# EFFECTS OF PHYTOGENIC COMPOUNDS ON GROWTH AND NUTRITIONAL PHYSIOLOGY OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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**By**

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# Declaration

This thesis has been composed entirely by the candidate and has not been submitted for any other degree. Except where specifically acknowledged, the work described in this thesis is the result of the candidate`s own investigations.

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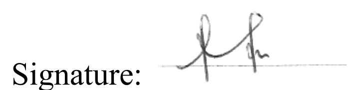
Signature: 

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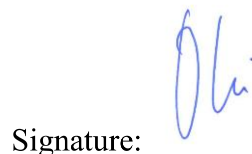
Signature: 

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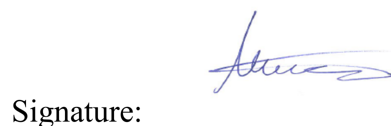
Signature: 

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Lastly, I thank the almighty God for his loving kindness, creating an opportunity for me to undertake this study and granting me the ability to complete it successfully. This is a great milestone in my career development.

# Abstract

With increasing world population, the demand for fish is growing thus there is a need to identify products with potential to increase the efficiency of fish production. Phytogenics are among the products being investigated as potential naturally derived growth promoters. The aim of this study was to identify phytogenic compounds and doses with growth-promoting effects in Nile tilapia and investigate relevant pathways underlying their growth promotion effects. The phytogenic compounds limonene, carvacrol and thymol, major constituents of essential oils from the plants citrus, oregano and thyme, respectively, were evaluated. Six Trials (Trials I, II, III, IV, V and VI) were carried out using diets supplemented with varying concentrations of the phytogenic compounds. In Trials I, II and III (Chapter 3), the effects of either limonene (Trial I), carvacrol (Trial II) or thymol (Trial III) on growth performance of Nile tilapia were investigated (objective 1) and performance parameters including final fish weight, daily growth coefficient, growth rate per metabolic body weight, percentage (%) weight gain, % survival, feed intake, feed conversion ratio and protein efficiency ratio were evaluated. Results from Trials I, II and III indicated that dietary supplementation of 400 and 500 ppm limonene and 750 ppm thymol had growth-promoting effects in Nile tilapia but the somatic growth was not associated with enhanced feed intake and feed utilisation efficiency. Trials IV and V (Chapter 4) investigated growth and nutritional physiology pathways in Nile tilapia regulated by individual phytogenic compounds (objective 2). This was accomplished by analysing the effects of limonene (Trial IV) and thymol (Trial V) supplemented diets on the expression of key genes participating in selected pathways of somatotropic axis-mediated growth, appetite regulation, nutrient digestion, absorption and transport, lipid metabolism, and antioxidant enzyme defence system. Limonene was supplemented in the diet at 0, 200, 400 and 600 ppm while thymol was supplemented at 0, 250 and 500 ppm. Trials IV and V found that growth-promoting effects of limonene (400 and 600 ppm) in Nile tilapia involved up-

regulation of key genes within pathways including somatotropic axis-mediated growth, nutrient digestion, absorption and transport, lipid metabolism and antioxidant enzyme defence system. Dietary thymol at 250 and 500 ppm did not significantly enhance growth of Nile tilapia nor regulate the nutritional physiology pathways listed above. In Trial VI (Chapter 5), the effects of combined phytogenic compounds (limonene and thymol) on growth and nutritional physiology of Nile tilapia was tested (objective 3) to establish if the compounds had synergistic or additive effects on the growth of the fish as well as complementary effects on the selected nutritional physiology pathways. A candidate gene approach was also used for the selected pathways. Results from Trial VI showed that a diet supplemented with a combination of limonene (400 ppm) and thymol (500 ppm) has neither synergistic nor additive effects on the growth performance of Nile tilapia, with limonene mainly influencing the attained somatic growth. The analysed candidate genes involved in the pathways of nutrient digestion, absorption and transport, lipid metabolism, antioxidant enzymes and somatotropic axis growth also showed no synergistic or additive effects of a dietary combination of limonene and thymol in Nile tilapia. Overall, results from the study suggest approaches for developing functional diets for Nile tilapia using limonene and thymol growth promoters.

# Abbreviations and Acronyms

**AGPs** antibiotic growth promoters
**ALP** alkaline phosphatase
**P-AMY** pancreatic alpha-amylase
**AP** amino peptidase
**ANOVA** analysis of variance
**AOAC** official methods of analysis
**ARDC** aquaculture research and development center
**B** titration volume for blank
**C** carbon
**CAT** catalase
**cDNA** complementary deoxyribonucleic acid
**CF** condition factor
**Ct** cycle threshold
**CTRA** chymotrypsin A-like
**d** day
**DGC** daily growth coefficient
**DNA** deoxyribonucleic acid
**dNTP** deoxy-nucleotide-triphosphate
**EOs** essential oils
**F** conversion factor for nitrogen to protein
**FAO** food and agriculture organisation of the united nation
**FAS** fatty acid synthase
**FCE** feed conversion efficiency
**FCR** feed conversion ratio
**FI** feed intake
**$FI_{MBW}$** feed intake per metabolic body weight
**FW** final fish weight
**GH** growth hormone
**GHRH** growth hormone regulating hormone
**GHR-I** growth hormone receptor-I
**GHR-II** growth hormone receptor-II
**GIT** gastro-intestinal tract
**GLUT2** glucose transporter 2
**GPX** glutathione peroxidase
**GR** glutathione reductase
**GR** growth rate
**$GR_{MBW}$** growth rate per metabolic body weight
**IEC** international electrotechnical commission
**IGF-I** insulin growth factor-I
**IOA** institute of aquaculture
**ISO** international organisation for standardisation
**LD50** lethal dose 50
**LDL** low-density-lipid
**LEP** leptin
**LEPR** leptin receptor variant XI
**LPL** lipoprotein lipase
**LXR** liver X receptor
**M** molarity of acid
**MBW** mean metabolic body weight

**mmt** million metric tonnes
**mRNA** messenger ribonucleic acid
**MUC** mucin-like protein
**NGPs** natural growth promoters
**NPY** pro-neuropeptide Y-like
**PEPT1** oligo-peptide transporter 1
**PLA2** phospholipase A2
**PPAR** peroxisome proliferator-activated receptor
**RNA** ribonucleic acid
**SOD** superoxide dismutase
**SREBF1**sterol regulatory element binding transcription factor 1
**SREBP-1C** sterol regulatory element binding protein 1 c

**Symbols (Units)**

**cm** centimetre
**$d^{-1}$** per day
**g $cm^{-3}$** gram per cubic centimetre
**g** gram
**g $kg^{-1}$** gram per kilogram
**h** hour
**kg** kilogram
**kj $g^{-1}$** kilojoule per gram
**L** litre
**m** metre
**mg $L^{-1}$** milligram per litre
**mg** milligram
**min** minute
**mL $kg^{-1}$** millilitre per kilogram
**mL** millilitre
**mol** molar
**ng** nanogram
**nm** nanometres
**pH** molar concentration of hydrogen ions
**pMol** picomole
**ppm** parts per million
**S** second
**%** percentage
**µl** microlitre
**µg** microgram
**$^{o}$C** degrees centigrade
**ß** beta
**α** alpha
**γ** gamma

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# Chapter 1:

# General Introduction

External appearance of Nile tilapia *Oreochromis niloticus* (Original picture)

"*I know the human being and fish can co-exist peacefully*"

**George W. Bush**

## 1.1 Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758)

Nile tilapia (*Oreochromis niloticus*) is a freshwater fish species that belongs to the family cichlidae (Fishbase, 2016). It is native to Africa and it`s distribution covers the Nile basin (including lake Albert, Edward and Tana), Jebel Marra, Lake Kivu, Lake Tanganyika, Awash River, various Ethiopian lakes, Omo River system, Lake Turkana, Suguta River and Lake Baringo (Trewavas, 1983). In West Africa, its native range covers the basins of the Senegal, Gambia, Volta, Niger, Benue and Chad (Teugels and Audenaerde, 2003). It also naturally occurs in coastal rivers of Israel (Trewavas and Teugels, 1991). It has been introduced to other parts in the world for aquaculture because of its tolerance to a wide range of water quality conditions (pH 3.7-11.0, temperature 8-42 $^{o}$C, and up to 0.1 mg $L^{-1}$ dissolved oxygen and 7.1 mg $L^{-1}$ ammonia-nitrogen), fast growth rate, ability to adapt to diets with a higher percentage of plant ingredients and lower protein and higher carbohydrate levels (Ross, 2000; El-sherif and El-feky, 2008; NRC, 2011; FAO, 2016a; Fishbase, 2016). For aquaculture of Nile tilapia, the ideal water quality conditions for optimum growth are pH 6-9, temperature 22 - 29 $^{o}$C, oxygen $\geq$ 3 mg $L^{-1}$ and ammonia-nitrogen $\leq$ 2 mg $L^{-1}$ (Lim and Webster, 2006; Delong *et al*., 2009). Figure 1.1 shows some of the principal Nile tilapia producing countries. According to FAO Yearbook (2014) and FAO (2014), global aquaculture production of Nile tilapia increased from about 1.66 million metric tonnes (mmt) in the year 2006 to 3.67 mmt in 2014 and it is expected to continue increasing with better farming practices.

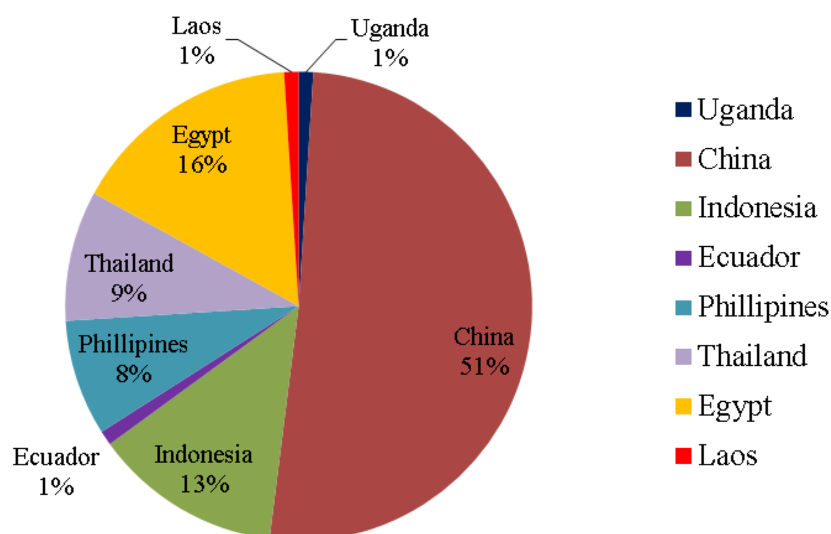



**Figure 1.1:** Major producers of Nile tilapia in the world (From Ng and Romano, 2013 with modifications).

## 1.2 Phytogenics

Phytogenics are a class of natural growth promoters (NGPs) or non-antibiotic growth promoters utilised as feed additives, obtained from herbs, spices or other plants (Hashemi and Davoodi, 2010; Yang *et al*., 2015; Yitbarek, 2015). They are normally considered as suitable alternatives to antibiotic growth promoters (AGPs) in livestock production (Costa *et al*., 2013). For many years phytogenics have been used for medical purposes, as food preservatives, and spices for human food (Wenk, 2003; Steiner, 2009; Williams and Losa, 2001). They derive their names from the plants from which they are obtained and are of different forms. They can be from dried and ground seeds, roots, leaves, peels, twigs, barks, stems, flowers and fruits from herbs and spices or extracts from different plant parts in the form of essential oils (EOs) (Cetingul *et al*., 2009; Gabor *et al*., 2012).

EOs are typically concentrated hydrophobic products and they do not or only partly dissolve in water but are soluble in ether, alcohol and most organic solvents (Steiner, 2009; Turek and Stintzing, 2013; Zeng *et al*., 2015). EOs are aromatic secondary

metabolites of plants, used by plants to provide defence against herbivores, pathogens, and abiotic stresses (Santos *et al*., 2011). The aroma from the EO is produced by specialised secretory cells in the glands located in the aerial part of the plants. Herbs contain between 0.1 and 30.0 g $kg^{-1}$ of EO (Hippenstiel *et al*., 2011).

EOs are active organic compounds categorised as terpenes belonging to different classes such as phenols, aldehydes, ketones, alcohols, esters and ethers (Windisch *et al*., 2008; Steiner, 2009; Santos *et al*., 2011). Terpenes are hydrocarbons produced from isoprene units, however, when they undergo chemical changes leading to formation of additional functional groups (hydroxyl and methyl groups), they are referred to as terpenoids (Hyldgaard *et al*., 2012). The terpenoids are derived biosynthetically from branched-chain five carbon units containing two saturated bonds comparable to units of isoprene (Turek and Stintzing, 2013). Terpenes are named based on the number of isoprene structures and can be classified as monoterpene, sesquiterpene, diterpene, triterpene, tetraterpene and polyterpene (Santos *et al*., 2011). EOs are made up mainly of active volatile bioactive compounds of the plants (Lee *et al*., 2004a; Janczyk *et al*., 2009). Each EO contains various bioactive compounds at different concentrations (Bassole and Juliani, 2012). The oils are mainly extracted through steam distillation (Figure 1.2) (Steiner, 2009, 2010; Applegate *et al*., 2010). The steam distillation process involves passing hot steam through plant material that makes the volatile components of the plant material to evaporate into the steam. The steam then passes through a condenser where it is cooled and enters a compartment that separates the water from the EO. Other methods for extracting EOs include cold pressing, solvent extraction, hydro-distillation and hydro-diffusion (Windisch *et al*., 2008; Steiner, 2009). The extraction methods differ in effectiveness and may affect the quantity, quality and chemical composition of the derived EOs (Yitbarek, 2015; Zeng *et al*., 2015).

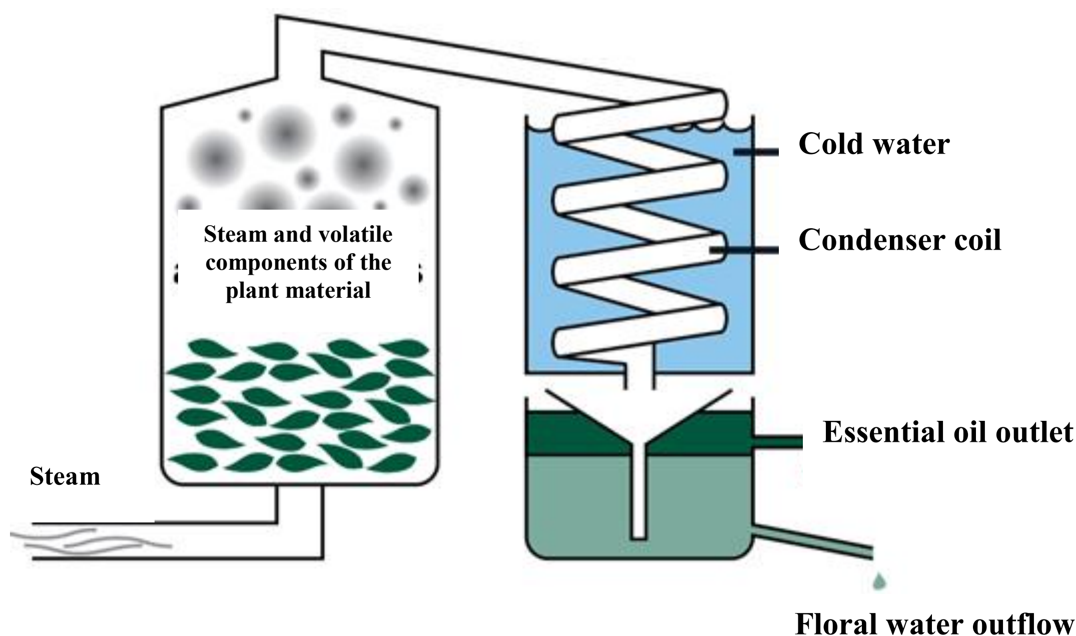



**Figure 1.2:** Basic set up of an essential oil steam distillation unit (modified from www.glorybee.com/process-of-essential-oil-extraction).

### 1.2.1 Major active compounds in some essential oils

An EO can contain up to 20 different bioactive phytogenic compounds with at least two compounds at a higher concentration (20-80 %) than the others (Windisch *et al*., 2008, Hippenstiel *et al*., 2011; Chakraborty *et al*., 2014). The compounds present in a higher proportion play a key role in determining the biological properties of the EO (Steiner, 2009). Major phytogenic compounds of EOs from some plants are provided in Table 1.1. The concentration of active compounds varies depending on the part of the plant from which they were extracted, plant species, physical and chemical soil condition, harvest period, stage of maturity at harvest, technology of drying, duration of storage, processing method and extraction process (Oluremi *et al*., 2007; Yang *et al*., 2009; Lawal *et al*., 2014; Zeng *et al*., 2015).

**Table 1.1:** Plants and their major bioactive compounds in the essential oil.

| Plant | Part used for essential oil extraction | Major active compounds |
|---|---|---|
| ***Aromatic herbs and spices*** | | |
| Nutmeg (*Myristica fragrans*) | Seed | Sabinene |
| Clove (*Syzygium aromaticum*) | Buds / flowers | Eugenol |
| Cinnamon (*Cinnamomum sp*) | Bark | Cinnaldehyde |
| Parsley (*Petroselinum crispum*) | Leaves | Apiol |
| Coriander (*Coriandrum sativum*) | Leaves and seeds | Linalol |
| Rosemary (*Rosmarinus officinalis*) | Leaves | Rosmarinic acid, Borneol, Linalool, Borynl acetate, Cineole |
| Garlic (*Allium sativum*) | Bulb | Allicin |
| Sage (*Salvia officinalis*) | Leaf | Cineole |
| Citrus (*Citrus sp)* | Citrus peels | Limonene |
| Oregano *(Origanum vulgare)* | Shoot | Carvacrol, Thymol |
| Thyme (*Thymus vulgaris*) | Leaves, flowers | Thymol |
| Eucalyptus (*Eucalyptus globulus*) | Leaves | Citronella, Oxides (1-8-cineol) |
| Maize (*Zea mays*) | Leaf | Ionone |
| Boronia (*Boronia sp*) | Flower | Ionone |
| Lemon grass (*Cymbopogon citratus*) | Grass | Citral, Citronella |
| ***Hot spices*** | | |
| Chilli pepper (*Capsicum sp*) | Fruit | Capsaicin |
| Pepper (*Piper nigrum*) | Fruit | Piperine |
| Horse radish *(Armoracia rusticana)* | Root | Allyl isothiocyanate |
| Ginger (*Zingiber Officinalis*) | Rhizome | Zingerol |

Source: Lee *et al*., 2004a; Castro, 2005; Gauthier, 2005; Mathe, 2009; Frankic *et al*., 2009

#### *1.2.1.1 Thymol, carvacrol and limonene*

In this Thesis three bioactive compounds, namely thymol, carvacrol and limonene have been evaluated because they are commercially available and economically feasible. They are major compounds of EOs derived from thyme, oregano and citrus, respectively (Table 1.1). Thymol and carvacrol are constituents of EOs from plants belonging to the family lamiaceae (Michiels *et al*., 2012). Carvacrol is a clear liquid with a molecular weight of 150.21 and its density is 0.975 g $cm^{-3}$ at 25 °C with a boiling point of 237 - 238 °C (Lee *et al*., 2004a; Suganthi and Manpal, 2013; Alagawany *et al*., 2015). On the other hand, thymol is white and crystalline with a strong aroma (Michiels *et al*., 2012) and has a molecular weight of 150, a density of 0.969 g $cm^{-3}$ and a boiling point of 233 °C (Lee *et al*., 2003b). Carvacrol and thymol are isomers differing mainly in the position of the hydroxyl group in the benzene ring as indicated in Figure 1.3. Lemonene is a major constituent of plants belonging to the genus *Citrus* (Voo *et al*., 2012). It is colourless, smells like oranges (Gamarra *et al*., 2006) and has a molecular weight of 136.23, a density of 0.8403 g $cm^{-3}$ at 25 ºC and a boiling point of 178 ºC (Mackay *et al*., 2006). Figure 1.3 shows the chemical structure of carvacrol, thymol and limonene.

