limiting amino acids in fish feeds (El-Sayed, 2006). The levels of both lysine and methionine were marginally higher in kikuyu grass compared to those in moringa leaves. This may have contributed to the superior performance of fish fed KLM-based diets.

High fibre levels in fish diets have been reported to reduce nutrient digestibility (Azaza *et al.*, 2008). The amount of fibre in the diets increased with increasing levels of leaf meals. Kikuyu leaf meal had higher fibre content than MLM. This did not seem to have a major impact on hindering nutrient utilisation as the kikuyu-based diets were utilised better by *Tilapia rendalli* than the moringa-based diets. The presence of anti-nutritional factors in the diet may reduce the availability or digestibility of dietary nutrients (Francis *et al.*, 2001). The poor growth performance observed in moringa-based diets may also be attributed to the higher concentration of anti-nutrients in MLM. MLM had higher concentrations of tannins and phenols than KLM. Tannins hamper the digestive process by binding digestive enzymes of feed components such as proteins and reduce the absorption of essential vitamins (Francis *et al.*, 2001). Phenolic substances are known to form phenolic-protein-enzyme complexes, which reduce protein digestibility and amino acid availability. Furthermore, saponins and phytate were also higher in moringa leaves than in kikuyu grass. Saponins have surface-active components, which may lead to the damage of biological membranes, resulting in increased permeability of the mucosal cells (Krogdahl *et al.*, 1995;

Bureau *et al.*, 1998). This may eventually inhibit active nutrient transport (Johnsen *et al.*, 1990). Phytate is an organically bound form of phosphorus in plants. Phytates in foods are known to bind with essential minerals (such as calcium, iron, magnesium and zinc) in the digestive tract, resulting in mineral deficiencies (Bello *et al.*, 2008). They bind minerals to form insoluble salts, thereby decreasing their bioavailability or absorption (Thompson, 1993; Muhammad *et al.*, 2011).

Feed intake decreased with increasing leaf meal inclusion in the diet and was poorer in fish fed moringa-based diets than in fish fed kikuyu diets. The low feed intake observed is an important factor that may also be responsible for the poor growth performance in fish fed moringa diets. Saponins and tannins have an astringent/bitter taste (Jansman, 1993; Francis *et al.*, 2001; Makker, 2003; Hostettman and Marston, 2005) which could have lowered palatability resulting in poor feed intake. Reduced feed intake with increasing moringa leaf meal levels in the diet was also reported by Afuang *et al.* (2003) in the Nile tilapia.

The adverse effects caused by the anti-nutrients found in diets with higher levels of plant meals are supported by the decrease in apparent digestibility of protein as the level of KLM and MLM in the diet increased. Apparent protein digestibility was lower in moringa-based diets. Several authors (Ray and Das, 1994; Bairagi *et al.*, 2004) who used leaf meals to replace fishmeal also reported a decrease in protein digestibility with increasing leaf meal inclusion. Olivera-Novoa *et al.* (2002) attributed the decrease in protein digestibility to a decline in the absorption of nutrients. The protein digestibility values obtained in the moringa-based diets are lower than that reported by Madalla (2008) where protein digestibility in *Oreochromis niloticus* ranged between 91% in the control to 84% when moringa leaf meal provided 60% of dietary protein feed. Digestibility is species specific and there are no studies available to compare the digestibility in *Tilapia rendalli* fed moringa or kikuyu leaf meals. The protein digestibility of kikuyu-based diets in this study fell within limits regarded as high i.e. 75-95% (Cho and Kaushik, 1990).

Intestinal villi are finger-like projections that serve as sites for the absorption of necessary nutrients and fluids into the body. The villi increase the small intestine's surface area, facilitating the absorption of nutrients. The intestinal villi displayed a

trend towards shorter villi with increasing MLM level in the diet. In kikuyu-based diets however, the villi height was not affected by the leaf meal inclusion. The shorter villi found in fish fed more MLM may have resulted in decreased efficiency of the absorptive process (Da Silva *et al.*, 2012; Caballero *et al.*, 2003) as a result of the reduced absorption surface area. This may also explain the poor growth performance of fish fed these diets. There was an increase in the number of goblet cells in the villi when the amount of leaf meal in the diet increased. The abundance of goblet cells was higher in fish fed moringa-based diets than in fish fed kikuyu-based diets. The increase in the number of goblet cells may be an indication of increased stress and irritation of the enterocytes as these cells produce mucous lining the brush border, which acts as a lubricant and provides protection against chemical and mechanical damage (Marchetti *et al.*, 2006).

The liver is a good indicator of nutritional status because of its role in metabolising products of the digestive system. Replacing fishmeal with plant meals led to a decrease in vacuolisation of the hepatocytes, displacement of the nuclei and sinusoidal reduction. The decrease in the number and size of vacuoles and the reduction in nuclei size as fishmeal in the diet was replaced by higher levels of moringa (75 -100%) indicates poor nutritional status of the fish (Mcfadzen *et al.*, 1997). This corresponds with the poor protein digestibility, low PER values and the resultant slow growth observed in *Tilapia rendalli* fed higher levels of moringa leaf meal in the diet.

Digestive enzyme activity in fish is correlated to diet composition (Kawai and Ikeda, 1973; Saha and Ray, 1998). The present results show that whilst all fish were fed diets with the same level of protein (30%), protease activity decreased with increasing leaf meal inclusion level. This decrease in protease activity may be attributable to the differences in the bioavailability of protein from the different diets. The high levels of fishmeal protein in the control may be more available than the protein from the leaf meals. The higher bioavailability of protein in the control diet may have induced higher protease activities in fish fed these diets. Secondly, as alluded to earlier, fish fed diets with high levels of leaf meals especially MLM had lower feed intake. The low feed intake may have reduced the amount of digestive enzyme secreted. Dietary nutrients especially proteins are important in increasing

feed intake and enzyme production (Hofer, 1982). The higher activities of protease (essential for the utilisation of protein from feed) in the fishmeal-based control may have contributed to the higher growth rate in these fish. Amylase activities in *Tilapia rendalli* intestines also decreased with increasing level of leaf meal in the diet. This decrease may be a result of the lower feed intake. German *et al.* (2004) also reported that amylase enzyme activity is affected by the quantity of the diet.

White blood cells participate directly in fish cell-mediated immune response and phagocytosis. Their increase with increasing levels of MLM may indicate compromised health. In the present study, RBC, Hb and HCT in *Tilapia rendalli* decreased when MLM levels increased in the diet. This decrease however, was only significant when 100% fishmeal was replaced with MLM. This decrease further confirms the signs of nutritional stress (observed in the liver) which was probably caused by the reduced bioavailability of proteins. Qiang *et al.* (2013) indicated that when dietary protein levels are low, physiological stress is induced and this increases damage to the liver leading to reduced RBC and Hb concentration. Similarly, Sakthivel (1988) and Abdel-Tawwab *et al.* (2010) reported a decrease in RBC and Hb in fish fed low protein levels in the diet. The replacement of fishmeal with KLM and MLM did not have a significant effect on plasma protein and BUN of *Tilapia rendalli*. However, plasma protein levels were lower in fish fed high MLM levels. The low plasma protein observed in fish fed high levels of MLM may be a consequence of decreased protein absorption emanating from the shorter villi and poor protein digestibility recorded for these fish.

Kikuyu-based diets had a higher profit index and lower incidence cost. Thus, in spite of the reduced growth performance, cost-benefit analysis indicates that KLM diets were economically superior to the fishmeal-based control. Feeding MLM based diets resulted in lower profit index and a higher incidence cost. This is because of the higher cost of moringa leaves compared to fishmeal. Moringa has been commercialised and its inclusion may not be sustainable in fish diets at least not in South Africa where claims on its human health benefits are on the rise. It is important to note that the fish used in this study had not reached market weight.

Kikuyu grass is a promising fishmeal substitute in *Tilapia rendalli* diets. It has an adequately balanced amino acid profile, relatively high protein and energy levels. Kikuyu leaf meal can replace up to 25% fishmeal and MLM <25% fishmeal without adverse effects on growth performance, intestine and liver histology or haematological parameters. Furthermore, unlike moringa, which is expensive, there was no financial value associated with this common lawn grass used in this study.

CHAPTER 5:

**THE EFFECT OF REPLACING FISHMEAL
WITH *PENNISETUM CLANDESTINUM* AND
MORINGA OLEIFERA IN THE DIET OF
*OREOCHROMIS MOSSAMBICUS***

5.1 INTRODUCTION

The ability of different fish species to utilise feed resources differs considerably as the capability to ingest, digest and assimilate dietary nutrients is species specific (Bowen *et al.*, 1995; Koumi *et al.*, 2011). Similarly, the sensitivity and response of different fish species to anti-nutrients found in plant-based diets also differs. Thus, for a plant protein source to be included in aquafeeds, its utilisation should be tested for each fish species. It is evident from the previous chapter, that kikuyu leaf meal can be used to replace up to 25% of fishmeal in the diet of *Tilapia rendalli* and that at this level, the kikuyu-based diet was well digested by the *Tilapia rendalli*. However, no information exists regarding the utilisation or digestibility of these plant diets for other fish species of economic importance in South Africa.

Tilapia production in South Africa is dominated by *Oreochromis mossambicus*. This fish species can be easily cultured and is highly adaptable to environmental changes making it an important species in developing countries. *Oreochromis mossambicus* is a native South African fish with a wide commercial acceptance and desirable traits for production (reproduces readily, relatively fast growth rate and disease resistance). Despite its wide availability and wide market acceptability, the culture of *Oreochromis mossambicus* in South Africa has not reached sustainable levels. High feed cost is one of the major limitations in aquaculture development in South Africa, especially among small-scale rural farmers.

Knowledge of the natural feeding habits and digestive system of different fish is an essential factor for the attainment of high productivity in aquaculture. *Tilapia rendalli* is a macrophagous herbivore in nature, feeding almost exclusively on macrophytes. *Oreochromis mossambicus* on the other hand, is a microphagous filter feeder and feeds mainly on phytoplankton in nature (El-Sayed 2006). It is described as an effective algal feeder and an opportunistic omnivore that feeds on a wide variety of dietary sources, including phytoplankton, zooplankton and detritus (Doupé *et al.*, 2010). *Oreochromis mossambicus* has closely spaced gill rakers on the branchial arch to aid filtration. For efficient plankton harvesting from the water column, *Oreochromis mossambicus* secretes mucous through the gills to trap plankton, the plankton-rich bolus is then swallowed.

Both *Tilapia rendalli* and *Oreochromis mossambicus* have dorsal and ventral pharyngeal plates, which bear teeth used to prepare the food for enzyme digestion. In *Tilapia rendalli*, the pharyngeal mill has fewer robust teeth to triturate the macrophytes they feed on (Fryer and Iles, 1972; Caulton, 1976). *Oreochromis mossambicus* on the other hand, being an algal feeder has numerous fine pharyngeal teeth that serve to break up aggregates.

The nutritive value of fish feed does not only depend on the nutrient composition of the individual feed components, but also on the ability of the fish to break down and absorb the nutrients (Falaye and Jauncey, 1999; Riche *et al.*, 2001). Macrophytes generally have lower protein contents (9-22% dry weight) and lipids (1-4% dry weight) (Hasan and Chakrabarti, 2009; Hlophe and Moyo, 2011). On the contrary, most algae species have a higher content of protein (40-60% dry weight), high lipid content (10-20% dry weight) (Becker, 1994; Richmond, 2013; Becker, 2007) and low fiber (Wegeberg and Felby, 2010).

The extent to which fish can digest and assimilate (expressed as a percentage of amount ingested) feed is important in aquaculture. Studies carried out on the assimilation efficiency of this fish species feeding on their natural diets indicate that *Oreochromis mossambicus* assimilates 60 % of its diet (Bowen, 1980). The macrophyte feeder, *Tilapia rendalli* assimilates 55% of its diet (Caulton, 1976). From these observations, it is clear that even though these fish feed selectively and possess special adaptations for digestion of their diets, their digestive capabilities differ. The replacement of fishmeal with low cost, readily available protein sources is important and has been a prioritised area of research for the past decades. Information on the nutritive value of non-conventional feedstuffs that could potentially replace fishmeal is vital (Edwards *et al.*, 2004; Naylor *et al.*, 2009). Thus, in this chapter, the ability of *Oreochromis mossambicus* to utilise kikuyu and moringa-based diets was determined and compared to that of *Tilapia rendalli*.

5.2 OBJECTIVES

The specific objectives of this study were to determine:

- i. the effect of partially and totally replacing fishmeal with different levels of kikuyu and moringa leaf meals on the growth performance, liver histology, intestine histology, digestive enzyme activities and haematological parameters in *Oreochromis mossambicus*.
- ii. incidence cost and profit index of partially and totally replacing fishmeal with different levels of kikuyu and moringa leaf meals in the diet of *Oreochromis mossambicus*.

5.3 MATERIALS AND METHODS

5.3.1 Fish acclimatisation

Oreochromis mossambicus used in this experiment was bred at the Aquaculture Research Unit. The fish were acclimatised to the experimental tanks (fibreglass tanks) for one month prior to start of experiment. During this period, fish were offered a standard commercial tilapia diet (34% crude protein, 17 MJ/kg energy and 3% lipid).

5.3.2 Feed preparation

Kikuyu grass was harvested from the lawn at the Aquaculture Research Unit and moringa leaves were purchased at Patient Wellness Centre in Lebowakgomo. The drying and milling of leaves was carried out as described in Chapter 4, Section 3.4.2.

Nine diets were prepared by replacing fishmeal with either KLM or MLM at 25, 50, 75 and 100%. These diets were isonitrogenous with 30% crude protein and isoenergetic having 20 MJ/kg, gross energy. Chromic oxide (Cr_2O_3) was added at 0.5% in all diets as an inert marker. Diets were prepared as described in Chapter 4, Section 3.4.2.

5.3.3 Experimental system and experimental design

The feeding experiments were carried out in the same experimental system described in Chapter 4, Section 3.4.3. Twenty seven tanks were selected for use in this completely randomised design experiment.

5.3.4 Water quality management

Water quality parameters monitored were temperature, dissolved oxygen, ammonia and pH as described in Chapter 4, Section 3.4.4.

5.3.5 Diet allocation and feeding

The diets were randomly assigned to three replicate tanks stocked with 15 *Oreochromis mossambicus* (mean initial weight = 12.50 ± 1 g). All fish were hand fed their allotted diet to apparent satiation three times a day (0900, 1300 and 1700 hours) for 60 days. Feed intake (g/fish/day) was recorded daily. Faeces were collected and pooled for each dietary treatment as outlined in Chapter 4, Section 3.4.5.

5.3.6 Proximate composition of leaf meals, experimental diets and faeces

Proximate composition for fishmeal, kikuyu grass, moringa leaves and all the experimental diets was determined. Dry matter, crude protein, crude lipid, crude fibre and gross energy were determined following procedures stipulated by the Official Analytical Chemists (AOAC International, 2012) as outlined in Chapter 4, Section 3.4.6.

5.3.7 Growth performance parameters

Fish were individually weighed at the beginning and at the end of the experiment. Fish from each tank were bulk weighed once every two weeks for the duration of the experiment. At the end of the experimental period, SGR, TGC, FCR, PER and ADC for protein were determined as described in Chapter 4, Section 3.4.7.

5.3.8 Histological analysis

Five fish from each tank (15 per dietary treatment) were sacrificed at the end of the experimental period for histological analysis of the liver and intestine. Liver and intestine samples were stored in 10% neutral buffered formalin soon after collection and were processed according to the procedures outlined in Chapter 4, Section 3.4.8.

5.3.9 Digestive enzyme analysis

On termination of the feeding experiments, five fish from in each dietary treatment were sacrificed for digestive enzyme analysis. The whole intestines was collected, pooled per dietary treatment and stored in marked plastic sample bags at -86°C until used for analysis of enzyme activity. Protease, amylase and lipase activities were determined as described in Chapter 4, Section 3.4.9.

5.3.10 Haematological analysis

At the end of the experiment, blood was collected from five fish in each tank (15 per dietary treatment) for haematological analysis. Blood was analysed for white blood cells, red blood cells, haemoglobin, hematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, plasma glucose and blood urea nitrogen as described in Chapter 4, Section 3.4.10.

5.3.11 Cost benefit analysis

Incidence cost and Profit index (described in Chapter 4, Section 3.4.11) were used to determine the economic impact of using KLM and MLM as protein sources in practical diets of *Oreochromis mossambicus*.

5.3.12 Statistical analysis

Statistical analysis was carried out as described in Chapter 4, Section 3.4.12.

5.4 RESULTS

5.4.1 Growth performance and apparent protein digestibility

5.4.1.1 Weight gain

Fish fed the control diet had the highest body weight gain ($P < 0.05$) in the treatment groups (Figure 5.1 and Figure 5.2). Increasing the level of KLM in the diet resulted in progressively lower body weight gain (Figure 5.1). Increasing the level of MLM in the diet also led to reduced ($P < 0.05$) weight gain with fish fed the highest MLM level showing the lowest gain (Figure 5.2). No mortality was observed for the duration of the experiment (60 days).

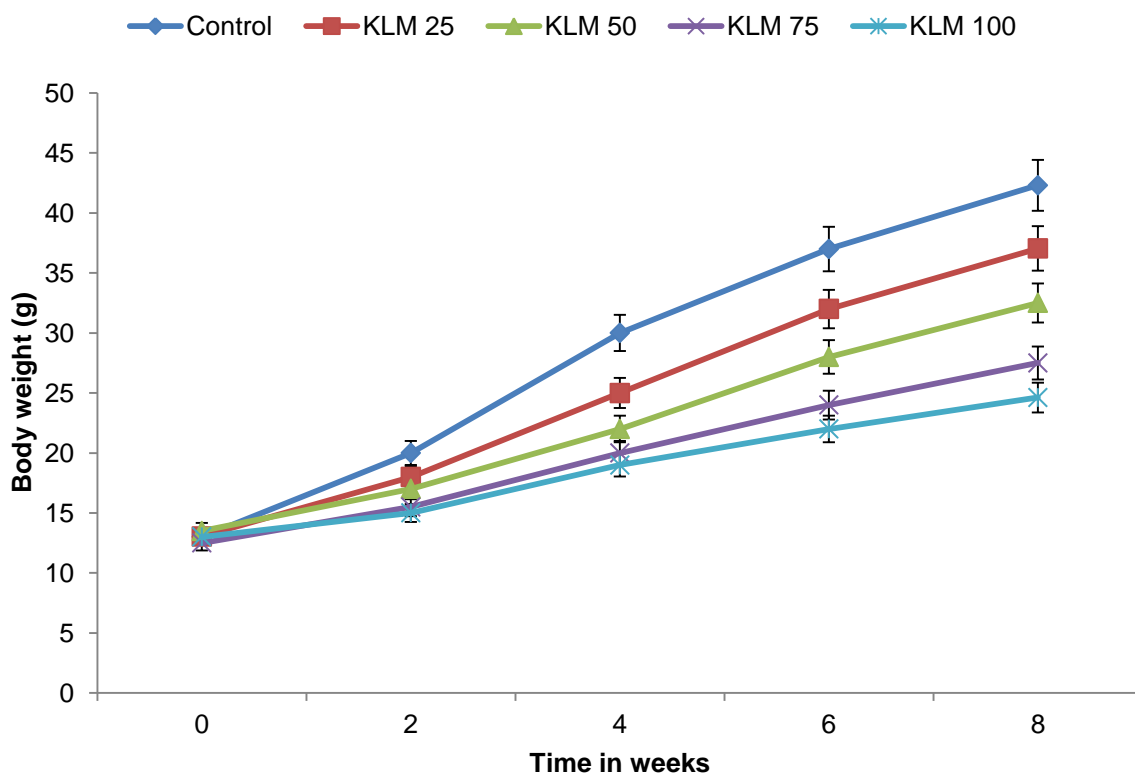


Figure 5.1: Change in body weight of *Oreochromis mossambicus* fed KLM diets

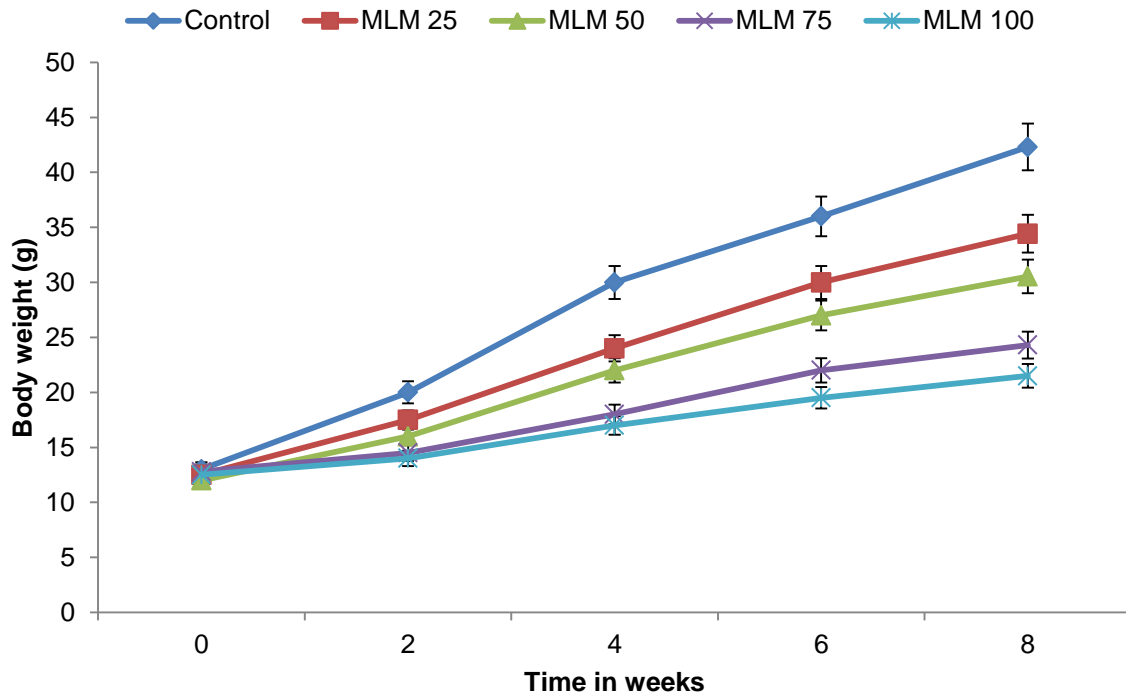


Figure 5.2: Change in body weight of *Oreochromis mossambicus* fed MLM diets

5.4.1.2 Feed intake

Feed intake was highest in *Oreochromis mossambicus* fed the control diet. A significant ($P < 0.05$) negative linear regression was observed with the addition of both KLM and MLM. Feed intake was higher for kikuyu-based diets (Figure 5.3A) than for moringa-based diets (Figure 5.3B) at all inclusion levels ($P < 0.05$, ANCOVA). In all treatment diets, *Oreochromis mossambicus* consumed less feed than *Tilapia rendalli* (Table 5.1).

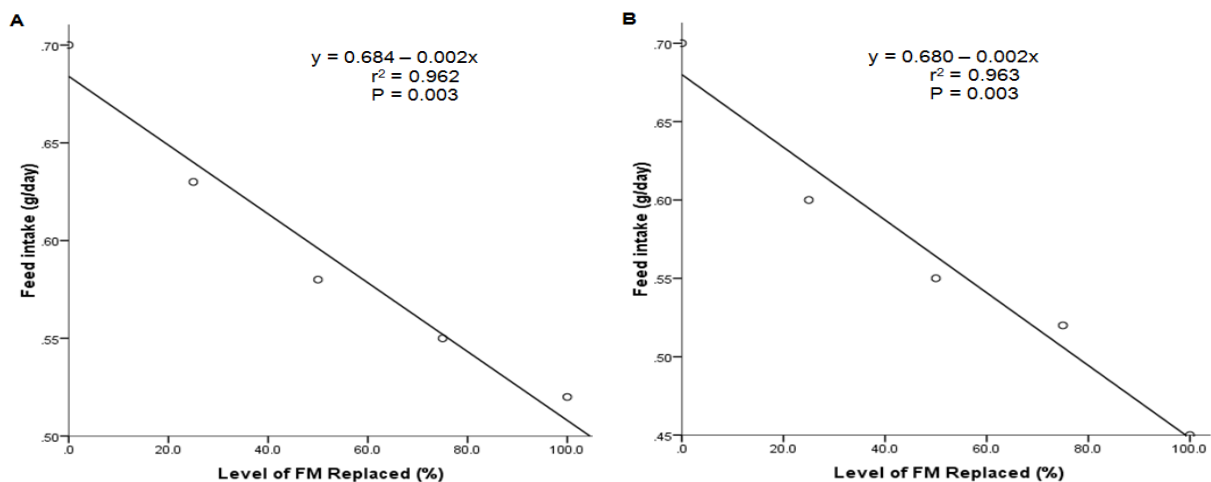


Figure 5.3: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on feed intake in *Oreochromis mossambicus*

Table 5.1: Comparison of the feed utilisation parameters between *Tilapia rendalli* and *Oreochromis mossambicus*

	Control	KLM 25	KLM 50	KLM 75	KLM 100	MLM 25	MLM 50	MLM 75	MLM 100
Feed intake (g/fish/day)									
<i>Tilapia rendalli</i>	0.80±0.2	0.77±0.1	0.72±0.2	0.70±0.1	0.61±0.1	0.85±0.2	0.82±0.2	0.70±0.1	0.64±0.1
<i>Oreochromis mossambicus</i>	0.83±0.3	0.63±0.1	0.58±0.2	0.55±0.2	0.52±0.2	0.64±0.1	0.55±0.1	0.52±0.3	0.45±0.2
FCR									
<i>Tilapia rendalli</i>	1.65±0.1	1.83±0.3	2.12±0.1	2.43±0.3	2.78±0.2	1.61±0.1	1.83±0.1	2.32±0.1	2.74±0.1
<i>Oreochromis mossambicus</i>	1.45±0.2	1.52±0.4	1.84±0.3	2.54±0.2	2.82±0.1	1.67±0.1	2.01±0.1	2.77±0.2	3.18±0.2
PER									
<i>Tilapia rendalli</i>	2.02±0.9	1.94±0.9	1.63±0.1	1.34±0.2	1.22±0.3	2.14±0.1	1.73±0.1	1.51±0.1	1.33±0.1
<i>Oreochromis mossambicus</i>	2.13±0.2	2.11±0.4	1.61±0.3	1.27±0.5	1.18±0.2	1.99±0.1	1.82±0.2	1.24±0.1	1.05±0.3
Protein ADC (%)									
<i>Tilapia rendalli</i>	82.13±1.1	80.91±2.5	79.62±3.0	77.10±2.1	76.21±1.5	82.18±2.0	79.34±1.5	76.54±0.9	74.12±1.2
<i>Oreochromis mossambicus</i>	84.25±0.5	82.14±1.2	79.35±2.0	78.24±1.5	75.30±0.8	81.51±2.2	78.61±3.0	74.52±1.5	72.10±0.5

5.4.1.3 Thermal growth coefficient

Thermal growth coefficient decreased significantly ($P < 0.05$) when the level of fishmeal in the diet decreased. The negative linear relationship between TGC and the level of fishmeal replaced was significant for both KLM (Figure 5.4A) and MLM (Figure 5.4B). Thermal growth coefficient was higher in fish fed KLM than in those fed MLM ($P < 0.05$, ANCOVA).