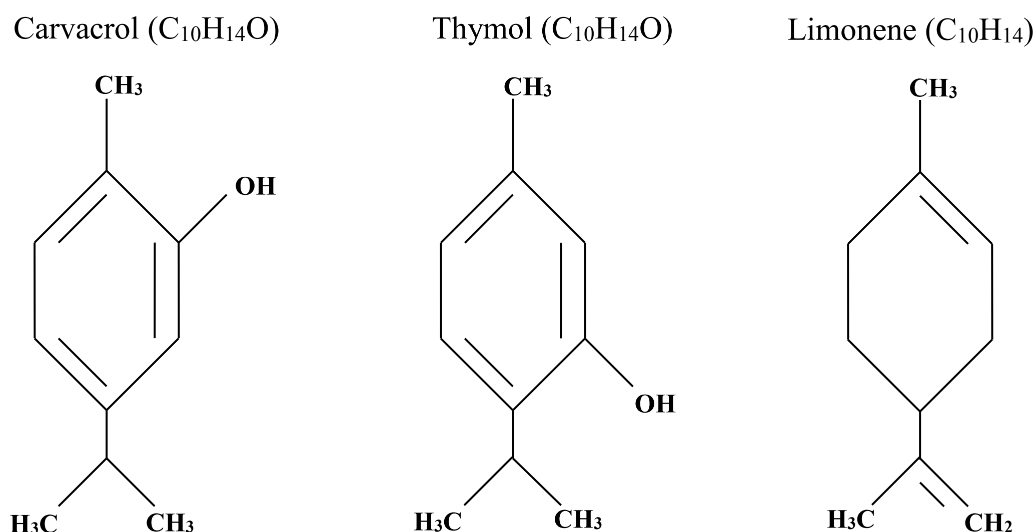



**Figure 1.3:** Chemical structure of carvacrol, thymol and limonene (Lee *et al*., 2003b; Turek and Stintzing, 2013).

EOs containing high concentration of limonene (a monoterpene), carvacrol or thymol (phenols classified as monoterpenoids) are reported to have strong biological effects (Ouwehand *et al*., 2010; Bassole and Juliani, 2012). However, carvacrol, thymol and limonene are volatile when subjected to high temperature or steam treatment, which can affect their effectiveness (Steiner, 2009; Turek and Stintzing, 2013). During feed formulation, a loss of 24 % thymol was reported when a diet for weaned piglets was pelleted at 65 $^{o}$C (European Food Safety Authority, 2012). An 18 % loss of carvacrol following steam treatment of the diet at 48-55 °C was also reported, whereas un-pelleted piglet diets displayed retention levels of 94.7 % carvacrol and 95.8 % thymol (Michiel *et al*., 2008). In addition, EOs need to be stored under limited light to avoid alterations of their composition (Burt, 2004).

Thymol, carvacrol and limonene are generally regarded as safe for consumption and have been used as preservatives and flavour in human food and beverages, ice cream, sweets, cakes, chewing gum, condiments and frozen dairy products (Lee *et al*., 2003b; Suntres *et al*., 2013). A maximum of 125 mg $kg^{-1}$ (ppm) thymol and carvacrol is recommended for use as flavour in food (European Food Safety Authority,

2012), and up to 2300 ppm limonene in chewing gum (Sun, 2007). Following daily administration of thymol and limonene to rats, no adverse effect levels (NOAELs) of administration of 61.55 and 250 ppm body weight $d^{-1}$, respectively, were found (Jidong, 2007; Gad, 2012). Indeed, acute oral toxicity levels in rats (lethal dose 50% - LD50) are reported to be 810, 980, and 4400 ppm body weight $d^{-1}$ for carvacrol, thymol and limonene, respectively, while in rabbits and cats the LD50 for thymol is 750 and 250 ppm body weight $d^{-1}$, respectively (Lee *et al*., 2003b; Suntres *et al*., 2013). This suggests that the LD50 and probably NOAELs for different phytogenic compounds differ from one type of animal to another. For instance, as stated above, the LD50 for thymol in cats is low (250 ppm) because carnivores (cats) are not efficient in eliminating chemical substances from their bodies (Allen, 2003).

Studies with rabbits and rats have shown that following oral uptake of thymol, carvacrol and limonene, the compounds are rapidly absorbed, the majority are metabolised and end products of catabolism are eliminated from the body mainly by the kidneys as glucuronides or exhaled as carbon dioxide limiting their accumulation in the body (Kohlert *et al*., 2000; Brenes and Roura, 2010), and, thus, ensuring safety of the consumer of the animal product. In piglets, Michiels *et al*. (2008) noted that carvacrol and thymol were mainly and almost completely absorbed in the stomach and the proximal small intestine. However, given that the phytogenic compounds are in concentrated forms, there is need to avoid direct contact with the skin and eyes because they could potentially cause irritation (Burt, 2004).

Although NOAELs and LD50 have not been reported for thymol, carvacrol and limonene in fish, studies conducted with diets containing 200-5000 ppm carvacrol (Rattanachaikunsopon and Phumkhachorn, 2010; Volpatti *et al*., 2013; Yılmaz *et al*., 2014; Yilmaz *et al*., 2015), 500 ppm thymol (Zheng *et al*., 2009), 200-3000 ppm carvacrol-thymol combination (Ahmadifar *et al*., 2011; Perez-Sanchez *et al*., 2015) and 1000-5000

ppm of citrus essential oil containing 83 % limonene (Acar *et al*., 2015) have been shown to have no detrimental effects on fish. Rather, some of these studies resulted in improved growth performance, nutrient utilisation efficiency and disease resistance. Therefore, in this Thesis thymol, carvacrol and limonene were supplemented within the concentration ranges mentioned above in order to avoid any potential negative effects of the phytogenic compounds such as histological abnormalities, nephropathy, cytotoxicity, alterations in haematological and biochemical parameters (Sun, 2007; Gad, 2012).

## 1.3 Phytogenics as potential growth promoters for monogastric animals

As the world human population increases from 6.9 billion in 2010 to a projected 9.15 billion in 2050, the demand for meat for human consumption is estimated to increase from about 200 mmt in 2009 to 470 mmt in 2050 (FAO, 2009; Alexandratos and Bruinsma, 2012). Fish production also needs to be increased in order to sustain or increase the world per capita fish supply of 20 kg recorded in 2014 when the population was about 7.2 billion people (FAO, 2016b). As a result, feed additives are being evaluated in the diets of monogastric animals such as pigs, chicken and fish in order to increase their growth and feed efficiency. AGPs and hormones were previously used as the main growth enhancers (Stephany, 2010; Done *et al.*, 2015). However, the use of sub-therapeutic quantities of antibiotics in animal feeds causes the risk of creating antibiotic resistance in bacteria pathogenic to animals and humans (Lee *et al*., 2004a; Melanie and Isabelle, 2008), while hormones might deposit in animal tissue and negatively affect human health (Jeong *et al*., 2010). Consequently, the World Health Organisation banned their use as growth promoters in animal feeds (Windisch *et al*., 2009). Alternative growth-promoting feed additives are therefore being evaluated, amongst which are phytogenics, enzymes, probiotics, prebiotics, acidifiers, minerals, organic acids, anti-microbial peptides and vitamins (Lalles *et al*., 2009; Steiner, 2009; Yang *et al*., 2015). Phytogenics are being investigated as potential

naturally derived growth promoters (Hernandez *et al*., 2004; Peric *et al*., 2009) as they are reported to be safer and healthier than synthetic products like antibiotics and hormones (Brenes and Roura, 2010; Sicuro *et al*., 2010; Gabor *et al*., 2012; Mathivanan and Edwin, 2012; Syahidah *et al*., 2015).

Growth performance has been traditionally used as a production indicator to show how the size of an animal changes over time (Ali *et al*., 2015). Growth can be measured in terms of change in weight, length, condition factor (for fish), survival rate and body composition parameters like energy, protein, fat or dry matter. Other growth performance indicators relate to feed utilisation efficiencies such as feed conversion ratio (FCR), feed conversion efficiency (FCE) and protein efficiency ratio (PER) among others (Buyukcapar *et al*., 2011).

### 1.3.1 Phytogenics on the growth performance of pigs and chicken

The growth-promoting potential of phytogenics has been widely studied in pigs and chicken. Positive effects of different phytogenic products (herbal extracts, EOs and bioactive compounds) and doses on growth rate, specific growth rate (SGR), weight gain, feed efficiency and survival rates have been reported (Jamroz *et al*., 2003; Amik and Bilkei, 2004; Bampidis *et al*., 2005; Cullen *et al.,* 2005; Cabuk *et al*., 2006; Liu *et al*., 2008; Al-kassie, 2009; Mathe, 2009; Cetingul *et al*., 2009; Steiner, 2009; Windisch *et al*., 2009; Tiihonen *et al.,* 2010; Darabighane *et al.,* 2011; Maenner *et al*., 2011; Li *et al*., 2012a; Bento *et al*., 2013; Hashemipour *et al*., 2013; Awaad *et al*., 2014; Bravo *et al*., 2014; Karimi *et al*., 2014; Hafeez *et al*., 2016). Some studies suggested that a phytogenic product can have different levels of efficacy when used in different forms and doses. For instance, Mehala and Moorthy (2008) fed broilers diets supplemented with *Aloe vera* and *Curcuma longa* powder at a concentration of 0.0 %, 0.1 % and 0.2 % for six weeks but no significant differences in feed intake and body weight gain were noted between treatments.

However, Darabighane *et al.* (2001) found a significant improvement in the growth of broilers fed on diets with 1.5 %, 2.0 % and 2.5 % of *A. vera* gel. In some studies, significant increase in the growth of chicken was observed at different ages (Hashemi and Davoodi, 2010; Raeesi *et al.,* 2010; Ari *et al*., 2012) suggesting that some phytogenics and doses are effective for a specific age. Some researchers noted that broilers attained an enhanced growth at the end of the experiment (Juin *et al.*, 2003; Raeesi *et al.,* 2010). This suggests that the animals required a period of adaptation to the phytogenic products before they became effective in enhancing growth. Besides, some phytogenic products are reported to significantly improve the growth of broilers without increasing feed intake and improving feed efficiency (Wallace *et al*., 2010).

On the contrary, some studies found no improvements in somatic growth of pigs and poultry fed on diets supplemented with garlic (*A. sativum*), thyme (*T. vulgaris*) (Amooz and Dastar, 2009), thymol (Hoffman-Pennesi and Wu, 2010) and EO mixture from oregano, cinnamon, and pepper (Hernandez *et al*., 2004) compared to the controls. Interestingly, Sinurat *et al*. (2003) and Vukic-Vranjes *et al*. (2013) found significant improvements in feed efficiency of poultry fed diets containing *A. vera* gel and a commercial product composed of EOs from oregano (*Oreganum sp*), anise (*Pimpinella sp*) and citrus (*Citrus sp*), but without a significant increase in growth compared to control diets. Diets supplemented with garlic and thyme extracts at 0.0 %, 0.3 % and 0.6 % (Amouzmehr *et al*., 2012) and ginger (*Z. officinale*) at 0.0 %, 2.5 %, 5.0 % and 7.5 % concentrations (Mohammed and Yusuf, 2011) also had no improvement in the growth of broilers. Similarly, Samadian *et al*. (2013) observed no significant improvement in feed intake, body weight and feed:gain ratio of chicks fed on diets supplemented with *Mentha piperita*, *T. vulgaris*, *Citrus* and *Carum copticum* EOs at 0, 50, 100 and 150 ppm of diet, respectively. Furthermore, a diet containing a blend of carvacrol and thymol, each supplemented at 250 ppm of feed also had no significant improvement in body weight

gain, feed intake and FCR of chicken (Akyurek and Yel, 2011). In the study conducted by Lee *et al*. (2003b) using diets supplemented with 200 ppm carvacrol, the feed intake and weight gain of female chicken was significantly lowered but FCR improved compared to the control diet. Some studies suggest that different gender may have different physiological responses to phytogenic products and this might cause differences in the growth performance of animals (Zhang *et al*., 2001; Humer *et al*., 2014).

### 1.3.2 Phytogenics on the growth of fish

#### *1.3.2.1 Effects of individual herbs and essential oils on fish growth*

Several studies have been conducted to investigate the impact of phytogenics on the growth performance of fish using individual herbs and EOs. Table 1.2 summarises some of the investigations carried out using Nile tilapia (*O. niloticus*) and other fish species. The results suggest that not all herbal products and doses can enhance the growth of fish and moreover, some results are inconsistent. For instance, Shalaby *et al*. (2006) found improved final weight of Nile tilapia with 10,000 and 20,000 ppm of garlic powder but Mesalhy *et al*. (2008) found no significant increase in final weight with 10,000 and 20,000 ppm garlic. Such apparent discrepancies in results are majorly associated with varying chemical composition of herbs and EOs (Applegate *et al*., 2010; Chakraborty *et al*., 2014). In some cases, it is also linked to different doses used and feeding duration (Yang *et al*., 2015). Costa *et al*. (2013) and Rainer and Lea (2015) argued that combinations of phytogenic products may have stronger growth-promoting effects than individual phytogenic products.

**Table 1.2:** Effects of individual herbs and essential oils on the performance of different fish species.

| Phytogenic product | Fish species | Dose assessed (ppm) | Duration of experiment (days) | Performance in comparison with the control diet | References |
|---|---|---|---|---|---|
| Garlic (*Allium sativum*) powder | Nile tilapia | 10,000, 20,000, 30,000 and 40,000 | 90 | Improved FW, mean WG, SGR, and PER with all diets containing garlic powder | Shalaby *et al*., 2006 |
| | Nile tilapia | 1,000 | 90 | Improved FW, mean WG, FI, FCR, and PER with dietary garlic powder | Soltan and El-Laithy, 2008 |
| | Nile tilapia | 10,000 and 20,000 | Either 30 or 60 | No significant increase in FW after either one or two months feeding on 10,000 or 20,000 ppm dietary garlic | Mesalhy *et al*., 2008 |
| | Sterlet sturgeon (*Acipenser ruthenus*) | 500 and 1,000 | 70 | FW, SGR, WG and FCR improved with dietary garlic | Lee *et al*., 2012 |
| | Hybrid Nile tilapia | 500 and 1,000 | 48 | Decreased WG with 500 ppm of garlic at 14 to 48 days | Ndong and Fall, 2011 |
| Fennel (*Foeniculum vulgare*) powder | Nile tilapia | 1,000 | 90 | Improved FW, mean WG, FI, FCR and PER with dietary fennel powder | Soltan and El-Laithy, 2008 |
| Thyme (*Thymus vulgaris*) powder from dried leaves | Nile tilapia | 2,500, 5,000, 7,500 and 10,000 | 105 | FW, SGR and WG increased with 2,500 and 5,000 ppm dietary thyme powder | El-Gendy *et al*., 2010 |
| Thyme, fenugreek (*Trigonela foenum graecum*) or neem (*Azadirachta indica*) | Nile tilapia | 1,000 | 42 | Highest FW was with the diet containing thyme | Antache *et al*., 2013 |

| | | | | | |
|---|---|---|---|---|---|
| Thyme oil | Nile tilapia | 250 and 500 | 90 | FW, SGR and WG improved with all diets containing thyme oil | Shehata *et al*., 2013 |
| Ginseng (*Panax ginseng*) herb root powder extract | Nile tilapia | 50, 100, 150, 200 and 250 | 119 | FW, WG, SGR and FI enhanced by all diets with ginseng<br><br>FCR improved with 50, 150 and 200 ppm ginseng extract in the diet | Ashraf and Goda, 2008 |
| | Nile tilapia | 500, 1,000, 2,000 and 5,000 | 56 | FW, WG and SGR improved with 1,000, 2,000 and 5,000 ppm ginger root powder | Abdel-Tawwab, 2012 |
| Ground marjoram (*Majorana hortensis*) leaves, caraway (*Carum carvi*) seed meal, chamomile (*Matricaria chamomilla*) flowers meal and fennel seed meal | Nile tilapia | 500 and 1,000 | 84 | FW, SGR and average WG enhanced by all diets with the phytogenic products | Khalafalla, 2009 |
| Fenugreek meal, fenugreek sprouts meal, eucalyptus (*Eucalyptus citriodora*) fresh leaves, hot pepper (*Capsicum frutescens*) meal, thyme seeds meal and chamomile flowers meal | Nile tilapia | 1,000 and 2,000 | 112 | FW, WG, SGR improved with 1,000 ppm dietary fenugreek sprouts meal | Zaki, 2012 |
| Red clove (*Trifolium pratense*) powder | Red tilapia (*Oreochromis aureus*) | 50, 100 and 200 | 90 | Increased GR, PER and better FCR with 100 ppm red clove | Turan, 2006 |

| | | | | | |
|---|---|---|---|---|---|
| Acetone extract powder from bermuda grass (*Cynodon dactylon sp*), beal (*Aegle marmelos*), winter cherry (*Withania somnifera*,) and ginger | Mozambique tilapia (*Oreochromis mossambicus*) | 1,000 | 45 | FW and SGR enhanced by the diets with 1,000 ppm of each of the phytogenic extracts | Immanuel *et al*., 2009 |
| Orange peel (*Citrus sinensis*) essential oil | Mozambique tilapia | 1,000, 3,000 and 5,000 | 98 | SGR, % WG and FCR were improved with 1,000 ppm of citrus essential oil | Acar *et al*., 2015 |
| *Yucca schidigera* extract powder | Stripped catfish (*Plotosus lineatus*) | 75, 100 and 150 | 84 | SGR and FCR improved with 150 ppm of *Yucca schidigera* extract | Guroy *et al*., 2012 |
| *Astragalus radix* or *Lonicera japonica* powder | Pikeperch (*Sander lucioperca*) | 1,000 | 56 | No improvement in growth performance | Zakes, 2008 |
| Encapsulated oregano extract | Red tilapia | 500, 1,000 and 2,000 | 84 | No improvement in growth performance | Kulthanaparame *et al*., 2011 |
| *Scutellaria baicalensis* aqueous extract | Olive flounder (*Paralichthys olivaceus*) | 5,000, 10,000, 20,000, 30,000 and 50,000 | 56 | No improvement in growth performance | Cho *et al*., 2013 |
| Ground lemon balm (*Melissa officinalis*) | Rainbow trout (*Oncorhynchus mykiss*), | 20,000 | | No improvement in somatic growth | Farahi *et al*., 2012 |

FW, final fish weight; WG, weight gain; % WG, percentage weight gain; GR, growth rate; SGR, specific growth rate; FI, feed intake and PER, protein efficiency ratio.

### *1.3.2.2 Effects of combinations of different herbs and essential oils on fish growth*

Some combinations of herbs and EOs are reported to have additive or synergistic effects arising from the interactions between active compounds in the different herbs and EOs (Hyldgaard *et al*., 2012; Costa *et al*., 2013). Additive and synergistic effects can promote fish growth above levels attained when using each herb or EO individually in

the diet (Windisch *et al*., 2008; Chakraborty *et al*., 2013; Zeng *et al*., 2015). An additive effect occurs when the combination of active compounds from the different phytogenic products (herbs and/or EOs) has a collective effect that is equal to the sum of the effect of the individual products (Hyldgaard *et al*., 2012). On the other hand, synergy takes place when a combination of herbs and/or EOs has an effect that is greater than the sum of the effects of the individual phytogenic products (Bassole and Juliani, 2012). In this sense, in a feeding trial conducted for 60 days by Xie *et al*. (2009) evaluating the effect of individual and combined Chinese herbal extracts (*Eucommia ulmoides, Scutellaria baicalensis* and *Lonicera japonica*) on growth performance in juvenile tilapia, the highest growth performance was found with fish fed on diets containing the combined Chinese herbal extracts compared to those fed the individual herbs and the control.

However, most studies evaluating effects of combined herbal products and/or EOs do not make comparisons with their individual herbs/essential oils although improved fish performance is reported compared to the controls. For instance, a combination of essential oils of oregano, anise and citrus peels increased growth of Pangasius catfish (*Pangasianodo hypothalamus*) and red tilapia (*Oreochromis niloticus* × *O. mossambicus*) with the highest growth recorded at 125 ppm for Pangasius catfish and 120 ppm for red tilapia (Pedro, 2009). Gabor *et al*. (2012) found that the combination of either garlic (2 %) and ginger (1 %) or oregano (1 %) and *Echinacea* (0.5 %) resulted in improved body weight gain, SGR and FCR of rainbow trout compared to the control. Despite these positive results, some blends of herbs and EOs have been reported not to enhance somatic fish growth. For example, a diet formulated with 750 ppm of *Echinacea* extract and 600 ppm of ginseng extract did not significantly improve growth of Nile tilapia compared to the control (El-Sayed *et al*., 2014). Similarly, diets containing a mixture of medicinal herb *Astragalus radix* and *Lonicera japonica* each at an inclusion level of 1000 ppm resulted in no improvement in growth, condition factor or feed efficiency of pikeperch

after 56 days feeding (Zakes, 2008). The apparent lack of effect of some combinations of herbal extracts and/or EOs on growth performance has been associated with potentially antagonistic effects of the active compounds in the herbs and EOs (Steiner, 2009; Costa *et al.*, 2013).

In summary, although some studies demonstrated growth-promoting potential of some combinations of herbs and EOs, it might be difficult to obtain consistent results because the concentration of bioactive compounds in the herbal products and EOs varies within and between plant species. This also makes it impossible to attribute the beneficial growth effects to a particular compound or compounds of the phytogenic product. Therefore, there is need to use a consistent quality of phytogenic products. This could be achieved by using purified bioactive compounds of EOs. Recent research on phytogenic products needs to be directed to evaluating the effects of different bioactive compounds to identify compounds and doses that could be used in the diets of different fish species to enhance growth performance.