5.4.1.4 Specific growth rate

The reduced feed intake observed with increasing levels of KLM and MLM in the diet was confirmed by the subsequent poor growth performance. The linear regression of SGR with increasing level of fishmeal replacement was significant ($P < 0.05$) for both KLM (Figure 5.5A) and MLM (Figure 5.5B). Specific growth rate was significantly higher ($P < 0.05$, ANCOVA) in fish fed the KLM-based diets than those on the MLM diets.

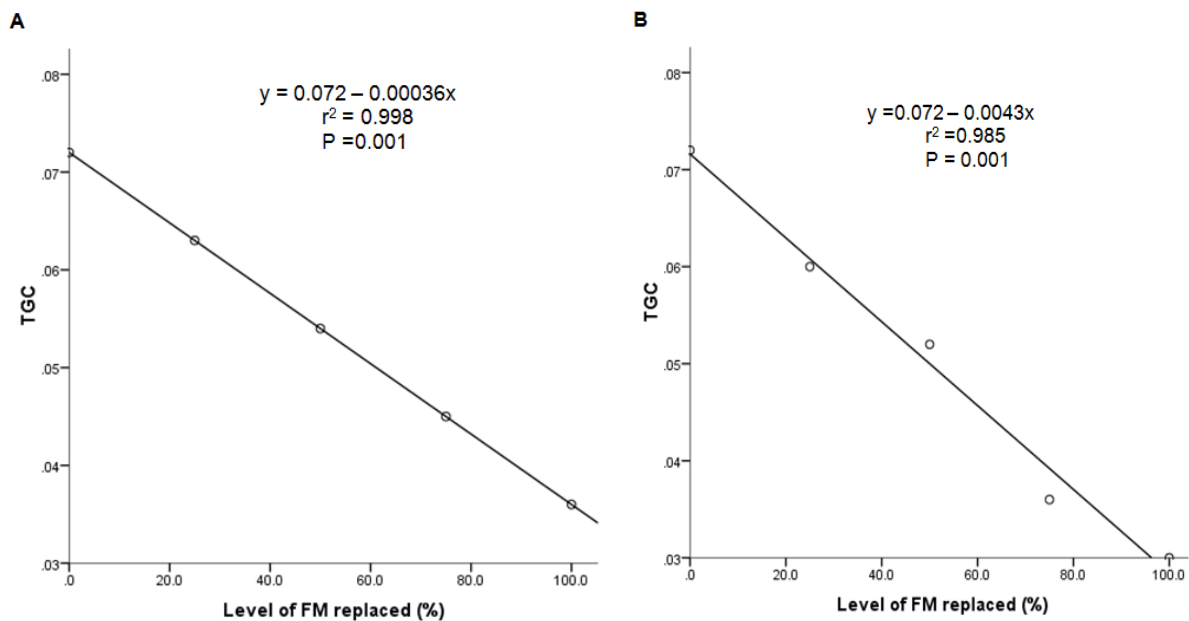


Figure 5.4: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on thermal-unit growth coefficient in *Oreochromis mossambicus*

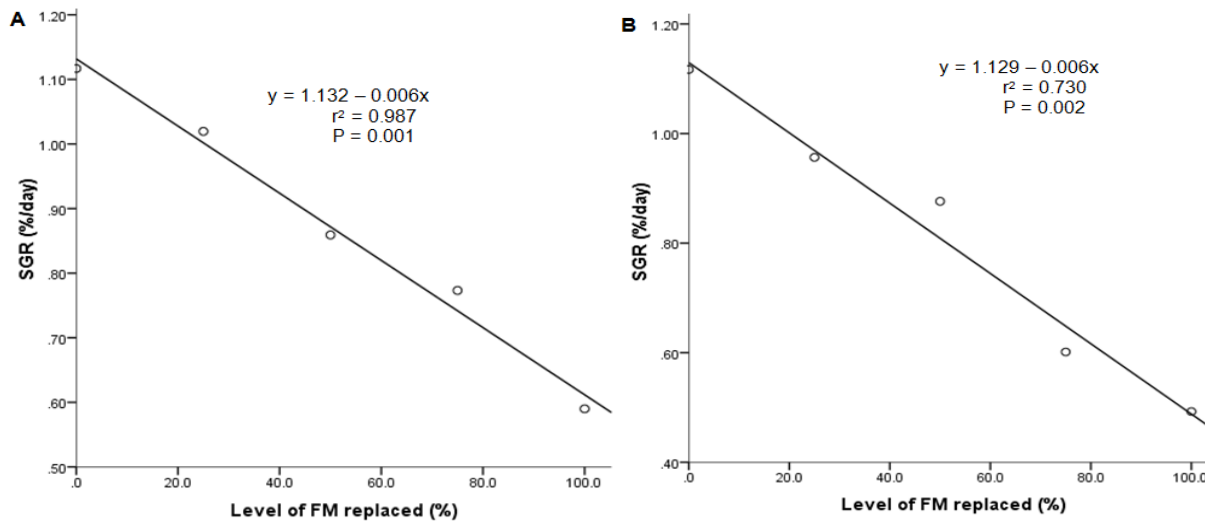


Figure 5.5: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on specific growth rate in *Oreochromis mossambicus*

5.4.1.5 Feed conversion ratio

Feed conversion ratio was lowest in fish fed the control diet and increased significantly ($P < 0.05$) with increasing levels of KLM and MLM (Figure 5.6). There was a significantly ($P < 0.05$) positive linear relationship between FCR and the level of fishmeal replaced by both KLM (Figure 5.6A) and MLM (Figure 5.6B). At all replacement levels FCR was significantly superior ($P < 0.05$, ANCOVA) in fish fed kikuyu-based diets than those fed on the moringa diets. Compared to *Tilapia rendalli*, *Oreochromis mossambicus* was inefficient in converting feed consumed into weight gained (higher FCR) when KLM replaced more than 50% fishmeal in the diet (Table 5.1).

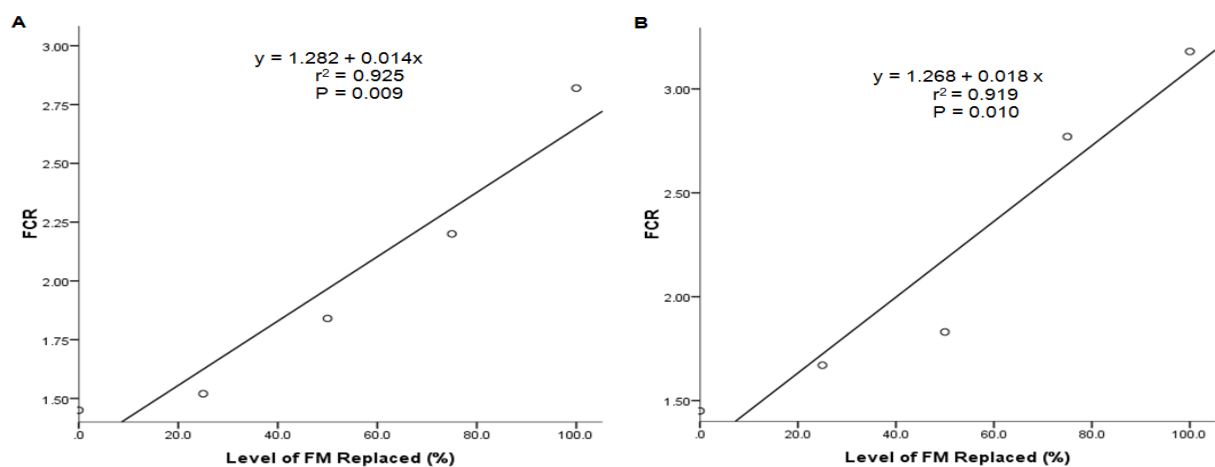


Figure 5.6: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on feed conversion ratio in *Oreochromis mossambicus*

5.4.1.6 Protein efficiency ratio

The highest PER was also recorded in fish fed the control diet. The negative linear relationship between PER and level of fishmeal replaced in the diet was significant ($P < 0.05$) for both KLM (Figure 5.7A) and MLM (Figure 5.7B) treatments. Analysis of covariance also confirmed that KLM protein is utilised better than MLM protein. PER was higher in *Oreochromis mossambicus* than in *Tilapia rendalli* when fed the control diet and KLM 25 (Table 5.1). When fishmeal was replaced with more than 25% KLM or MLM, PER was higher in *Tilapia rendalli* than in *Oreochromis mossambicus* (Table 5.1).

5.4.1.7 Apparent digestibility

Oreochromis mossambicus fed the fishmeal-based control had the highest protein digestibility values (Figure 5.8). There was a linear relationship ($P < 0.05$) between protein ADC for both KLM (Figure 5.8A) and MLM (Figure 5.8B). Protein digestibility was significantly higher ($P < 0.05$, ANCOVA) in KLM-based diets than in MLM diets. *Tilapia rendalli* was able to utilise the protein in both leaf meals better than *Oreochromis mossambicus*, this was evidenced by the higher protein digestibility values (Table 5.1).

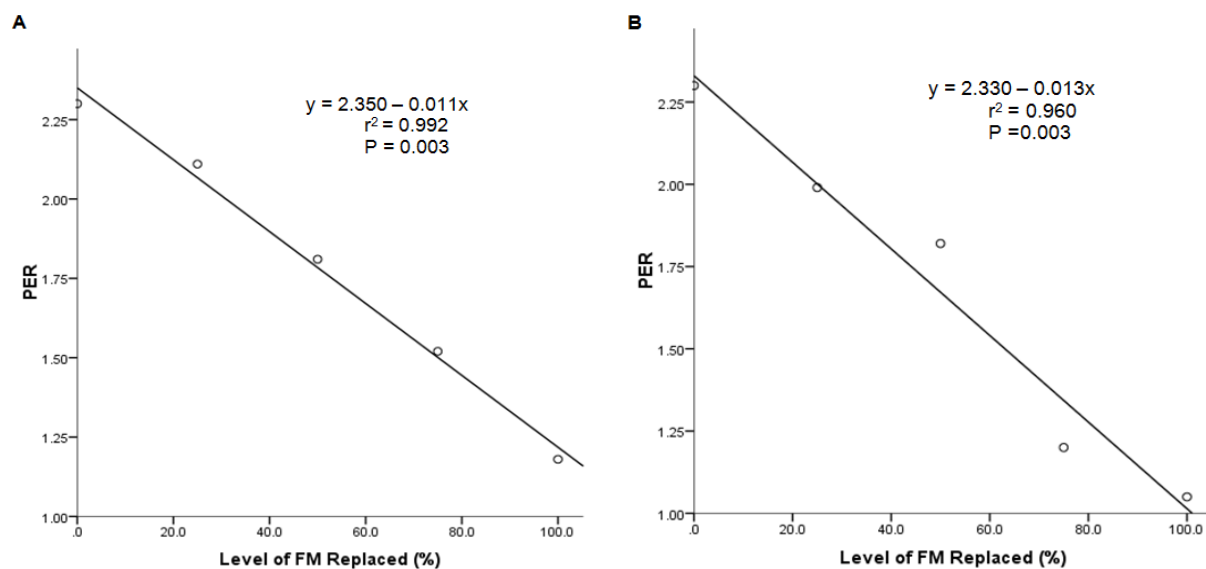


Figure 5.7: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on protein efficiency ratio in *Oreochromis mossambicus*

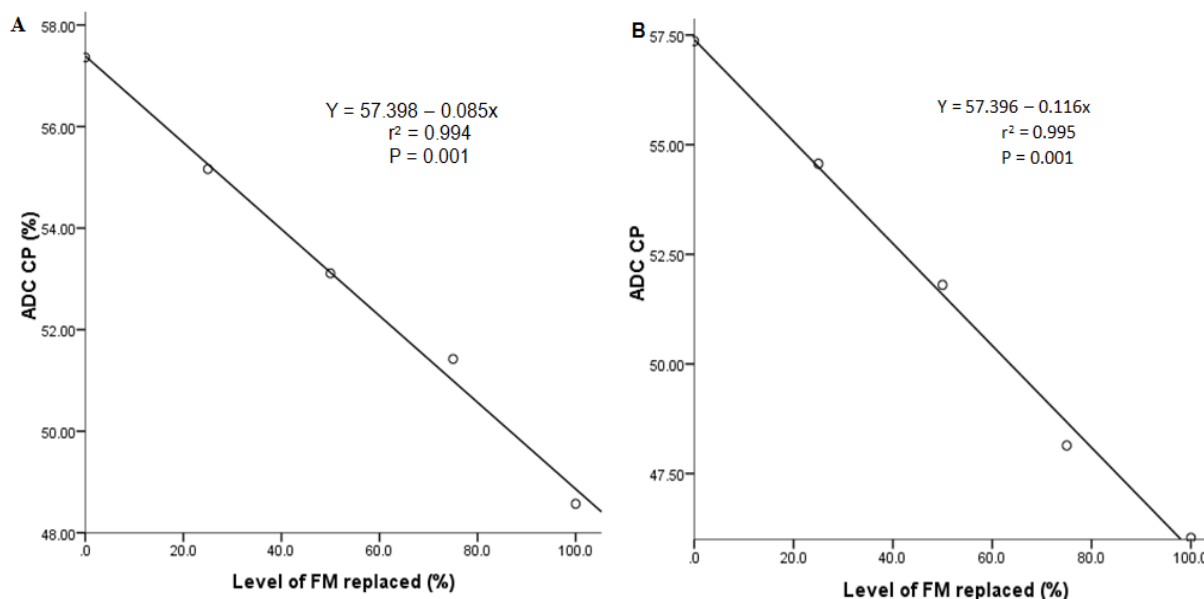


Figure 5.8: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on apparent digestibility coefficient for protein in *Oreochromis mossambicus*

5.4.2 Effect of kikuyu and moringa-based diets on intestine histology

Villi height in *Oreochromis mossambicus* intestines decreased when the level of leaf meal in the diet increased (Table 5.2). Villi height was lower in fish fed MLM compared to those fed KLM at similar dietary inclusion levels. Villi width on the other hand, was not significantly affected by leaf meal inclusion in the diet ($P > 0.05$). In *Oreochromis mossambicus* fed kikuyu-based diets the increase in goblet cell number was not statistically significant ($P > 0.05$). However, the number of goblet cells increased significantly ($P < 0.05$) with increasing levels of MLM in the diet (Table 5.2).

Table 5.2: Intestine histology values *Oreochromis mossambicus* fed different inclusion levels of kikuyu and moringa leaf meals

		% of fishmeal replaced by the plant meals in the diet				
		Control	25	50	75	100
<i>Villi length (μm)</i>						
KLM			514.71±14 ^a	508.50±10 ^a	497.75±12 ^a	488.59±10 ^a
MLM	517.43±9 ^a		502.50±10 ^a	492.50±5 ^a	485.32±9 ^b	477.40±10 ^b
<i>Villi width (μm)</i>						
KLM			60.27±15 ^a	60.14±10 ^a	61.00±12 ^a	59.75±11 ^a
MLM	60.10±10 ^a		60.31±12 ^a	62.01±9 ^a	62.21±14 ^a	60.54±13 ^a
<i>Goblet cell number</i>						
KLM			372±11 ^a	375±12 ^a	382±10 ^a	395.15 ^a
MLM	365±15 ^a		372±10 ^a	399±15 ^a	416±10 ^b	427±12 ^b

Values are mean ± standard deviations (sample size = 15 fish per treatment). Values in the same rows with the same superscripts are not significantly different (P>0.05)

No degenerative alterations were observed in the intestine of fish fed lower levels (KLM 25 and KLM 50) of KLM (Figure 5.9). In fish fed MLM, enterocyte degradation was observed when more than 25% fishmeal was replaced. This was confirmed by a higher incidence of goblet cells, degeneration of the microvilli and apical nuclear displacement within enterocytes (especially in *Oreochromis mossambicus* fed diets MLM 75 and MLM 100). These abnormalities were not seen in *Tilapia rendalli* (Chapter 4).

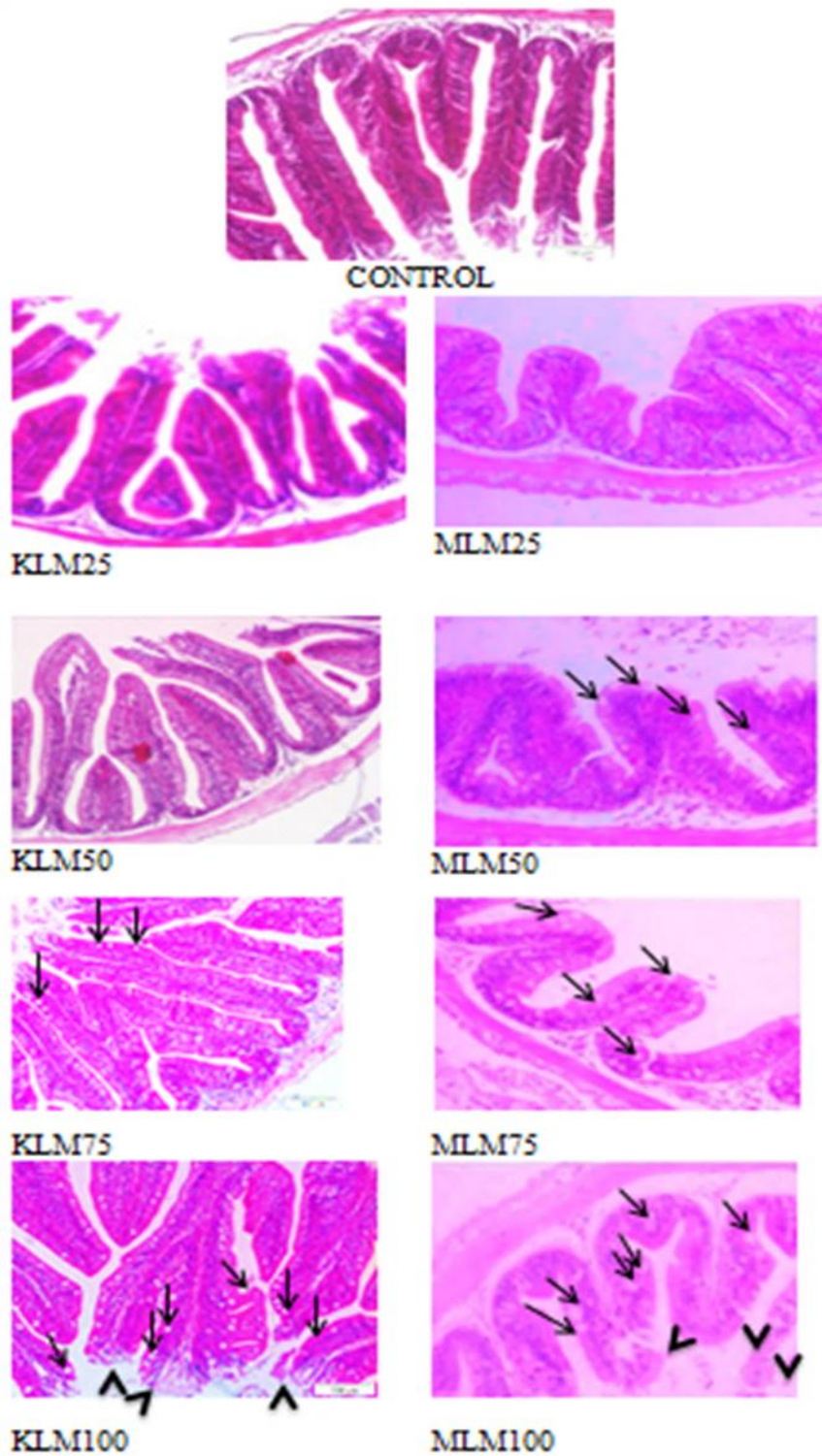


Figure 5.9: Effect of KLM and MLM on intestine histology of *Oreochromis mossambicus*. Notable is the higher incidence of goblet cells (arrows) and microvilli degeneration (arrow heads) in fish fed high levels of leaf meals

5.4.3 Effect of kikuyu and moringa diets on liver histology

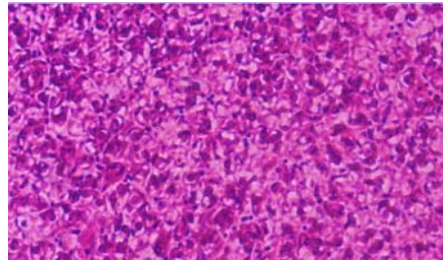
Liver histology indicated that increasing the level of both KLM and MLM led to increasingly poor hepatocyte condition compared to fish fed the control diet (Table 5.3). *Oreochromis mossambicus* fed high levels of KLM in the diet showed poor hepatocyte vacuolation with significantly higher ($P<0.05$) hepatocyte degradation scores (Table 5.3). In *Oreochromis mossambicus* fed the MLM-based diets, hepatocyte degradation was more pronounced when compared to that of fish fed KLM diets at the same inclusion level. Even at the lowest inclusion level of MLM in the diet, hepatocyte degradation scores were significantly ($P<0.05$) higher than the control (Table 5.3).

Table 5.3: Hepatocyte degradation scores of *Oreochromis mossambicus* fed different inclusion levels of kikuyu and moringa leaf meals (100 hepatocytes per treatment)

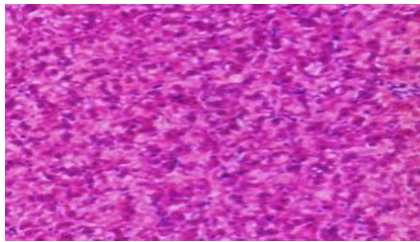
	% of fishmeal replaced by the plant meals in the diet				
	0	25	50	75	100
<i>Hepatocyte degradation score</i>					
KLM	1.25±0.1 ^a	1.40±0.1 ^a	1.80±0.3 ^b	2.20±0.4 ^c	2.58±0.4 ^d
MLM	1.25±0.1 ^a	1.90±0.3 ^b	2.40±0.3 ^c	2.60±0.2 ^c	2.86±0.1 ^d

Values in the same row with the same superscripts are not significantly different ($P>0.05$).

There was no significant difference in the nucleus of fish fed the control diet and KLM 25 (Figure 5.10). The staining of the cytoplasm was increasingly uneven as the level of KLM increased in the diet in fish fed KLM 75 and KLM 100 (Figure 5.10). Hepatocytes of *Oreochromis mossambicus* fed higher levels of MLM (75-100) in the diet include signs of atrophy such as darkly stained nuclei, disordered hepatic cell cords and hyaline cytoplasm with small pyknotic displaced nuclei (Figure 5.10). These abnormalities were not seen in fish fed the control or KLM 25 and KLM 50 (Figure 5.10).



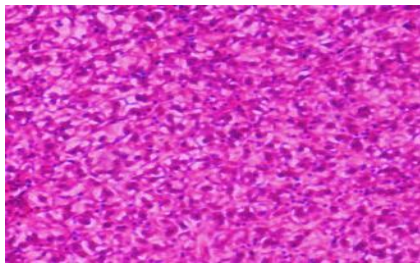
CONTROL



KLM25



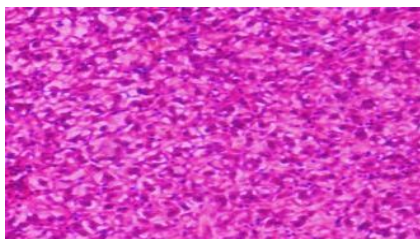
MLM25



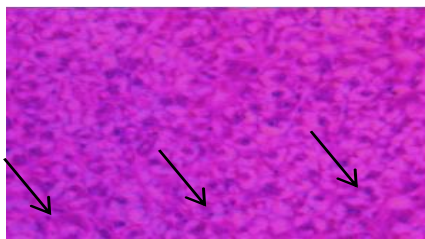
KLM50



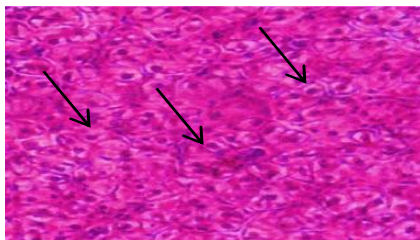
MLM50



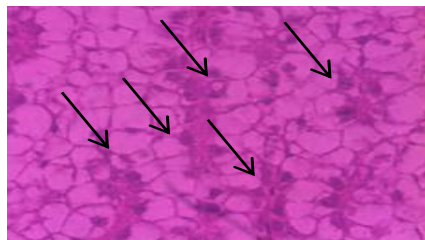
KLM75



MLM75



KLM100



MLM100

Figure 5.10: Effect of KLM and MLM on liver histology of *Oreochromis mossambicus*. Notable are the small pyknotic displaced nuclei (arrows) in fish fed high levels of leaf meals

5.4.4 Effect of plant diets on the activities of digestive enzymes

5.4.4.1 Protease activity

Protease activity in *Oreochromis mossambicus* decreased when fishmeal was replaced with both KLM and MLM (Figure 5.11). Replacing up to 25% of fishmeal with KLM did not cause any significant ($P>0.05$) decrease in protease activity in *Oreochromis mossambicus* intestines (Figure 5.11A). When KLM replaced more than 25% fishmeal however, protease activity was significantly ($P<0.05$) reduced. In fish fed the MLM diets, protease activity decreased significantly ($P<0.05$) even at the lowest inclusion level of MLM (Figure 5.11B). When compared to *Tilapia rendalli* (Chapter 4), the effect of leaf meal inclusion in the diet was more pronounced in *Oreochromis mossambicus*.

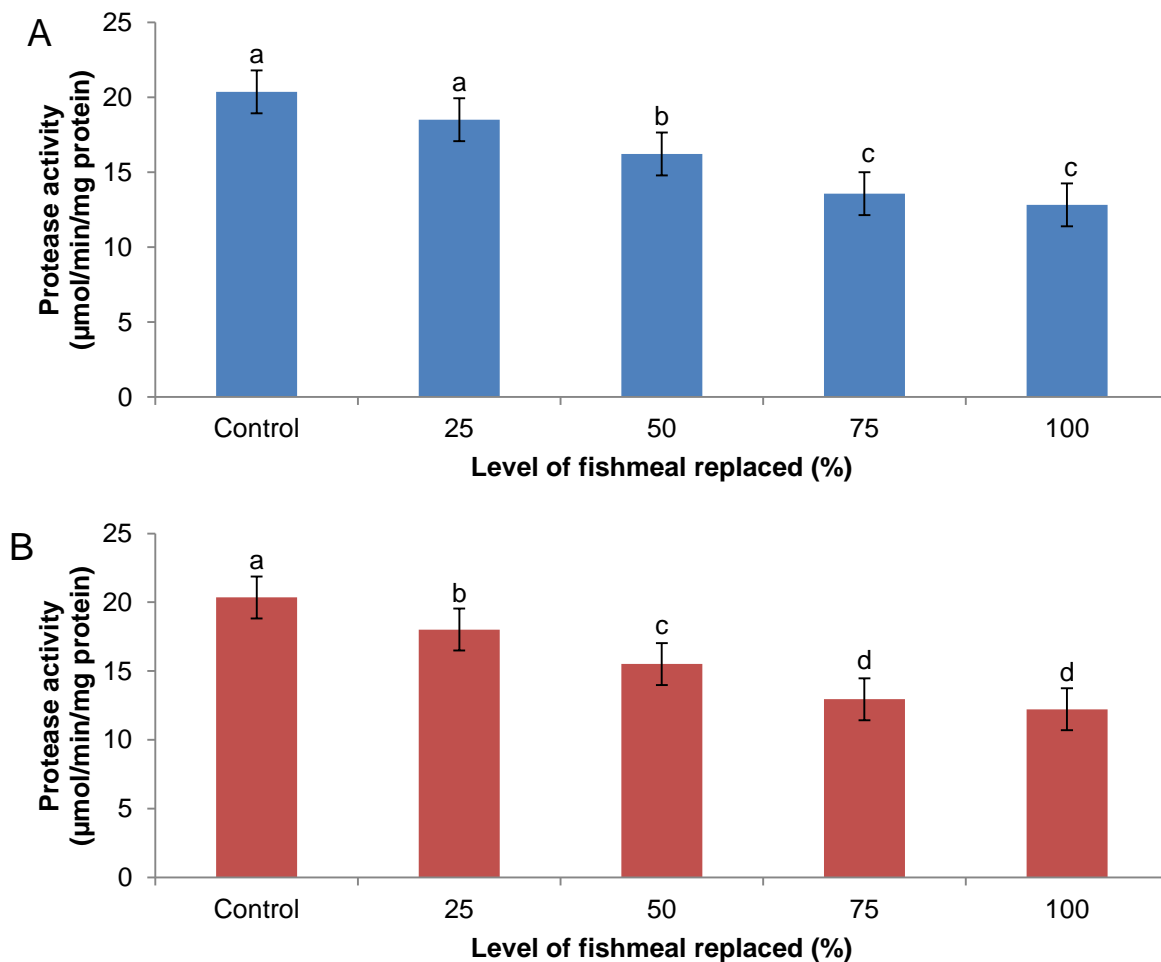
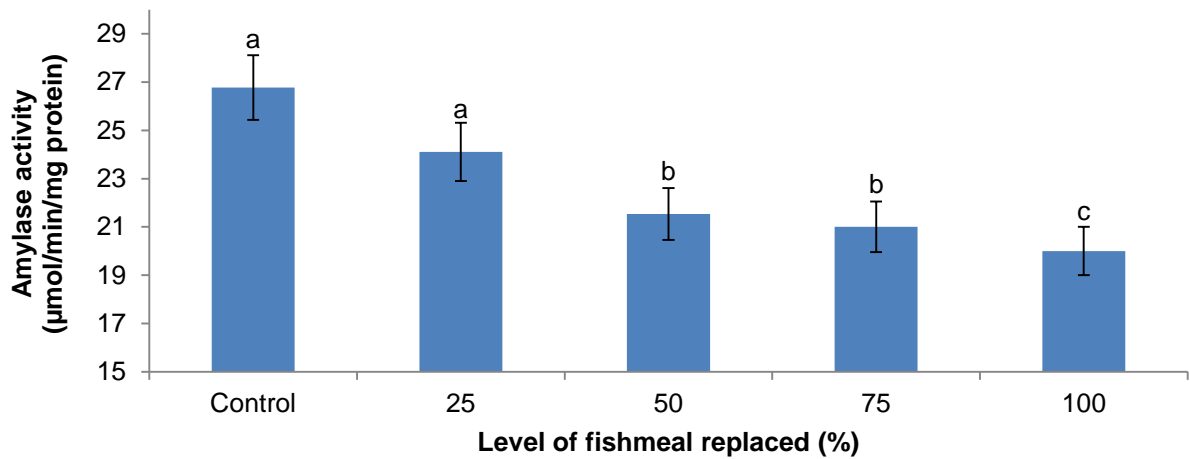


Figure 5.11: Effect of replacing fishmeal with KLM (A) and MLM (B) on the protease activity in *Oreochromis mossambicus* intestine. Bars with different letters are significantly different ($P<0.05$)

5.4.4.2 Amylase activity

Amylase activity also decreased with increasing levels of leaf meals in *Oreochromis mossambicus* diet (Figure 5.12). Adding up to 25% KLM in the diet did not have a significant effect ($P>0.05$) on amylase activities (Figure 5.12A). In the MLM diets however, a significant decrease ($P<0.05$) in amylase activity was evident even at the lowest inclusion level (MLM 25) (Figure 5.12B).

A



B

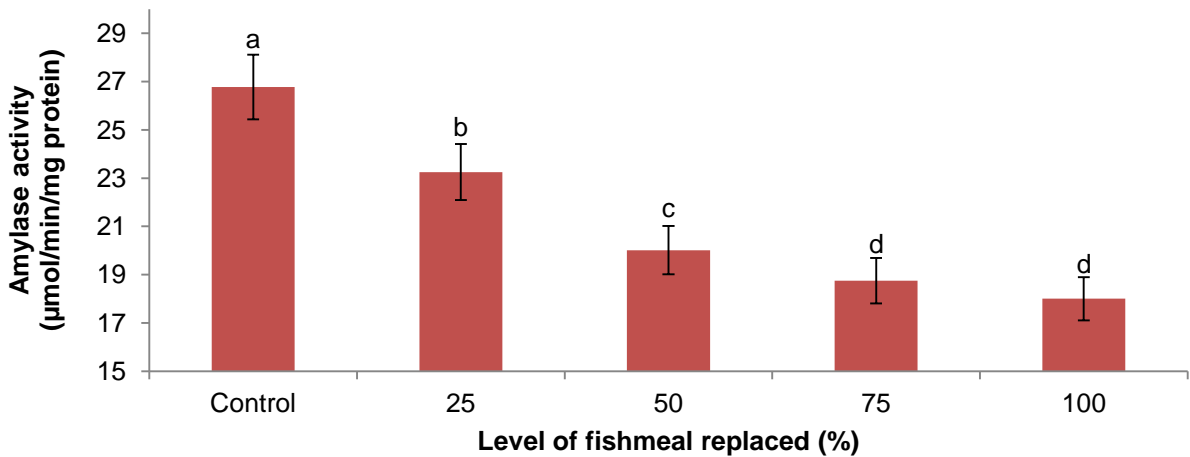


Figure 5.12: Effect of replacing fishmeal with KLM (A) and MLM (B) on the amylase specific activity in *Oreochromis mossambicus* intestine. Bars with different letters are significantly different ($P<0.05$)

5.4.4.3 Lipase activity

The activity of lipase from *Oreochromis mossambicus* intestines was highest (24.55 $\mu\text{mol}/\text{min}/\text{mg}$ protein) in fish fed the fishmeal-based control (Figure 5.13A and B). Lipase activity decreased significantly when dietary fishmeal was replaced with more than 25% KLM (Figure 5.13A). In the MLM diets, the decrease in lipase activity was significant even at the lowest inclusion level (Figure 5.13B).

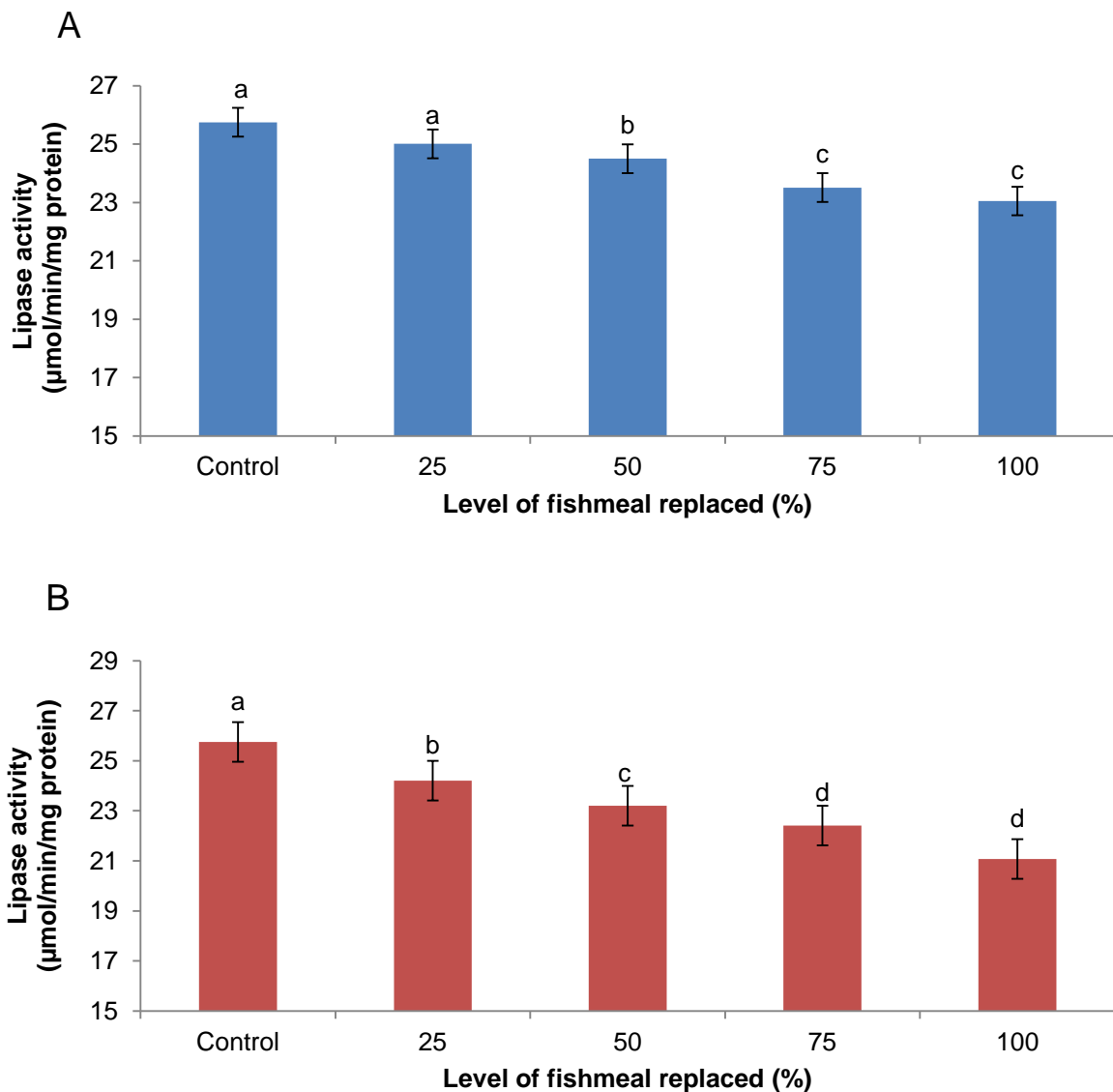


Figure 5.13: Effect of replacing fishmeal with KLM (A) and MLM (B) on the lipase specific activity in *Oreochromis mossambicus* intestine. Bars with different letters are significantly different ($P < 0.05$)

5.4.5 Effect of diet on haematological parameters

Hematological analysis indicated a significant ($P < 0.05$) increase in white blood cells (WBC) when KLM replaced more than 25% fishmeal in the diet (Table 5.4). When MLM replaced fishmeal on the other hand, WBC were significantly lower than the control diet even at the lowest (25%) inclusion level (Table 5.5). Red blood cells (RBC) and hematocrit (HCT) counts in KLM fed fish were not significantly different from the control at all inclusion levels (Table 5.4). In the moringa diets, a significant ($P < 0.05$) decrease in RBC and HCT was recorded in fish fed the highest level of MLM in the diet. Compared to the control diet, the inclusion of both KLM and MLM did not have any significant effect ($P > 0.05$) on the haemoglobin (Hb) concentration in *Oreochromis mossambicus* (Table 5.4; Table 5.5).

The mean cell volume (MCV) decreased with increasing KLM levels in the diet and was significantly lower than the control in fish fed KLM 100 (Table 5.4). In the MLM-based diets, MCV was also significantly lower ($P < 0.05$) in fish fed MLM 100 (Table 5.5). Mean cell haemoglobin (MCH) concentration in *Oreochromis mossambicus* decreased significantly with increasing levels of KLM, this decrease was significantly lower ($P < 0.05$) than the control when KLM replaced more than 25% fishmeal in the diet. In MLM diets however, a significant decrease in MCH was observed even at the lowest level of MLM (Table 5.5). Plasma glucose, plasma protein and blood urea nitrogen in fish fed all KLM diets (Table 5.4) and the MLM-based diets (Table 5.5) were not significantly different from those of fish fed the control diet

Table 5.4: The Effect of kikuyu- based diets on haematological parameters of *Oreochromis mossambicus* (n = 15)

	CNTL	KLM 25	KLM 50	KLM 100
WBC ($10^3\mu\text{l}$)	498.04±9.10 ^a	500.86±7.40 ^a	521.2±2.80 ^b	527.30±7.10 ^b
RBC ($10^6\mu\text{l}$)	1.42±0.20 ^a	1.39±0.18 ^a	1.30±0.23 ^a	1.29±0.24 ^a
HTC (l/l)	0.29±0.11 ^a	0.27±0.10 ^a	0.28±0.21 ^a	0.28±0.12 ^a
Hb (g/dl)	6.52±0.52 ^a	6.50±0.34 ^a	6.00±0.57 ^a	5.90±0.57 ^a
MCV (fL)	204.23±9.20 ^a	190.60±7.80 ^a	201.4±5.60 ^a	139.53±5.20 ^b
MCH (pg)	45.92±3.20 ^a	46.80±2.40 ^a	43.17±1.70 ^b	42.63±6.80 ^b
MCHC (g/dL)	22.48±2.10 ^a	24.50±3.40 ^a	21.4±2.20 ^a	30.56±4.10 ^b
Plasma glucose (g/dl)	8.20±1.5 ^a	8.70±0.90 ^a	8.50±1.00 ^a	7.90±0.90 ^a
BUN (mmol/L)	0.95±0.10 ^a	0.90±0.20 ^a	0.80±0.10 ^a	0.80±0.10 ^a
Plasma protein (g/L)	36.00±2.50 ^a	36.05±1.50 ^a	34.50±2.00 ^a	32.12±1.50 ^a

Values are mean of five determinations ± standard deviations. Values in the same rows with the same superscripts are not significantly different ($P>0.05$). WBC- white blood cells; RBC- red blood cells; HCT- hematocrit; Hb- hemoglobin; MCV- mean corpuscular volume; MCH- Mean corpuscular hemoglobin; MCHC- mean corpuscular haemoglobin concentration; BUN- Blood urea nitrogen

Table 5.5: The Effect of moringa-based diets on haematological parameters of *Oreochromis mossambicus* (n = 15)

	CNTL	MLM 25	MLM 50	MLM 100
WBC ($10^3\mu\text{l}$)	498.04±9.10 ^a	513.0±5.20 ^b	528.12±5.80 ^c	537.9±6.30 ^d
RBC ($10^6\mu\text{l}$)	1.42±0.20 ^a	1.60±0.15 ^a	1.32±0.18 ^a	0.60±0.34 ^b
HTC (l/l)	0.29±0.11 ^a	0.24±0.10 ^a	0.26±0.11 ^a	0.20±0.22 ^b
Hb (g/dl)	6.52±0.52 ^a	6.30±0.71 ^a	5.84±0.92 ^a	6.20±0.41 ^a
MCV (fL)	204.23±9.20 ^a	148.15±7.20 ^a	196.97±6.10 ^a	143.80±5.30 ^b
MCH (pg)	45.92±3.20 ^a	44.15±6.20 ^b	43.48±5.70 ^b	44.10±8.30 ^b
MCHC (g/dL)	22.48±2.10 ^a	26.25±4.80 ^a	22.08±5.00 ^a	34.30±3.40 ^b
Plasma glucose (g/dl)	8.20±1.50 ^a	7.80±1.00 ^a	6.60±0.70 ^a	5.80±0.80 ^a
BUN (mmol/L)	0.95±0.10 ^a	0.85±0.20 ^a	0.80±0.20 ^a	0.75±0.10 ^a
Plasma protein (g/L)	36.00±2.50 ^a	35.75±1.50 ^a	35.20±0.90 ^a	34.00±1.50 ^a

Values are mean of five determinations ± standard deviations. Values in the same rows with the same superscripts are not significantly different ($P>0.05$). WBC- white blood cells; RBC- red blood cells; HCT- hematocrit; Hb- hemoglobin; MCV- mean corpuscular volume; MCH- Mean corpuscular hemoglobin; MCHC- mean corpuscular haemoglobin concentration; BUN- Blood urea nitrogen

5.4.6 Water quality parameters

All the water quality parameters monitored were within the acceptable range for *Oreochromis mossambicus* optimal growth. These were not different between treatments for the duration of the experimental period. Water temperature was between 25.0-28.0°C (average 26.1 ± 2.2°C). The pH values ranged between 7.2-7.7. Dissolved oxygen and total ammonia ranged between 6.24-7.10 mg/L and 0.62-0.87 ppm respectively.

5.4.7 Cost benefit analysis

Increasing levels of KLM in the diet resulted in lower costs incurred in making the diets as seen in the low incidence cost (Table 5.6). On the contrary, incidence cost increased with increasing levels of MLM. The profit index increased with increasing levels of KLM in the diet. Adding more MLM in the diet on the other hand, resulted in a low profit index (Table 5.6).

Table 5.6: Cost benefit analysis of the experimental diets used

	% of fishmeal replaced in the diet				
	0	25	50	75	100
<i>Incidence cost</i>					
KLM	0.73	0.63	0.56	0.48	0.46
MLM	0.73	0.70	0.70	0.71	0.66
<i>Profit index</i>					
KLM	1.27	1.32	1.37	1.42	1.48
MLM	1.27	1.12	1.04	0.97	0.91

5.5 DISCUSSION

The effects of adding KLM and MLM on the growth performance of *Oreochromis mossambicus* diets followed the same trend as that reported for *Tilapia rendalli* (Chapter 3). The best growth performance (TGC, SGR), feed utilisation (FCR, PER) and protein digestibility was obtained in *Oreochromis mossambicus* fed the control diet. *Oreochromis mossambicus* showed better growth performance than *Tilapia rendalli* when fed the control diet. The superior performance observed in *Oreochromis mossambicus* fed the control diet compared to *Tilapia rendalli* may be genetic. Several workers have reported that *Oreochromis mossambicus* grows faster and attains a larger maximum size than *Tilapia rendalli* in the wild (El-Sayed, 2006; Pauly *et al.*, 1988). However, it is important to note that growth performance was higher in *Tilapia rendalli* than in *Oreochromis mossambicus* when the level plant meal increased above 25% in the diet. This indicates that although both fish species are herbivores in nature, *Tilapia rendalli* is more capable of utilising leaf meal-based diets than *Oreochromis mossambicus*.

Another factor that may have contributed to the poor growth in fish fed higher levels of plant meals in the diet is the reduction in palatability of the diets. Fishmeal is a highly palatable and highly digestible protein source that contains pro-nutritional factors, which improve palatability, growth and health of fish (Gaylord *et al.*, 2006; Lunger *et al.*, 2007). Its reduction in the diet may have caused the reduced feed intake and the subsequent poor growth observed. Furthermore, fishmeal is an excellent source of high quality protein and has an essential amino acid (EAA) profile that is almost identical to the known dietary EAA requirements of farmed fish. Fishmeal is also an excellent source of digestible energy for fish and has an essential fatty acid (EFA) profile, which approximates the dietary requirements for EFA in fish. It is a good source of essential minerals and trace elements, including calcium, phosphorus, magnesium, iodine, zinc, manganese, selenium, and trivalent chromium. Fishmeal is also a good source of essential vitamins, including choline, vitamin B₁₂, inositol, vitamin A, vitamin D₃ (Anderson *et al.*, 1995). It is therefore most probable that addition of the leaf meals in the diet led to a reduction of these nutrients, decreasing palatability of the diets and adversely affecting the growth performance.

In treatments where dietary fishmeal was replaced with KLM, *Oreochromis mossambicus* showed greater ability to utilise protein compared to those fed MLM at the same dietary inclusion level. The low PER values recorded are probably due to an inferior biological value for the MLM protein. Similarly, protein digestibility was higher in *Oreochromis mossambicus* fed KLM than in fish fed MLM at the same dietary inclusion level. The poor digestibilities and growth recorded in the diet with sole plant protein diet (100% fishmeal replaced) for both *Tilapia rendalli* and *Oreochromis mossambicus* indicate the necessity of fishmeal protein in plant-based diets to improve the essential amino acid balance. Low digestibility of plant-based diets has been attributed to a preponderance of complex and structural carbohydrates (Appler and Jauncey, 1983). The low protein digestibility and the subsequent poor levels of feed utilisation obtained for both fish species with increasing plant meal levels may be attributed in part to the presence of indigestible materials such as structural carbohydrates. This explains the better performance in *Tilapia rendalli* as it is more equipped (higher protease and amylase activities) to utilising complex protein and carbohydrate sources than *Oreochromis mossambicus*.

The inability of *Oreochromis mossambicus* to effectively utilise MLM protein explains the lower growth rate obtained in fish fed MLM compared to those fed KLM in the diet. The lower feed intake, slower growth performance and poorer utilisation of protein in *Oreochromis mossambicus* fed the moringa-based diets compared to those fed kikuyu-based diets may be attributed to the high levels of anti-nutrients in MLM. As indicated in the previous chapter, plant ingredients contain inherent anti-nutritional (ANFs) that reduce palatability, hinder feed utilisation efficiency and ultimately compromise growth rates of fish (Francis *et al.*, 2001). These anti-nutrients were higher in MLM than in KLM. Tannins and phytate hinder the digestive process by binding digestive enzymes such as protease, reducing their bioavailability and decreasing the absorption of nutrients (Francis *et al.*, 2001). Polyphenols form phenolic-protein-enzyme complexes, decreasing protein digestibility and the availability of amino acids (Francis *et al.*, 2001). Saponins may have adversely affected nutrient absorption by causing damage to biological membranes (Bureau *et al.*, 1998).

Evaluating the histological structure of the digestive organs of fish fed unconventional feed resources provides valuable information about their digestibility and possible health effects in a specific fish species (Cabellero *et al.*, 2003). In *Oreochromis mossambicus* fed high levels of MLM in the diet, a significant decrease in villi height was evident. In *Tilapia rendalli*, a slight (statistically insignificant) decrease in villi length was also observed in fish fed higher levels of MLM. In fish fed the fishmeal-based control, the greater efficiency in the utilisation of feed may have resulted in an enhanced development of intestinal morphology (higher villi). These findings are in agreement with Santin *et al.* (2001) who reported that the development of the morphology of gastrointestinal tract is greatly influenced by the diet of the animal. Vidanaravhvi *et al.*, 2006 observed that the intestine can change its surface by increasing or decreasing the height of its villi. Reduced villi height results in the loss of surface for digestion and absorption of nutrients, while longer villi increases the surface area for nutrient absorption. Villi height can thus be used as an effective indicator of the digestive capability in the intestine.

The reduced villi height in *Oreochromis mossambicus* fed higher levels on MLM may have resulted in a decrease in the absorption efficiency of dietary nutrients, which explains the poor SGR, FCR and PER of fish fed the MLM diets compared to those fed KLM diets. In addition, the number of goblet cells in the enterocytes was higher in fish fed MLM than in those fed KLM. Goblet cells excrete mucus which lubricate the intestines and provide protection (Krogdahl *et al.*, 1995; Bureau *et al.*, 1998). The significant increase in goblet cell number suggests that high levels of MLM in the diet cause irritation of the enterocytes because of the higher levels of anti-nutrients. The production of more mucus may also adversely affect growth performance by hindering the absorption of nutrients.

Normal liver histology was seen in *Oreochromis mossambicus* fed the control and KLM-based diets. These hepatocytes were characterised by centrally located nuclei and homogenous cytoplasm. However, fish fed MLM diets showed atrophied nuclei with irregular staining of the cytoplasm. Hepatocyte degradation increased in fish fed high levels of MLM (75 -100%). Hepatocyte degradation may be an indication of compromised health because of nutritional imbalances (Mosconi-Bac, 1990). The malnutrition signs observed in the liver of fish fed MLM diets are corroborated by the

lower feed utilisation; poor protein digestibility and impaired absorption efficiency observed in fish fed these diets. Hepatocyte degradation was more pronounced in *Oreochromis mossambicus* fed higher levels of MLM than in *Tilapia rendalli* fed the same diets. In *Oreochromis mossambicus*, disordered hepatic cell cords, hyaline cytoplasm, pyknotic and displaced nuclei were observed in fish fed MLM 75 and MLM 100. These abnormalities were not observed in *Tilapia rendalli* fed the same diets. The inability of *Oreochromis mossambicus* compared to *Tilapia rendalli* to effectively digest and utilise diets with higher levels of plant meals may have caused the higher levels of hepatocyte degradation in this fish.