#### *1.3.2.3 Effects of phytogenic compounds on fish growth*

There is scanty literature on the effects of phytogenic compounds on the growth performance of different fish species. Besides, some of the studies that have been conducted do not mention the level of purity of the phytogenic compounds used in the experiments. Among the studies conducted, channel catfish (*Ictalurus punctatus*) of 50 g attained a significantly higher final weight than the control when fed diets formulated with 500 ppm of carvacrol, and a combination of carvacrol-thymol but not with thymol alone and the control (Zheng *et al.*, 2009). On the other hand, rainbow trout (*Oncorhynchus mykiss*) of 8.4 g attained significantly higher body weight and feed conversion efficiency than the control when fed on a diet containing thymol-carvacrol powder at 3000 ppm for 45 days (Ahmadifar *et al.*, 2011). Similarly, juvenile European sturgeon (*Huso huso*) of

43.6 g had a significant increase in final weight and feed utilisation efficiency with dietary thymol-carvacrol powder at 1000, 2000 and 3000 ppm after 60 days (Ahmadifar *et al*., 2014). On the contrary, Rattanachaikunsopon and Phumkhachorn (2010) investigated the growth of Nile tilapia fed on diets formulated with carvacrol, cymene and their combination at 200 ppm and observed no significant differences in weight gain and survival among treatments. Cymene is a constituent of oregano EO and is a precursor of carvacrol. Freccia *et al*. (2014) also found no improvement in the growth of Nile tilapia broodstock fed for 180 days on diets supplemented with 0, 50, 100, 150, and 200 ppm of a microencapsulated blend of EOs including carvacrol, cinnamaldehyde, 1,8-cineol and pepper oleoresin. In another study, Yilmaz *et al*. (2015) found no improvement in the growth of juvenile rainbow trout fed on diets formulated with 1000, 3000 and 5000 ppm of carvacrol for 60 days compared with the control. Furthermore, rainbow trout fed on a diet with phytogenic additive containing either a high proportion of thymol (6000 ppm thymol) or carvacrol (12000 ppm carvacrol) at 1000 ppm had significantly improved feed utilisation efficiency but not body weight gain (Giannenas *et al*., 2012). In addition, a study evaluating the growth performance of European sea bass (*Dicentrarchus labrax*) fed on diets supplemented with 250 and 500 ppm carvacrol found no significant differences in final weight, weight gain, feed intake and feed utilisation efficiency, although the diet with 500 ppm showed a tendency to improve performance (Volpatti *et al*., 2013).

Overall, the findings detailed above suggest that different phytogenic compounds, dosage, duration of experiment and age of fish do cause different growth responses in fish. Thus, there is need to identify phytogenic compounds and doses that can be used in the diets of different fish species/ages to enhance growth. Besides, there is need to understand the physiological mechanisms underlying the growth-promoting effects of phytogenic compounds in fish. This information will help in the formulation of functional fish diets containing phytogenic compounds. It can also guide policy makers in developing

enabling regulations for the use of phytogenic compounds (limonene, thymol and carvacrol) in the diet of Nile tilapia and potentially other fish species.

## 1.4 Effects of phytogenic compounds on nutritional physiology of fish and other monogastric animals

The effects of phytogenics on the physiology of fish have mainly been studied in relation to immune-stimulation and resistance to diseases (Yin *et al*., 2006; Ardo *et al*., 2008; Schwerin *et al*., 2009; Caruana *et al*., 2012; Perez-Sanchez *et al*., 2015; Ran *et al*., 2016). Recent studies have shown that phytogenic products also have growth-enhancing effects in fish (Zheng *et al*., 2009; Ahmadifar *et al*., 2011; Ndong and Fall, 2011; Guroy *et al*., 2012; Lee *et al*., 2012; Zaki, 2012; Ahmadifar *et al*., 2014; Acar *et al*., 2015). Nutritional physiology plays a role in regulating fish growth but the literature is inconsistent in addressing the mechanisms by which phytogenic compounds exert growth promotion. Although it is suggested that the mode of action by phytogenics involves stimulating growth through the somatotropic axis, increasing appetite, nutrient digestion, digestive secretions, improving nutrient absorption, lipid metabolism and antioxidant enzyme status (Sections 1.4.1 - 1.4.6), some studies do not show a linkage between some of these pathways and an enhanced growth of fish. This could possibly be because different forms of phytogenic compounds have different biological properties hence they might be stimulating different physiological responses making it difficult to have a generalised mode of action for all phytogenic compounds. Therefore, there is need to explore the effect of different phytogenic compounds on the nutritional physiology of different fish species. Given that there is scant information on the effects of phytogenic compounds on the nutritional physiology of fish, most of the literature reviewed in this section is from studies conducted with other monogastric animals, particularly pigs, chicken, rats and humans.

### 1.4.1 Growth stimulation by phytogenics through the somatotropic axis

The somatotropic axis is composed of growth hormone (GH) and insulin growth factor I (IGF-I) that are core in regulating and controlling fish growth (Fox *et al*., 2010; Qiang *et al*., 2012). Interestingly, the nutritional status of fish can directly or indirectly influence the production and secretion of GH and IGF-I. GH is produced by the pituitary gland and takes part in controlling many physiological processes besides somatic growth (Reinecke *et al*., 2005), with the liver being the major site for its endocrine activity in fish (Pierce *et al*., 2012). Figure 1.4 shows a schematic representation of the GH-IGF-I axis.

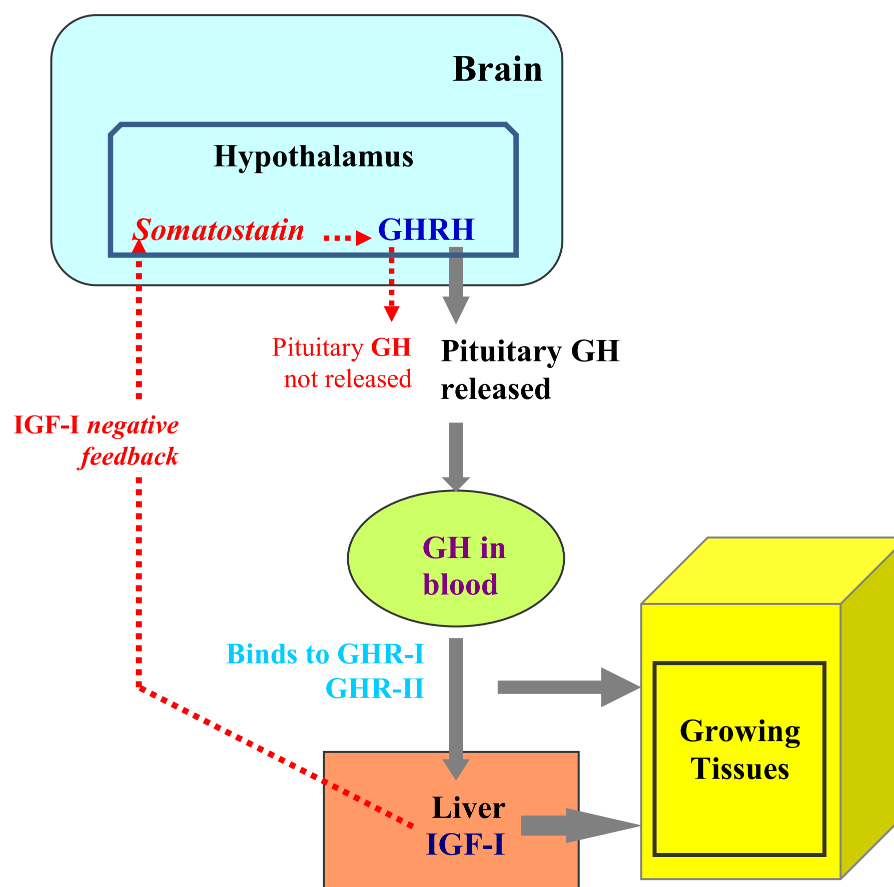



**Figure 1.4:** Schematic representation of growth hormone (GH) - insulin growth factor-I (IGF-I) regulation in fish (Modified from Moriyama *et al*., 2000; Reinecke, 2010).

Just as in other vertebrates, fish GH is synthesised and released from the pituitary by growth hormone releasing hormone (GHRH) into the blood stream where it binds to growth hormone receptors I and II (GHR-I, GHR-II), especially GHR-I, and may act directly to stimulate growth in tissues and/or indirectly by activating the liver to produce and release IGF-I into the blood to stimulate somatic growth (Fox *et al*., 2010; Pierce *et al*., 2012). It is noted that GH mainly influences growth through its actions on IGF-I (Qiang *et al*., 2012). An increase in *igf-I* mRNA expression in fish tissue enhances IGF-I secretion in the liver and promotes tissue growth (Liu *et al*., 2008; Reinecke *et al*., 2005). Given that IGF-I is the final determinant of somatic growth, it has been traditionally used as an indicator of growth (Dyer *et al*., 2004). Under optimal dietary conditions, fish of

the same species, age and husbandry conditions attain heavier weights corresponding with a high mRNA expression of *ghr-I*, *igf-I* and low *gh* (Perez-Sanchez and Le Bail, 1999; Cameron *et al*., 2007; Picha *et al*., 2008; Reindl and Sheridan, 2012). It is known that IGF-I can have a negative feedback on GH in order to maintain an appropriate growth homeostasis by stimulating release of somatostatin, which inhibits GHRH that ultimately stimulates secretion of GH (Picha *et al*., 2008).

Some phytogenics have been reported to influence the levels of GH and IGF-I in monogastric animals (Park *et al*., 2000; Liu *et al*., 2008; Li *et al*., 2012b; Devi *et al*., 2015). In weaned pigs, a diet supplemented with 100 ppm of a phytogenic product containing 18 % thymol and cinnamaldehyde significantly increased plasma levels of IGF-I compared with the control but the growth performance of the pigs was not determined (Li *et al*., 2012b). Miller *et al*. (2013) also found a significant increase in *igf-I* in women administered limonene. With other phytogenic products such as herbs and EOs, Liu *et al*. (2008) found a significant increase in the weight and *igf-I* mRNA levels within the liver, stomach, duodenum and muscle of pigs fed on a diet containing 250 ppm of a blend of extracts from the herbs *Astragalus membranaceus*, *Lycium barbarum*, *Atractylodes macrocephala, Shenqu* sp and *Glycyrrhiza uralensis* compared with the control. This suggested that the herbal extracts influenced IGF-I secretion, which in turn promoted tissue growth. Likewise, Devi *et al*. (2015) fed pigs on diets with herbal extracts from *Phlomis umbrosa*, *Cynanchum wilfordii*, *Z. officinale* and *Platycodi radix* and obtained a higher daily weight gain and IGF-I concentration in the plasma with 500 and 1000 ppm of herbal extracts compared with the control. The same herbs were also reported to effectively promote IGF-I secretion in studies with humans, rats and broiler chicken (Choi *et al*., 2002; Kim *et al*., 2002; Begum *et al*., 2014).

### 1.4.2 Influence of phytogenic compounds on appetite of monogastric animals

The taste, aroma and flavour of a feed are among the key sensory qualities that determine feed acceptance (palatability) and appetite (Kulczykowska *et al.*, 2010; Michiels *et al.*, 2012). In animal nutrition studies, appetite is usually simplified to consideration of feed intake (Volpatti *et al.*, 2013). The effect of adding phytogenics to diets has, however, produced variable results on feed intake and growth of pigs, chicken and fish (Steiner, 2009; Applegate *et al.*, 2010; Franz *et al.*, 2010; Yang *et al.*, 2015; Abd El-Hack *et al.*, 2016). Some EOs with smells are reported to improve palatability of the feed thus enhancing appetite, feed intake and growth (Steiner, 2009; Syahidah *et al.*, 2015; Hippenstiel *et al.*, 2011). However, increased feed intake by the phytogenic-fed animals may not always result into higher weight gains than those attained by the control groups. Some studies report lower or similar feed intake with controls but better FCR and somatic growth with phytogenic-fed animals compared to controls (Hashemipour *et al.*, 2013; Bravo *et al.*, 2014). Chicken fed on diets containing an equal mixture of thymol and carvacrol at 0, 60, 100 and 200 ppm had a significant linear decrease in feed intake, but the highest body weight gain and feed efficiency was observed in broilers offered 200 ppm of the phytogenic product (Hashemipour *et al.*, 2013). Bravo *et al.* (2014) found no difference in feed intake of chicken fed diets with a mixture of carvacrol, cinnamaldehyde and capsicum at 100 ppm compared with the control. However, there was a significantly higher weight gain and lower FCR with the diet containing the phytogenic compounds (Bravo *et al.*, 2014). Conversely, some phytogenic compounds like thymol, carvacrol and eugenol have strong smells and may be hot, restricting their feed intake (Windisch *et al.*, 2008; El-Gendy *et al.*, 2010; Hippenstiel *et al.*, 2011; Michiels *et al.*, 2012; Colombo *et al.*, 2014). This may cause dose-related decrease in feed intake or require some time for the animals to adapt to the diet (Steiner, 2009; Costa *et al.*, 2013). In this sense, it is proposed that fish use a learning process based on experience to associate their sensory properties (taste,

smell and texture) with the type of feed consumed thereby adapting to a diet (Kulczykowska *et al*., 2010).

The brain is the central site for stimulating feed intake using appetite-regulating peptides (Kulczykowska *et al*., 2010; He *et al*., 2013). The process is complex and involves components that either stimulate or inhibit feed intake (Volkoff *et al*., 2005). Neuropeptide Y (NPY) related peptides play key roles in enhancing the appetite of fish (Cerda-Reverter and Larhammar 2000; Lin *et al.*, 2000; Kiris *et al*., 2007). They are located in the pituitary and GIT of fish (Kulczykowska *et al*., 2010; Zhou *et al*., 2013) and up-regulation of mRNA levels of *npy* has been associated with high appetite levels in goldfish (*Carassius auratus*) (Volkoff *et al*., 2006). Kiris *et al*. (2007) also reported stimulated feed intake in Nile tilapia following intraperitoneal administration of NPY. However, enhanced growth in fish fed phytogenics might not always be accompanied by increased expression of *npy* gene. This was recently demonstrated in a study by Zeppenfeld *et al*. (2015) where silver catfish (*Rhamdia quelen*) were fed on a diet supplemented with a herbal extract, lemon verbena (*Aloysia triphylla*), at 0, 0.25, 0.5, 1.0, 2.0 ml $kg^{-1}$ of which limonene was one of the most abundant bioactive compound. Significantly higher final body weight, weight gain and SGR were found with fish fed on 2.0 ml $kg^{-1}$ of the additive in feed but there was no difference in *npy* expression levels among treatments. This might suggest that the fish in all the treatments had the same appetite. On the other hand, leptin (LEP), known as the "satiety hormone", is known to also regulate appetite in fish. It is synthesised and released mainly from the liver of fish into the blood circulatory system where it binds to leptin receptors and enters the hypothalamus to perform its functions (Prokop *et al*., 2012). Leptin receptor variant XI (LEPR) is one of the key mediators of the actions of LEP (Ronnestad *et al*., 2010). Figure 1.5 illustrates how fish appetite is regulated by LEP and NPY.

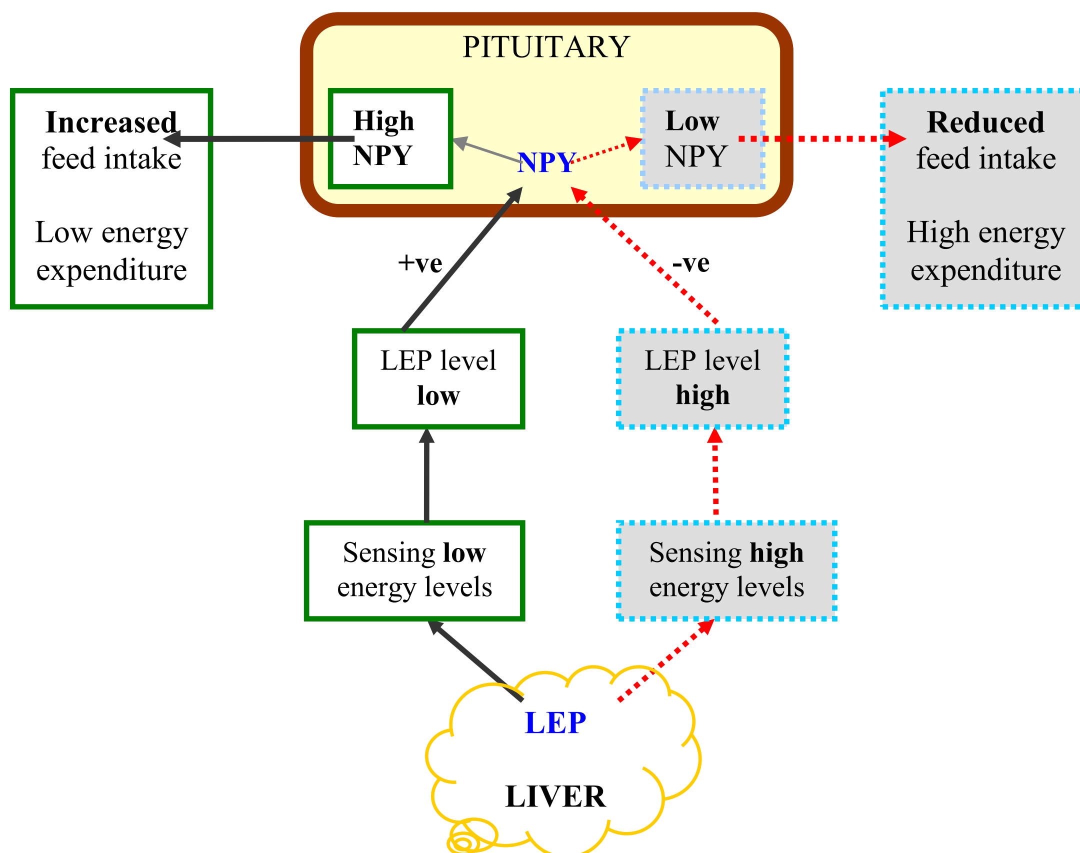



**Figure 1.5:** Diagram illustrating regulation of fish appetite and feed intake by leptin (LEP) and neuropeptide Y (NPY) hormone in the brain (Modified from Volkoff *et al*., 2005; Kulczykowska and Vazquez, 2010).

Leptin is produced mainly in the liver of fish where it is released to the blood and influences NPY in the pituitary to regulate feed intake. When LEP senses low energy levels in the body, plasma concentration of LEP is reduced then LEP signals NPY to increase feed intake leading to high NPY concentration in the brain and reduced energy expenditure until energy levels are restored. Whereas when LEP senses high energy levels in the body, LEP concentration in the blood increases and LEP signals NPY to reduce feed intake resulting into low concentration of NPY in the brain and increased energy expenditure.

Leptin regulates appetite by controlling energy balance through a reduction in appetite and feed intake when energy reserves are high in the body (Murashita *et al*., 2011; He *et al*., 2013). This is achieved by LEP sensing high energy levels and signalling NPY to reduce appetite (Copeland *et al*., 2011). This action leads to high mRNA *lep* and low *npy* levels (Fuentes *et al*., 2012). The regulation of feed intake by LEP has been reported in gold fish, rainbow trout and grass carp (Copeland *et al*., 2011). On the other hand, studies with common carp (*Cyprinus carpio*), zebrafish (*Danio rerio*), coho salmon (*Oncorhynchus*

*kisutch*) and green sunfish (*Lepomis cyanellus*) found either limited or no link between LEP levels and feed intake (Copeland *et al*., 2011; Murashita *et al*., 2011).

### 1.4.3 Digestibility of diets supplemented with phytogenic compounds

The main dietary nutrients include protein, lipid and carbohydrates (Rust, 2003; Krogdahl *et al*., 2005). Efficient digestion of nutrients increases their availability for absorption in the GIT and use to stimulate growth (Rust, 2003; Habte-Tsion *et al*., 2015). Some studies have shown that phytogenic compounds can enhance the digestibility of diets and this could be a potential mechanism for enhancing growth (Li *et al*, 2012; Bravo *et al*., 2014). For instance, the commercial diet Digestarom P. E. P MGE containing carvacrol, thymol, anethol and limonene at either 100, 200, 300, or 400 ppm improved apparent digestibility of crude protein and lipid, as well as growth and feed efficiency of Nile tilapia (Khaled and Megahad, 2014). Digestibility of dry matter and crude protein were also significantly improved in pigs fed on a diet containing thymol and cinnamaldehyde compared to the control (Li *et al*., 2012b). The phytogenic compounds also significantly increased IGF-I levels in the plasma suggesting that improved digestibility of the nutrients could have contributed to improved growth of the pigs since higher IGF-I levels are associated with improved somatic growth. Furthermore, 100 ppm of a dietary mixture of 5 % carvacrol, 3 % cinnamaldehyde and 2 % capsicum improved fat digestibility, weight gain and FCR of broiler chicken compared to the control (Bravo *et al*., 2014). The enhanced digestibility of fat could have potentially increased the availability of lipid for use as an energy source for the broilers thereby sparing protein for enhancing growth. Hafeez *et al*. (2016) reported enhanced digestion of crude protein in broilers corresponding with improved feed efficiency and body weight gain with a dietary mixture of carvacrol, thymol and limonene. In a comparative study by Lee *et al*. (2004b), cinnamaldehyde but not thymol had a strong tendency to improve the digestibility of fat and growth of female

broilers. In addition, a genomic study by Colombo *et al*. (2014) observed that when 50 mg of thymol per kilogram of young pig body weight was introduced into the stomach of the pigs, it significantly up-regulated genes involved in digestion within the pyloric mucosa suggesting that thymol has potential to improve digestion of nutrients at the pyloric mucosa. Conversely, the digestibility of nutrients (crude protein, fibre and amino acids) was not significantly improved in birds fed on diets supplemented with 100 ppm of a combination of capsaicin, cinnamaldehyde and carvacrol compared with the control (Jamroz *et al*., 2005). Although the body weight was not enhanced, the FCR of birds fed on diets with the phytogenic compounds improved. Muhl and Liebert (2007a,b) also found no effect of 500, 1000 and 1500 ppm of a blend of inulin, carvacrol and thymol on the digestion of protein and amino acids in pigs following a 35-day feeding experiment. It was argued that, when diets with highly digestible ingredients are used, phytogenics may not cause any increase in the digestibility of nutrients (Lee *et al*., 2003a; Applegate *et al*., 2010; Fascina *et al*., 2012).