Protease, amylase and lipase enzyme activities in *Oreochromis mossambicus* fed the experimental diets were lower than those recorded for *Tilapia rendalli* fed the same diets. This may imply that *Tilapia rendalli* is better equipped to utilise plant-based diets. The lower protease, amylase and lipase activities recorded in *Oreochromis mossambicus* fed the experimental diets correspond with the low digestibility values obtained for these diets. These results concur with (Peres *et al.*, 1998) and German *et al.* (2004) who reported that digestive enzyme activities in fish vary not only according to the stages of ontogenic development of animals, but also according to the species and the quantity and composition of the diet.

Haematological parameters are valuable tools for assessing the health status and for monitoring stress in fish. Svobodova *et al.* (1991) suggested that ichthyohaematology is useful in the assessment of feed composition, nutritional status in relation to environmental conditions affecting fish. Leucocytes play an important role in protecting fish body and preventing diseases infection. White blood cells count increased when the level of both leaf meals increased in the diet. It is therefore most probable that the increase in WBC when higher levels of leaf meal (especially MLM) in the diet increased may be the fish's reaction against infections or foreign substances, which can alter the normal physiological processes and enhance non-specific immune function (Misra *et al.*, 2006). Thus, the lower levels of WBC in fish fed control diet and KLM 25 imply optimal immunity.

When KLM and MLM replaced fishmeal in the diet, RBC counts in blood were lower compared to the control diet, possibly due to inhibition or injury of erythrocytes. The

higher RBC count in fish fed the control diet may have occurred because of its release from the storage pool in the spleen, facilitating the synthesis of Hb in the erythrocytes after RBC release into circulation (Pulsford *et al.*, 1994). The results of this experiment indicate lower levels of RBC, Hb and HCT in fish fed MLM compared to those fed KLM at similar dietary inclusion levels. Qiang *et al.* (2013) also reported lower levels of RBC in *Oreochromis niloticus* fed diets with protein levels that were below or above the optimal range. Low levels of RBC, Hb and HCT may be a consequence of the compromised health status of the liver. It is important to note that all the diets used in this study were isonitrogenous. Thus, the poorer health performance in *Oreochromis mossambicus* fed moringa diets compared to *Tilapia rendalli* may be a function of unavailability of protein caused by the inability of *Oreochromis mossambicus* to breakdown the complex cell structure of the plant when more than 25% fishmeal is replaced. Furthermore, the lower plasma protein observed in these fish may be caused by the poor protein utilisation (PER), poor digestibility resulting in poor absorption of digested nutrients because of shorter villi. The liver is the main source of urea and the lower BUN levels in *Oreochromis mossambicus* fed moringa diets confirm the compromised functioning of the liver.

The cost benefit analysis undertaken confirms the results obtained in Chapter 3 that KLM is a cheaper protein source than MLM for use in *Oreochromis mossambicus* diets. The results show that although replacing fishmeal with both kikuyu and moringa leaf meals resulted in reduced growth performance, kikuyu-based diets were more economical compared to the moringa-based diets.

The results of this study indicate that *Oreochromis mossambicus* may have evolved to grow more rapidly and achieve a larger size than *Tilapia rendalli*. This is supported by the faster growth rates and feed utilisation when both fish species are fed the fishmeal-based control and lower levels of KLM. Furthermore, *Oreochromis mossambicus* fed these diets had higher digestive enzyme activities than *Tilapia rendalli*. Digestive enzymes are crucial for optimum utilisation of dietary nutrients. However, when the fishmeal was replaced with higher levels (>25%) of KLM and MLM, feed intake, growth performance parameters and digestive enzymes were higher in *Tilapia rendalli* compared to *Oreochromis mossambicus*. This observation confirms the assertion that although *Tilapia rendalli* may have a slower growth rate

compared to *Oreochromis mossambicus*; it is genetically predisposed to utilising plant-based diets. However, adverse effects were observed in the intestines and liver of both fish fed high MLM, this implies that the content of anti-nutrients was higher than the critical values tolerated by fish; therefore, feeding MLM to fish should be done with caution.

CHAPTER 6:

**THE EFFECT OF REPLACING FISHMEAL
WITH *PENNISETUM CLANDESTINUM* AND
MORINGA OLEIFERA IN THE DIET OF
*CLARIAS GARIEPINUS***

6.1 INTRODUCTION

The African catfish, *Clarias gariepinus* is eurytopic and one of the important freshwater fish species cultured in South Africa. The superior growth performance of *Clarias gariepinus* compared to other *Clarias* species has probably contributed to its wide introduction to areas outside its natural range (Verreth *et al.*, 1992). Clariidae catfishes are the second most important group of cultured fish in the world (Fapohunda, 2012). They feed on a wide range of artificial and natural food items, have high growth rates and tolerate poor water quality (Oresegun *et al.*, 2007).

As the price of fishmeal increases, the need to use plant-based diets in aquafeeds also increases. From an economic perspective it may appear advantageous to use plant protein sources as an alternative to fishmeal. However, it is imperative to consider any long term tissue or organ level damage that may occur. Fishmeal free formulations for herbivorous fish such as tilapias have been reported and used in practical diets (Shiau *et al.*, 1990; Goda *et al.*, 2007), but seldom studied on other omnivorous or carnivorous fish. Herbivorous fish (such as tilapias) ingest food items that are often morphologically and chemically encased in largely indigestible (at least by endogenous enzymes) fibrous cell walls, and that are considered nutrient poor (Horn, 1989). The digestive morphology of herbivorous fish indicates their adaptation to utilising plant-based diets. Herbivores depend on their long intestines to increase the amount of time for the feed to be in contact with digestive enzymes to maximise digestion and absorption of dietary nutrients. The longer digestive tracts observed in herbivorous fish also allow for an increase the volume of food that can be ingested per feeding bout.

Predatory/carnivorous fish (such as *Clarias gariepinus*) on the other hand, have a strong masculine stomach and shorter intestines. Predatory fishes depend on a high-protein, low-fiber, animal-based, nutrient-rich diet that is easily digestible. There is need to replace fishmeal in their diets for sustainable aquaculture production. However, adding plant-based protein sources in feeds for predatory/carnivorous fish without adequate knowledge of the ingredient's potential for negative health effects may result in unwanted consequences including reduced growth, inflammatory reactions in the intestine and compromised health.

From the previous chapters, the ability of two closely related fish species to utilise plant-based diets was determined and it was evident that nutrient digestibility varies among fish species. The morphology of the digestive system and its digestive enzymes play a key role in the differences observed. It is evident that large species differences exist and data from studies on one species cannot be implemented with success to another. Consequently, the utilisation of a plant protein source should be tested in different fish species to determine each fish species' ability to use the protein source and its response to the anti-nutrients found in plant protein sources (Francis *et al.*, 2001; Chong *et al.*, 2002; Gatlin *et al.*, 2007; Collins *et al.*, 2012; Chaudhuri *et al.*, 2012). Thus, the aim of this chapter was to evaluate growth performance, protein utilisation and digestibility in a predatory/carnivorous fish, *Clarias gariepinus*, fed kikuyu and moringa-based diets. The effects of these diets on digestive enzyme activities, liver and intestine histology as well as blood parameters were also determined.

6.2 OBJECTIVES

The specific objectives in this chapter were to determine:

- i. the effect of partially and totally replacing fishmeal with different levels of kikuyu and moringa leaf meals on the growth performance, liver histology, intestine histology, digestive enzyme activities and haematological parameters in *Clarias gariepinus*.
- ii. incidence cost and profit index of partially and totally replacing fishmeal with different levels of kikuyu and moringa leaf meals in the diet of *Clarias gariepinus*.

6.3 MATERIALS AND METHODS

6.3.1 Fish acclimatisation

Clarias gariepinus used in this experiment was obtained from the Sand River, in Polokwane, Limpopo province. The fish were acclimatised to the experimental tanks (fibreglass tanks) for one month. During this period, fish were offered a standard commercial catfish diet (34% crude protein, 17 MJ/kg energy and 3% lipid). After acclimatisation, fish were weighed individually (30.50 ± 2 g) and stocked at 20 fish per tank.

6.3.2 Feed preparation

Kikuyu grass and moringa leaves were dried and milled as described in Chapter 4, Section 4.4.2. Nine isonitrogenous (30% crude protein) and isoenergetic (20 MJ/kg, gross energy) diets were prepared by replacing fishmeal at 25, 50, 75 and 100% with KLM in diets KLM 25, KLM 50, KLM 75 and KLM 100 respectively. In diets designated as MLM 25, MLM 50, MLM 75 and MLM 100 fishmeal was replaced with MLM at 25, 50, 75 and 100%. Chromic oxide (Cr_2O_3) was added at 0.5% in all diets as an inert marker. Diets were prepared as described in Chapter 4, Section 3.4.2.

6.3.3 Experimental system and experimental design

A completely randomised design experiment was set up and twenty seven tanks were used (three per dietary treatment). The feeding experiments were carried out in the same experimental system described in Chapter 4, Section 3.4.3.

6.3.4 Water quality management

Water quality parameters monitored were temperature, dissolved oxygen, ammonia and pH as described in Chapter 4, Section 3.4.4.

6.3.5 Diet allocation and feeding

Each diet was randomly assigned to three replicate tanks. All fish were hand fed their allotted diet to apparent satiation three times a day (0900, 1300 and 1700 hours) for 60 days. Feed intake (g/fish/day) was recorded daily. Faeces were collected and pooled for each dietary treatment as outlined in Chapter 4, Section 3.4.5.

6.3.6 Proximate composition of leaf meals, experimental diets and faeces

Proximate composition for fishmeal, kikuyu grass, moringa leaves and all the experimental diets was determined. Dry matter, crude protein, crude lipid, crude fibre and gross energy were determined following procedures stipulated by the Official Analytical Chemists (AOAC International, 2012) as outlined in Chapter 4, Section 3.4.6.

6.3.7 Growth performance parameters

Fish were individually weighed at the beginning and at the end of the experiment. Fish from each tank were bulk weighed once every two weeks for the duration of the experiment. At the end of the experimental period, SGR, TGC, FCR, PER and ADC for protein were determined as described in Chapter 4, Section 3.4.7.

6.3.8 Histological analysis

Five fish from each tank (15 per dietary treatment) were sacrificed at the end of the experimental period for histological analysis of the liver and intestine. Liver and intestine samples were stored in 10% neutral buffered formalin soon after collection and were processed according to the procedures outlined in Chapter 4, Section 3.4.8.

6.3.9 Digestive enzyme analysis

Intestine samples were collected from five fish in each treatment for digestive enzyme analysis. Protease, amylase and lipase activities were determined following procedures outlined in Chapter 4, Section 3.4.9.

6.3.10 Haematological analysis

At the end of the experiment, blood was collected from five fish in each tank (15 per dietary treatment) for haematological analysis. Blood was analysed for white blood cells, red blood cells, haemoglobin, hematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, plasma glucose and blood urea nitrogen as described in Chapter 4, Section 3.4.10.

6.3.11 Cost benefit analysis

Incidence cost and Profit index (described in Chapter 4, Section 3.4.11) were used to determine the economic impact of using KLM and MLM as protein sources in practical diets of *Clarias gariepinus*.

6.3.12 Statistical analysis

Statistical analysis was carried out as described in Chapter 4, Section 3.4.12.

6.4 RESULTS

6.4.1 Growth performance and apparent digestibility

6.4.1.1 Weight gain

Clarias gariepinus feeding on the fishmeal-based control had the highest ($P<0.05$) body weight gain over the 60-day experimental period. Adding KLM led to a reduction in weight gain and fish fed KLM 100 had the lowest weight gain in the kikuyu-based diets (Figure 6.1). Replacing dietary fishmeal with MLM also led to reduced body weight gain in *Clarias gariepinus*. Weight gain decreased ($P<0.05$) with increasing MLM levels in the diet (Figure 6.2).

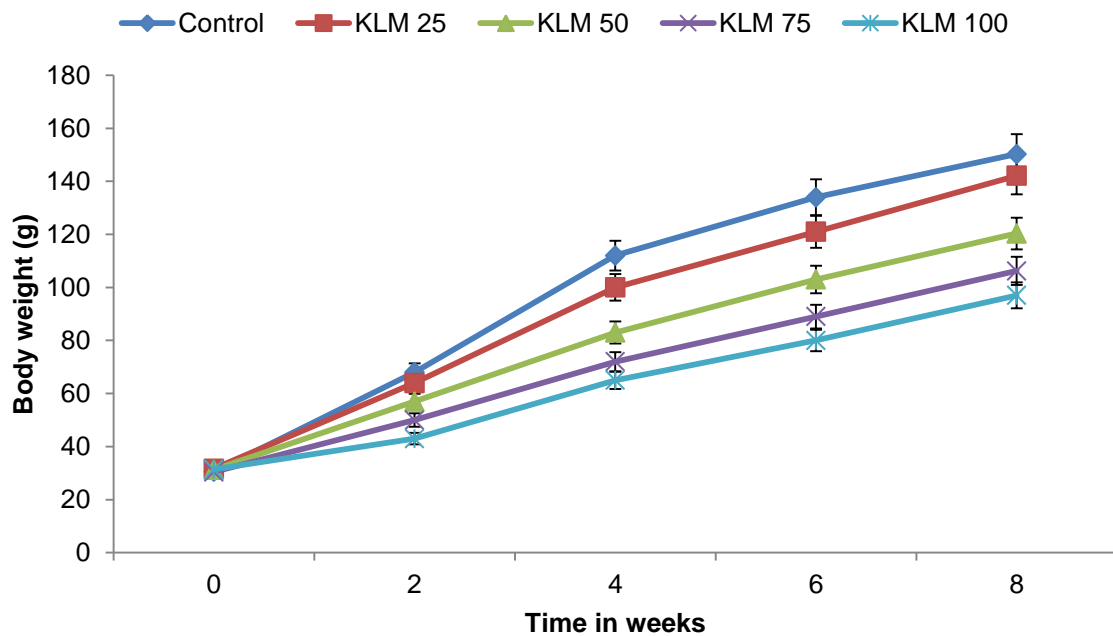


Figure 6.1: Change in body weight of *Clarias gariepinus* fed KLM diets

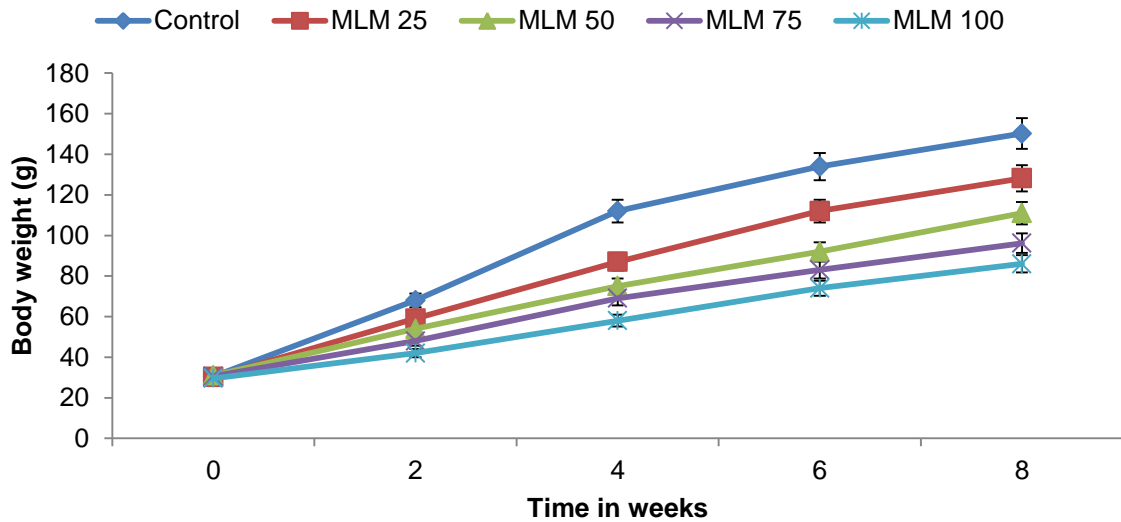


Figure 6.2: Change in body weight of *Clarias gariepinus* fed MLM diets

6.4.1.2 Feed intake

All feeds were accepted at the start of the feeding trial, however, feed intake was significantly ($P < 0.05$) reduced as the levels of both leaf meals in the diet increased (Figure 6.3A and Figure 6.3B). A negative linear regression described the relationship between feed intake and the level of KLM (Figure 6.3A) and MLM (Figure 6.3B). This regression was significantly higher ($P < 0.05$; ANCOVA) in KLM diets compared to MLM diets.

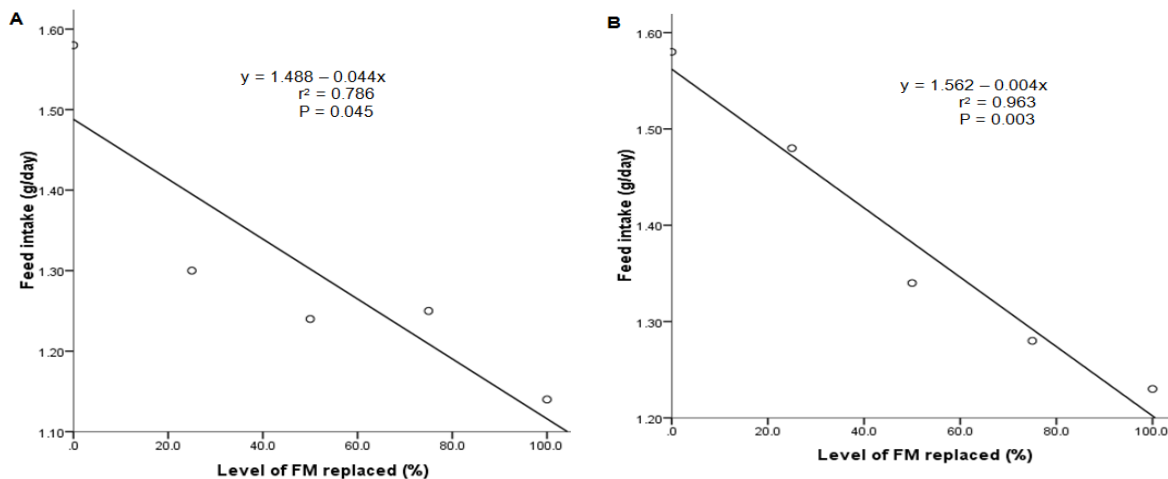


Figure 6.3: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on feed intake in *Clarias gariepinus*

6.4.1.3 Growth performance

The highest TGC was recorded in *Clarias gariepinus* fed the control diet. TGC decreased linearly with increasing levels of both KLM (Figure 6.4A) and MLM (Figure 6.4B) in the diet. Fish fed KLM-based diets had significantly higher ($P < 0.05$, ANCOVA) TGC than those fed the MLM diets. SGR was also significantly higher ($P < 0.05$, ANCOVA) in KLM (Figure 5.5A) fed fish compared to those fed the MLM (Figure 6.5B) diets. The negative linear regression explaining the relationship between SGR and the level of fishmeal replaced in the diet was significant ($P < 0.05$) for both leaf meals.

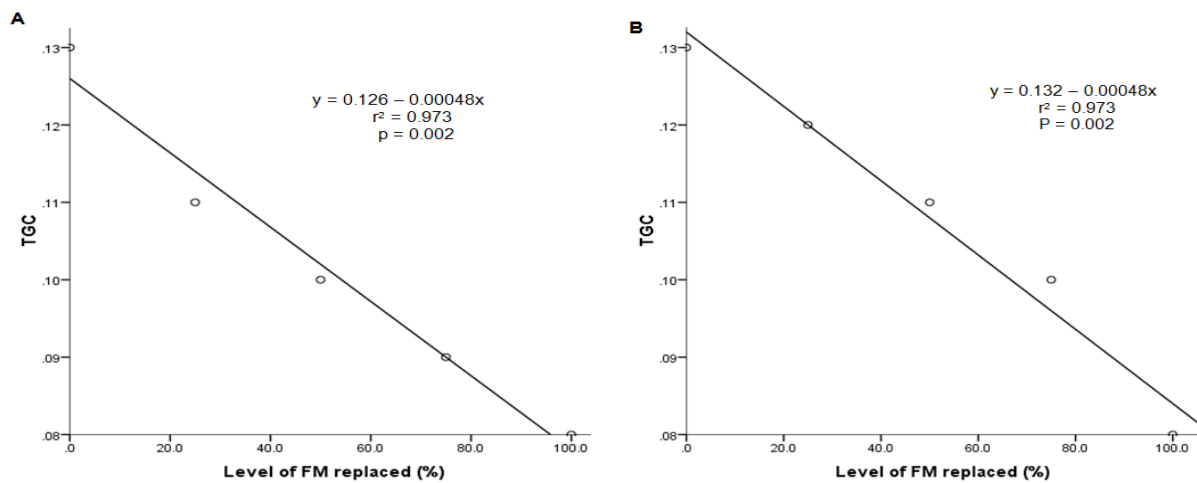


Figure 6.4: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on thermal growth coefficient in *Clarias gariepinus*

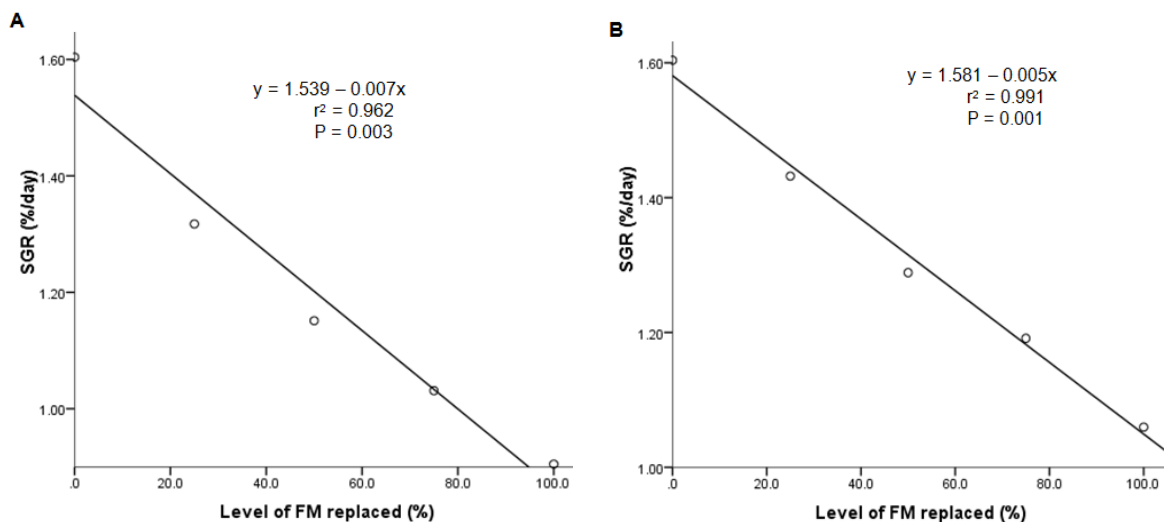


Figure 6.5: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on specific growth rate in *Clarias gariepinus*

6.4.1.4 Feed utilisation

The best FCR was recorded in fish fed the control diet and increased significantly ($P < 0.05$) with leaf meal inclusion (Figure 6.6A; Figure 6.6B). Increasing KLM to replace more than 50% fishmeal led to FCR values that were significantly ($P < 0.05$) higher than the control (Figure 6.6A). In the MLM diets, when MLM replaced more than 25% fishmeal, the FCR values were significantly ($P < 0.05$) higher than the control (Figure 6.6B). Fish fed the KLM-based diets had significantly lower FCR ($P < 0.05$, ANCOVA) than those fed MLM diets.

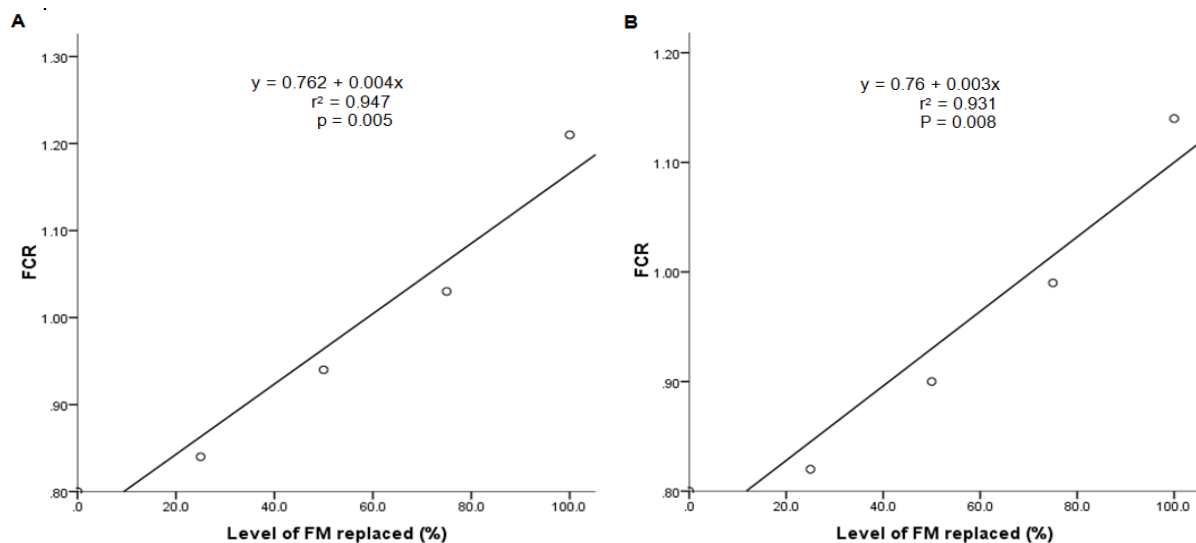


Figure 6.6: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on feed conversion ratio in *Clarias gariepinus*

6.4.1.5 Protein utilisation

Clarias gariepinus ability to utilise dietary protein decreased significantly ($P < 0.05$) when levels of both leaf meals increased in the diet. The PER of *Clarias gariepinus* fed KLM-based (Figure 6.7A) diets was significantly higher ($P < 0.05$) than of fish fed MLM-based (Figure 5.7B) diets. Apparent digestibility coefficient for protein also decreased as the level of both KLM (Figure 6.8A) and MLM (Figure 6.8B) in the diet increased and was significantly higher ($P < 0.05$, ANCOVA) in fish fed KLM than those fed MLM diets.