### 1.4.4 Effects of phytogenic compounds on digestive secretions, nutrient absorption and transportation

The activities and quantities of digestive secretions can vary depending on the type of diet eaten by the fish (Panserat and Kaushik, 2010). For instance, phytogenic feed additives have been proposed to increase the quantity and activities of digestive secretions such as saliva, bile, mucus and enzymes (Hashemi and Davoodi, 2010; Syahidah *et al*., 2015). An increased rate of secretion of digestive enzymes suggests more efficient digestion of nutrients in the diet and more nutrients available for absorption to enhance growth (Nya and Austin, 2011). Increased digestion and absorption of nutrients can also improve feed utilisation efficiency and growth (Panserat and Kaushik, 2010). The stomach and intestine are key locations where phytogenics are metabolised along the GIT (Michiels

*et al*., 2008). Among others, these are potentially major organs where phytogenic products moderate digestive secretions.

In Nile tilapia, the digestion of protein starts in the stomach. The gastric glands secrete gastric juice, mucus, hydrochloric acid and pepsinogen. The acid in the stomach facilitates the conversion of pepsinogen to the enzyme pepsin, which then facilitates the breakdown of protein molecules into polypeptides, peptides and free amino acids (Rust, 2003). Through peristaltic movements, the chyme from the stomach is moved into the small intestine. This stimulates the secretion of digestive enzymes by the gall bladder (bile salts), hepatopancreas and mucosal cells of the intestine. The gall bladder, a sac associated with the liver produces and secretes bile salts, which aid in neutralising the pH in the intestine (optimal pH 6 to 8) and thus emulsifying fats (Denbow, 2000; Rust, 2003). In the intestine, digestive enzymes such as pancreatic alpha-amylase that is secreted by the hepatopancreas breakdown starch into monosaccharides (Krogdahl *et al*., 2005; Zambonino-Infante and Cahu, 2007; Nya and Austin, 2011) and proteases like trypsin, chymotrypsin and aminopeptidases complete the breakdown of proteins into di/tri-peptides and amino acids that can be readily absorbed (Natalia *et al*., 2004). The intestine also contains lipases that breakdown emulsified fats into free fatty acids and monoglycerides, and phospholipases that digest phospholipids into lyso-phospholipids (Rust, 2003). Among phospholipases, phospholipase A2 is the main enzyme involved in the digestion of dietary phospholipids (Zambonino-Infantea and Cahu (2007). It is secreted by the hepatopancreas in reaction to the presence of phospholipds in the intestinal lumen.

Phytogenics have been demonstrated to increase the secretion of digestive enzymes (Windisch *et al*., 2008). Curcumin supplemented in the diet at 5,000 and 10,000 ppm increased the activity of α-amylase, protease and lipase in Mozambique tilapia (Midhun *et al*., 2016). Jamroz *et al*. (2005) found enhanced lipase activity in the pancreas and intestinal wall of broilers with a dietary combination of 100 ppm of capsaicin,

carvacrol and cinnamaldehyde. Hashemipour *et al*. (2013) suggested that there is an age at which some phytogenic compounds are more effective in enhancing the digestion of nutrients. In their study, broilers were fed on a combination of thymol and carvacrol at 0 (control), 60, 100 and 200 ppm. A significant increase in intestinal and pancreatic trypsin, lipase, and protease activities was noted in 24-day-old but not in 42-day-old birds. Conversely, up to 1500 ppm of a mixture of carvacrol and thymol in the diet did not improve the activity of trypsin and α-amylase in piglets (Muhl and Liebert, 2007a,b). Lee *et al*. (2003a) also found no difference in digestive enzyme activity in pancreatic tissue, feed intake and weight gain between female broilers fed on diets containing either thymol, cinnamaldehyde, a commercial phytogenic product, CRINA® Poultry and a control.

Phytogenics have been also reported to enhance the secretion of mucus in the GIT (Windisch *et al*., 2008). In monogastric animals, the epithelium of the GIT is covered by a thin layer of mucus comprising bacteria, water and mucin glycoproteins (Schroers *et al*., 2009; Dezfuli *et al*., 2010). The layer of mucus protects the epithelium against acidic chyme, digestive enzymes and pathogens (Tsirtsikos *et al*., 2012). In addition, it lubricates the GIT and aids nutrient translocation (Kamali *et al*., 2014). Mucin glycoproteins (mucins) are the major component of mucus, giving mucus its viscous property (Marel *et al*., 2012). There are mainly two different types of mucins, namely gel forming/secreted and membrane-bound mucins (Aliakbarpour *et al*., 2012; Perez-Sanchez *et al*., 2013). Mucin 2 gene, a mucin protein is the main secretory gel forming mucin and is most abundant in the posterior intestine of fish (Neuhaus *et al*., 2007; Marel *et al*., 2012; Perez-Sanchez *et al*., 2015). Mucus is synthesised and secreted by goblet cells located in the mucosa of the epithelium of the intestine (Neuhaus *et al*., 2007). The type of diet eaten influences the quantity of mucus secreted and expression of *muc* gene. Mucin glycoprotein is made up of the amino acids threonine, serine and proline, and five oligosaccharides (N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and sialic acids) (Perez-

Sanchez *et al*., 2013; Schroers *et al*., 2009). It is known that oligosaccharides are selectively fermented by bacteria in the distal intestine and this stimulates proliferation of *Bifidobacteria* and *Lactobacilli*, which have probiotic effects on microbial pathogens in the intestine (Mahious *et al*., 2006; Neuhaus *et al*., 2007; Schroers *et al*., 2009; Aliakbarpour *et al*., 2012). The probiotic bacteria kill pathogenic bacteria attached to the mucosa of the intestine. This action increases the intestinal surface area for absorption of nutrients and reduces competition for nutrients between the animal and intestinal microflora (Windisch *et al*., 2008; Steiner, 2010). The enzyme phospholipase A2 (PLA2) has been also noted to limit the load of pathogenic bacteria by degrading their lipid cell membranes (Nevalainen *et al*., 2008).

The positive effects of phytogenics towards increasing mucus secretion are demonstrated in studies conducted with pigs, chicken and rats. Jamroz *et al*. (2006) noted increased mucus secretion in the stomach of chicken fed diets containing carvacrol, cinnamaldehyde and capsaicin. Poultry fed on diets supplemented with the herbs turmeric, thyme (containing thymol) and cinnamon had an increased expression of a mucin protein, mucin 2 (*muc2*) in the small intestine, thus demonstrating increased mucus secretion (Kamali *et al*., 2014). In rats, dietary limonene (245 ppm) and EO from *Citrus aurantum* (250 ppm) also increased gastric mucus production (Moraes *et al*., 2009). Additionally, the positive effects of phytogenics in enhancing mucus secretion in the intestine increase the efficiency by which digested nutrients are transported into the blood stream for use in metabolic processes including growth (Smirnor *et al*., 2006).

Nutrient absorption and transport takes place mainly at the fore intestine (Rust, 2003). The digested nutrients are transported majorly by diffusion and active transport (Bakke *et al*., 2010). Lipids are largely transported by simple diffusion across a lipid-bilayer, while glucose (a monosaccharide), amino acids and peptides are transported by facilitated diffusion and active transport aided by transporter proteins (Rust, 2003). The

nutrient transporters are either associated with the brush border or basolateral membrane of the intestine. An increase in the expression of genes regulating the nutrient transporter proteins and enzymes indicates enhanced nutrient transportation and their potential absorption into the blood stream (Speier *et al*., 2012). Digested protein is transported from the intestinal lumen as free amino acids by numerous brush border membrane amino acid transporters (including amino peptidase, AP) and, as di/tripeptides, by the brush border membrane transporter oligo-peptide transporter 1 (PEPT1) into the enterocytes and subsequently to the blood stream (Verri *et al*., 2000, 2011; Rønnestad *et al*., 2007). An increase in *pept1* mRNA levels is associated with increased availability of protein for enhancing fish growth (Terova *et al*., 2013). It is reported that fish utilise dietary di/tripeptides more efficiently than amino acids to sustain metabolic processes and enhance growth (Tengjaroenkul *et al*., 2000; Verri *et al*., 2011). Carbohydrates are transported as monosaccharide sugars such as glucose, fructose, maltose, galactose and sucrose (Krogdahl *et al*., 2005). Glucose, the most common monosaccharide, is mainly transported by sodium dependent glucose and galactose transporter (SGLT1) located in the brush border membrane and glucose transporter 2 (GLUT2) through facilitated diffusion across the basolateral membrane (Rust, 2003; Bakke *et al*., 2010). Alkaline phosphatase (ALP) is also involved in facilitating the transport of protein and carbohydrate from the intestine to the hepatic portal vein into the liver where they are transferred into the blood stream (Tengjaroenkul *et al*., 2000). It is argued that a decline in the activity of ALP could also be due to a high increase in soluble protein rather than from a lowering in the enzyme's activity (Kvale *et al*., 2007). Fatty acids, monoglycerides and lyso-phospholipids derived from lipid digestion are emulsified by bile salts to form micelles and transported into enterocytes where they are re-esterified into triglycerides, then combined with protein and secreted as part of chylomicrons (lipoproteins) into intestinal lymph vessels into the blood

stream (Tocher, 2003). The enzyme lipoprotein lipase (LPL) hydrolyses chylomicrons to facilitate diffusion of triglycerides into body cells for use in metabolic processes.

### 1.4.5 Effects of phytogenic compounds on lipid metabolism

Lipids can be classified into three main groups; neutral lipids, phospholipids and glycolipids (Prabhakara Rao *et al*., 2013). Neutral lipids are composed mainly of triglycerides or triacylglycerides (TAGs), which constitute the largest proportion of dietary lipids. On the other hand, phospholipids are polar lipids possessing a phosphate group, while glycolipids are lipids with a carbohydrate attached. Lipids are a major source of energy for fish and provide essential fatty acids and phospholipids that are required for normal growth and development (Rust, 2003). The liver is a key regulatory organ in lipid metabolism in fish (Tocher, 2003).

Lipid metabolism involves lipolysis and lipogenesis processes (Tocher, 2003; Rui, 2014). Fatty acids modulate the expression of genes involved in regulation of lipid metabolism, especially transcription factors including sterol regulatory element binding protein 1 c (*srebp-1c*) and peroxisome proliferator-activated receptor (*ppar*) α, β and γ, which belong to the PPAR subfamily of hormone receptors (Kleveland *et al*., 2006; Georgiadi and Kersten, 2012; Rui, 2014; Zheng *et al*., 2015). *Pparα* is expressed mostly in the liver where it stimulates lipolysis (oxidation) of the lipid absorbed from the diet in form of chylomicrons or lipoproteins (Tocher, 2003; Rui, 2014). Lipolysis is mediated by the enzyme lipoprotein lipase (*lpl*) resulting in release of fatty acids that are taken up by tissues for energy production (Tian *et al*., 2015; Yan *et al*., 2015) and thereby sparing protein for enhancing somatic growth. Surplus fatty acids are deposited as TAG and stored as lipid droplets in adipose tissue, liver and muscle (Rust, 2003). The stored TAG can be metabolised when required for energy production. An increase in expression of *pparα* gene leads to a rise in plasma cholesterol and a decline in TAG levels resulting in a decrease in

the levels of free fatty acids in the tissue (Skiba-Cassy *et al*., 2012). This means *ppar*s are essential for adapting to low energy levels by stimulating the genes for fatty acid oxidation (Li *et al*., 2015). They are also involved in regulating the synthesis of long-chain ($\geq C_{20}$) polyunsaturated fatty acids (PUFAs) to counteract low energy levels in the body (Tocher, 2003).

Lipogenesis, on the other hand, involves the synthesis of fatty acids through an acetyl-CoA unit derived mainly from carbohydrates (glucose) and non-essential amino acids. Acetyl-CoA is converted into malonyl-CoA by the enzyme acetyl-CoA carboxylase (Park and Chalfant, 2014). Saturated fatty acid (SFA) are then synthesised from the malonyl-CoA mediated by the enzyme fatty acid synthase (*fas*). This is followed by reactions that lead to elongation or desaturation of the SFA forming longer-chain SFA and monounsaturated fatty acid (MUFA), respectively (Skiba-Cassy *et al*., 2012). The activities of *fas* are regulated by liver X receptor (*lxr*) and *srebp-1c* (Tocher, 2003) that is encoded by sterol regulatory element binding transcription factor 1 (*srebf1*) (Grarup *et al*., 2008). Diets with high fat content may stimulate the release of *srebp-1c*, which in turn increases lipogenesis leading to fatty liver (Zheng *et al*., 2015). However, PUFAs suppress induction of *fas* and low-density lipoprotein (LDL) by inhibiting the expression and processing of *srebp-1c* (Yan *et al*., 2015).

Phytogenics have been found to minimise intracellular lipid accumulation in adipose tissues by decreasing adipogenesis in monogastric animals. Diets containing an equal mixture of thymol and carvacrol at 0, 60, 100, and 200 ppm were fed to chicken, and the chicken fed 200 ppm of dietary phytogenic additive had higher PUFA and lower total MUFA levels corresponding with a higher weight gain compared to the control (Hashemipour *et al*., 2013). Lillehoj *et al*. (2011) noted that some pathways involved in lipid metabolism were activated by carvacrol compared to cinamaldehyde and capsicum oleoresin in chicken. Correspondingly, Suganthi and Manpal, (2013) reported that

carvacrol reduced the expression of *pparγ*, *lpl* and *lep* (leptin) in mice fed high fat diet thereby reducing adipogenesis. Kim *et al*. (2013) reported that dietary carvacrol at 1000 ppm prevented high-fat diet (HFD) -induced hepatic steatosis by decreasing expression of *lxrα*, s*rebp1c*, *fas* and *lepr* genes involved in lipogenesis in mice fed HFD. Curcumin an active compound of turmeric, and eugenol a major bioactive compound of clove oil increased skeletal muscle activity of *lpl* gene in rats (Kochhar, 1999), suggesting increased transport of lipids to muscles for energy production. In addition, high expression of *lpl* gene was found in the ileum of pigs fed on a diet with *Tumeric oleoresin* herbal extract containing curcumin (Liu *et al*., 2014). Despite the information provided herein, there is still a gap in understanding how different phytogenic compounds influence the key drivers of lipid metabolism (such as *ppar*α, *srebp-1c*, *fas*, and *lpl*) and how this affects the growth performance of fish including Nile tilapia.

### 1.4.6 Antioxidant effects of phytogenic compounds

In aquaculture, antioxidants play two key roles. First, they protect the lipid in the diet from oxidative damage (Hashemipour *et al*., 2013) because, when fed on lipid-oxidised diets, fish exhibit reduced growth, increased incidence of skeletal deformities and histological alterations (Tocher, 2003). Secondly, antioxidants are involved in defending the fish against reactive oxygen intermediates (ROIs) that can inhibit growth when accumulated in high quantities in the body (Zheng *et al*., 2009; Saccol *et al*., 2013). ROIs are generated during normal metabolic processes and include superoxide, hydroxyl radicals and hydrogen peroxide (Mueller *et al*., 2012). High levels of ROIs in fish tissue can cause damage and loss of cellular functions but their levels can be controlled by antioxidants (Rajat and Panchali, 2014). When the concentrations of ROIs reaches levels that antioxidants cannot detoxify, oxidative stress takes place (Lesser, 2006; Kelestemur *et al*., 2012). It leads to oxidative damage of proteins, nucleic acids and lipid peroxidation of

PUFAs in the cell membrane, which can ultimately affect growth (Tocher, 2003; Rajat and Panchali, 2014). The antioxidant defence response in fish is triggered when the fish encounter stressful conditions that increase metabolic processes such as rough handling, poor water quality, toxic substances, poor diet, disease infections or parasites (Lesser, 2006; Trenzado *et al*., 2006; Costa *et al*., 2013). The extent of the response depends on the duration and magnitude of exposure to such conditions (Lesser, 2006; Kelestemur *et al*., 2012).

The antioxidant defence system in fish is composed of enzymes and non-enzymes. The enzymes comprise superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPX), while the non-enzymes are vitamin E, vitamin C, vitamin A, selenium, carotenoids and flavonoids among others (Kelestemur *et al*., 2012; Hashemipour *et al*., 2013; Rajat and Panchali, 2014). Antioxidant enzymes convert ROIs into none harmful compounds. Superoxide dismutase catalyses the breakdown of superoxide anion to oxygen and hydrogen peroxide, while CAT breaks down the hydrogen peroxide into oxygen and water (Lesser, 2006). Glutathione peroxidase breaks down hydrogen peroxide and lipid hydroperoxides leading to the production of oxidised glutathione (Zheng *et al*., 2009). It has been reported that some phytogenic components have potential antioxidant effects (see Table 1.3) (Steiner, 2009). However, most of the studies evaluating antioxidant properties of phytogenics have been conducted *in vitro* and with the overall aim to improve the storage quality of fish (Velasco and Williams, 2011; Albarracin *et al*., 2012; Alvarez *et al*., 2012, Giannenas *et al*., 2012; Hernandez *et al*., 2014a,b). With regards to *in vivo* studies investigating antioxidant properties of phytogenic compounds, Giannenas *et al*. (2012) found significantly increased activity of CAT in the blood of rainbow trout following feeding on a diet with either thymol or carvacrol compared with the control. Furthermore, Zheng *et al*. (2009) found enhanced level of SOD in fish fed on a diet with 500 ppm of a mixture of carvacrol and

thymol compared with the control. In broiler chicken, a mixture of carvacrol and thymol at either 60, 100 or 200 ppm in the diet increased SOD and GPX in a dose-dependent manner, attaining the highest body weight gain and FCR with 200 ppm of the phytogenic compounds (Hashemipour *et al.*, 2013). Hoffman-Pennesi and Wu (2010), however, reported improved antioxidant status of broilers fed on a diet with 4000 ppm thymol but this did not correspond with enhanced growth performance and feed conversion efficiency. Similarly, 250 ppm of dietary thymol and 300 and 500 ppm of carvacrol increased the activity of GPX in the serum of heat stressed broiler chicken (Shad *et al.*, 2016). However, carvacrol did not improve the performance of the chicken, while thymol increased the body weight gain and feed conversion efficiency (Shad *et al.*, 2016). This suggests that an improved antioxidant status may not always contribute to better somatic growth. In addition, only specific doses of different phytogenic compounds can have beneficial effects on both antioxidant enzyme activity and growth performance.

**Table 1.3:** Some essential oil components with potential antioxidant properties (adapted from Steiner, 2009 with modifications).

| **Essential oil component** | **Plant Species** | | | |
|---|---|---|---|---|
| | *Monarda citriodora* | *Myristica fragrans* | *Origanum vulgare* | *Thymus Vulgaris* |
| α-pinene | √ | √ | | √ |
| ß-pinene | √ | √ | | √ |
| terpinene-4-ol | √ | √ | | √ |
| ß-caryophyllene | √ | | √ | √ |
| *p*-cymene | √ | | √ | √ |
| 1,8-cineole | √ | | | √ |
| Carvacrol | √ | | √ | √ |
| Limonene | √ | √ | | |
| Linalool | | √ | √ | √ |
| Thymol | √ | | √ | √ |
| Isoeugenol | | √ | | √ |
| Camphene | | | | √ |
| Borneol | | | | √ |
| Sabinene | | √ | √ | |
| Myrcene | √ | | | √ |

## 1.5 Conclusion

The literature reviewed indicates that some herbs and EOs have potential to improve the growth performance of fish when they are supplied in the diet at certain doses. Combinations of different herbs or EOs are suggested to have stronger growth enhancing effects than when used in the diets individually. However, the concentration of bioactive compounds in herbs and EOs is variable making it difficult to obtain consistent and comparable results. This may be attributed to differences in plant parts from which they were extracted, species of plant, physical and chemical soil condition, harvest period, stage of maturity at harvest, duration of storage, processing method and extraction process. As a result, there is need to use phytogenic products with a consistent quality. This could be attained by using purified compounds of EOs, i.e. phytogenic compounds. Presently, there is still little information on the effect of phytogenic compounds on the performance of fish. Although a few studies indicate that phytogenic compounds have potential to enhance the growth of fish, fish species seem to response differently to a variety of phytogenic compounds and doses. Therefore, there is need to identify appropriate phytogenic compounds and doses to use to enhance the growth of different fish species. In addition, there is little and inconsistent information on the mode of action of phytogenic compounds in enhancing the growth of fish and other monogastric animals. Moreover, although nutritional physiology is known to play a central role in modulating the growth of fish, the pathways by which phytogenic compounds exert their actions remain unclear. Given that phytogenic compounds have different chemical composition, it is hypothesised that they activate different pathways such as those involved in somatotropic-axis growth-mediation, appetite regulation, nurient digestion, absorption and transport, lipid metabolism and antioxidant enzyme defence system, to enhance the growth of fish.

## 1.6 Objectives of the study

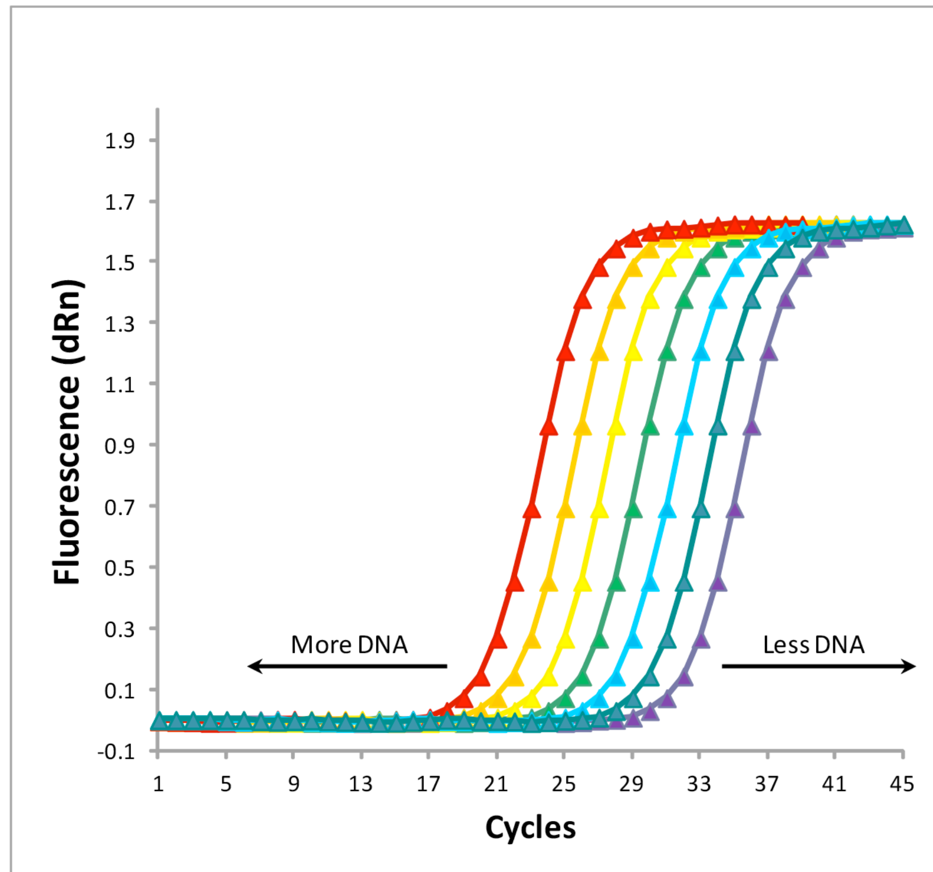
Based on the research gaps described above, this research project aimed at investigating the effect of different phytogenic compounds on the growth and nutritional physiology of Nile tilapia. In particular, the study was carried out to investigate the effects of phytogenic compounds and their dietary doses on growth of Nile tilapia, as well as to undertand the mode of action by which these compounds operate. This information is vital for guiding the formulation of functional Nile tilapia diets supplemented with phytogenic compounds.

The specific objectives of the research included:

1. To determine the effects of selected phytogenic compounds (limonene, carvacrol and thymol) on the growth performance of Nile tilapia.
2. To explore the effects of those phytogenic compounds found to promote growth on the expression of genes of the somatotropic axis, appetite regulation, nutrient digestion, absorption and transport, lipid metabolism and antioxidant enzyme status in Nile tilapia.
3. To investigate the effects of combined phytogenic compounds on growth performance and genes of nutrient digestion, absorption and transport, lipid metabolism, antioxidant enzyme activity and somatotropic axis in Nile tilapia.

# Chapter 2:

# Materials and methods




An example of qPCR amplification curves
(Source: IDEXX RealPCR Technical Guide).

*"Theories might inspire you, but experiments will advance you"*

**Amit Kalantri**

## 2.1 Overview of the research

In this research project, six Trials (Trials I, II, III, IV, V and VI) were carried out. Trials I, II, III and VI were carried out at the Aquaculture Research and Development Centre (ARDC) in Wakiso District, Uganda. Trials IV and V were conducted at the Institute of Aquaculture (IOA), University of Stirling, UK. The Trials were conducted in accordance with the Uganda (Trials I, II, III and VI) or British Home Office (Trials IV and V) guidelines regarding research on experimental animals. In Trials I, II and III (Chapter 3), the effects of selected phytogenic compounds (limonene, carvacrol and thymol) on the growth performance of Nile tilapia was determined (Objective 1). Given that limonene and thymol showed growth-promoting effects, the pathways in the nutritional physiology of Nile tilapia underlying their growth-promoting effects were investigated in Trials IV and V (Chapter 4, Objective 2). This was done by assessing the expression of key genes participating in selected pathways regulating appetite, nutrient digestion and absorption, lipid metabolism, antioxidant enzyme activity and somatotropic axis-mediated growth. Trial VI (Chapter 5) investigated the effects of a dietary combination of phytogenic compounds (Limonene and thymol) on growth performance and expression of genes involved in nutrient digestion and absorption, lipid metabolism, antioxidant enzyme activity and somatotropic axis in Nile tilapia (Objective 3).

## 2.2 Experimental diets

Commercial fish feeds were used as the standard diets to which the phytogenic compounds were coated. In Trials I, II and III, Nile tilapia commercial diets were obtained from Ugachick Poultry Breeders Ltd and ARDC in Uganda. For Trials IV and V, the feed was obtained from BioMar Ltd., UK. The phytogenic compounds limonene (97 % purity), thymol (99.5 % purity) and carvacrol (98 % purity) were obtained from

Sigma Aldrich Company Ltd. UK. In Chapter 3, Trials I, II and III were carried out with either limonene, carvacrol or thymol supplemented diets. In Chapter 4, Trials IV and V were conducted with either thymol or limonene supplemented diets. Trial VI (Chapter 5) was undertaken with diets supplemented individually with either limonene, thymol or a combination of limonene and thymol. The phytogenic compounds were coated onto the diets at increasing concentrations (in ppm of feed) as described in Chapters 3, 4 and 5. The inclusion levels of the phytogenic compounds in the Trials were adapted from previous authors (Zheng *et al*., 2009; Rattanachaikunsopon and Phumkhachorn, 2010; Volpatti *et al*., 2013; Yilmaz *et al*., 2014; Acar *et al*., 2015). For each diet, the amount of phytogenic compound to supplement was dissolved in 100 mL of absolute ethanol and sprayed on to 1 kg of the standard feed. This was done while mixing the feed to ensure even distribution of the phytogenic solution within the diet. The controls were also added similar volume of ethanol as the other diets to ensure that the only difference between the diets was the concentration of the phytogenics. The diets were spread out and air-dried for one day at room temperature. The proximate composition of the experimental diets is provided in the corresponding Chapter (3, 4 or 5) where the diets were used.

## 2.3 Experimental fish and facilities

Nile tilapia juveniles were used in this research. For each experiment, fish from the same spawning batch / cohort were used. The fish were size graded to select those with similar sizes. Size grading was carried out by taking individual fish weight using a digital measuring scale (precision 0.1 g). Before grading the fish, they were anaesthetised with a low dose of clove oil or benzocaine as detailed in Chapters 3, 4 and 5, and kept in aerated water. The fish were reared in tanks of 20 to 60 L. Figure 2.1 shows the experimental facility used at the IOA.

**Figure 2.1:** Closed circulation tanks (60 L) used at the Institute of Aquaculture, University of Stirling.

The water quality was monitored every week during the Trials in order to maintain it within the requirements for Nile tilapia growth. Dissolved oxygen and water temperature were measured using an oxygen meter (HQ40D model, Hach Ltd Germany in Trials I, II III and VI, and OaKton DO 6+, Eutech Instruments Pte Ltd, The Netherlands in Trials IV and V). The ammonia-nitrogen and pH levels were measured with fresh water test kits from LaMotte Company Ltd, USA (Trials I, II and III), Tropic Marin Company Ltd., Germany (Trials IV and V) and API Company Ltd UK (Trial VI) following the instructions from the manufacturers.

## 2.4 Experimental design

The layout and conditions of the Trials is indicated in Table 2.1. All treatments were allocated using a complete randomised design.

**Table 2.1:** Layout and conditions of the trials investigating the effects of diets supplemented with phytogenic compounds on growth and nutritional physiology of Nile tilapia.

| Trial | Phytogenic compound | Number of treatments or concentrations of phytogenic compound | Number of fish rearing tanks | Number of replicates | Number of fish per tank | Average weight of fish* (g) |
|---|---|---|---|---|---|---|
| I | Limonene | 6 | 24 | 4 | 40 | 3.7 ± 0.0 |
| II | Carvacrol | 5 | 20 | 4 | 51 | 1.1 ± 0.0 |
| III | Thymol | 5 | 15 | 3 | 30 | 0.3 ± 0.0 |
| IV | Limonene | 4 | 12 | 3 | 37 | 1.5 ± 0.0 |
| V | Thymol | 3 | 9 | 3 | 25 | 1.5 ± 0.0 |
| VI | Limonene and thymol | 4 | 16 | 4 | 38 | 1.6 ± 0.0 |

* *refers to fish weight ± standard error*

At the start of each Trial, fish of similar sizes were stocked to ensure there was no significant difference in initial weights between treatments. In each Trial, the fish in the different treatments were fed their respective diets twice a day at 10:00 am and 4:00 pm by hand to apparent satiation. In Trials IV, V and VI, biometric measurements of length and weight (precision 0.1 cm and 0.1 g, respectively) were taken from each fish after every two or three weeks to assess growth trends. Figure 2.2 illustrates how weight and length of the fish were measured.

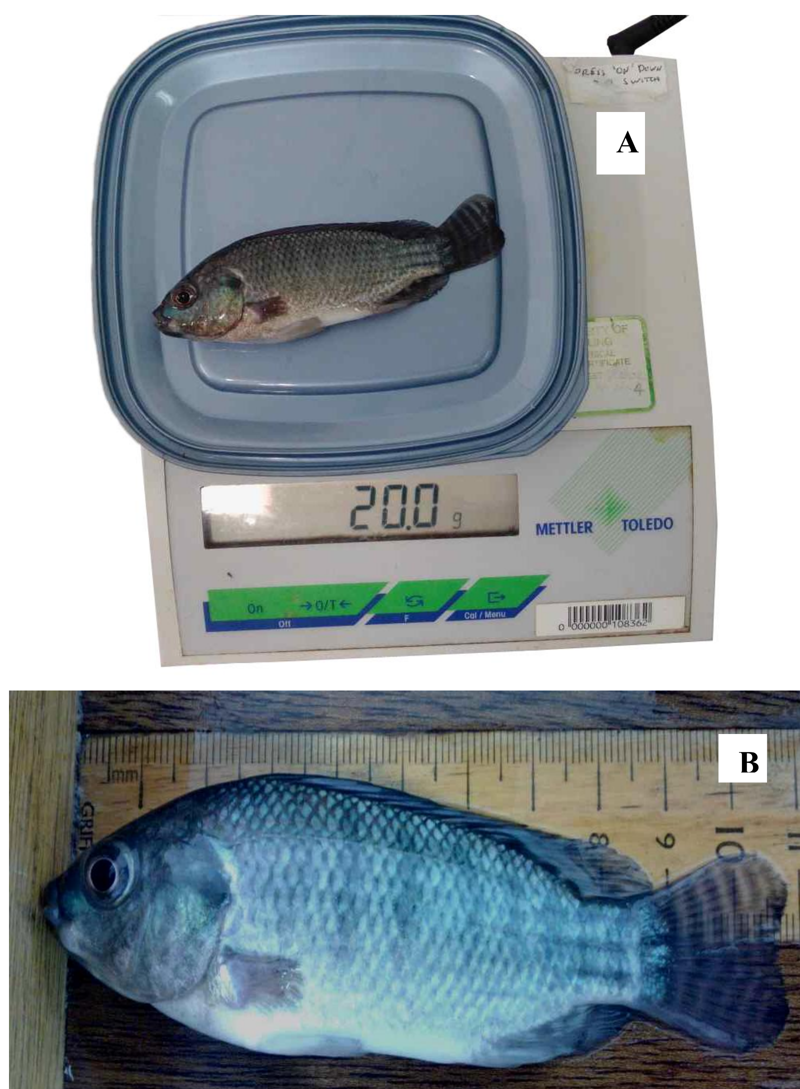



**Figure 2.2:** Illustration on the measurement of fish weight using a weight scale (A) and total length (B) using a calibrated meter ruler.

Before measuring the fish, they were anesthetised with a low dose of clove oil or benzocaine and kept in aerated water. A small number of fish was taken at a time to ensure that they did not stay too long under anaesthesia before measurements (length and weight). Minimal stress was ensured during fish handling and the fish were allowed to recover before being transferred to the rearing tanks. At the end of the growth Trials (Trials I, II, III, IV, V and VI) within Chapter 3, 4 and 5, the total length and weight (precision 0.1 cm and 0.1 g, respectively) of each fish were taken except for Trial III (Chapter 3) where total lengths could not be recorded. Thereafter, the fish were killed humanely with an over dose of anaesthetic. Samples of liver, fore intestine and brain were collected and put in 1.5 mL

tubes containing RNAlater from IOA in Trials IV and V (N = 3 per tank, 9 per treatment) and Sigma Aldrich, Uganda in Trial VI (N = 3 per tank, 12 per treatment). Figure 2.3 shows a fish with part of the digestive sytem exposed, ready for collection of tissues of liver and fore intestine. The samples were kept at 4 ºC overnight and transferred to a -20 ºC freezer until analysed to determine the expression of genes.

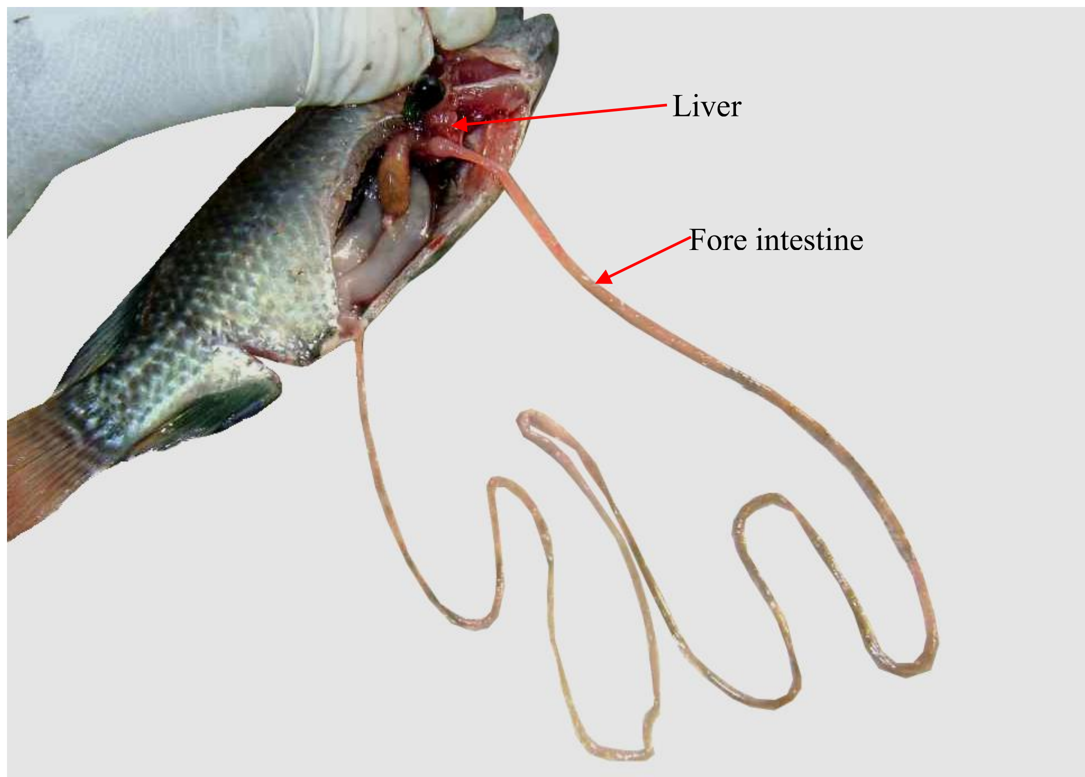



**Figure 2.3:** An example of a fish prepared to collect liver and fore intestine tissues.

## 2.5 Laboratory analytical procedures

### 2.5.1 Proximate analysis of diets

The proximate composition (crude protein, lipid, fibre, ash, moisture, dry matter and gross energy) of the experimental diets was determined following the methods described in AOAC (1990) and ISO/IEC 17025.

#### *2.5.1.1 Moisture*

Feed samples were ground into a powder using a mortar and pestle and homogenised. A sample of 1-5 g was weighed into a pre-weighed dish and placed in a drying oven at 110 °C overnight. The sample was removed from the oven, cooled in a desiccator and reweighed. The moisture content of the samples was calculated as:

$$\% \ \ Moisture = \frac{Sample\ weight\ (g)\ -\ Dried\ weight\ (g)}{Sample\ weight\ (g)}\ X\ 100$$

#### *2.5.1.2 Dry matter*

The dry matter content was calculated as:

$$Dry\ matter\ (\%) = 100\ -\ \%\ moisture\ content$$

#### *2.5.1.3 Ash*

One gram of homogenised ground diet sample was weighed into a pre-weighed porcelain crucible and placed into a muffle furnace at 600 ºC for 20-28 hours (h). The samples were cooled at room temperature in a desiccator and reweighed. The ash content was calculated as:

$$\% \text{ ash} = \frac{\text{Ash weight (g)}}{\text{Sample weight (g)}} \ X\ 100$$

#### *2.5.1.4 Crude protein*

Crude protein content was determined by the Kjeldahl method (nitrogen x 6.25) using a Tecator Kjeltec Autoanalyser (Foss, Warrington, UK) (Figure 2.4).

**Figure 2.4:** Tecator Kjeltec Autoanalyser for crude protein analysis.

Duplicate samples of 250 mg were weighed onto 42.5 mm filter paper disc and placed into a Kjeldahl digestion tube. A glycine standard was made by weighing 250 mg of glycine in duplicate and transferring each to a Kjeldahl digestion tube. Two blank tubes were also prepared. A 42.5 mm filter paper was added to all the standards and blanks. Two copper Kjeltabs (BDH Chemicals Ltd, UK) and 5 mL of concentrated sulphuric acid were added to each sample and glycine standard tubes. The tubes with the samples were placed into a digestion block at 420 ºC for 1 h. The tubes were then removed from the block and cooled

inside a fume cupboard for at least 20 minutes (min). Subsequently, 20 mL of de-ionised water was added to each digestion tubes and vortex mixed. The content in the tubes were distilled using a Kjeltec 2300 Auto analyser and the titration values were recorded. All samples were analysed in duplicate. For each batch of samples, two glycine standard tubes and two blank tubes were analysed. The protein content of the samples was calculated as:

$$\%\ Nitrogen = \frac{(T\text{-}B) \times M \times 14.007 \times 100}{Weight\ of\ sample\ (mg)}$$

% protein = $N \times F$

Whereby:

$T$ = titration volume for sample (ml)

$B$ = titration volume for blank (ml)

$M$ = molarity of acid

$F$ = conversion factor for nitrogen to protein (6.25)

#### *2.5.1.5 Crude lipid*

The samples were acid hydrolysed using a Tecator soxtec (Hydrotec 8000, Foss, Warrington, UK) method as follows. A sample of 1.0 to 3.0 g wet weight and celite of the same weight were placed in a hydrolysis tube into which 100 mL of 3 M hydrochloric acid solution were added. Each tube was inserted into a hydrolysing unit and glass thimbles were placed into the supports. The samples were boiled gently for 1 h and 100 mL of de-ionised water was added to each tube. The solution was filtered through the glass thimbles and each tube was washed with 5 x 50 mL of hot deionised water using a spray gun. The thimble was dried at 60 °C for 16-18 h. The lipid in the samples was thereafter extracted with soxhlet extraction apparatus (2050 Soxtec, Foss, Warrington, UK) using chloroform:methanol (2:1, v/v) (Figure 2.5).

**Figure 2.5:** Soxhlet extraction apparatus for lipid extraction.

A homogenised sample of 0.5 to 3.0 g was weighed into an extraction thimble and the same weight of celite was added, mixed with a spatula and covered with cotton wool. An extraction cup containing 5 to 10 glass balls was weighed for each thimble and 80 ml of chloroform:methanol (2:1, v/v) was added to each extraction cup. The soxtec extraction unit was run at 135 $^{o}$C with pre-set boiling and rinsing times. After the run, the cups were removed, dried in an oven at 100-105 $^{o}$C for 1 to 2 h, cooled in a desiccator over fresh silica gel and reweighed. All samples were analysed in triplicate. The crude lipid content was calculated as:

$$\%\ \text{lipid} = \frac{\textit{Weight of cup and sample after drying (g)} - \textit{Weight of cup alone (g)}}{\textit{Weight of sample (g)}}$$

#### *2.5.1.6 Crude fibre (Foss FeiberCap System)*

Feed samples of 0.5-1.0 g (to four decimal places) were weighed into pre-weighed fibrecap capsules, placed in a small holding carousel and de-fatted by agitating the fibrecaps in a beaker containing 120 mL of petroleum ether for 30 seconds (s) three times. The procedure was repeated in two beakers with fresh petroleum ether. Hydrolysable material was then removed from the feed samples by sequential boiling in an extraction vessel with 350 mL of 1.25 % sulphuric acid ($H_2SO_4$) for 30 min while gently agitating occasionally. The liquid was disposed of and the capsules and allowed to drain out before boiling in the extraction vessel with 350 mL of de-ionised water for 1 min while agitating. The samples were boiled a further two times with fresh water. The procedure followed when boiling the samples in $H_2SO_4$ was also followed using 1.25 % sodium hydroxide (NaOH) solutions and finally acetone. The samples were dried in a drying oven for at least 5 h and cooled to room temperature in a desiccator before re-weighing (Capsule + sample dry weight). The capsules were placed in re-weighed crucibles and ashed in a muffle furnace for at least 4 h at 600 °C. The crucibles were cooled to room temperature in a desiccator and re-weighed (crucible + ashed sample weight). The quantity of fibre in each sample was calculated as:

$$\%\,fibre = \frac{\{(\text{“Capsule + sample dry weight”} \times 1.0011) - \text{“Capsule weight”} - ((\text{Crucible + ashed sample weight}) - 0.0025)\}}{\text{Sample weight}} X\,100$$

1.0011 = correction factor to account for loss of mass of the capsule itself, solvent and acid washing

0.0025 = mean ash residue of a washed, empty capsule

### 2.5.1.7 *Gross energy*

The gross energy content of the diets and fish was determined using a 6200 bomb calorimeter (Parr 6100 Calorimeter, Foss, Warrington, UK) (Figure 2.6).