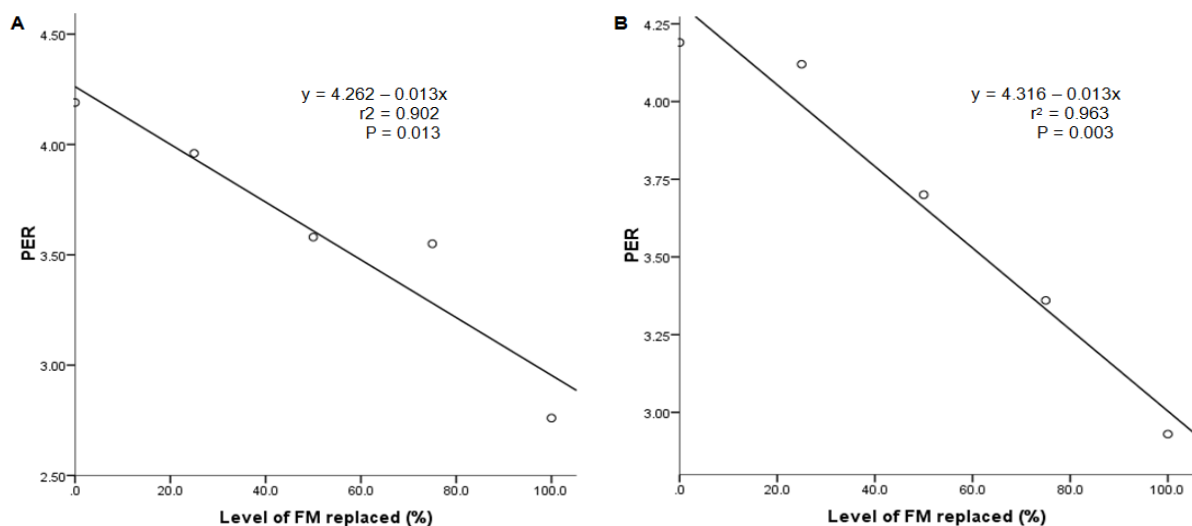


Figure 6.7: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on protein efficiency ratio in *Clarias gariepinus*

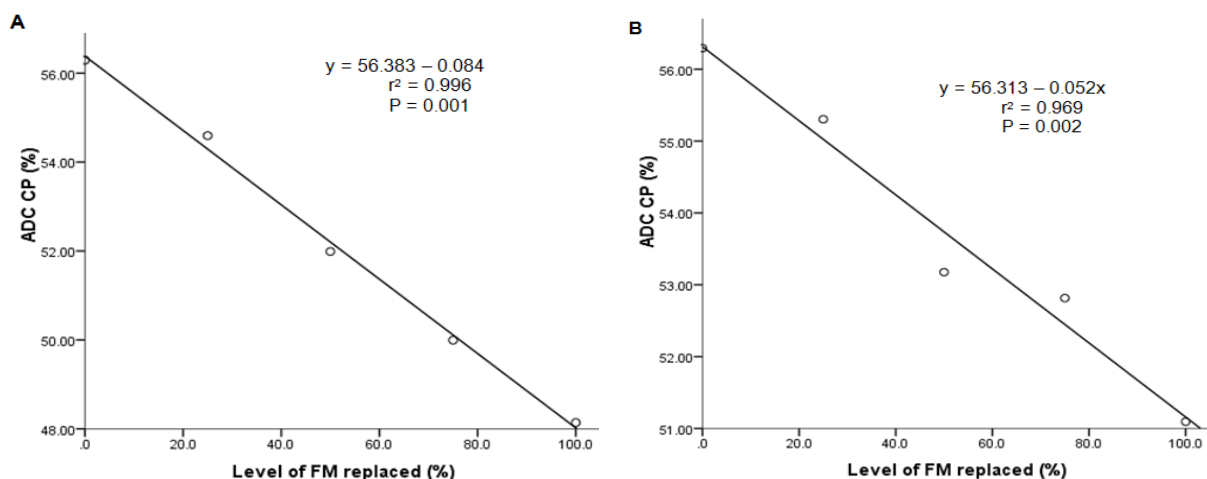


Figure 6.8: Linear regression analysis of fishmeal replacement with KLM (A) and MLM (B) on apparent digestibility coefficient for protein in *Clarias gariepinus*

6.4.2 Effect of kikuyu and moringa diets on intestine histology

Villi height decreased with increasing levels of KLM and MLM in the diet (Table 6.1). In KLM fed fish, this decrease was only significant ($P < 0.05$) at the highest inclusion level (KLM 100). In the fish fed MLM-based diets, this decrease was significant ($P < 0.05$) in fish fed diets MLM 75 and MLM 100. There was an increase in villi width with increasing leaf meal inclusion in *Clarias gariepinus* diet (Table 6.1). Villi width was significantly lower in fish fed diets where $>50\%$ fishmeal was replaced. Furthermore, a significant increase in goblet cells number was observed in treatment groups (Figure 6.9). Goblet cell number was significantly higher ($P < 0.05$) when MLM replaced more than 50% of fishmeal in *Clarias gariepinus* diets (Table 6.1).

Table 6.1: Intestine histology values of *Clarias gariepinus* fed different inclusion levels of kikuyu and moringa leaf meals

		% of fishmeal replaced by the plant meals in the diet				
		Control	25	50	75	100
<i>Villi length (μm)</i>						
KM			624.51±6 ^a	617.41±8 ^a	602.55±9 ^a	600.38±12 ^b
MM	634.48±10 ^a		621.75±10 ^a	614.52±10 ^a	593.89±30 ^b	576.75±13 ^b
<i>Villi width (μm)</i>						
KM			82.71±14 ^a	82.89±12 ^a	75.74±9 ^b	73.38±12 ^b
MM	83.12±10 ^a		84.02±11 ^a	83.59±13 ^a	71.50±11 ^b	67.45±15 ^c
<i>Goblet cell number (per 100 epithelial cells)</i>						
KM			462.±15 ^a	478±12 ^a	492±10 ^b	503±14 ^b
MM	450.02±14 ^a		485±10 ^a	498±7 ^b	502±11 ^b	527±16 ^b

Values are mean of five determinations ± standard deviations. Values in the same rows with the same superscripts are not significantly different (P>0.05)

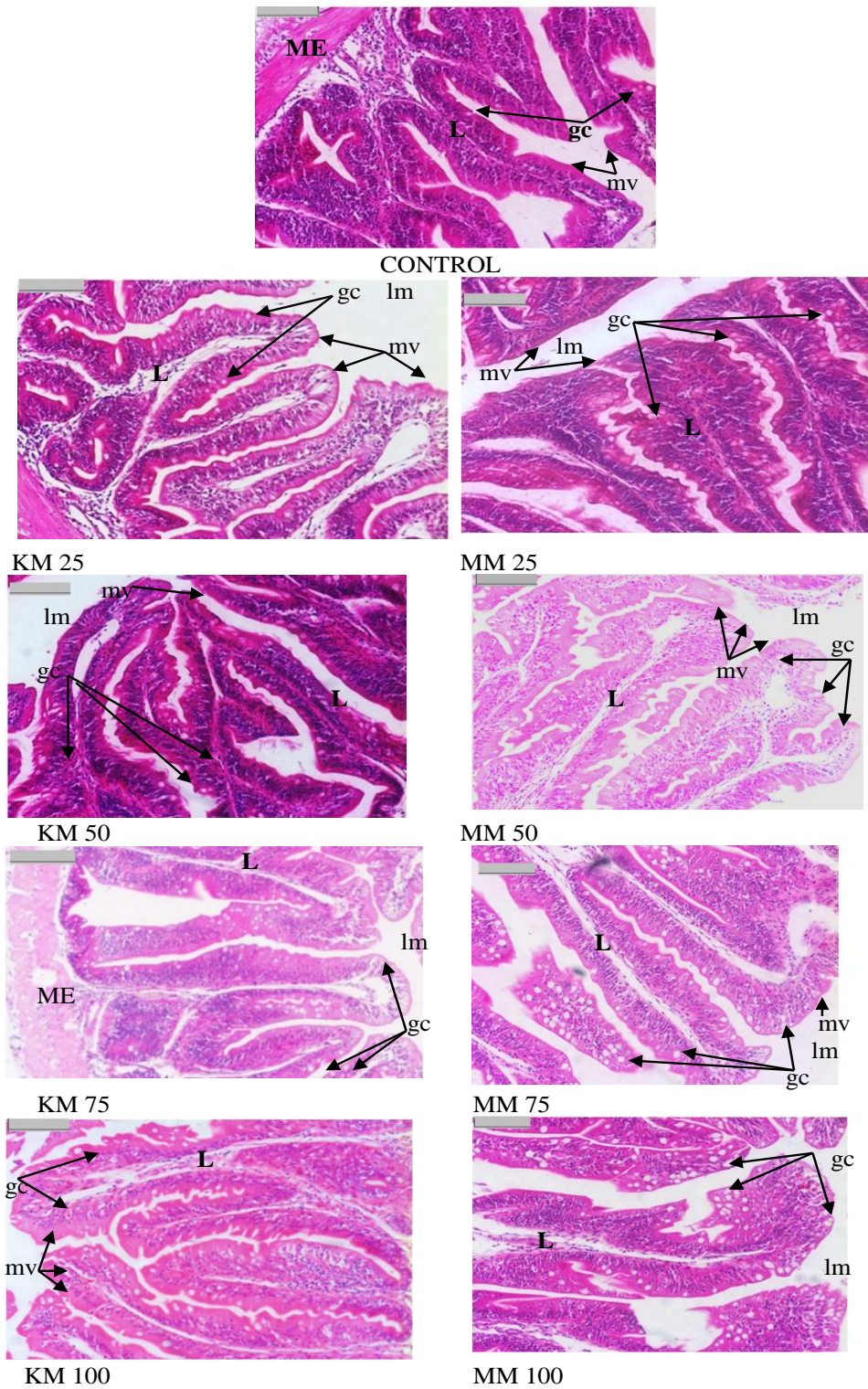


Figure 6.9: Effect of kikuyu and moringa leaf meals on intestine histology of *Clarias gariepinus*. Scale bar = 100 μ m. ME-muscularis externa, L-lamina propria, lm-lumen; arrows point to microvilli (mv), goblet cells (gc)

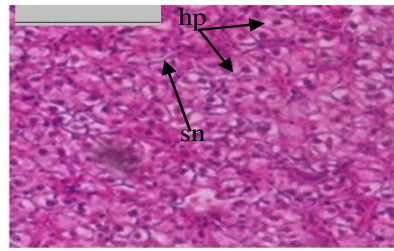
6.4.3 Effect of kikuyu and moringa leaf meals on liver histology

In *Clarias gariepinus*, the hepatocyte condition followed the same trend as reported for the tilapias, however, degradation was more pronounced in *Clarias gariepinus*. Histological analysis of *Clarias gariepinus* fed the control diet showed hepatocytes of regular shape with large centrally located nuclei and homogenous cytoplasmic lipid content and distinct boundaries (Figure 6.10). A slight degradation in hepatocytes integrity was observed with increasing levels of KLM in the diet. The hepatocytes of fish fed high levels of MLM in the diet had poorly visible cell membranes and hyaline cytoplasm, small pyknotic nuclei pushed to the periphery of the cell and higher incidence of Kupffer cells (Figure 6.10). Fish fed up to 75% KLM did not show any significant increase in hepatocyte degradation scores (Table 6.2). Fish fed MLM had higher degradation scores than those fed KLM at the same replacement level (Table 6.2). Hepatocytes of *Clarias gariepinus* fed MLM-based diets were unevenly shaped with smaller nuclei and showed significantly higher ($P < 0.05$) hepatocyte degradation when MLM replaced $>25\%$ of fishmeal. *Clarias gariepinus* fed MLM100 had the highest hepatocyte degradation score of 3.00 (Table 6.2).

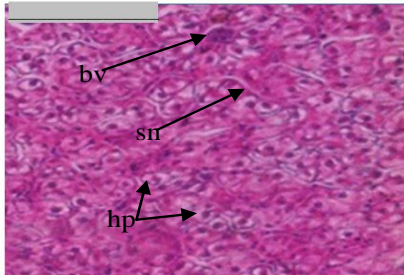
Table 6.2: Mean hepatocyte degradation score for *Clarias gariepinus* fed different inclusion levels of kikuyu and moringa leaf meals (100 hepatocytes per treatment)

	% fishmeal replaced by plant meal in the diet				
	20	25	50	75	100
KLM	1.20±0.4 ^a	1.50±0.2 ^b	1.92±0.5 ^c	2.40±0.5 ^d	2.64±0.3 ^e
MLM	1.20±0.4 ^a	1.80±0.3 ^b	2.50±0.6 ^c	2.80±0.2 ^d	3.00±0.1 ^e

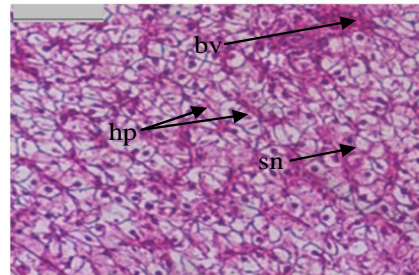
Values in the same row with the same superscripts are not significantly different ($P > 0.05$)



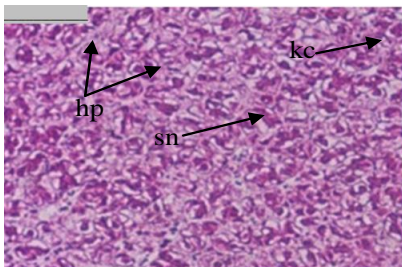
CONTROL



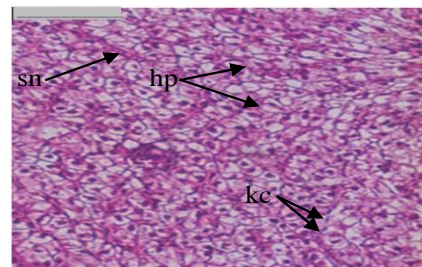
KM 25



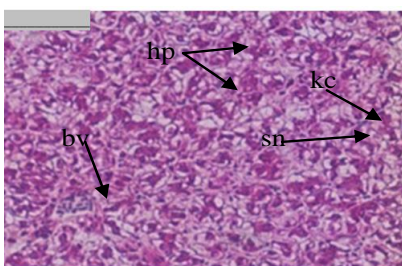
MM 25



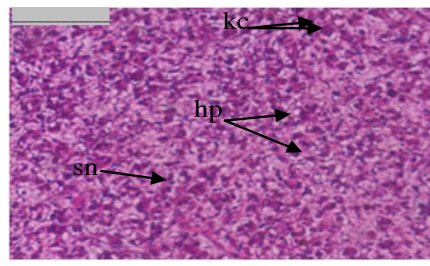
KM 50



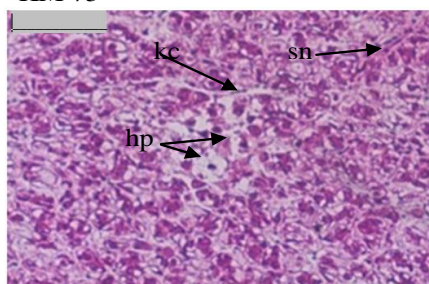
MM 50



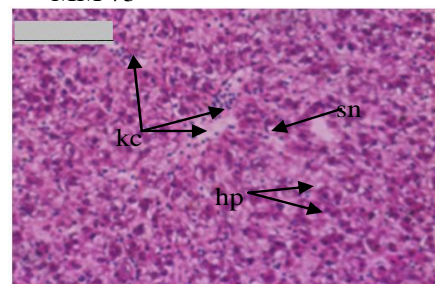
KM 75



MM 75



KM 100



MM 100

Figure 6.10: Effect of kikuyu and moringa leaf meals on liver histology in *Clarias gariepinus*. Scale bar = 100 μ m. arrows point to hepatocytes (hp), sinusoids (sn), Kupfer cells (kc) and blood vessels (bv)

6.4.4 Effect of kikuyu and moringa leaf meals on digestive enzyme activities

6.4.4.1 Protease activity

Protease activity was highest in *Clarias gariepinus* fed the fishmeal-based control and decreased with increasing leaf meal inclusion (Figure 6.11A and B). When KLM was added up to 25% in the diet, protease activity did not differ significantly ($P < 0.05$) from the control (Figure 6.11A). Significantly lower ($P < 0.05$) protease activity was recorded in fish fed diets where fishmeal was replaced with more than 25% KLM. For MLM diets on the other hand, protease activity decreased significantly ($P < 0.05$) at the lowest inclusion level (MLM 25) (Figure 6.11B).

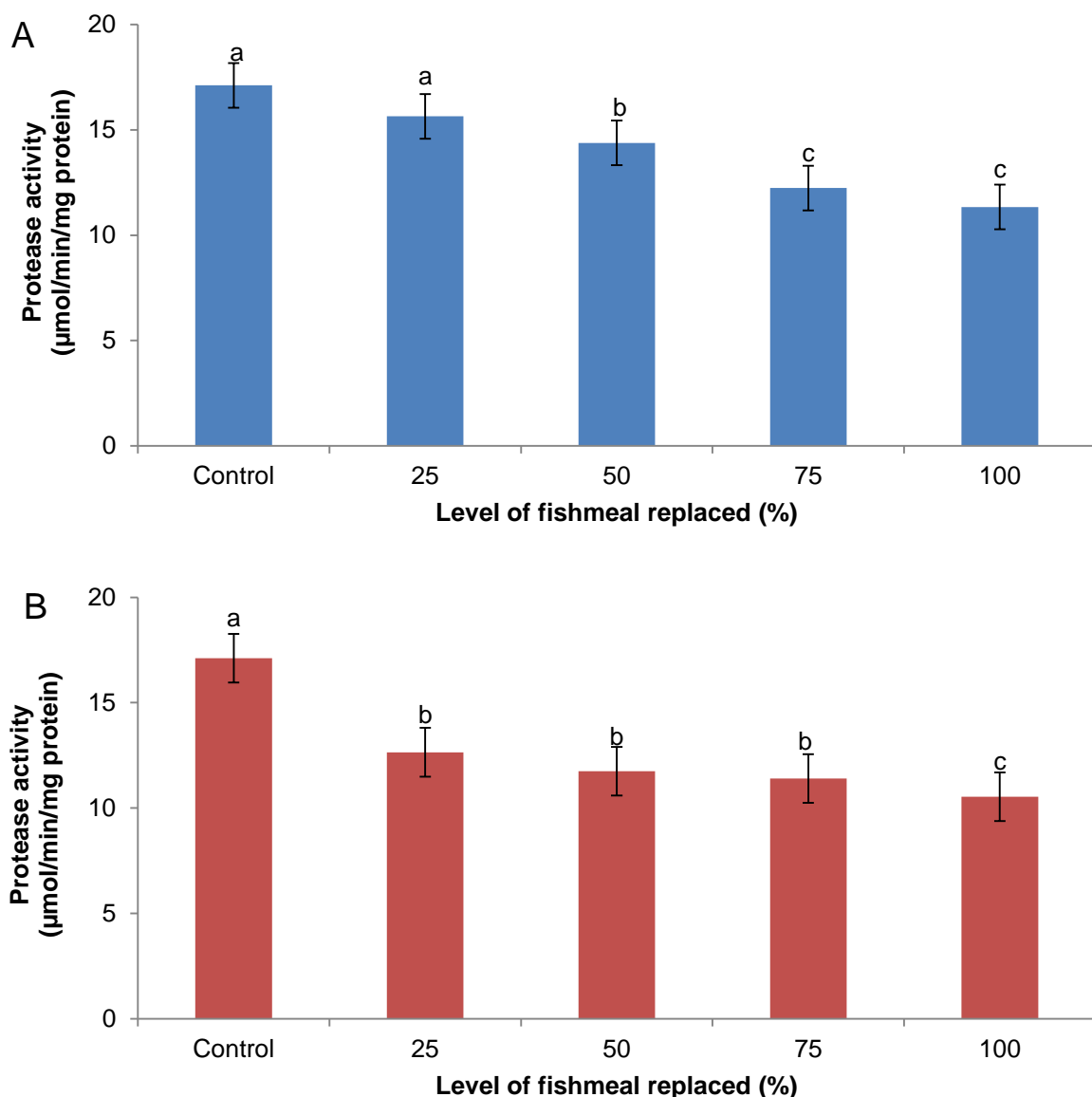


Figure 6.11: Effect of replacing fishmeal with KLM (A) and MLM (B) on the protease activities in *Clarias gariepinus* intestine. Bars with different letters are significantly different ($P < 0.05$)

6.4.4.1 Amylase and lipase activity

Amylase activity in *Clarias gariepinus* was lower than that recorded for the tilapias. A significant decrease ($P < 0.05$) in amylase activity was evident when more than 50% fishmeal was replaced by KLM (Figure 6.12A). In the moringa-based diets, a significant decrease ($P < 0.05$) in amylase activity in *Clarias gariepinus* intestines was recorded when MLM replaced more than 25% fishmeal in the diet (Figure 6.12B) in the diet.

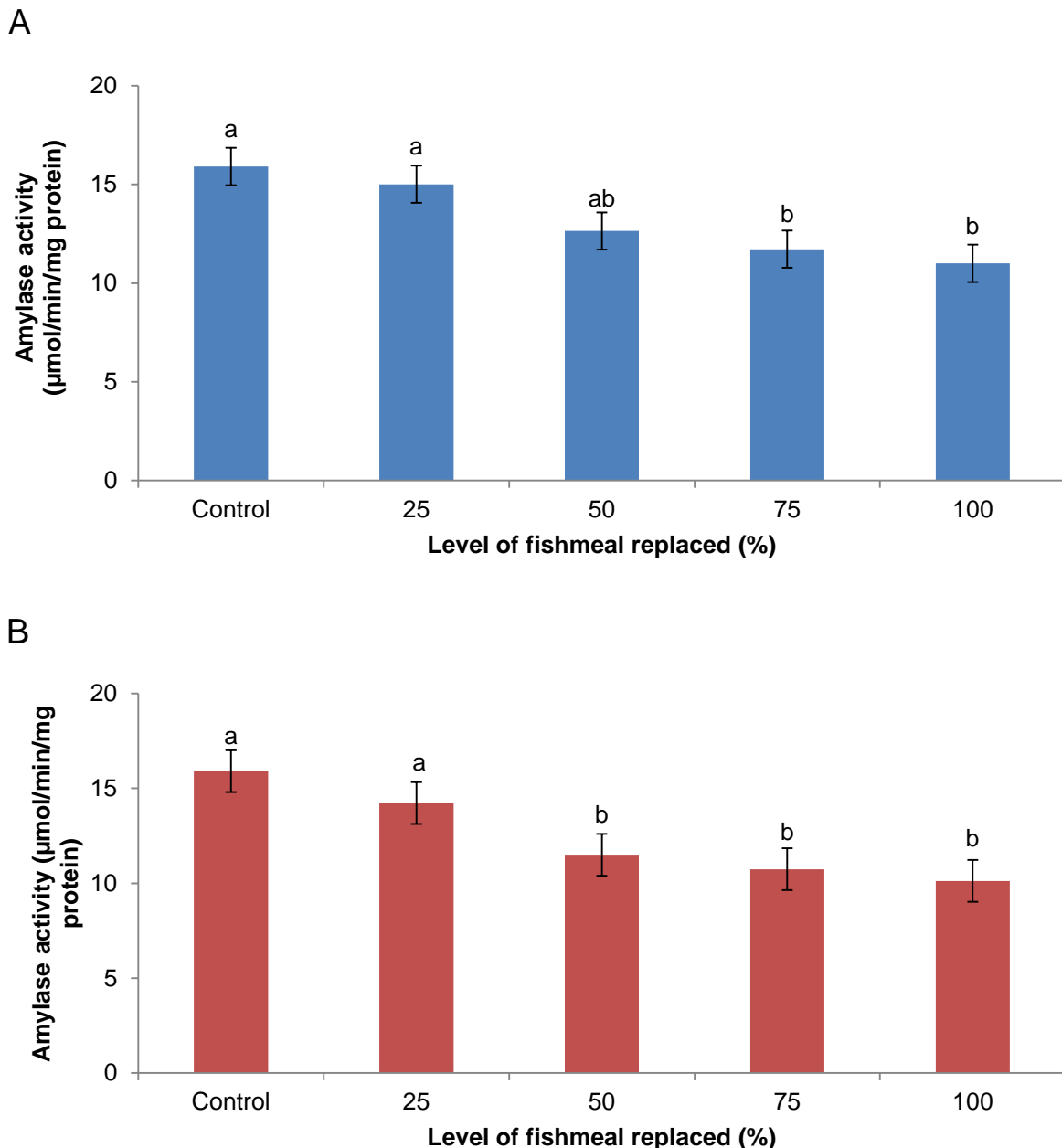


Figure 6.12: Effect of replacing fishmeal with KLM (A) and MLM (B) on the amylase activities in *Clarias gariepinus* intestine. Bars with different letters are significantly different ($P < 0.05$)

Lipase activities on the other hand, only showed a significant decrease ($P < 0.05$) when fishmeal was totally replaced by KLM (Figure 6.13A). For the MLM diets, lipase activity was significantly lower than the control when MLM replaced more than 50% dietary fishmeal (Figure 6.13B). Lipase activity in *Clarias gariepinus* intestines was higher than that recorded for the tilapias (Chapter 4 and 5).