**Figure 2.6:** Bomb calorimeter used for measuring gross energy content.

A dried sample of 1 g was placed into a crucible and pressed to make a firm pellet. The crucible was then placed into an electrode assembly holder. A firing wire was attached between the electrodes and a loop was formed above the pellet. The electrode assembly was placed into the bomb calorimeter. The water jacket of the bomb was filled with tap water and weighed to exactly 2812.8 g. The calorimeter vessel was placed into the water jacket. Prior to firing the calorimetry bomb, the thermometer reading was recorded as the initial temperature. The bomb was fired and the energy value (kj $g^{-1}$) was recorded. The samples were analysed in duplicate.

### 2.5.2 Molecular analyses

#### *2.5.2.1 RNA extraction*

Tissue samples were homogenised by putting 100 mg of each tissue (liver, brain or fore intestine) in 1 ml of TRI Reagent (Sigma Aldrich Company Ltd, UK) in 1.5 mL screw cap tubes as detailed in Chapter 4 and 5. For samples less than 100 mg, the volume of TRI Reagent used was adjusted accordingly. The tissues were incubated on ice for 30-60 min depending on the toughness of the tissue. Then, samples were homogenised in a mini-bead beater-24 (BioSpec Products Inc, USA) for 30-60 s. The homogenised samples were incubated at room temperature for 5 min. For every millilitre of TRI Reagent used during the homogenisation phase, 100 µl of chloroform was added to the samples. The tubes containing the samples were shaken vigorously by hand for 15 s and then incubated at room temperature for 15 min. The samples were centrifuged at 20,000 × g for 15 min at 4 ºC. The aqueous (upper) layer that developed at the surface of each sample was removed slowly with a pipette and transferred to a new 1.5 ml tube. The volume of the supernatant transferred was recorded. RNA precipitation solution and isopropanol were added to the supernatant, each at half the volume of the supernatant. The samples were gently inverted 4-6 times, incubated for 10 min at room temperature and centrifuged at 20,000 × g for 10 min at 4 °C. The RNA precipitate formed a gel like pellet on the side/bottom of the tube. The supernatant was removed by pipetting leaving behind the pellet, which was washed for 30 min at room temperature with 1 ml of 75 % ethanol in water (v/v). This was accomplished by flicking and inverting the tube a few times to lift the pellet from the bottom and ensure the entire surface of the pellet and tube were washed. The samples were centrifuged at 20,000 x g for 5 min at room temperature. Thereafter each supernatant was removed using a separate pipette. The samples were spun for 3-5 s and

any remaining ethanol was removed with a pipette. The RNA pellets were then air dried at room temperature for 3-5 min until all visible traces of ethanol were gone and the pellets were re-suspended in an appropriate amount of RNase free water. The tubes were flicked to ensure the pellets completely dissolve in the RNase free water. For each sample, the RNA concentration and purity were measured at absorbance wavelength ratios of 260/280 nm and 260/230 nm, respectively, by spectrophotometry using a NanoDrop ND 1000 (Thermo Scientific, Wilmington, USA). The integrity of the RNA was confirmed by agarose gel electrophoresis using 1 % agarose gel in 0.5 x TAE buffer containing 0.3 - 0.5 μl ethidium bromide (10 mg $mL^{-1}$) and Ultra-Violet fluorescent detection (INGENIUS, Syngene Bio imaging, Cambridge, UK). Figure 2.7 shows an example of a set of RNA samples from liver after extraction following the procedure described above. The RNA solution was stored at -70 $^{o}$C prior to further analysis.

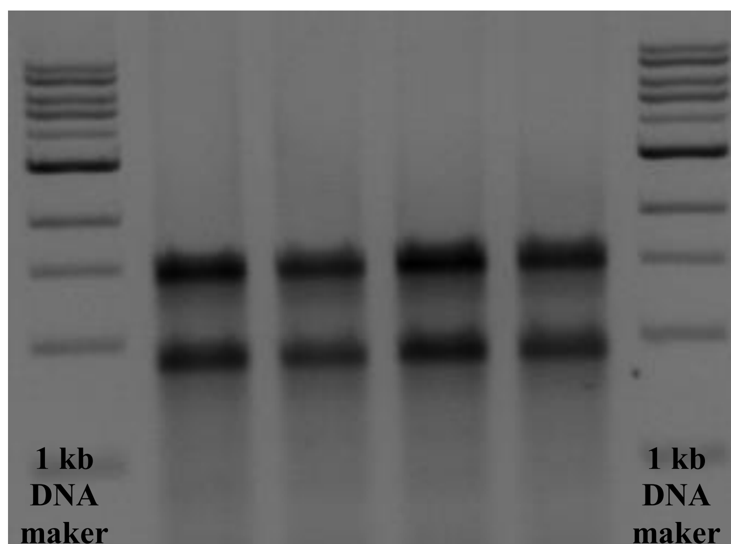



**Figure 2.7:** An example of gel electrophoresis of Nile tilapia liver RNA isolated using TRI Reagent protocol.

#### *2.5.2.2 Complementary DNA (cDNA) synthesis*

RNA from the tissue samples was reverse transcribed to cDNA using a high capacity reverse transcription kit from AB Applied Biosystems (Warrington, UK). Briefly, 2 μg of total RNA of each sample were made up to a volume of 10 μl with nuclease-free

water in 0.2 mL tubes, vortex mixed, centrifuged and incubated on ice. To each sample, 10 µl of the reverse transcription mixture, containing 2 µl of 10 × reverse transcription (RT) buffer, 0.8 µl of 25 × dNTP mix, 2 µl of 10 × random primer in the molar ratio of 1.5:0.5 for the random to oligo-dT primer, respectively, 1.0 µl of reverse transcriptase and 4.2 µl of nuclease free water was added. The 20 µl mixture was vortex mixed, centrifuged and placed in a thermocycler (Biometra Analytik Jena, Germany) to synthesise cDNA following the thermal cycling protocol provided by the manufacturer. The cDNA was stored at -20 $^{o}$C until further analysis.

#### *2.5.2.3 Quantitative real-time Polymerase Chain Reaction (qPCR)*

The expression of genes was determined by quantitative real-time polymerase chain reaction (qPCR) using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) (Figure 2.8).

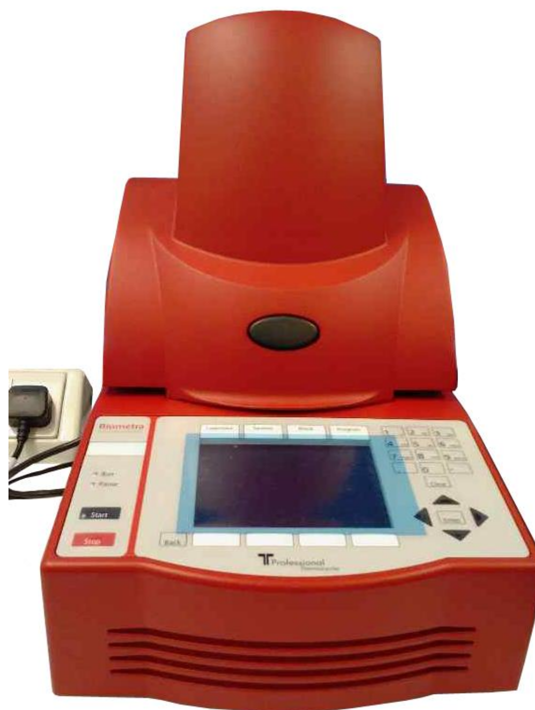



**Figure 2.8:** Biometra TOptical Thermocycler used for running qPCR analyses.

The efficiency of the primers was tested by generating standard dilution curves, assessing the melting curves, cycle threshold (Ct) values and electrophoresis of PCR products. Five different serial dilutions (1/5, 1/10, 1/20, 1/50, 1/100, 1/200 and 1/500 in duplicate) of pooled cDNA sample solutions were used. Each qPCR cycle consisted of pre-heating samples at 50 °C for 2 min, initial denaturing at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 30 s. The Ct value was considered the number of cycles at which the fluorescence emission monitor exceeded the threshold limit. The standard curves were established by plotting the Ct 20 values against the log10 of the five dilutions of cDNA by applying the equation: $E = 10^{(-1/slope)}$.

Efficient primers were considered to have values between 0.80 - 1.10 and PCR melting curves with a single melting peak in the reactions. In addition, the electrophoresis of the resulting PCR product was carried out to check for production of a single clear band comparable to the predicted size when designing the primers. Any bands appearing below a size marker of 100 base pairs were considered as being primer-dimers. The nucleotide sequence of the primers for each gene is shown in Table 2.2. Quantitative PCR (qPCR) analysis for each gene was performed on nine (Trials IV and V) and six (Trial VI) cDNA replicates as detailed in Chapters 4 and 5 respectively. Every qPCR was run in a total volume of 20 µl that contained 5 µl of 20-fold (1/20) diluted cDNA and 15 µl of qPCR master mix comprising 3 µl nuclease-free water, 1 µl (10 pmol) each for the forward and reverse PCR primer, and 10 µl of Luminaris Color HiGreen qPCR Mix (Thermo Scientific, Hemel Hempstead, UK). In addition, a 20-fold dilution of pooled cDNA from all the samples and a non-template control (NTC) containing no cDNA were included in each run as a calibrator between plates and to control any genomic DNA contamination. The thermal cycling procedure was the same as the one used for testing the primers.

**Table 2.2:** Nucleotide sequences of primers used for quantitative real-time PCR analyses.

| Functional group | Gene symbol | Oligonucleotide sequences (5'- 3') | Size (bp) | Accession number* |
|---|---|---|---|---|
| Nutrient digestion, absorption and transport | *muc* | F: TGCCCAGGAGGTAGATATGC<br>R: TACAGCATGAGCAGGAATGC | 101 | XM_005466350.2 |
| | *pept1* | F: CAAAGCACTGGTGAAGGTCC<br>R: CACTGCGTCAAACATGGTGA | 196 | XM_013271589 |
| | *Alp* | F: CTTGGAGATGGGATGGGTGT<br>R: TTGGCCTTAACCCCGCATAG | 200 | XM_005469634.2 |
| | *Ctra* | F: AGTGCCGAGAACATCCAGAC<br>R: GAAGTCTCGGCCACACAAAC | 155 | XM_003437588.3 |
| | *pla2* | F: CTCCAAACTCAAAGTGGGCC<br>R: CCGAGCATCACCTTTTCTCG | 177 | XM_005451846 |
| | *glut2* | F: TCTAAAGGGGCCGCATGATC<br>R: GAAAGGTGCATCATGAGGGC | 153 | FJ914656 |
| | *Ap* | F: TTACCACTCCGAACCAGACC<br>R: GAGTAGTTCCCTCCTGCCTC | 238 | XM_005449270 |
| | *p-amy* | F: TGGAGGCCCTGGTATCAAAG<br>R: TCCTGTTCCACCACCAGATC | 168 | XM_003448471.2 |
| Lipid metabolism | *Lpl* | F: TGCTAATGTGATTGTGGTGGAC<br>R: GCTGATTTTGTGGTTGGTAAGG | 217 | NM_001279753.1 |
| | *Pparα* | F: CTGATAAAGCTTCGGGCTTCCA<br>R: CGCTCACACTTATCATACTCCAGCT | 106 | NM_001290066.1 |
| | *srebf1* | F: TGCAGCAGAGAGACTGTATCCGA<br>R: ACTGCCCTGAATGTGTTCAGACA | 102 | XM_005457771.2 |
| | *Fas* | F: TGAAACTGAAGCCTTGTGTGCC<br>R: TCCCTGTGAGCGGAGGTGATTA | 144 | GU433188 |
| Appetite regulation | *Lepr* | F: TCTGTTCCTTTGGGCATAG<br>R: CCCTCCTGATGTCTTTTCCA | 143 | XM_005460446.1 |
| | *Npy* | F: CCTCTTCCTTACGCATCAGC<br>R: CCCCTCCTCCACTTTACGAT | 115 | XM_003448854.2 |
| Antioxidant enzyme activity | *Gpx* | F: ACAAGTGACATCGAGGCAGA<br>R: CAAACCCAGGCCTGCTATAA | 186 | NM_001279711.1 |
| | *Cat* | F: TCCTGGAGCCTCAGCCAT<br>R: ACAGTTATCACACAGGTGCATCTTT | 79 | JF801726 |
| | *sod2* | F: CTCCAGCCTGCCCTCAA<br>R: TCCAGAAGATGGTGTGGTTAATGTG | 58 | XM_003449940.3 |
| Somato-tropic axis growth mediation | *Gh* | F: TCGGTTGTGTGTTTGGGCGTCTC<br>R: GTGCAGGTGCGTGACTCTGTTGA | 90 | XM_003442542 |
| | *ghr-I* | F: ATGGCTCTCTCGCCCTCCTCTAA<br>R: ATGTCGTGTGGTCCCAGTCAGTGA | 109 | NM_001279601 |
| | *igf-I* | F: GTCTGTGGAGAGCGAGGCTTT<br>R: CACGTGACCGCCTTGCA | 70 | NM_001279503 |
| Reference genes | *ef-1α* | F: GCACGCTCTGCTGGCCTTT<br>R: GCGCTCAATCTTCCATCCC | 250 | NM_001279647 |
| | *ß-actin* | F: TGGTGGGTATGGGTCAGAAAG<br>R: CTGTTGGCTTTGGGGTTCA | 217 | XM_003443127.3 |

*muc* mucin-like protein, *pept1* oligo-peptide transporter 1, *alp* alkaline phosphatase, *ctra* chymotrypsinogen A-like, *pla2* phospholipase A2, *glut2* glucose transporter 2, *ap* aminopeptidase N-like, *p-amy* pancreatic alpha-amylase, *lpl* lipoprotein lipase, *pparα* peroxisome proliferator-activated receptor alpha, *srebf1* sterol regulatory element binding transcription factor 1, *fas* fatty acid synthase, *lepr* leptin receptor variant X1, *npy* pro-neuropeptide Y-like , *gpx* glutathione peroxidase, *cat* catalase, *sod2* superoxide dismutase 2, *gh* growth hormone, *ghr-I* growth hormone receptor I, *igf-I* insulin growth factor I, *ef-1α* elongation factor 1α, *ß-actin* beta-actin.

*GenBank (http://www.ncbi.nlm.nih.gov/), bp base pairs.

## 2.6 Data computation

### 2.6.1 Growth performance parameters

Final average fish weight (FW), weight gain (WG), percentage (%) weight gain (% WG), growth rate (GR), daily growth coefficient (DGC), mean metabolic body weight (MBW), growth rate per metabolic body weight ($GR_{MBW}$), condition factor (CF) and percentage (%) fish survival were determined using the formulae below (Jobling, 1994; Workagegn *et al.*, 2014):

$$\textbf{\textit{FW}}\ (g) = \frac{FB}{N_f}$$

*FB* (g) is total fish biomass at end of the trial (g)

$N_f$ is number of fish at end of the trial

$$\textbf{\textit{WG}} = W_t - W_o$$

$W_t$ is the average weight (g) of fish at the end of the trial

$W_o$ is the average weight (g) of fish at the beginning of the trial

$$\textbf{\% \textit{WG}} = \frac{(W_t - W_o)}{W_o} X\ 100$$

$W_t$ is the average weight (g) of fish at the end of the trial

$W_o$ is the average weight (g) of fish at the beginning of the trial

$$\textbf{\textit{GR}}\ (g\ d^{-1}) = \frac{W_t - W_o}{d}$$

$W_t$ is the average weight (g) of fish at the end of the trial

$W_o$ is the average weight (g) of fish at the beginning of the trial

*d* is duration of trial in days

$$\textbf{DGC}\ (\%\ d^{-1}\ fish^{-1}) = \frac{W_t^{(1/3)} - W_o^{(1/3)}}{d} X\ 100$$

$W_t$ is the average weight (g) of fish at the end of the trial

$W_o$ is the average weight (g) of fish at the beginning of the trial

$d$ is duration of trial in days

$$\textbf{MBW}(Kg^{-0.8}\ fish^{-1}) = ((W_t/1000)^{0.8} + (W_o/1000)^{0.8})\ /\ 2$$

$W_t$ is the average weight (g) of fish at the end of the trial

$W_o$ is the average weight (g) of fish at the beginning of the trial

$$\textbf{GR}_{\textbf{MBW}}\ (g\ Kg^{-0.8}\ d^{-1}) = (W_t - W_o)\ /\ MBW)\ /\ d$$

$W_t$ is the average weight (g) of fish at the end of the trial

$W_o$ is the average weight (g) of fish at the beginning of the trial

$MBW$ is the mean metabolic body weight

$d$ is duration of trial in days

$$\textbf{CF} = \frac{W_t}{L^{\wedge 3}} X\ 100$$

$W_t$ = final mean fish weight at end of the trial

$L$ = final mean fish length at the end of the trial

$$\textbf{\% survival} = \frac{N_f}{N_o} X\ 100\ \%$$

$N_f$ is the number of surviving fish by the end of the trial

$N_o$ is the number of fish at the start of the trial

In addition, the length-weight relationship of the fish was analysed to predict the body form of the fish. This was done by generating scatter plot diagrams and using the regression line equation $W = aL^b$ (Froese, 2006) where:

W is the weight of the fish (g)

L is the total length of the fish (cm)

a is the intercept that describes the rate of change of weight with total length

b is the slope of the power regression line of weight and total length and describes the weight at unit length

The results were interpreted based on the cube law which states that an increase in fish weight to the third power of its length results into isometric growth with the value of ‘b’ equal to 3 (Froese, 2006). In that case, the weight of a fish increases proportionally to its length. Values of ‘b’ higher than 3 are associated with positive allometric growth whereby fish weight increases more than the length and when ‘b’ value is less than 3, fish are said to have negative allometric growth characterised by the length increasing more than weight.

The feed intake as a percentage of body weight (% FI) and expressed per metabolic body weight ($FI_{MBW}$), feed conversion ratio (FCR), feed conversion efficiency (FCE), protein intake (PI) and protein efficiency ratio (PER) were calculated using the formulae (Jobling, 1994; Bodin *et al*., 2012):

$$\textbf{\% FI} \ (\% \text{ body weight } d^{-1}) = 100 \times (F / ((W_o \pm W_t) / 2)) / d$$

F is the average feed intake (g) by each fish during the trial

$W_t$ is the average weight (g) of fish at the end of the trial

$W_o$ is the average weight (g) of fish at the beginning of the trial

$d$ is duration of trial in days

$$\boldsymbol{FI_{MBW}}\ (g\ Kg^{-0.8}\ d^{-1}) = \frac{F}{MBW \times d}$$

*F* is the average feed intake (g) by each fish during the trial

*MBW* is the mean metabolic body weight (kg$^{-0.8}$ fish$^{-1}$)

*d* is duration of trial in days

$$\boldsymbol{FCR} = \frac{F}{(W_t - W_o)}$$

F is the average feed intake (g) by each fish during the trial

$W_t$ is the average weight (g) of fish at the end of the trial

$W_o$ is the average weight (g) of fish at the beginning of the trial

$$\boldsymbol{FCE}\ (\%) = \frac{(W_t - W_o)}{F}\ X\ 100$$

$W_t$ is the average weight (g) of fish at the end of the trial

$W_o$ is the average weight (g) of fish at the beginning of the trial

*F* is the average feed intake (g) by each fish during the trial

$$\boldsymbol{PI} = F \text{ x } p$$

*F* is the average feed intake (g) by each fish during the trial

*p* is the fraction of crude protein in the feed

$$\boldsymbol{PER} = \frac{(W_t - W_o)}{F \times p}$$

$W_t$ is the average weight (g) of fish at the end of the trial

$W_o$ is the average weight (g) of fish at the beginning of the trial

*F* is the average feed intake (g) by each fish during the trial

*p* is the fraction of crude protein in the feed

### 2.6.2 qPCR gene expression

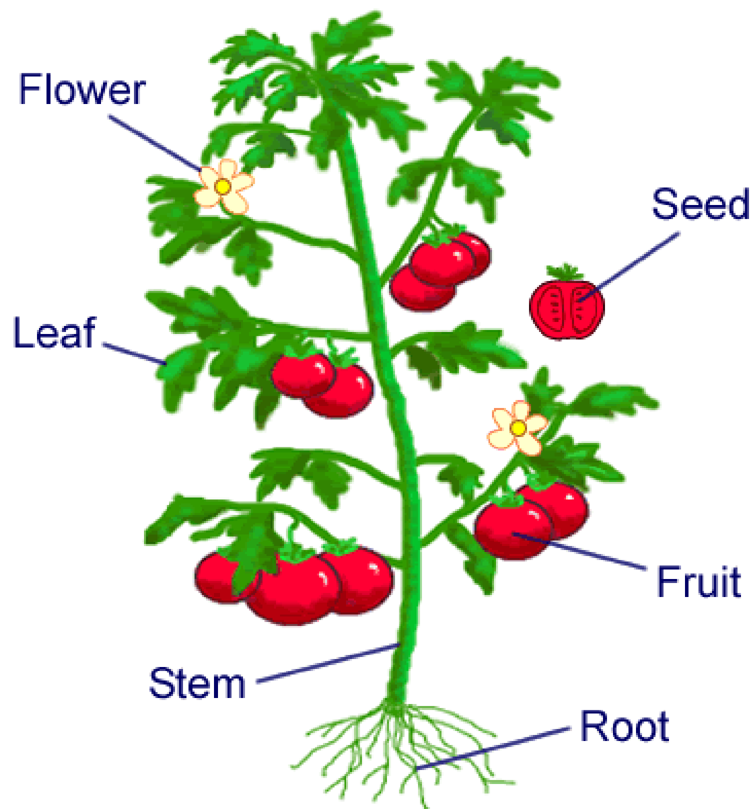
The relative expression for each gene was determined based on the PCR efficiency and the Ct 20 value of every sample compared with the control, and expressed in relation to the reference genes (*β-actin* and *ef-1α*) following the mathematical model of Pfaffl (Pfaffl, 2001). The data were normalised using the geometric mean expression of the references genes (*β-actin* and *ef-1α*).

## 2.7 Data analysis

Statistical analyses for all experiments were performed using SPSS version 19, Chicago, USA (Landau and Everitt, 2004). Data on performance measures and gene expression per treatment were expressed as means ± standard error. The data were first checked for normality of distribution using Kolmogorov-Smirnov test. Data that were not normally distributed was transformed using square root (final weight), natural logarithm (gene expression) or arcsin square-root (data in percentages and ratios as specified in Chapter 3, 4 and 5). To determine differences among treatments, one-way ANOVA was performed on the data followed by Tukey's test. Data that were not homogenous were analysed using Welch's test and Game-Howell's test to establish differences among treatments. The Pearson's correlation analysis was carried out on some performance parameters as detailed in Chapter 3, 4 and 5. Significant differences were considered at P value < 0.05.

# Chapter 3:

# Effects of selected phytogenic compounds on growth performance of Nile tilapia




Plant showing parts from which phytogenics can be derived
(Image from www.ncsu.edu/project/agronauts/mission2)

"*In every walk with nature, one receives far more than he seeks*"

**John Muir**

## 3.1 Introduction

The effects of phytogenics on the growth performance of fish have mainly been evaluated using herbs and EOs. Some authors report significant improvements in growth performance (Shalaby *et al*., 2006; Turan, 2006; Ji *et al*., 2007; Ashraf and Goda, 2008; Soltan and El-Laithy, 2008; Immanuel *et al.,* 2009; Khalafalla, 2009; Ndong and Fall, 2011; Guroy *et al*., 2012; Lee *et al*., 2012; Zaki, 2012; Maniat *et al*., 2014) while other found no enhanced growth (Mesalhy *et al*., 2008; Zakes, 2008; Kulthanaparamee *et al*., 2011; Ndong and Fall, 2011; Farahi *et al*., 2012; Yilmaz *et al*., 2012; Cho *et al*., 2013; Kim *et al*., 2013; Takaoka *et al*., 2016). These studies clearly demonstrated that not all herbs, EOs and doses have growth enhancing effects in every fish. In addition, because the concentration of bioactive compounds in herbs and EOs varies due to plant species and part used, chemistry of the soil where the plant was grown, season of harvest, method of processing, extraction and storage, it makes it difficult to obtain consistent results (Windisch *et al*., 2008; Applegate *et al*., 2010; Yang *et al*., 2015). Consequently, research is required to evaluate bioactive compounds of phytogenics in an effort to standardise the dose of active compounds.

Presently, few authors have investigated the effects of phytogenic compounds such as limonene, thymol and carvacrol on the growth of different fish species (Zheng *et al*., 2009; Rattanachaikunsopon and Phumkhachorn, 2010; Ahmadifar *et al*., 2011; Giannenas *et al*., 2012; Volpatti *et al*., 2013; Ahmadifar *et al*., 2014; Acar *et al*., 2015; Peterson *et al*., 2014, 2015; Perez-Sanchez *et al*., 2015; Yilmaz *et al*., 2015). Besides, there is scant literature on the effects of these compounds on the growth of Nile tilapia. Rattanachaikunsopon and Phumkhachorn (2010) fed Nile tilapia a diet with 200 ppm carvacrol but the fish did not attain improved somatic growth. In Mozambique tilapia (*Oreochromis mossambicus*), the weight gain of the fish was significantly improved

compared to the control with a diet formulated with 1000 ppm of an EO containing 83 % limonene (Acar *et al*., 2015). With channel catfish, rainbow trout and sturgeon, significant improvements in fish growth were reported with diets containing either carvacrol, thymol or their combination at different doses and feeding duration (Zheng *et al*., 2009; Ahmadifar *et al*., 2011; Ahmadifar *et al*., 2014). Conversely, no significant improvement in somatic growth of sea bass (*Dicentrarchus labrax*) and rainbow trout was found following feeding on diets supplemented with carvacrol at 250 ppm and 500 ppm for sea bass (Volpatti *et al*., 2013) or 1000, 3000 and 5000 ppm for rainbow trout (Yilmaz *et al*., 2015). On the other hand, Giannenas *et al*. (2012) reported significant improvement in the efficiency of feed utilisation but not body weight gain in rainbow trout fed on diets with 1000 ppm of phytogenic additive composed of either 6000 ppm thymol or 12000 ppm carvacrol. Overall, the literature indicates that there is an information gap on the impact of limonene, thymol and carvacrol on the growth of Nile tilapia. The aim of this experiment was therefore to investigate the effects of dietary supplementation of limonene, thymol and carvacrol on growth performance of Nile tilapia. These investigations are a crucial preliminary step for identification of specific phytogenic compounds and doses that can be used in the diet of Nile tilapia in order to enhance somatic growth.

## 3.2 Materials and methods

Three Trials (Trials I, II, and III) were carried out at the Aquaculture Research and Development Centre (ARDC) in Uganda. In each Trial, the effect of one individual phytogenic compound (limonene, carvacrol or thymol) on the growth performance of Nile tilapia was investigated. Trial I was conducted for 56 days, while Trials II and III were carried out for 42 days.

### 3.2.1 Experimental diets and fish

Two commercial Nile tilapia fingerling diets A - CP40 (Trials I and II) and B - CP35 (Trial III) produced in Uganda by Ugachick Poultry Breeders Ltd and at ARDC, respectively, were used as standard diets. These were the commercial diets available in Uganda at the time that the Trials were carried out. Specifically, during Trials I and II diet A was in supply (on the market) while diet B was the one available during Trial III. The phytogenic compounds were obtained from Sigma Aldrich Supplier, Uganda. In Trial I, limonene (97 %) was supplemented in diet A at 0, 100, 200, 300, 400, and 500 ppm denoted as L0, L100, L200, L300, L400 and L500 respectively. In Trial II diet A was supplemented with 0 (C0), 250 (C250), 500 (C500), 750 (C750) and 1000 (C1000) ppm of carvacrol (99 %). For Trial III, diet B was supplemented with 0 (T0), 250 (T250), 500 (T500), 750 (T750), 1000 (T1000) ppm of thymol (99.5 %). The required concentration of each phytogenic in the diet was prepared in 100 mL of absolute ethanol and evenly dispensed to 1 kg of feed and air-dried at room temperature for one day. The proximate composition of the experimental diets is provided in Table 3.1.

**Table 3.1:** Proximate composition of diet A (CP40) used in Trials I and II and diet B (CP35) during Trial III.

| Analysis | Diet A | Diet B |
|---|---|---|
| Dry matter (%) | 85.8 | 92.2 |
| Moisture (%) | 14.2 | 7.8 |
| Crude protein (%) | 41.4 | 33.5 |
| Crude lipid (%) | 10.0 | 5.3 |
| Crude ash (%) | 12.9 | 7.6 |
| Crude fibre (%) | 6.8 | 10.5 |
| Gross energy (Kj $g^{-1}$) | 19.0 | 16.8 |

Nile tilapia juveniles obtained from the same spawning batch were used in each Trial. The fish were acclimatised for two weeks before the start of the Trials. The initial weight of fish was 3.7 ± 0.0 g, 1.1 ± 0.0 g and 0.3 ± 0.0 g (mean ± standard error) for Trials I, II and III, respectively. The fish were measured individually to obtain those with similar initial weight between treatments. This was done while the fish were anaesthetised with a low dose of 0.02 g $L^{-1}$ clove oil in aerated water to reduce stress.

### 3.2.2 Experimental design

Trials I and II were conducted in 60 L glass tanks in a flow through system (1-2 L $min^{-1}$) from an underground water source with the water first pumped into a storage tank. Trial III was conducted in 20 L glass tanks because the fish were smaller than those used in Trials I and II. Each supplementation level of the phytogenic compound in the diet constituted a treatment and each treatment was tested in four replicate tanks in Trials I and II and three replicate tanks in Trial III. The treatments were distributed to the experimental tanks following a complete randomised design. In Trial I, each tank was stocked with 40 fish, whereas 51 and 30 fish were stocked in Trials II and III, respectively. The fish were fed to satiation by hand between 9:00 - 10:00 am and 4:00 - 5:00 pm. The quality of the inflowing water was monitored weekly during the Trials in order to maintain it within the optimal ranges for Nile tilapia growth. Dissolved oxygen, pH, water temperature and ammonia-nitrogen were measured as described in section 2.2. In Trials I and II, water in each fish rearing tank was heated using electric aquaria heating rods, the temperature of the water in the tanks was also monitored and regulated. Water quality parameters recorded during the three Trials are presented in Table 3.2 (means ± standard deviation).

**Table 3.2:** Dissolved oxygen, pH, temperature and ammonia-nitrogen levels measured in the water used for rearing Nile tilapia during Trials I, II and III.

| | Dissolved oxygen (mg $L^{-1}$) | pH | Temperature (°C) | Ammonia-nitrogen (mg $L^{-1}$) |
|---|---|---|---|---|
| *Trial I* | 7.6 ± 0.3 | 7.3 ± 0.2 | 27.3 ± 0.9 | < 0.2 |
| *Trial II* | 7.3 ± 0.2 | 7.0 ± 0.1 | 27.0 ± 1.0 | < 0.2 |
| *Trial III* | 7.1 ± 0.2 | 7.2 ± 0.2 | 24.5 ± 0.2 | < 0.2 |

*Trial I*, N = 28 measurements per water quality parameter
*Trial II* and *III*, N = 10 measurements per water quality parameter

When the Trials were terminated, the weight (precision 0.1 g) and total length (0.1 cm) for every fish was measured and recorded. These biometric measurements were carried out while the fish was anesthetised using clove oil at 0.02 g $L^{-1}$ of water for 3-5 min. A digital weighing scale measuring maximum 200 g (precision 0.1 g) was used to determine the weight of the fish. A smooth wooden board with a metre ruler was used for measuring the total length (cm) of the fish except for Trial III. The number of live fish in each tank was recorded for computing fish survival rates.

### 3.2.3 Proximate analysis of feed

The proximate composition (crude protein, lipid, fibre, ash, moisture, dry matter and gross energy) of the experimental diets was analysed following the procedures described by the AOAC (1990) and ISO / IEC 17025 as stated in section 2.4. Briefly, moisture content was considered the percentage (%) loss in weight of a sample placed in a vacuum oven at 105 °C to a constant weight while percentage dry matter was computed as the % retained weight of the original sample. Crude protein content was determined using the Kjeldahl method through a digester and distillation unit and the amount of crude lipid was determined gravimetrically using soxhlet extraction apparatus. Crude fibre content

was analysed as the amount of sample (insoluble residues) resistant to acid / alkaline hydrolysis. The quantity of crude ash was estimated by incineration of a sample in a muffle furnace and determination of ashed weight with an analytical balance. Gross energy content was analysed using a bomb calorimeter.

### 3.2.4 Growth performance computations

The final average weight of the fish (FW), daily growth coefficient (DGC), mean metabolic body weight (MBW), growth rate per metabolic body weight ($GR_{MBW}$), percentage (%) weight gain (% WG), percentage (%) fish survival, feed intake as a percentage of body weight per day (% FI), feed intake per metabolic body weight ($FI_{MBW}$), feed conversion ratio (FCR), and protein efficiency ratio (PER) were determined using the formulae:

FW (g) = total fish biomass at the end of the trial (g) / number of fish;

DGC = ((final average fish weight$^{(1/3)}$ (g) - initial average fish weight$^{(1/3)}$ (g)) / trial duration (d) x 100;

MBW = ((initial average fish weight/1000)$^{0.8}$ + (final average fish weight/1000)$^{0.8}$) / 2;

$GR_{MBW}$ = (body weight gain / ((initial average fish weight/1000)$^{0.8}$ + (final average fish weight/1000)$^{0.8}$) / 2) / trial duration (d);

% WG = ((final average fish weight (g) - initial average fish weight (g)) / initial average fish weight (g)) × 100;

% fish survival = (number of alive fish at end of the trial / initial number of fish stocked) × 100;

% FI = (100 × (average feed intake fish$^{-1}$ / ((initial average body weight ± final average body weight) / 2))) / trial duration (d);

$FI_{MBW}$ = average feed intake fish$^{-1}$ / metabolic body weight;

FCR = feed intake / weight gain;

PER = weight gain / protein intake.

### 3.2.5 Statistical data analysis

The data on growth and feed efficiency indicators were analysed using the Statistical Package for the Social Sciences (SPSS) version 19 (Chicago, USA). Kolmogorov-Smirnov test was performed to check if the data were normally distributed. The square-root (FW) and arcsin square-root (DGC, $GR_{MBW}$, % WG, % fish survival, % FI, $FI_{MBW}$, FCR, and PER) transformations were performed on data that were not normally distributed. Levene`s test for homogeneity of variance of the data was performed for each variable. One-way ANOVA and Tukey’s test were carried out to identify statistical differences between treatments. Data with heteroscedasticity of variances between treatments were subjected to Welch's and Game-Howell's tests to determine differences between treatments. All results are presented as means ± standard error with significant differences determined at P value < 0.05. Moreover, Pearson’s correlation analysis was carried out on the data from Trials I and III to show the relationship and level of correlation between different performance indicators. Specifically, the correlations between limonene concentration in the diet and either DGC as well as between FW and DGC were analysed in Trial I. In Trial III, the correlation between DGC and either % WG or $FI_{MBW}$ as well as between $FI_{MBW}$ and either % WG or $GR_{MBW}$ was assessed. The significance level of correlation was considered at P < 0.05.

## 3.3 Results

### 3.3.1 Effects of dietary limonene on the performance of Nile tilapia (Trial I)

The performance of Nile tilapia fed on diets with increasing quantities of limonene, 100 (L100), 200 (L200), 300 (L300), 400 (L400), 500 (L500) ppm and a control without limonene (L0) is presented in Table 3.3. Fish from treatments L400 and L500 had significantly ($P < 0.01$) higher final mean weights than those of the control and L200. The final fish weight, in absolute values, appeared to increase linearly with increasing concentration of limonene in the diet except for the lower fish weight with treatment L200. Although there were no statistical differences in daily growth coefficient (DGC) between the limonene supplemented diets and the control ($P = 0.847$), there was a significant positive correlation ($r = 0.851$, $P = 0.032$) between DGC and concentration of limonene in the diet; as the concentration of dietary limonene increased, DGC increased. In addition, DGC had a positive correlation ($r = 0.945$, $P = 0.004$) with final fish weight. Besides, growth rate per metabolic body weight ($GR_{MBW}$) and percentage weight gain (% WG) of the fish showed a tendency towards an increasing trend ($GR_{MBW}$; $P = 0.073$ and % WG; $P = 0.091$ ) in a limonene dose dependant pattern, although there were no significant differences between the diets supplemented with limonene and the control. Conversely, fish from treatment L200 had significantly lower $GR_{MBW}$ and % WG ($P = 0.045$ and 0.045 respectively) compared with L500 but had statistically similar values with the control. In this study (Trial I), dietary limonene did not significantly increase the survival of the fish compared to the control ($P = 0.076$).

**Table 3.3:** Initial mean weight, final mean weight, daily growth coefficient (DGC), growth rate per metabolic body weight ($GR_{MBW}$), percentage (%) weight gain (% WG), % survival, % feed intake per day (% FI), feed intake per metabolic body weight ($FI_{MBW}$), feed conversion ratio (FCR) and protein efficiency ratio (PER) of Nile tilapia fed on diets with increasing levels of limonene for 56 days (Trial I).

| **Parameter** | **Experimental diets** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **L0** | **L100** | **L200** | **L300** | **L400** | **L500** | **P value** |
| Initial mean weight (g) | 3.8 ± 0.0 | 3.7 ± 0.0 | 3.8 ± 0.0 | 3.8 ± 0.0 | 3.7 ± 0.0 | 3.8 ± 0.0 | NS |
| Final mean weight (g fish$^{-1}$) | 17.1 ± 0.4$^{ab}$ | 17.6 ± 0.3$^{bc}$ | 16.2 ± 0.3$^{a}$ | 18.0 ± 0.3$^{bc}$ | 18.8 ± 0.36$^{c}$ | 18.9 ± 0.3$^{c}$ | 0.000 |
| DGC (% d$^{-1}$ fish$^{-1}$) | 1.8 ± 0.0$^{ab}$ | 1.9 ± 0.1$^{ab}$ | 1.8 ± 0.0$^{a}$ | 1.9 ± 0.1$^{ab}$ | 2.0 ± 0.1$^{ab}$ | 2.0 ± 0.0$^{b}$ | 0.044 |
| $GR_{MBW}$ (g Kg$^{-0.8}$ d$^{-1}$) | 9.5 ± 0.0$^{ab}$ | 9.7 ± 0.4$^{ab}$ | 9.2 ± 0.1$^{a}$ | 9.8 ± 0.4$^{ab}$ | 10.1 ± 0.5$^{ab}$ | 10.2 ± 0.2$^{b}$ | 0.045 |
| % WG | 357.4 ± 2.9$^{ab}$ | 370.0 ± 27.8$^{ab}$ | 334.1 ± 9.9$^{a}$ | 382.1 ± 27.9$^{ab}$ | 402.1 ± 38.4$^{ab}$ | 403.9 ± 12.5$^{b}$ | 0.045 |
| % survival | 92.5 ± 5.0 | 95.0 ± 0.0 | 99.2 ± 0.8 | 98.1 ± 1.9 | 93.8 ± 1.6 | 96.3 ± 1.6 | NS |
| % FI (% body weight d$^{-1}$) | 4.9 ± 0.2 | 4.7 ± 0.2 | 4.8 ± 0.1 | 4.5 ± 0.2 | 4.5 ± 0.2 | 4.4 ± 0.1 | NS |
| $FI_{MBW}$ (g Kg$^{-0.8}$ d$^{-1}$) | 14.3 ± 0.2$^{ab}$ | 14.5 ± 0.4$^{ab}$ | 13.4 ± 0.2$^{a}$ | 14.9 ± 0.3$^{b}$ | 15.1 ± 0.2$^{b}$ | 15.0 ± 0.2$^{b}$ | 0.008 |
| FCR | 1.5 ± 0.0 | 1.5 ± 0.0 | 1.5 ± 0.0 | 1.5 ± 0.0 | 1.5 ± 0.0 | 1.5 ± 0.0 | NS |
| PER | 1.6 ± 0.0 | 1.6 ± 0.1 | 1.7 ± 0.0 | 1.6 ± 0.1 | 1.6 ± 0.1 | 1.6 ± 0.0 | NS |

*Experimental diets include: 0 (L0), 100 (L100), 200 (L200), 300 (L300), 400 (L400) and 500 (L500) ppm limonene. All values are means of treatments ± standard error. abc mean values with different superscripts in the same row are significantly different from each other at $P< 0.05$. NS refers to not significantly different values. For each treatment, N = 160 for initial mean weight; for final mean weight, N = number of fish alive at the end of the trial and N = 4 replicates for DGC, $GR_{MBW}$, % WG, % survival, % FI, FCR and PER.*

Feed intake as a percentage of body weight (% FI) did not differ between the treatments with limonene and the control (P = 0.504). Similarly, feed intake per metabolic body weight ($FI_{MBW}$) of the fish was not significantly different between the control and diets supplemented with limonene but treatment L200 had a significantly lower (P = 0.008) $FI_{MBW}$ from L300, L400, and L500. Dietary limonene did not improve feed conversion ratio (FCR) and protein efficiency ratio (PER) compared with the control (P = 0.363 and 0.345 respectively).

### 3.3.2 Effects of dietary carvacrol on the performance of Nile tilapia (Trial II)

Table 3.4 shows the performance of Nile tilapia fed on diets supplemented with carvacrol at 250 (C250), 500 (C500), 750 (C750), and 1000 (C1000) ppm as well as a control (C0) without carvacrol (Trial II).