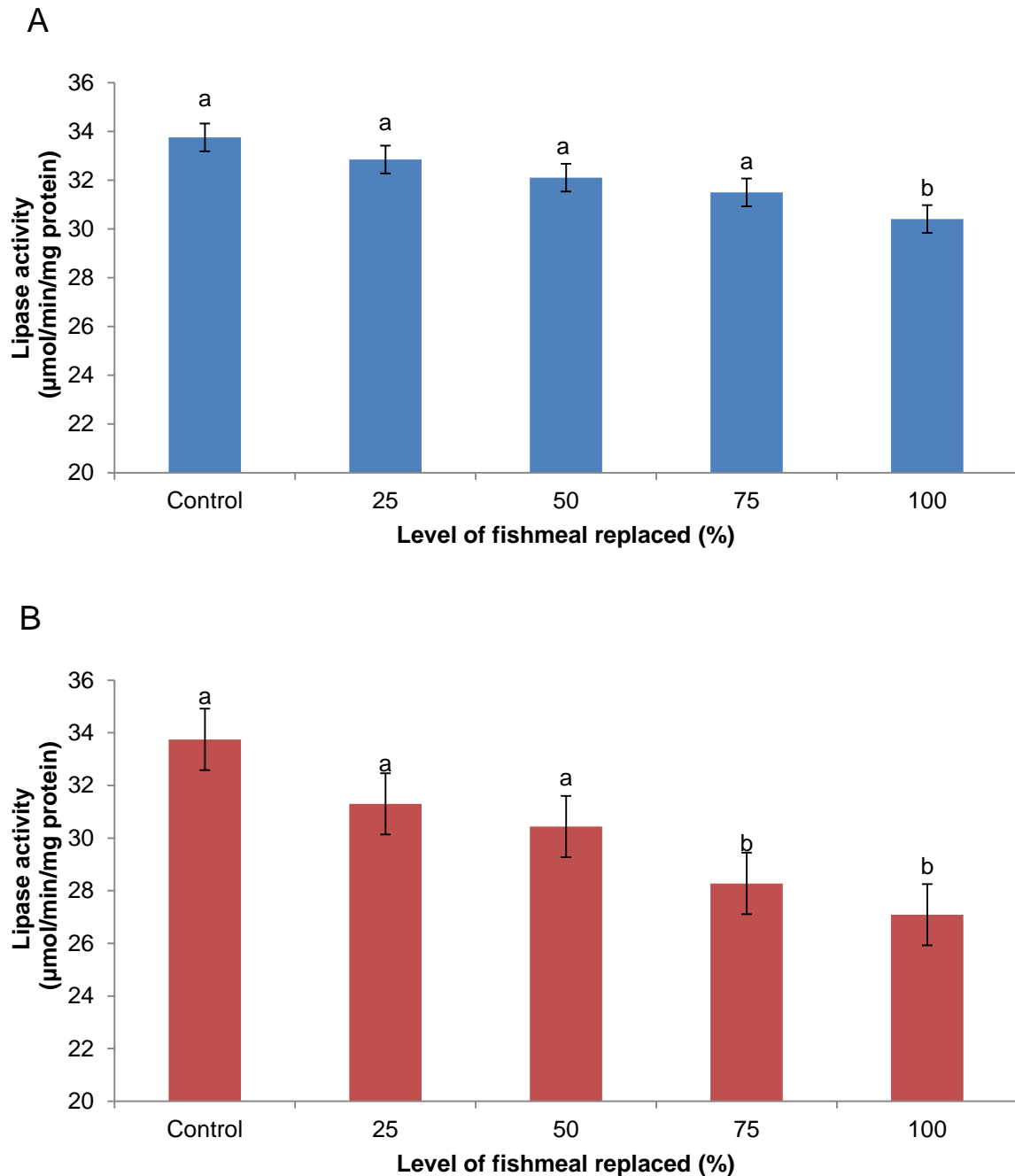


Figure 6.13: Effect of replacing fishmeal with KLM (A) and MLM (B) on the lipase activities in *Clarias gariepinus* intestine. Bars with different letters are significantly different ($P < 0.05$)

6.4.5 Effect of kikuyu and moringa leaf meals on haematological parameters

White blood cell count in *Clarias gariepinus* increased with increasing KLM level in the diet and was significantly ($P>0.05$) higher than the control in fish fed KLM 100 (Table 6.3). In the moringa treatments, WBC count was significantly higher than the control in fish fed diets where MLM replaced more than 25% fishmeal (Table 6.4). RBCs and HCT were not significantly different ($P>0.05$) from the control at all levels of KLM inclusion (Table 6.3). However, in the MLM diets, RBCs and HCT were significantly lower than the control in fish fed MLM100.

Mean corpuscular volume, MCH, MCHC and plasma glucose were not significantly different ($P>0.05$) from the control for all KLM (Table 6.3) and MLM (Table 6.4) inclusion levels. Plasma protein and BUN were not significantly different ($P>0.05$) from the control in all KLM diets (Table 6.3). In the MLM diets, plasma protein and BUN decreased with increasing MLM levels and were significantly lower ($P<0.05$) than the control in fish fed more than 25% MLM in the diet (Table 6.4).

Table 6.3: The Effect of kikuyu-based diets on haematological parameters of *Clarias gariepinus* (n =15)

	Control	KLM 25	KLM 50	KLM 100
WBC ($10^3 \mu\text{l}$)	609.80±9.2 ^a	615.81±9.6 ^a	617.64±7.1 ^a	625.24±8.7 ^b
RBC($10^6 \mu\text{l}$)	2.85±0.19 ^a	2.72±0.17 ^a	2.67±0.2 ^a	2.68±0.15 ^a
HTC (l/l)	0.39±0.1 ^a	0.37±0.1 ^a	0.35±0.2 ^a	0.32±0.1 ^a
Hb (g/dl)	10.33±2.1 ^a	10.32±1.5 ^a	10.30±2.7 ^a	10.28±1.9 ^a
MCV (fL)	136.84±7.5 ^a	136.03±5.6 ^a	131.10±4.5 ^a	119.40±7.1 ^a
MCH (pg)	36.24±2.1 ^a	37.94±3.1 ^a	38.60±2.1 ^a	38.36±2.5 ^a
MCHC (g/dl)	26.48±2.4 ^a	27.89±3.5 ^a	29.40±1.6 ^a	32.13±2.3 ^a
Plasma Glucose (g/dl)	2.40±0.2 ^a	3.40±1.2 ^a	2.70±0.1 ^a	1.80±0.3 ^a
Total protein (g/l)	41.00±1.1 ^a	40.00±2.3 ^a	39.60±2.1 ^a	34.00±1.5 ^b
BUN	1.00±0.1 ^a	0.95±.02 ^a	0.80±0.1 ^a	0.85±0.1 ^a

Values are mean of five determinations ± standard deviations. Values in the same rows with the same superscripts are not significantly different ($P>0.05$). WBC- white blood cells; RBC- red blood cells; HCT- hematocrit; Hb- hemoglobin; MCV- mean corpuscular volume; MCH- Mean corpuscular hemoglobin; MCHC- mean corpuscular haemoglobin concentration; BUN- Blood urea nitrogen

Table 6.4: The Effect of moringa-based diets on haematological parameters of *Clarias gariepinus* (n =15)

	Control	MLM 25	MLM 50	MLM 100
WBC ($10^3 \mu\text{l}$)	609.80±9.2 ^a	611.87±9.8 ^a	629.74±8.5 ^b	635.21±7.2 ^b
RBC($10^6 \mu\text{l}$)	2.85±0.19 ^a	2.65±0.20 ^a	2.64±0.19 ^a	2.62±0.15 ^b
HTC (l/l)	0.39±0.1 ^a	0.36±0.1 ^a	0.34±0.2 ^a	0.31±0.1 ^b
Hb (g/dl)	10.33±2.1 ^a	10.25±2.2 ^a	10.25±2.1 ^a	10.26±2.8 ^b
MCV (fL)	136.84±7.5 ^a	135.85±9.6 ^a	128.79±4.8 ^a	118.32±7.2 ^a
MCH (pg)	36.24±2.1 ^a	38.83±2.8 ^a	38.83±1.5 ^a	39.10±3.8 ^a
MCHC (g/dl)	26.48±2.4 ^a	28.47±4.1 ^a	30.14±2.5 ^a	33.09±3.5 ^a
Plasma glucose (g/dl)	2.40±0.2 ^a	1.80±0.2 ^a	3.00±0.2 ^a	2.40±0.3 ^a
Total protein (g/l)	41.00±1.1 ^a	39.00±1.0 ^a	35.00±2.0 ^b	34.50±1.4 ^b
BUN	1.00±0.1 ^a	0.85±.011 ^a	0.75±0.2 ^b	0.70±0.1 ^b

Values are mean of five determinations ± standard deviations. Values in the same rows with the same superscripts are not significantly different ($P>0.05$). WBC- white blood cells; RBC- red blood cells; HCT- hematocrit; Hb- hemoglobin; MCV- mean corpuscular volume; MCH- Mean corpuscular hemoglobin; MCHC- mean corpuscular haemoglobin concentration; BUN- Blood urea nitrogen

6.4.6 Cost benefit analysis

The cost benefit analysis show that adding KLM in *Clarias gariepinus* diets resulted in lower incidence cost and higher profit index when compared to the control (Table 6.5). The inclusion of MLM on the other hand, resulted in increasingly higher incidence costs and lower profit index with increasing levels of MM in the diet (Table 6.5).

Table 6.5: Cost benefit analysis on feeding *Clarias gariepinus* on kikuyu and moringa-based diets

	% fishmeal replaced by the plant meals in the diet				
	0	25	50	75	100
<i>Kikuyu-based diets</i>					
Incidence cost	0.01	0.01	0.01	0.01	0.01
Profit index	2.02	2.07	2.18	2.26	2.36
<i>Moringa-based diets</i>					
Incidence cost	0.01	0.01	0.01	0.02	0.02
Profit index	2.02	1.79	1.66	1.55	1.44

6.5 DISCUSSION

The growth performance parameters (TGC and SGR) and feed utilisation (FCR, PER and protein ADC) in *Clarias gariepinus* fed the experimental diets decreased as the level of both leaf meals in the diet increased. *Clarias gariepinus* is a predatory omnivore that is pre-adapted to utilising animal-based diets. Protein digestibility was higher in *Clarias gariepinus* than in the tilapias for the control group. However, in fish fed the the leaf meal-based diets; *Clarias gariepinus* had lower levels of protein digestibility and utilisation compared to *Tilapia rendalli* and *Oreochromis mossambicus* feeding on the same diets. This may be attributed to their natural feeding habits and digestive morphology; both *Tilapia rendalli* and *Oreochromis mossambicus* have longer intestines compared to *Clarias gariepinus*. This increases the time the digesta is in contact with the digestive enzymes as well as the surface area for nutrient absorption, which may have helped contribute to the higher ADC for protein recorded in the plant-based diets.

Intestine histology showed an increase in goblet cell number, microvilli degeneration, infiltration of inflammatory cells in *Clarias gariepinus* fed diets where >50% fishmeal was replaced. The damage to the intestinal cells was more pronounced in *Clarias gariepinus* than in the tilapias. This implies that the predatory fish may not be equipped to utilise high levels of plant meal in the diet. These results concur with several authors who reported morphological changes and damage of the intestinal tract in *Salmon salar* a (predator) fed high levels of plant-based diets (Baeverfjord and Krogdahl, 1996; Bakke-McKellep *et al.*, 2007; Kraugerud *et al.*, 2007; Uran *et al.*, 2008).

In rainbow trout, significantly shorter (distal intestine) and less densely packed (proximal intestine) microvilli on the enterocyte surfaces of fish fed a soybean meal diet than fish fed the fishmeal diet Merrifield *et al.* (2009). These studies indicate that high levels of plant-based diets may have detrimental effects on the digestive tract in predatory fishes. The morphological changes sustained may affect the exchange of substances across the barriers including facilitated uptake of pathogenic bacteria.

Efficiency of food conversion and absorption also depend on the availability of digestive enzymes in the digestive tract (Jobling, 1995). Amylase activities were also

higher in the tilapias compared to those recorded in *Clarias gariepinus* in all the experimental diets. This explains the adaptability of tilapias to utilising plant-based diets. Several authors have also reported that amylase activity is dependent on the natural diet of each species, and that herbivorous fish have higher activity than carnivores (Hidalgo *et al.*, 1999; Hofer *et al.*, 1982).

Intestinal protease activities recorded in *Clarias gariepinus* decreased with increasing leaf meal inclusion in the diet. This decrease in protease activity is an important factor in the ability to utilise dietary protein. Some anti-nutrients present in plant diets hamper digestive enzyme activities in predatory fish. Chong *et al.*, (2002) reported that plant-based diets had an inhibitory effect on activity of protease in *Symphysodon* spp. Anti-trypsin inhibitors present in soybean have also been proven to reduce the capability to digest proteins in rainbow trout (Krogdahl *et al.*, 1994) and coho salmon (Olli *et al.*, 1994). In a separate study, Lemieux *et al.* (1999) reported that trypsin activity limits the digestive capacity in cod and its activity was directly related to food conversion efficiency and growth. Interestingly, protease activity was lower in the catfish intestine than in the tilapias regardless of the diet. This may be a genetic adaptation by the tilapias to secrete higher protease levels for the digestion of the plant proteins, which are more difficult to breakdown compared to the animal protein which dominates the catfish diet.

Understanding the brush border enzymatic capability, and adjusting the diets to the digestive capacity of the different fish, is paramount in the selection of suitable feed ingredients for fish diets. It is therefore, concluded that digestive enzymes could potentially set a limit upon the digestive capacity of individual fish and determine their maximal growth rate and food conversion efficiency. These results show that the different fish species exhibit distinctly different patterns of brush-border enzyme activity.

The higher hepatocyte degradation and higher stress level (indicated by the haematological parameters) in *Clarias gariepinus* compared to both tilapias confirm the poor ability of the predatory fish to utilise plant-based diets. Liver histology indicated that *Clarias gariepinus* fed higher MLM levels had necrotic signs associated with poor nutritional status (Fontagne *et al.*, 1998; Power *et al.*, 2000;

Ostaszewska *et al.*, 2005; Tusche *et al.*, 2012). The liver is the main source of urea, thus the lower BUN levels confirm the compromised functioning of the liver. This implies that because of the lower digestibility and poor absorption, a larger portion of dietary nutrients was excreted in *Clarias gariepinus*; hence the necrosis of the hepatocytes which is a sign of malnutrition. *Clarias gariepinus* feeding on the high MLM diets had the lowest growth rate and the most apparent change in tissue morphology than the tilapias. This suggests that *Clarias gariepinus* was less equipped to utilising the anti-nutritional factors in the plant diets.

Cost benefit analysis showed that KLM diets are economically superior to both the fishmeal-based control and MLM diets. Feeding KLM diets resulted in higher profit index and lower incidence cost than feeding the Fishmeal-based control whereas, feeding MLM gave lower profit index and a higher incidence cost. Widespread claims on the health benefits in humans have increased the demand and the price for moringa leaves in South Africa, making its use in fish diets unsustainable.

This study showed that adding even the lowest level of leaf meal to *Clarias gariepinus* diets led to pathological changes in the digestive organs that increased in severity as the level of leaf meal increased. The morphological changes observed in the liver and intestine may increase susceptibility to diseases. Optimal health and disease resistance is dependent not only on optimal balance of nutrients but also on optimal function of the digestive tract and associated organs. This is corroborated by the reduced growth in fish fed higher levels of leaf meal in the diet. The presence of the morphological changes as well as the decrease in the enzyme activities in the intestine of fish fed the MLM diets indicate that this protein source may not be suitable for *Clarias gariepinus*.

CHAPTER 7:

**EFFECT OF EXOGENOUS ENZYME
SUPPLEMENTATION ON THE GROWTH
PERFORMANCE AND DIGESTIVE ENZYME
ACTIVITIES IN *OREOCHROMIS
MOSSAMBICUS***

7.1 INTRODUCTION

Fish diets generally contain high levels of fishmeal. However, the increasing costs and unstable supply of fishmeal have led to its replacement with plant protein sources. Plant diets contain anti-nutrients that inhibit their utilisation and interfere with digestive processes. Therefore, the identification and destruction of anti-nutritional factors that inhibit nutrient utilisation is an imperative in the successful use of plant-based protein for fish feed. Exogenous enzymes may be used to inactivate anti-nutritional factors and enhance the nutritional value of plant-based protein in fish feeds (Dalsgaard *et al.*, 2012).

Exogenous enzymes are widely used in the diets of terrestrial animals such as pigs and poultry (Cowieson *et al.*, 2006). With exception of phytase, there are few studies on the use of exogenous enzymes in fish feeds. A feeding trial conducted with *Oreochromis niloticus* fingerlings showed that fish fed a commercial phytase enzyme (Natuphas) exhibited higher weight gain and a better feed conversion ratio than those fed a control diet (Feord, 1996). Additionally, Cao *et al.* (2007) reported that the inclusion of phytate in fish feeds reduces eutrophication by making the bound phosphorus and other nutrients available to the fish for growth.

Results on using other exogenous enzymes alone or in combination are contradictory. Drew *et al.* (2005) reported an increase in the specific growth rate (1.27% to 1.33%), an improvement in feed conversion ratio (1.21 to 1.12) and an increase in apparent nutrient digestibility when supplementing a commercial protease at 0.25 g/kg to rainbow trout (*Oncorhynchus mykiss*) diet containing a mixture of rapeseed and pea meals. Similarly, Ng *et al.* (2002) reported a significant increase in the weight gain (297.5% to 338.5%), specific growth rate (1.97% to 2.11%), protein efficiency ratio (2.07 to 2.25), net protein utilisation (31.2% to 31.1%), ADC of dry matter (52.3% to 62.3%), ADC of protein (71.2% to 74.5%), ADC of lipid (69.6% to 75.6%) and ADC of energy (58.4% to 68.2%) and also a significant improvement in the feed conversion ratio (1.56 to 1.41) when 0.1% of a commercial feed enzyme cocktail (Allzyme Vegpro™), was supplemented to palm kernel-based diets of red hybrid tilapia (*Oreochromis Sp.*). On the contrary, Ogunkoya *et al.* (2006) did not find any effect on the growth and feed efficiency after adding graded levels of a commercial enzyme cocktail to rainbow trout diets containing up to 200 g soybean

meal/kg DM of diet. In another study, Farhangi and Carter (2007) reported that adding commercial enzymes: Energex™, Bio-Feed™ Pro and Alpha galactosidase™ separately or in combination to a diet containing 50% of de-hulled blue lupin for rainbow trout was ineffective.

Studies on exogenous enzyme supplementation in fish diets have focussed on carnivorous fish including trout and salmon (Ogunkoya *et al.*, 2006; Farhangi and Carter, 2007). Work done on herbivorous fish is mostly on the fast growing Nile tilapia (Lin *et al.*, 2007; Tahoun *et al.*, 2011). Moreover, in most of the studies, exogenous enzyme supplementation was done on legume meal-based diets. There are no studies on efficacy of exogenous enzyme supplementation to leaf meal-based diets such as kikuyu leaf meal. It is, therefore, important to determine the effect of enzyme supplementation to a kikuyu leaf meal-based diet as KLM has potential to partially replace fishmeal in tilapia diets.

Natuzyme50[®] (Bioproton (PTY LTD), Sunnybank, Queensland, Australia) is a commercial multi-enzyme containing protease, alpha-amylase, phytase, cellulase and xylanase. The use of a multi enzyme such as Natuzyme50[®] may be beneficial in improving the digestibility of KLM-based diets, as high levels of KLM led to a decrease in endogenous enzyme activities. Furthermore, fish lack the enzyme cellulase, which is vital in the digestion of plant diets. Kikuyu leaf meal as previously stated contains anti-nutritional factors including tannins, saponins, phytate, cellulose and fibre.

In the previous chapters, tilapias showed a greater ability to utilise plant-based diets than the catfish. When fed a control diet or the lowest level of kikuyu inclusion (KLM 25), *Oreochromis mossambicus* out-performed *Tilapia rendalli*. *Oreochromis mossambicus* was selected and fed the best performing plant diet (KLM 25) supplemented with increasing levels of Natuzyme50[®]. Thus, the aim of this chapter was to investigate the effect of Natuzyme50[®] on the growth performance, protein ADC, digestive enzyme activities and gastric evacuation rates in *Oreochromis mossambicus* fed a kikuyu-based diet. Natuzyme50[®] supplementation levels for optimal productivity of *Oreochromis mossambicus* were also determined.

7.2 OBJECTIVES

The specific objectives of this chapter were to determine the effect of Natuzyme50[®] supplementation on:

- i. growth performance, feed utilisation and digestive enzyme activities in *Oreochromis mossambicus* fed a kikuyu-based diet.
- ii. gastric evacuation rate in *Oreochromis mossambicus* fed a kikuyu-based diet.

7.3 MATERIALS AND METHODS

7.3.1 Fish acclimatisation

Oreochromis mossambicus (15±2.3 g) were used for this experiment. Fish were acclimatised to the experimental tanks 2 weeks before the start of the feeding trial. During this period fish were fed a commercial tilapia diet. At the start of the experiment, a total number of 225 healthy fish were selected. The fish were weighed and randomly distributed into 15 experimental tanks (15 fish in each).

7.3.2 Feed preparation

The best performing diet (KLM 25) was selected for use in this experiment. This diet was used as a control; four diets were formulated by adding a multi enzyme complex (Table 7.1) Natuzyme50[®] (Bioproton, Australia). Natuzyme50[®] was added at a rate of 0.25, 0.5, 0.75 and 1 g/kg DM diet and the diets were coded NT 25, NT 50, NT 75 and NT 100, respectively. The control diet was coded NT 0 (Table 7.2).

Table 7.1: Composition of the multi-enzyme cocktail (Natuzyme50[®]) form Bioproton (PTY LTD), Australia

Enzyme	Activity
Alpha-Amylase	3 457 cu/g
Beta-Glucanase	6 462 xu/g
Phytase	12 975 bu/g
Cellulase	48 750 U/g
Xylanase	82 750 U/g
Protease	5 376 U/g

Table 7.2: Ingredients and proximate composition of experimental diets used

	NT 0	NT 25	NT 50	NT 75	NT 100
*Natuzyyme50 [®]	0.00	0.025	0.05	0.075	0.100
KLM	7.50	7.50	7.50	7.50	7.50
Fish meal	7.71	7.71	7.71	7.71	7.71
Soybean meal	7.32	7.32	7.32	7.32	7.32
Canola meal	18.00	18.00	18.00	18.00	18.00
Sunflower meal	16.80	16.80	16.80	16.80	16.80
Maize gluten	12.00	12.00	12.00	12.00	12.00
Wheat middlings	2.17	2.17	2.17	2.17	2.17
Maize meal	23.00	23.00	23.00	23.00	23.00
Canola oil	1.00	1.00	1.00	1.00	1.00
Mineral + vitamin premix	1.00	1.00	1.00	1.00	1.00
Methionine	1.00	1.00	1.00	1.00	1.00
Binder	2.00	2.00	2.00	2.00	2.00
Chromic oxide	0.50	0.50	0.50	0.50	0.50
Dry matter (%)	92.40	92.09	92.32	91.32	91.40
Crude protein (%)	29.87	30.15	30.06	29.74	30.07
Energy (MJ kg ⁻¹)	20.34	20.55	20.28	20.63	21.02
Fat (%)	4.65	4.61	4.73	4.61	4.52
Crude fibre (%)	8.07	8.02	7.88	8.21	7.91

*Multi-enzyme complex Natuzyyme[®] (Bioprotin, Australia), contains protease, lipase, α amylase, cellulase, amyloglucosidase, β -glucanase, pentosonase, hemicellulase, xylanase, pectinase, acid phosphatase and acid phytase (Table 7.1)

7.3.3 Experimental system

This experiment was conducted in the recirculating system described in Chapter 3, Section 3.4.4.

7.3.4 Water quality management

Dissolved oxygen, water temperature and ammonia were monitored once daily using a hand held YSI (556 MPS) water quality multi parameter instrument. pH was monitored biweekly using a pH meter (CG 840, Schott). Photoperiod was natural.

7.3.5 Experimental design, diet allocation and feeding

A completely randomised design was used in this experiment. At the beginning of the experiment, fish were individually weighed and stocked at 15 fish per tank. Each diet was randomly allocated to triplicate tanks. All fish were hand-fed their allocated diet three times daily at 0900, 1300 and 1700 hours to apparent satiation. The feeding experiment lasted for 60 days.

7.3.6 Proximate composition of diets and faecal analysis

The experimental diets were analysed for dry matter, crude protein, gross energy, crude lipid, crude fibre and chromic oxide (Furukawa and Tsukahara, 1966). Crude protein and chromic oxide levels in the faeces were also determined. All analyses were done according to AOAC International (2012) guidelines as described in Chapter 4, Section 3.4.6.

7.3.6 Growth performance parameters

All fish were weighed at the beginning of the experiment and, thereafter, fortnightly. At the end of the experimental period (60 days), SGR, TGC, FCR, PER and ADC for protein were determined as described in Chapter 4, Section 3.4.7.

7.3.7 Digestive enzyme analysis

On termination of the feeding experiments, five fish in each dietary replicate were sacrificed for the analysis of digestive enzymes. The whole intestines was collected, pooled per dietary treatment and stored in marked plastic sample bags at -86°C until analysed. Protease, amylase, lipase and cellulase activities were determined as described in Chapter 4, Section 3.4.9.

7.3.8 Gastric evacuation rate

The gastric evacuation rate of the different experimental diets was determined at the end of the feeding period. The method used was the serial slaughter technique (Windell, 1971). This method involves sacrificing fish at regular time intervals after feeding and measuring the amount of feed remaining in the stomach.

Two fish were sampled from each tank at the beginning of the serial slaughter trial to ensure that the stomach was empty. All remaining fish were fed their allocated diet to

apparent satiation for 1 hour. After the 1 hour feeding period, all uneaten feed was removed from all the tanks. Two fish were sampled from each tank at 4-hour intervals for analysis. Fish were killed by placing on ice and then dissected to remove the gut.

The stomach content (all material between the posterior end of the oesophagus and the anterior end of the small intestine) was removed and placed in pre-weighed oven-proof containers, the stomach content in wet mass was recorded, stomach content was dried to constant weight at 90°C for 24 hours and dry mass measured.

The gastric evacuation rate for the different plant diets was expressed by the exponential equation (De Silva and Anderson, 1995):

$$S_t = S_o \cdot e^{-bt}$$

where: S_t = weight of stomach content

S_o = weight of meal eaten

t = time in hours

b = constant

7.3.9 Statistical analysis

Normality and homogeneity of variance was confirmed using the Shapiro-Wilk normality test and Levene test, respectively (SAS, 2008). Data on feed intake, growth performance parameters (TCG, SGR), feed utilisation parameters (FCR, PER) and apparent digestibility of protein and activities of digestive enzymes was subjected to one-way analysis of variance. Natuzyme50[®] supplementation level for optimal weight gain was estimated by quadratic regression analysis ($y = ax^2+bx+c$). Analysis of covariance (ANCOVA) was used to determine if there were any significant ($P<0.05$) differences in the gastric evacuation rate of *Oreochromis mossambicus* fed the experimental diets. Significance was accepted at probabilities < 0.05 . All statistical analysis was carried out on SAS (SAS, 2008).

7.4 RESULTS

7.4.1 Effect of Natuzyme50[®] supplementation on growth performance and protein digestibility

7.4.1.1 Weight gain

Oreochromis mossambicus fed the diet containing 0.50 g/kg enzyme cocktail (Natuzyme50[®]) had the highest ($p < 0.05$) body weight from the second week of feeding (Figure 7.1). Fish fed the control diet (0 g/kg Natuzyme50[®]) had the lowest ($P < 0.05$) body weight. Adding Natuzyme50 resulted in higher ($P < 0.05$) body weights. However, adding more than 0.50 g Natuzyme50[®]/kg DM diet did not improve weight gain (Figure 7.1). Quadratic regression analysis showed that Natuzyme50[®] supplementation level for optimal weight gain was 0.62 g/kg MD diet (Figure 7.2).

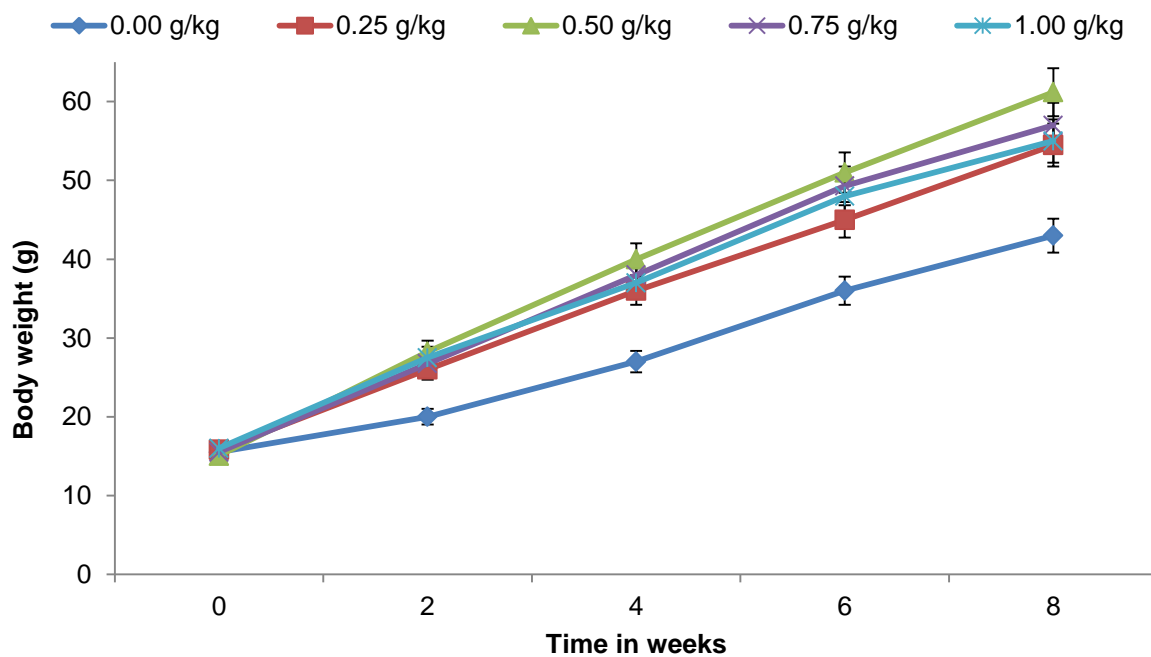


Figure 7.1: Change in body weight in *Oreochromis mossambicus* fed kikuyu-based diets supplemented with increasing levels of Natuzyme50[®]

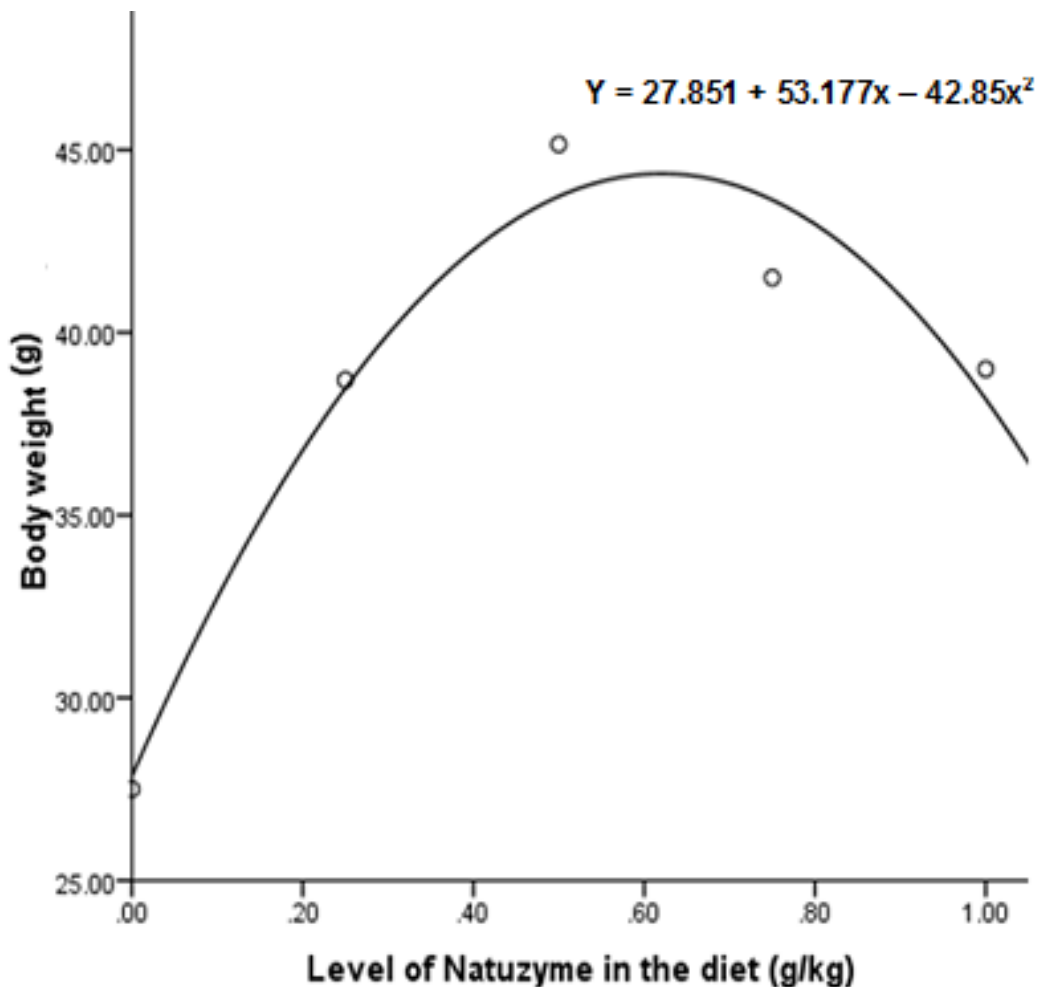


Figure 7.2: The effect of Natuzyme50[®] supplementation on body weight gained in *Oreochromis mossambicus*

7.4.1.2 Feed intake

Natuzyme50[®] supplementation increased ($P < 0.05$) feed intake by *Oreochromis mossambicus*. Fish on diets NT50, NT 75 and NT 100 had higher ($P < 0.05$) diet intakes than those on diets NT 25 and NT 0. Similarly, fish on diet NT 25 ate more ($P < 0.05$) than those fed diet NT 0. However, feed intake of fish fed on diets NT 50, NT 75 and NT 100 was not significantly different ($P > 0.05$; Table 7.3).

7.4.1.3 Specific growth rate

Oreochromis mossambicus on a diet supplemented with 0.5 g Natuzyme50[®] per kg DM had higher ($P < 0.05$) SGR than those on diet NT0, NT75 and NT 100. Similarly, fish on Diets NT 25, NT 75 and NT 100 had higher ($P < 0.05$) SGR than those on Diet

NT 0. However, the fish on NT 25, NT 75 and NT 100 had similar ($P>0.05$) SGR values (Table 7.3).

7.4.1.4 Feed conversion ratio

The best FCR was recorded in fish fed diets supplemented with 0.5 g Natuzyme50[®] per kg DM diet (Table 7.3). Feed conversion ratio improved significantly ($P<0.05$) with increasing Natuzyme50[®] levels up to 0.5 g/kg in the diet. When the level of Natuzyme50[®] increased above 0.5 g/kg in the diet, FCR declined significantly ($P<0.05$).

7.4.1.5 Protein efficiency ratio

The highest PER was also recorded in fish fed 0.5 g Natuzyme50[®] per kg DM diet (Table 7.3). Fish fed Natuzyme50[®] in the diet had significantly higher ($P<0.05$) PER than those fed the control diet. Increasing the level of Natuzyme50[®] in the diet above 0.5 g Natuzyme50[®] per kg DM diet led to lower ($P<0.05$) PER in *Oreochromis mossambicus* feeding on KLM-based diets.

7.4.1.6 Apparent digestibility

Fish fed the control diet with no enzyme supplementation had the lowest protein apparent digestibility values (Table 7.3). The addition of Natuzyme50[®] in the diet led to higher ($P<0.05$) ADC for protein in *Oreochromis mossambicus*. Fish fed 0.5 g Natuzyme50[®] per kg DM diet had the best ADC for protein. Increasing Natuzyme50[®] levels above 0.5 g Natuzyme50[®] per kg DM diet led to lower ($P<0.05$) ADC for protein in *Oreochromis mossambicus* (Table 7.3).

Table 7.3: Effect of Natuzyme50[®] supplementation level on growth parameters of *Oreochromis mossambicus*

	Control	NT 25	NT 50	NT 75	NT 100
Initial weight (g)	15.50±1.2	15.80±2.0	15.05±1.5	15.50±1.4	16.00±1.0
Final weight (g)	43.00±2.2	54.50±1.5	60.20±2.1	52.80±2.6	50.50±1.8
Feed intake (g/day)	0.70±0.1 ^a	0.85±0.1 ^b	0.92±0.1 ^c	0.94±0.1 ^c	0.95±0.1 ^c
SGR (g/day)	1.70±0.2 ^a	2.05±0.6 ^b	2.52±0.4 ^c	2.04±0.2 ^b	1.92±0.3 ^b
FCR	1.53±0.7 ^c	1.32±0.5 ^b	1.22±0.1 ^a	1.36±1.0 ^b	1.46±0.8 ^c
PER	2.18±0.5 ^a	2.53±0.4 ^b	2.73±0.5 ^c	2.45±0.3 ^b	2.28±0.7 ^b
ADC (%)	82.50±3.5 ^a	88.00±2.6 ^b	92.50±2.4 ^c	86.50±1.8 ^d	85.50±1.3 ^d

a,b,c,d: Treatment means with the same small letter within a row were not significantly different ($P < 0.05$)

7.4.2 Effect of Natuzyme50[®] supplementation on digestive enzyme activities in *Oreochromis mossambicus*

7.4.2.1 Protease activity

Natuzyme50[®] supplementation increased protease activity (Figure 7.3). Fish on NT 50 had highest protease activities. Increasing Natuzyme50[®] supplementation from 0.00 to 0.25 g/kg DM increased ($P < 0.05$) protease activity. However, when Natuzyme50[®] supplementation increased above 0.5 g/kg DM, protease activity declined. Fish on diets supplemented with 0.75 or 1.00 g Natuzyme50[®]/kg had similar ($P > 0.05$) protease activity (Figure 7.4).

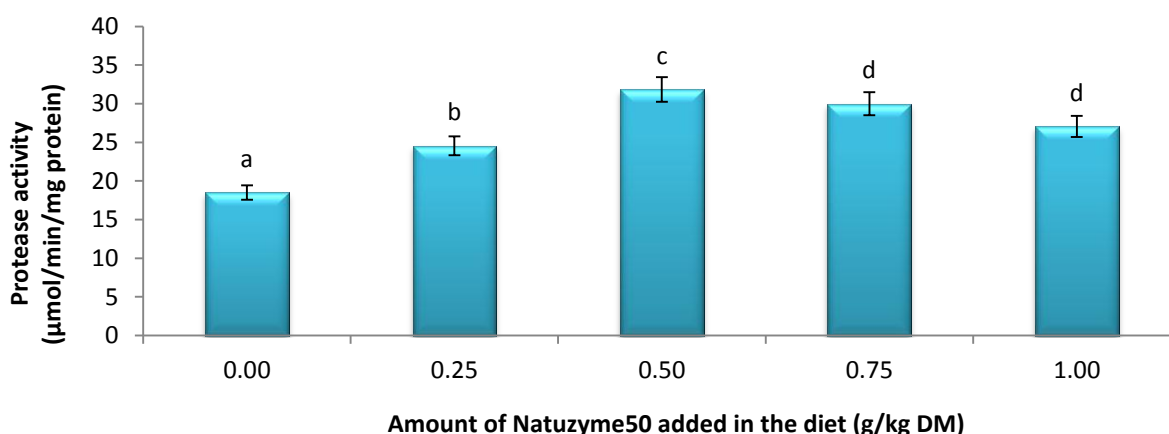


Figure 7.3: The effect of Natuzyme50[®] supplementation on protease activity in *Oreochromis mossambicus* intestines. Bars with different letters are significantly different ($P < 0.05$)

7.4.2.2 Amylase activity

Natuzyme50[®] supplementation increased amylase activity (Figure 7.4). Fish on NT 75 and NT 100 had more amylase activity than those on NT 50, NT 25 or NT 0. Similarly, fish on 0.50 g of Natuzyme50[®]/kg DM feed had more ($P<0.05$) amylase activity than those on 0.25 or 0.00 g of Natuzyme50[®]/kg DM feed. Increasing Natuzyme50[®] supplementation from 0.00 to 0.25 g/kg DM increased ($P<0.05$) amylase activity. However fish on diets supplemented with 0.50, 0.75 or 1.00 g Natuzyme50[®]/kg had similar ($P>0.05$) amylase activity (Figure 7.4).

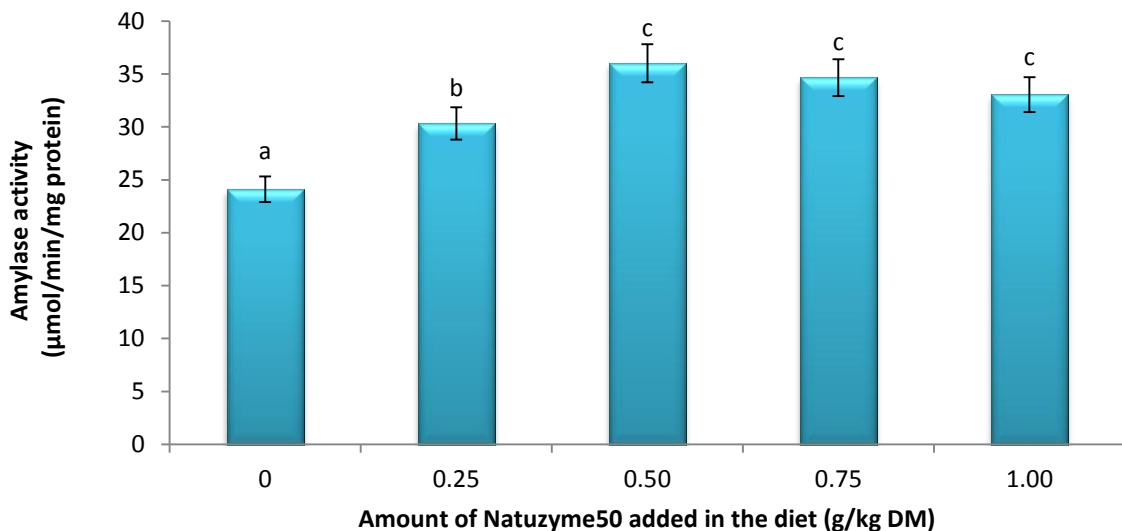


Figure 7.4: The effect of Natuzyme50[®] supplementation on amylase activity in *Oreochromis mossambicus* intestine. Bars with different letters are significantly different ($P<0.05$)

7.4.2.3 Lipase activity

Natuzyme50[®] supplementation increased ($P<0.05$) lipase activities in *Oreochromis mossambicus*. Fish on Diets NT 50, NT 75 and NT 100 had higher ($P<0.05$) lipase activities than those on Diets NT 0 and NT 25. Similarly, fish on Diet NT 25 had higher ($P<0.05$) lipase activity than those on Diet NT 0. However, fish on Diets NT 50, NT 75 and NT 100 had similar lipase activities (Figure 7.5).

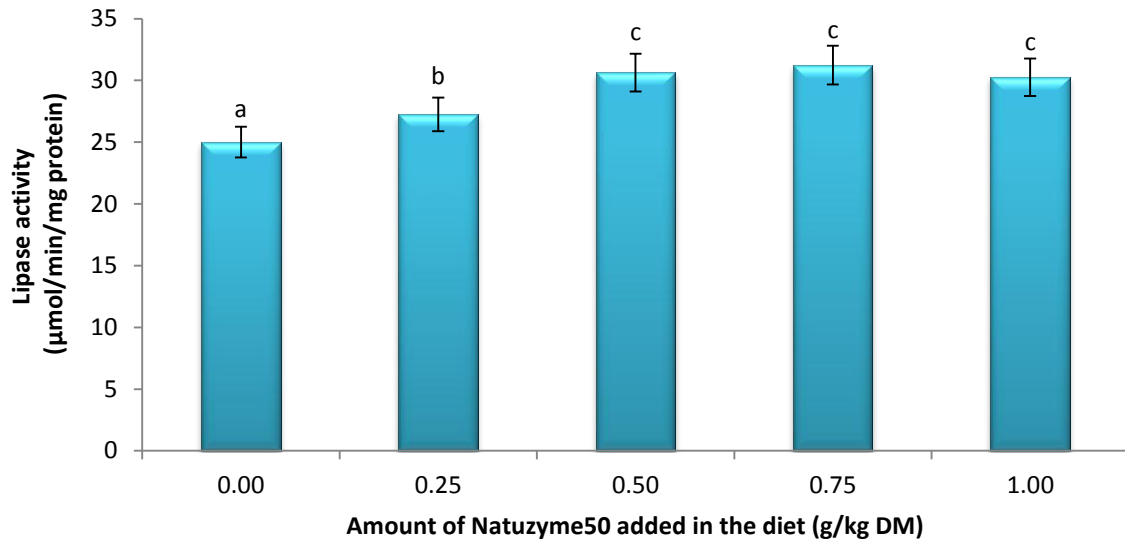


Figure 7.5: The effect of Natuzyme50[®] supplementation on lipase activity in *Oreochromis mossambicus* intestine. Bars with different letters are significantly different ($P < 0.05$)

7.4.2.4 Cellulase activity

Natuzyme50[®] supplementation also increased ($P < 0.05$) cellulase activity in *Oreochromis mossambicus* intestines. Fish fed on Diet NT 0 had negligible levels of cellulase. Fish fed on NT 25 had higher cellulase activity than those fed NT 0. Similarly, fish on NT 75 and NT 100 had similar cellulase activities (Figure 7.6).

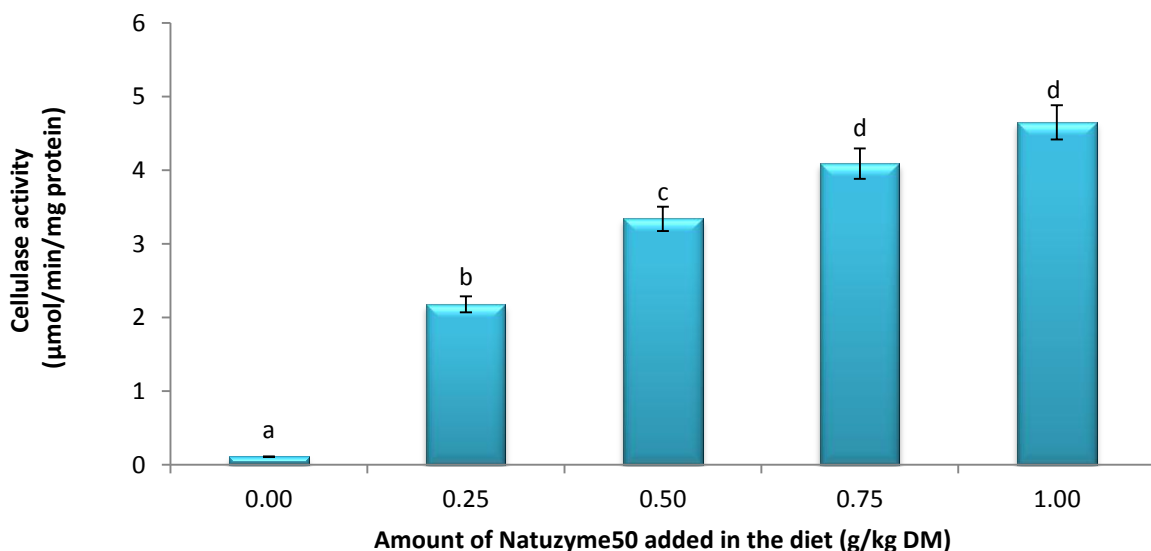


Figure 7.6: The effect of Natuzyme50[®] supplementation on cellulase activity in *Oreochromis mossambicus* intestine. Bars with different letters are significantly different ($P < 0.05$)

7.4.3 Effect of Natuzyme50[®] supplementation on gastric evacuation rates in *Oreochromis mossambicus*

Gastric evacuation rate for *Oreochromis mossambicus* fed 0.00, 0.25, 0.50, 0.75 and 1.00 g Natuzyme50[®]/kg DM diet is represented (Figure 7.4). The gastric evacuation rates were represented by the exponential equations: $S_t = 0.695 * \exp^{-0.069t}$ (Figure 7.8), $S_t = 0.701 * \exp^{-0.079t}$ (Figure 7.9), $S_t = 0.604 * \exp^{-0.086t}$ (Figure 7.10), $S_t = 0.619 * \exp^{-0.086t}$ (Figure 7.11) and $S_t = 1.036 * \exp^{-0.149t}$ (Figure 7.12), respectively. Gastric evacuation rates in fish fed 0.75 and 1.00 g/kg were significantly higher ($P < 0.05$) than that of fish fed the control diet (ANCOVA). No significant differences ($P > 0.05$) were observed in the gastric evacuation rate of fish fed the other diets when compared to the control.

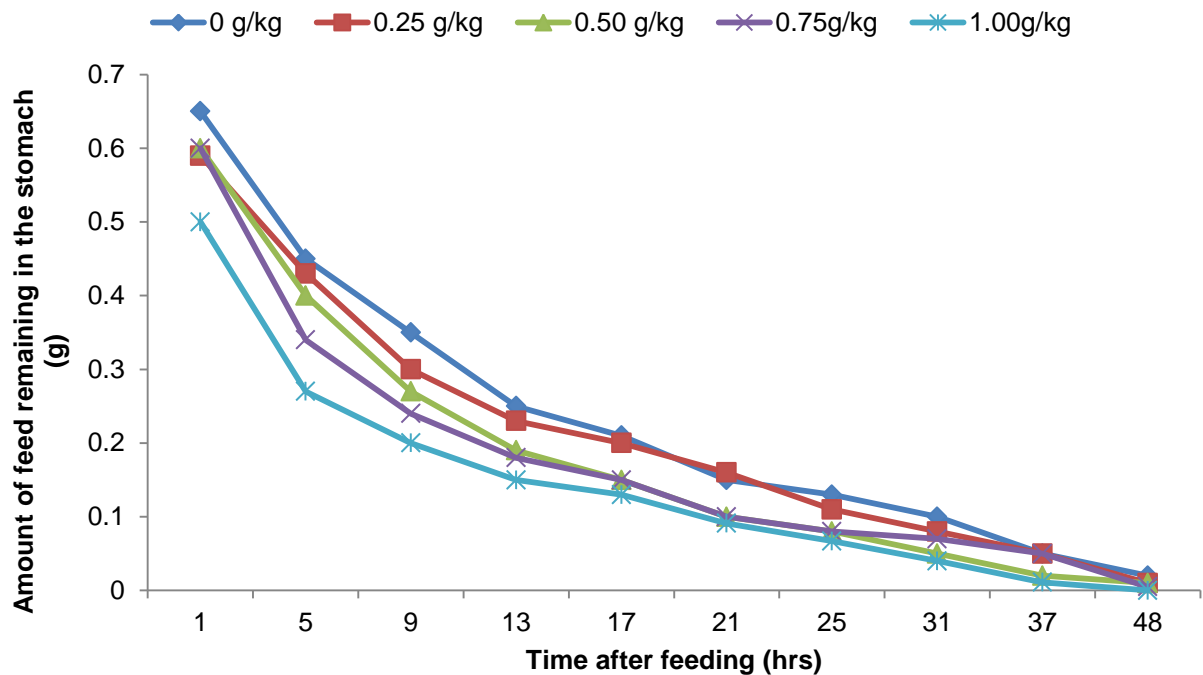


Figure 7.7: Gastric evacuation rate in *Oreochromis mossambicus* fed kikuyu-based diets supplemented with increasing levels of Natuzyme50[®]

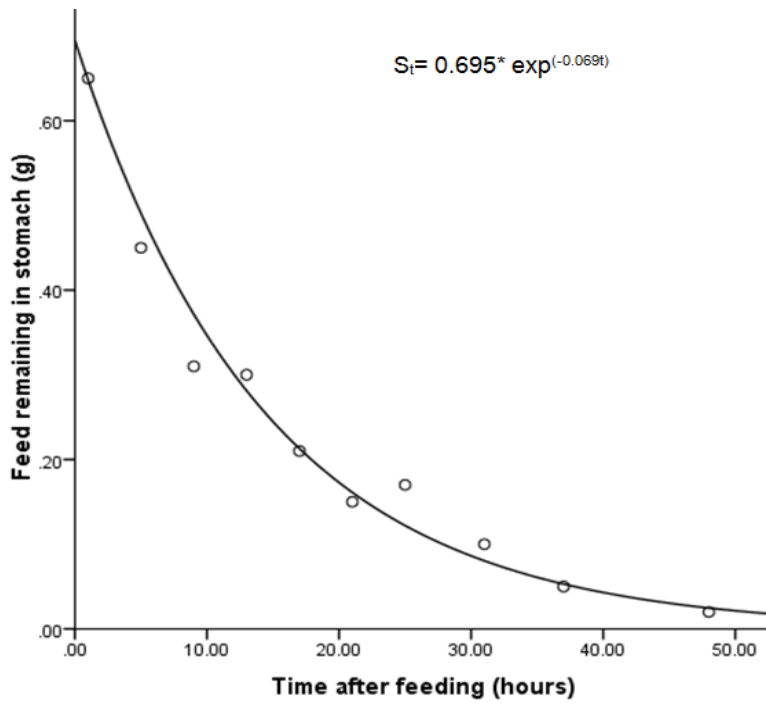


Figure 7.8: Exponential curve representing gastric evacuation rate of *Oreochromis mossambicus* fed a kikuyu-based control

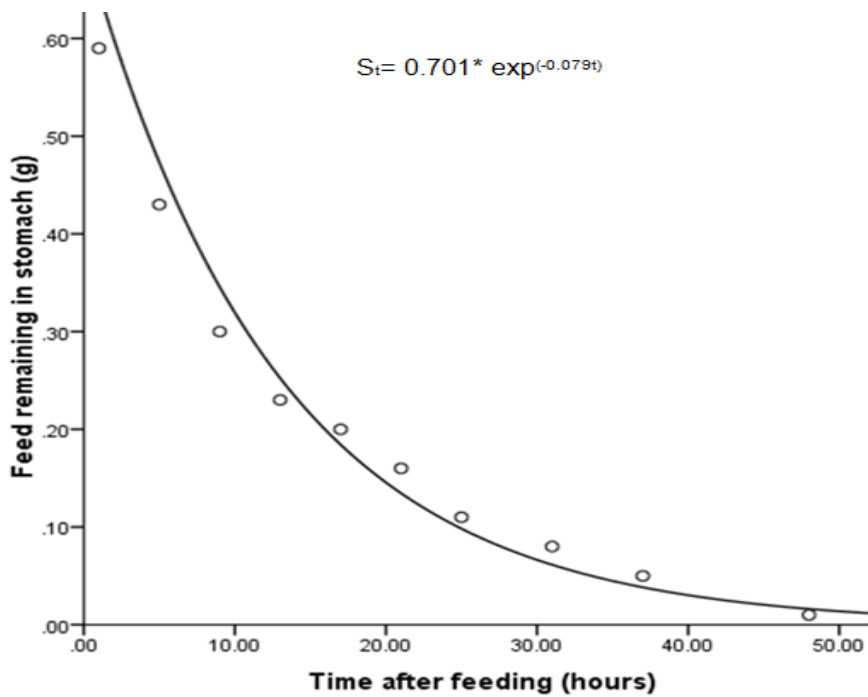


Figure 7.9: Exponential curve representing gastric evacuation rate of *Oreochromis mossambicus* fed a kikuyu-based diet supplemented with 0.25 g Natuzyme50[®]/kg DM feed

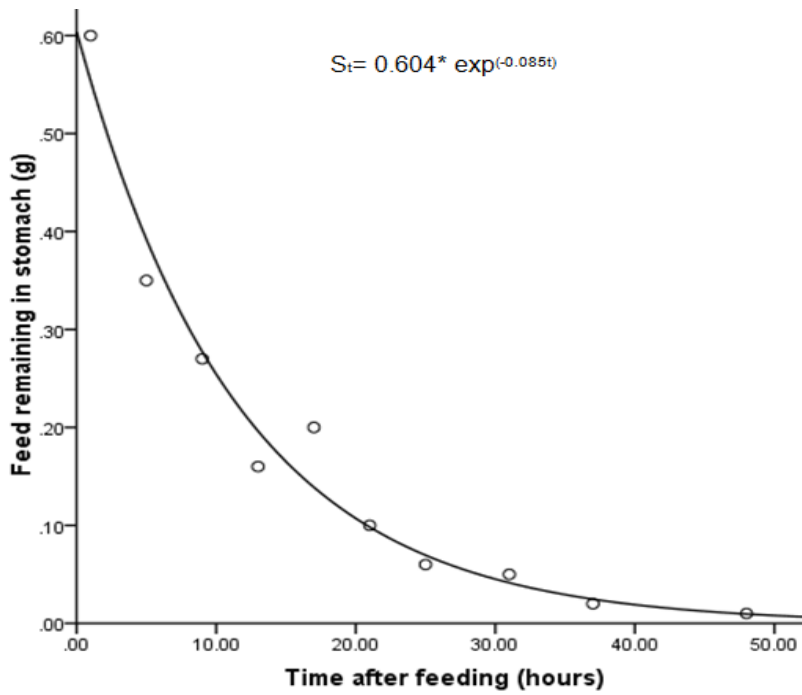


Figure 7.10: Exponential curve representing gastric evacuation rate of *Oreochromis mossambicus* fed a kikuyu-based diet supplemented with 0.50 g Natuzyme50[®]/kg DM feed

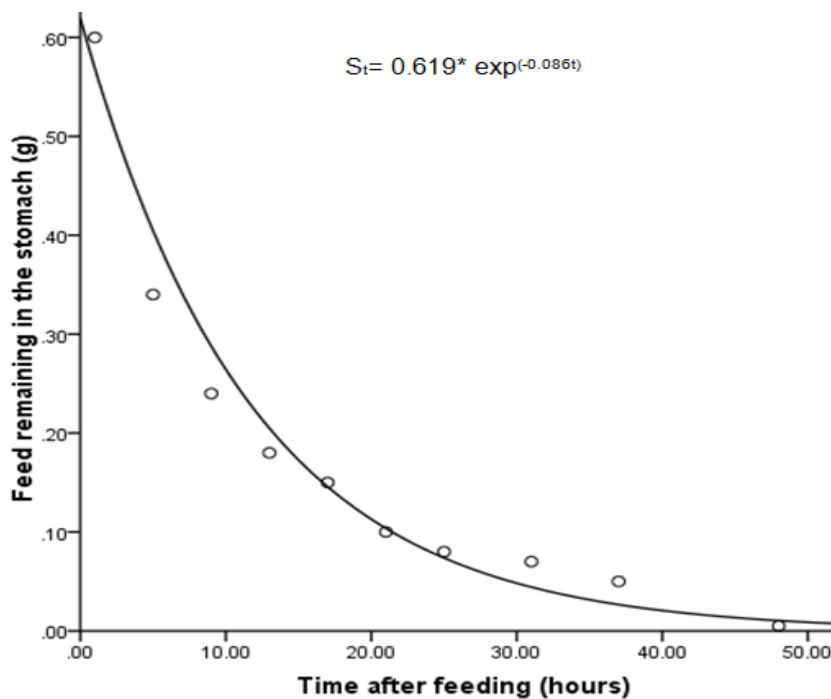


Figure 7.11: Exponential curve representing gastric evacuation rate of *Oreochromis mossambicus* fed a kikuyu-based diet supplemented with 0.75 g Natuzyme50[®]/kg DM feed

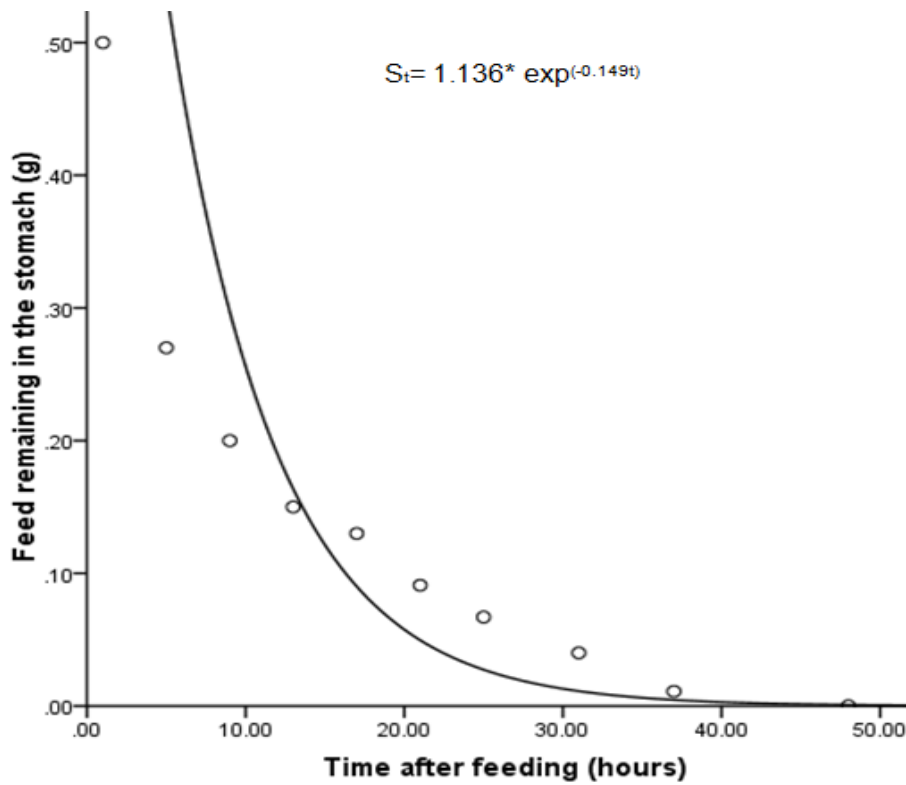


Figure 7.12: Exponential curve representing gastric evacuation rate of *Oreochromis mossambicus* fed a kikuyu-based diet supplemented with 1.00 g Natuzyme50[®]/kg DM feed

7.5 DISCUSSION

Dietary supplementation with increasing levels of Natuzyme50[®] led to increased growth performance in *Oreochromis mossambicus* up to an optimal level. All fish fed diets supplemented with the exogenous enzyme cocktail recorded significantly higher growth, protein efficiency and protein digestibility than fish fed the control diet. The best SGR was recorded in fish fed diets supplemented with 0.5 g Natuzyme50[®]/kg DM feed. This suggests that the addition of Natuzyme50[®] enhanced growth performance.

Several authors reported that exogenous enzymes eliminate the effects of anti-nutritional factors and improve the utilisation of dietary energy and amino acids, resulting in improved growth performance of fish (Farhangi and Carter, 2007; Lin *et al.*, 2007; Soltan, 2009). The improved growth performance observed with Natuzyme50[®] supplementation is an indication that exogenous enzymes in Natuzyme50[®] can alleviate some of the negative effects of anti-nutrients associated with KLM diets.

Kikuyu grass contains high levels of non-starch polysaccharides (NSP) such as cellulose (26.9%), hemicellulose (22.2%) and lignin (5.88%) (López Vásquez, 2010) which reduce its nutritive value. *Oreochromis mossambicus* does not produce endogenous enzymes such as cellulase (Chapter 3) to digest these carbohydrates. The addition of Natuzyme50[®] containing cellulase, xylanase and glucanase is vital in plant-based diets with high fibre and cellulose content. Tahir *et al.* (2008) reported that cellulases are capable of cleaving the resistant galacturonic acid and rhamnose bonds in plant-based protein sources. The cellulase in Natuzyme50[®] may have broken down the fibrous components and released bound nutrients for digestion by the fish. Thus, the improved growth of fish fed Natuzyme50[®] supplemented diets may be attributed in part, to the presence of cellulase, xylanase and glucanase in this cocktail.

Another important anti-nutrient in kikuyu grass is phytate. Phytate binds to dietary nutrients (phosphorous, amino acids, calcium, magnesium iron and zinc) consequently decreasing their bioavailability. *Oreochromis mossambicus* like most fish does not possess phytase to break down phytate and release the bound

nutrients. These essential nutrients, therefore, pass through the fish undigested. Exogenous phytase supplementation not only releases phosphorous from the phytate but also releases amino acids and minerals, ensuring maximum utilisation of nutrients. Phytase hydrolyses the ester bond between carbon and the associated phosphate group, liberating the phosphate for utilisation by the fish (Adeola and Cowieson, 2011). The presence of phytase in Natuzyme50[®] may have contributed to the release of bound nutrients, resulting in improved nutrient utilisation, improved protein digestion and the observed increase in growth performance. These results are in agreement with several authors who have reported that phytase supplementation improves phytate-phosphorous availability, consequently improving growth performance in fish (Cain and Garling, 1995; Yu and Wang, 2000; Rodehutscord and Pfeffer, 1995; Dalsgaard *et al.*, 2012).

Exogenous enzymes such as protease and amylase can also be used to complement endogenous enzymes produced by the fish to enhance the digestibility of plant protein and improve starch digestibility. Lin *et al.* (2007) reported that supplementing a mixture of enzymes containing neutral protease, β -glucanase and xylanase to a plant-based diet promoted the secretion of endogenous protease and amylase in the Nile tilapia. The presence of protease and alpha-amylase in Natuzyme50[®], therefore, may have been instrumental in improving the protein and starch digestibility. Increased protease activity is essential to increased protein digestibility, fast absorption and increased growth (Majed, 2008). Van Weerd *et al.* (1999) and Ng and Chen (2002) reported similar results, indicating that exogenous enzyme supplementation promoted the secretion of endogenous enzymes and enhanced nutrient digestion. Carter *et al.* (1994) also reported a positive effect on growth performance and feed efficiency in Atlantic salmon smolt (*Salmo salar*) when supplementing a combination of proteolytic enzymes and carbohydrases to a diet containing 340 g SBM/kg DM.

The use of multiple enzyme activities (such as Natuzyme50[®]) to target different anti-nutritive compounds in feedstuffs may be important to obtain maximum benefit as opposed to the use of a single enzyme. Farhangi and Carter (2007) supplemented protease and carbohydrases alone or in combination to de-hulled, lupin-based, juvenile rainbow trout diets. No effects on performance were observed when single

enzymes were supplemented, but the combination significantly improved PER and apparent digestibility of dry matter and protein. Similarly, Zamini *et al.* (2014) reported that a combination of two multi-enzyme complexes (Natuzyne[®] and Hemicell[®]) resulted in a higher growth rate than in the control. These authors concluded that using the two multi-enzymes was more effective.

Natuzyne50[®] enhanced the activities of the digestive enzymes (Protease, amylase, lipase and cellulase). Jiang *et al.* (2014) reported that enzyme supplementation improved the cholecystokinin content in the intestine. Cholecystokinin is a hormone that controls the release of digestive enzymes and bile from the pancreas into the intestinal lumen. Cholecystokinin secretion is regulated by dietary protein (Cahu *et al.*, 2004). Therefore, the increased intestinal digestive enzyme activities with Natuzyne50[®] supplementation may be partly because of the increased availability of protein.

There was a decrease in protease, amylase and lipase activity when more than 0.5 g/kg Natuzyne50[®] was added in the diet. However, this decrease was only significant for protease activity. This decrease in activity of these enzymes may be an indication that homeostasis was reached, thus endogenous production was reduced. Cellulase activity, on the other hand, continued to increase with increasing exogenous enzyme supplementation because there is no endogenous cellulase production in *Oreochromis mossambicus*.

The growth rate increased up to an optimal level while food intake continued to increase when Natuzyne level in the diet increased. This suggests that the capacity for growth may be constrained by the digestion and transport of nutrients (Houlihan *et al.*, 1988). This is confirmed by the increased gastric evacuation rates with increasing levels of Natuzyne50[®] in the diet. Adding Natuzyne50[®] up to 0.5 g/kg provided a balance between feed intake and faecal excretion. However, increasing the level of Natuzyne50[®] above 0.5 g/kg led to higher gastric evacuation rates, implying that at this level Natuzyne50[®] may have a mild laxative effect. This is corroborated by the reduced growth performance. Reduced growth performance and decreased enzyme activities have recently been reported in carp fed diets with exogenous enzymes above the optimal level (Jiang *et al.*, 2014). Poor weight gain

was also reported in broiler chickens fed high levels of exogenous enzymes (Mendes *et al.*, 2013). Excess enzyme supplementation has been reported to result in osmotic diarrhoea or poor performance in chickens (Schutte, 1990).

The results of this experiment demonstrate that Natuzyme50[®] improved apparent protein digestibility, enhanced intestinal enzyme activities and increased growth performance in *Oreochromis mossambicus* fed a kikuyu leaf meal-based diet. The Natuzyme50[®] dietary level for optimal growth in *Oreochromis mossambicus* was found to be 0.62 g/kg DM feed.

CHAPTER 8:

GENERAL DISCUSSION, RECOMMENDATIONS AND CONCLUSION

The warm water aquaculture industry in South Africa has failed to reach sustainable levels. One of the main challenges facing this industry is expensive fish feed. The development of commercial aquaculture feeds is based on fishmeal (Nguyen *et al.*, 2009). However, as the price of fishmeal continues to increase, it is imperative to replace fishmeal with less expensive protein sources. Thus, the aim of this thesis was to evaluate the efficacy of kikuyu and moringa leaf meals as fishmeal replacers in the diets of three warm water fish species in South Africa. Both kikuyu and moringa leaf meals have relatively high protein content and well-balanced amino acid profiles, which are critical in the selection of plant ingredients to be used as fishmeal replacers (Tacon *et al.*, 2011).

The control diet used in this study contained 10.62% fishmeal. Kikuyu leaf meal and MLM replaced 25, 50, 75 and 100% fishmeal in the experimental diets. Graded levels of both KLM and MLM were tested in the diets of *Tilapia rendalli* (Chapter 4), *Oreochromis mossambicus* (Chapter 5) and *Clarias gariepinus* (Chapter 6). The results of these experiments indicate that in all these fish species, the growth performance of fish fed the lowest inclusion level of KLM (25%) was comparable to that of fish fed the control diet. It is significant to note that only 10% fishmeal was used in the control diet yet the results obtained for kikuyu at the lowest inclusion level were comparable to growth rates obtained using higher levels of fishmeal in soybean-based diets. This diet (KLM 25) had high protein digestibility and minimal adverse effects on the histology of the digestive organs and health status of the fish. On the contrary, in fish fed even the lowest level (25%) of MLM, growth rate and protein digestibility decreased significantly and showed extensive histological alterations of the digestive organs. It is evident that kikuyu is a promising plant-based protein that can replace fishmeal in the diet of herbivorous fish like *Tilapia rendalli* and *Oreochromis mossambicus*. In this study, kikuyu grass used was collected from cuttings of the university lawn. Therefore, had no commercial value and its inclusion in fish diets will drastically reduce production costs. However, the major limitation to its inclusion in fishmeal diets is the bioaccumulation of heavy metals. Steps will have to be undertaken to ensure that it is grown in places where there are low levels of heavy metals. Furthermore, feasibility studies on the possibility of producing enough quantities of kikuyu grass for the fish feed industry must be undertaken.

To formulate practical diets for fish, it is important to determine the nutrient composition of the ingredients used in the diet and their biological availability to fish. In the current study, however, the digestibility values reported are for the complete diet, which contained the test ingredients (KLM and MLM) as well as other ingredients. Therefore, in future studies on the development of KLM-based pellets; investigations should be conducted to determine the digestibility of protein; amino acids and energy in the different ingredients prior to feed formulation for practical diets.

The results also indicate that at higher inclusion levels of KLM in the diet, the growth performance decreased and this was attributed to the presence of anti-nutritional factors in the leaf meal. Several studies also confirm that the use of plant ingredients containing tannins and saponins in fish feeds negatively affects texture and taste leading to reduced palatability and consequently poor feed intake (De Silva and Gunasekera, 1989; El-Sayed, 1999; Francis *et al.*, 2001). These anti-nutritional factors act as deterrents to keep away herbivores (Makkar and Becker, 1999). The presence of deterrents in both KLM and MLM was confirmed by low feed intake in all three fish species fed diets with increasing levels of these leaf meals. It is, however, not clear at what level (concentration) polyphenols, tannins, saponins and phytate affect palatability and feed intake. With the exception of phytate very few studies with diets containing increasing levels of purified anti-nutrients have been conducted. Spinelli *et al.* (1983) reported that 0.5% phytic acid reduced growth and feed efficiency in rainbow trout *Oncorhynchus mykiss*. In common carp (*Cyprinus carpio*) adding up to 0.5% phytic acid also led to reduced growth, lower feed and protein efficiency, lower protein digestibility as well as reduced calcium and zinc bioavailability, hypertrophy and vacuolization of intestinal epithelium (Hossain and Jauncey, 1993). However, no work has been done on the effect of purified anti-nutrients in *Tilapia rendalli*, *Oreochromis mossambicus* or *Clarias gariepinus*. Therefore, feeding experiments using purified individual anti-nutrients are needed to determine the threshold limits that will not adversely affect productivity. Furthermore, financial feasibility of these studies is also important.

In our study, reduced feed intake may be the overriding factor responsible for poor growth in fish fed KLM-based diets. Histological analysis of the liver in all species

showed that the fish were in poor condition at high plant inclusion levels. It is, therefore, prudent to look at how palatability and subsequently intake of the kikuyu pellets may be improved. This could be achieved by the addition of attractants in the diet. According to Kasumyan and Doving (2003), attractants can be grouped into 3 categories: incitants, stimulants and enhancers based on their effects on the oral and extra-oral taste systems. Incitants act on the extra-oral taste system while stimulants and enhancers act on the oral taste system. Incitants induce food capture by evoking actions like suction, grasping, snapping, biting, tearing or pinching. Stimulants act by initiating or continuing feeding activity only when fish come in direct contact with the feed and they are characterised by high ingestion rate. Enhancers act by accentuating flavour of food causing an increase in their consumption. In the development of KLM-based pellets, it is important to determine suitable attractants that can be used to enhance feed utilisation. However, the cost benefit analysis of putting these enhancers must be evaluated.

Although KLM is a promising low cost replacement for fishmeal in fish diets, the presence of anti-nutritional factors limits its utilisation. It was, therefore, important to develop strategies to reduce the effect of these anti-nutrients in fish fed KLM diets. Consequently, an exogenous enzyme cocktail (Natuzyyme50[®]) was used to counteract the effects of anti-nutrients and enhance enzyme production in *Oreochromis mossambicus* (Chapter 7). The results indicate that the addition of Natuzyyme50[®] improved protein digestibility, feed utilisation and the growth performance in *Oreochromis mossambicus*. It is important to note that Natuzyyme50[®] was added in the diet that had the lowest KLM level. Increasing the level of KLM to replace more than 25% of fishmeal in the diet may require additional measures to neutralise the anti-nutrients in the grass.

This is the first study on the use of Natuzyyme 50[®] in leaf meal-based fish diets. This contrasts with exogenous enzyme use in the diets of swine and poultry dating at least back to the 1960s (Warden and Schaible, 1962). The present study showed that supplementing Natuzyyme 50[®] to a KLM-based *Oreochromis mossambicus* diet significantly improved the apparent digestibility of protein and improved digestive enzyme activities. The usefulness of exogenous enzymes in the diets of fish is an emerging area that warrants further research in field trials. Future research on

exogenous enzyme supplementation in fish feed should focus on effects of enzymes on amino acid availability, specific enzyme modes of action including interactions with endogenous enzymes during digestion and effects on target substrates. Kikuyu leaf meal used in this study was collected from lawn cuttings and, therefore, available at no financial cost, making KLM pellets cheaper than the fishmeal-based control. It is therefore necessary to undertake a cost-benefit analysis when digestive enzymes are added in the diet to maintain a low cost fish feed.

Despite the potential of KLM as a protein source, several questions remain regarding best production and harvesting practices to maximise nutrient composition and reduce anti-nutrient levels. According to Onyeonagu and Ukwueze (2012), the anti-nutritional contents of grass could be manipulated by management. No research was found on the effect of cutting frequency and fertiliser (Nitrogen) application on the anti-nutritional components of kikuyu grass. Therefore, it is recommended that the best combination of nitrogen fertiliser application and harvesting stage be determined before kikuyu-based pellets can be developed for commercial use.

In addition, several pre-treating techniques may be used to destroy anti-nutritional factors in kikuyu grass after harvesting. In this study, kikuyu grass was only dried under a shade before processing. Other processing techniques including wet heating, boiling, extracting with water and extracting the protein concentrate have been widely and successfully used to reduce the concentration of anti-nutrients in plant ingredients. The effect of these treatment methods on anti-nutrient levels in kikuyu grass needs to be explored. However, caution needs to be exercised when selecting the treatment method to minimise any adverse effects on the nutritional quality of the feed material. For example, heat treatment reportedly alters the chemical nature and decreases the nutritional quality of proteins and carbohydrates (Maina *et al.*, 2007).

It is, also, recommended that the pellet water stability of the kikuyu pellet be tested. Pellet water stability is an important parameter in the manufacture of aquaculture feeds. High pellet water stability is defined as the retention of pellet physical integrity with minimal disintegration and nutrient leaching while in the water until consumed by the fish (Obaldo *et al.*, 2002).

This study has demonstrated that kikuyu grass can be used as low cost protein source in the diets of commonly cultured tilapia species (*Tilapia rendalli* and *Oreochromis mossambicus*), in South Africa. However, the kikuyu grass-based diet is not a suitable feed for *Clarias gariepinus*. Fish species like *Tilapia rendalli*, on the other hand, have the right enzyme profile to utilise plant-based diets. The digestibility of the kikuyu-based diet can be enhanced through enzyme supplementation. Supplementing 0.62 g Natuzyme50[®] per kg DM feed resulted in optimal growth of *Oreochromis mossambicus*. Despite the current hype about moringa in South Africa, the utilisation of moringa-based diets was very poor in all species because of the high anti-nutrient levels in this plant.

CHAPTER 9:

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