**Table 3.4:** Initial mean weight, final mean weight, daily growth coefficient (DGC), growth rate per metabolic body weight ($GR_{MBW}$), percentage (%) weight gain (% WG), % survival, % feed intake per day (% FI), feed intake per metabolic body weight ($FI_{MBW}$), feed conversion ratio (FCR) and protein efficiency ratio (PER) of Nile tilapia fed on diets with increasing levels of carvacrol for 42 days (Trial II).

| **Parameter** | **Experimental diets** | | | | | |
|---|---|---|---|---|---|---|
| | **C0** | **C250** | **C500** | **C750** | **C1000** | **P value** |
| Initial mean weight (g) | 1.1 ± 0.2 | 1.1 ± 0.1 | 1.1 ± 0.2 | 1.1 ± 0.2 | 1.1 ± 0.1 | NS |
| Final mean weight (g $fish^{-1}$) | 6.0 ± 0.1 | 6.1 ± 0.1 | 6.1 ± 0.1 | 6.3 ± 0.1 | 6.1 ± 1.1 | NS |
| DGC (% $d^{-1}$ $fish^{-1}$) | 1.8 ± 0.1 | 1.9 ± 0.1 | 1.9 ± 0.0 | 1.9 ± 0.1 | 1.9 ± 0.0 | NS |
| $GR_{MBW}$ (g $Kg^{-0.8}$ $d^{-1}$) | 10.9 ± 0.2 | 11.1 ± 0.2 | 11.1 ± 0.1 | 11.2 ± 0.3 | 11.0 ± 0.1 | NS |
| % WG | 424.2 ± 17.6 | 436.7 ± 18.5 | 438.9 ± 6.1 | 450.9 ± 25.8 | 433.6 ± 5.6 | NS |
| % survival | 94.1 ± 1.8 | 95.6 ± 2.0 | 91.7 ± 2.8 | 89.7 ± 3.4 | 98.0 ± 1.4 | NS |
| % FI (% body weight $d^{-1}$) | 4.0 ± 0.1 | 4.2 ± 0.2 | 4.3 ± 0.2 | 4.0 ± 0.2 | 4.4 ± 0.1 | NS |
| $FI_{MBW}$ (g $Kg^{-0.8}$ $d^{-1}$) | 13.6 ± 0.3 | 13.8 ± 0.1 | 14.7 ± 0.6 | 14.6 ± 0.5 | 14.2 ± 0.6 | NS |
| FCR | 1.3 ± 0.1 | 1.3 ± 0.1 | 1.4 ± 0.1 | 1.2 ± 0.1 | 1.4 ± 0.0 | NS |
| PER | 1.9 ± 0.1 | 1.82 ± 0.1 | 1.8 ± 0.1 | 2.0 ± 0.1 | 1.7 ± 0.0 | NS |

*Experimental diets include: 0 (C0), 250 (C250), 500 (C500), 750 (C750) and 1000 (C1000) ppm carvacrol. All values are means of treatments ± standard error. NS refers to not significantly different values. N = 204 for initial mean weight; for final mean weight, N = number of fish alive at the end of the trial and N = 4 replicates for DGC, $GR_{MBW}$, % WG, % survival, % FI, FCR and PER.*

The final fish weight, DGC, $GR_{MBW}$, % WG, % survival, % FI, $FI_{MBW}$, FCR, and PER were not significantly different between the dietary treatments containing carvacrol and the control.

### 3.3.3 Effects of dietary thymol on the performance of Nile tilapia (Trial III)

The performance of Nile tilapia fed on diets with different concentrations of thymol, 250 (T250), 500 (T500), 750 (T750) and 1000 (T1000) ppm as well as a control diet without thymol (T0) is presented in Table 3.5. A significant ($P = 0.001$) increase in final mean fish weight was found with Nile tilapia fed on diet T750 compared with the control and diets T250 and T1000. There was a strong significant positive correlation ($r = 1.0$, $P < 0.001$) between the concentration of thymol in the diet and the final weight of the fish up to the concentration of 750 ppm with increasing quantity of thymol in the diet (250, 500 and 750 ppm). Although no significant difference in DGC, $GR_{MBW}$, % WG, % fish survival, % FI, $FI_{MBW}$ and feed utilisation efficiency indices (FCR, and PER) were found between the treatments, using one-way ANOVA, there was a significant positive correlation between $FI_{MBW}$ and either DGC, % WG or $GR_{MBW}$ ($r = 1.000$, $P < 0.01$; $r = 0.961$, $P = 0.039$ and $r = 1.000$, $P < 0.01$ respectively) up to 750 ppm thymol.

In Trials I, II and III, the highest PER values were found in Trial III (dietary thymol) followed by Trial II (dietary carvacrol) and lastly Trial I (dietary limonene) corresponding with the size of the fish (smaller, medium and larger, respectively).

**Table 3.5:** Initial mean weight, final mean weight, daily growth coefficient (DGC), growth rate per metabolic body weight ($GR_{MBW}$), percentage (%) weight gain (% WG), % survival, % feed intake per day (% FI), feed intake per metabolic body weight ($FI_{MBW}$), feed conversion ratio (FCR) and protein efficiency ratio (PER) of Nile tilapia fed on diets with increasing levels of thymol for 42 days (Trial III).

| Parameter | Experimental diets | | | | | |
|---|---|---|---|---|---|---|
| | **T0** | **T250** | **T500** | **T750** | **T1000** | **P value** |
| Initial mean weight (g) | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0. 1 | 0.3 ± 0.1 | NS |
| Final mean weight (g fish$^{-1}$) | 1.0 ± 0.3$^{a}$ | 1.1 ± 0.4$^{a}$ | 1.2 ± 0.4$^{ab}$ | 1.3 ± 0.4$^{b}$ | 1.1 ± 0.3$^{a}$ | 0.001 |
| DGC (% d$^{-1}$ fish$^{-1}$) | 0.82 ± 0.1 | 0.9 ± 0.1 | 1.0 ± 0.1 | 1.0 ± 0.1 | 0.9 ± 0.0 | NS |
| $GR_{MBW}$ (g Kg$^{-0.8}$ d$^{-1}$) | 1.3 ± 0.1 | 1.4 ± 0.1 | 1.4 ± 0.1 | 1.5 ± 0.1 | 1.4 ± 0.0 | NS |
| % WG | 258.0 ± 44.4 | 317.0 ± 54.3 | 334.3 ± 77.2 | 346.0 ± 49.1 | 283.0 ± 19.3 | NS |
| % survival | 74.4 ± 1.9 | 77.8 ± 4.84 | 83.3 ± 16.7 | 75.0 ± 3.2 | 75.6 ± 1.1 | NS |
| % FI (% body weight d$^{-1}$) | 5.9 ± 0.4 | 5.5 ± 0.4 | 5.2 ± 1.3 | 5.2 ± 0.4 | 5.5 ± 0.3 | NS |
| $FI_{MBW}$ (g Kg$^{-0.8}$ d$^{-1}$) | 2.5 ± 0.3 | 2.8 ± 0.3 | 2.8 ± 0.4 | 2.9 ± 0.2 | 2.7 ± 0.1 | NS |
| FCR | 2.2 ± 0.1 | 2.2 ± 0.0 | 2.2 ± 0.1 | 2.2 ± 0.0 | 2.3 ± 0.0 | NS |
| PER | 2.2 ± 0.2 | 2.0 ± 0.2 | 2.0 ± 0.2 | 2.0 ± 0.1 | 2.0 ± 0.1 | NS |

*Experimental diets include: 0 (T0), 250 (T250), 500 (T500), 750 (T750) and 1000 (T1000) ppm thymol. All values are means of treatments ± standard error. ab mean values with different supercripts in the same row are significantly different from each other at $P < 0.05$. NS refers to not significantly different values. N = 90 for initial mean weight; for final mean weight, N = number of fish alive at the end of the trial and N = 3 replicates for DGC, $GR_{MBW}$, % WG, % survival, % FI, FCR and PER.*

## 3.4 Discussion

Previous research on the impact of phytogenics on fish performance has mainly been conducted using herbs and EOs. Both significant (Shalaby *et al*., 2006; Turan, 2006; Ashraf and Goda, 2008; Mesalhy *et al*., 2008; Soltan and El-Laithy, 2008; Immanuel *et al.,* 2009; Khalafal, 2009; Ndong and Fall, 2011; Guroy *et al*., 2012; Lee *et al*., 2012; Zaki, 2012) and no significant (Zakes, 2008; Farahi *et al*., 2011; Kim *et al*., 2013) improvements in fish growth have been reported. This apparent discrepancy of results has been generally attributed to changing concentrations of the active compounds found in herbs and EOs making it difficult to obtain consistent results (Applegate *et al*., 2010; Yang *et al*., 2015). Therefore, research on the potential of active compounds of herbs and EOs to enhance fish performance has become a topical area (Applegate *et al*., 2010). For this reason, this experiment was conducted to investigate the effect of diets supplemented with limonene, thymol and carvacrol on the growth performance of Nile tilapia. The results indicated that, compared to unsupplemented diets (controls), diets supplemented with 400 ppm (L400) and 500 ppm (L500) limonene, as well as 750 ppm (T750) thymol had significant growth-promoting effects on Nile tilapia as discussed below.

The final mean weight of Nile tilapia fed on diets L400 and L500, as well as T750 ppm were higher than those of fish fed the control diets, but these diets did not enhance daily growth coefficient (DGC), growth rate per metabolic body weight ($GR_{MBW}$), percentage (%) weight gain (WG) and % fish survival compared with the control. Growth-promoting effects of limonene have also been reported in other fish species and monogastric animals (Juin *et al*., 2003; Acar *et al*., 2015; Dalkilic *et al*., 2015; Ngugi *et al*., 2016). With Japanese quails, 300 ppm of dietary orange EO containing 92.3 % limonene enhanced FW of quails from day 14-35 compared to the control (Dalkilic *et al*., 2015). Nor-Spice AB, a commercial product from concentrated citrus extract comprising mainly

limonene, also significantly improved the live weight of broilers compared to the control at a dietary concentration of 1000 ppm but had no effect on weight gain (Juin *et al*., 2003). With Mozambique tilapia, Acar *et al*. (2015) reported that a diet supplemented with 1000 ppm of an EO extract from sweet orange peels containing 83 % limonene improved the % WG of the fish compared to the control. In addition, 1000, 2000, 5000 and 8000 ppm of bitter lemon (*Citrus limon*) EO containing 81.4 % limonene improved FW, WG, SGR and FCR of ningu (*Labeo victorianus*) fingerlings (Ngugi *et al*., 2016).

With dietary thymol, Zheng *et al*. (2009) also did not find growth-promoting effects at 500 ppm in channel catfish after a 56-day feeding experiment. Similarly, Giannenas *et al*. (2012) reported no significant increase in the weight of adult rainbow trout (111.3 - 111.8 g) after 56 days feeding on a diet containing 1000 ppm of a phytogenic product containing 6000 ppm thymol. In the present study, the somatic growth (fold-increase) of the fish in Trial III that evaluated the effects of dietary thymol was however lower than the growth of Nile tilapia reported in other studies with fish fry of the same size range (Newman and Popma, 1995; Santos *et al*., 2013). This could be attributed to the temperature (24.5 ± 0.2 $^{o}$C) of the water in which the fish were reared. It is argued that although Nile tilapia can shrive at temperatures ranging from 22 - 28 $^{o}$C, the growth rate is lower at lower temperatures (Newman and Popma, 1995; El-sherif and El-feky, 2009).

No significant increment in fish weight or any other evaluated performance parameter was found with the diets supplemented with carvacrol. In agreement with the present results, Rattanachaikunsopon and Phumkhachorn (2010) did not find any significant improvement in the weight and survival of Nile tilapia fed on diets supplemented with 200 ppm carvacrol but higher inclusion levels were not investigated. Diets supplemented with 250 and 500 ppm carvacrol also did not significantly improve the final weight and feed utilisation of sea bass (Volpatti *et al*., 2013). Similarly, Yilmaz *et al*.

(2015) did not observe an improvement in growth performance (FW, WG, FCR and SGR) of rainbow trout fed on diets supplemented with 1000, 3000 and 5000 ppm carvacrol. Contrary to this study, channel catfish of 50 g attained a significantly higher final weight than the control with diets formulated with 500 ppm of carvacrol and a blend of carvacrol-thymol (Zheng *et al*., 2009). The reason for the discrepancy in results could not be explained.

In the present study, feed intake (as a percentage of body weight (% FI) and per metabolic body weight ($FI_{MBW}$)) was not increased by dietary limonene, carvacrol and thymol. This suggested that the improved growth of Nile tilapia found with diets supplemented with 400 and 500 ppm limonene, as well as 750 ppm thymol was not due to improved appetite (feed intake). Similarly, limonene did not influence the feed intake of Japanese quails (Dalkilic *et al*., 2015). Anderson *et al*. (2012) reported no influence of thymol at 67 and 201 ppm on feed intake of swine. With sea bass, carvacrol supplemented to the diet at 250 and 500 ppm did not also improve feed intake (Volpatti *et al*., 2013). Although some literature has stated that phytogenics can increase feed intake (appetite) of monogastric animals, the effects on feed intake are inconsistent (Applegate *et al*., 2010; Franz *et al*., 2010, Steiner and Syed, 2015; Yang *et al*., 2015; Zeng *et al*., 2015). Therefore, there is need to complement the information collected on feed intake in this study (Chapter 3) with investigations with other approaches such as gene expression. Specifically, the effects of the diets on the expression of genes regulating feed intake and appetite in fish (Volkoff *et al*., 2009), an investigation that was carried out in Chapter 4. In the present study (Chapter 3), feed utilisation efficiency (feed conversion ratio (FCR) and protein efficiency ratio (PER) was not improved by the diets supplemented with limonene, thymol and carvacrol. This suggested that the enhanced somatic growth of Nile tilapia found in this study with limonene and thymol supplemented diets was not due to efficient utilisation of nutrients in the diets but possibly a result of other nutritional factors like enhanced

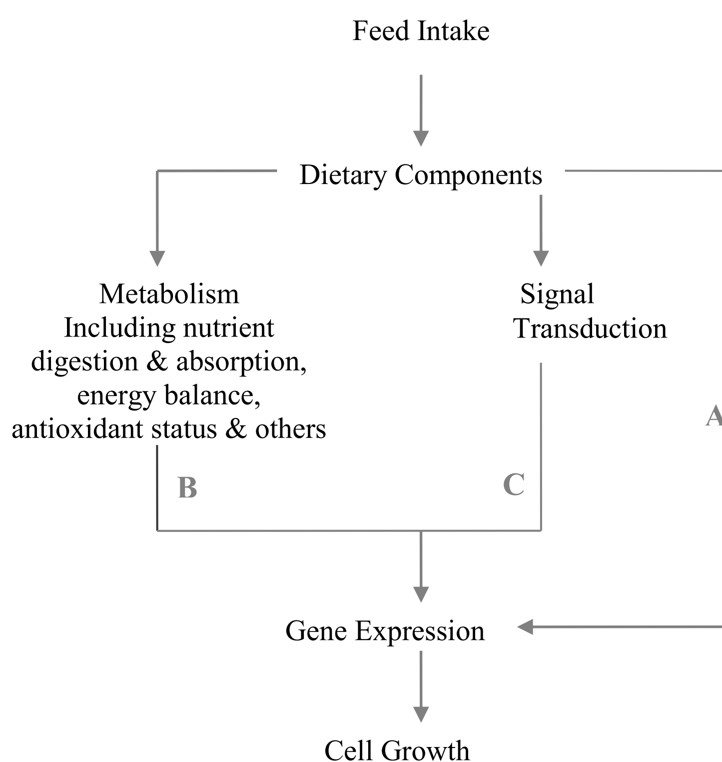
nutrient transportation and absorption, lipid and energy metabolism and antioxidant activity among others (Hashemi and Davoodi, 2010; Muthusamy and Sankar, 2015; Steiner and Syed, 2015; Yitbarek, 2015; Zeng *et al*., 2015; Abd El-Hack, *et al*., 2016). It is reported that phytogenics may use more than one mode of action to exert their effects on growth performance and the modes of actions are often interrelated (Hashemi and Davoodi, 2010; Rainer and Lea, 2015; Steiner and Syed, 2015).

The higher PER values found with thymol-fed fish compared to those fed on carvacrol and limonene supplemented diets is attributed to a physiological age/size-related effect with smaller fish utilising protein more effectively than bigger fish because they are in an active growth stage (Loum *et al*., 2013). In addition PER is known to be lower with higher quantity of protein in the diet (Sweilum *et al*., 2005). This is because at high protein levels in the diet, less feed is eaten to maintain a balance between energy for growth and metabolism (Miller, 2004).

In summary, the results of this experiment indicated that Nile tilapia diets supplemented with limonene at 400 and 500 ppm and thymol at 750 ppm had growth-promoting effect on Nile tilapia. No differences were observed in feed intake and feed utilisation efficiency thus, there is need to determine the pathways/mechanisms underlying the growth-promoting effects of limonene and thymol. Given that carvacrol did not show growth-enhancing effects, further experiments were not considered with carvacrol.

# Chapter 4:

## Effects of individual phytogenic compounds on the pathways of growth and nutritional physiology in Nile tilapia




Nutritional physiology schematic diagram
(Modified from http://nutrigenomics.ucdavis.edu/)

"*The goal of physiological research is functional nature*"

**Walter Rudolf Hess**

## 4.1 Introduction

Nutritional physiology mechanisms underlying the growth enhancing effects of phytogenic compounds are not fully understood. Although it is known that diets can influence appetite, nutrient digestion, absorption and transport, lipid metabolism, antioxidant enzyme activity and somatotropic axis-mediated growth among other processes (Rust, 2003; Qiang *et al*., 2012), there is little literature on the pathways activated by phytogenic compounds. In addition, different phytogenic compounds seem to have different effects on the physiology of animals (Lee *et al*., 2003a, b, 2004b; Lillehoj *et al*., 2011), thus potentially activating different pathways to enhance growth. Presently, most of the studies that have investigated the mode of action of phytogenic compounds in fish, pigs and chicken have been carried out using diets containing combinations of phytogenic compounds (Jamroz *et al*., 2003, 2005; Muhl and Liebert, 2007; Zheng *et al*., 2009; Ahmadifar *et al*., 2011; Li *et al*., 2012; Matysiak *et al*., 2012; Hahemipour *et al*., 2013; Awaad *et al*., 2014; Bravo *et al*., 2011, 2014; Perez-Sanchez *et al*., 2015; Gharib, 2014; Jiang *et al*., 2015; Hafeez *et al*., 2016; Hashemipour *et al*., 2016). From these studies, it is difficult to elucidate the specific pathways activated by individual compounds. Other studies, however, have investigated phytogenic compounds (such as carvacrol, thymol, cinnamaldehyde and capsicum oleoresin) individually (Lee *et al*., 2003a, b, 2004; Lillehoj *et al*., 2011; Galeotti *et al*., 2012; Yilmaz *et al*., 2014), although there is still limited knowledge on pathways that different phytogenic compounds influence in nutritional physiology, particularly for fish. For instance, carvacrol influenced lipid metabolism by lowering plasma triglyceride concentration but not plasma cholesterol in female broiler chicken, while thymol increased plasmatic levels of cholesterol (Lee *et al*. 2003b). Additionally, Lee *et al*. (2004a) found that cinnamaldehyde but not thymol had a trend towards improved lipid digestion in female broiler chicken. The effect of either carvacrol, cinnamaldehyde or capsicum oleoresin on the genome-wide profile of broiler chicken was

investigated by Lillehoj *et al*. (2011) who reported that some pathways related to lipid metabolism were activated by carvacrol compared to cinamaldehyde and capsicum oleoresin in the chicken. Carvacrol also down-regulated expression of oxidative stress markers (antioxidant enzymes) while cinnamaldehyde seemed to activate the pathways for carbohydrate metabolism to a further extent than the other tested compounds.

Amongst the studies that have been carried out on fish, Yilmaz *et al*. (2014) noted that carvacrol influenced the bioavailability of trace elements (copper, manganese and zinc) in rainbow trout. These micro-nutrients are important components of hormones and enzymes and activate enzymes that mediate nutritional physiology processes including feed intake, digestion, absorption and lipid metabolism among other functions (NRC, 2011). Meanwhile Galeotti *et al*. (2012) found no effect of diets with 250 and 500 ppm carvacrol on antioxidant enzyme status (SOD2) of juvenile European sea bass after a 63-days feeding experiment. Overall, the specific pathways activated by different phytogenic compounds in the nutritional physiology of fish are still poorly understood and to the best of our knowledge, there appear to be no such studies in Nile tilapia. In the previous experiment in Chapter 3, the phytogenic compounds limonene and thymol were found to have growth enhancing effects in Nile tilapia, whereas carvacrol did not exert any positive effect on fish performance. Therefore, the aim of this experiment was to identify pathways in the nutritional physiology of Nile tilapia underlying the growth-promoting effects shown by limonene and thymol in the previous trials. Specifically, the present study investigated the expression of genes regulating appetite, nutrient digestion and transport, lipid metabolism, antioxidant enzyme status and somatotropic axis-mediated growth in Nile tilapia after 63 days of feeding on diets with either limonene or thymol along with control diets.

## 4.2 Materials and methods

### 4.2.1 Experimental diets and fish feeding

Two feeding Trials (Trials IV and V) were carried out at the Institute of Aquaculture, University of Stirling, UK with the approval of the Divisional Ethics Committee of the Institute. In each Trial, the effects of either limonene (Trial IV) or thymol (Trial V) supplemented diets on the expression of genes of appetite, nutrient digestion and transport, lipid metabolism, antioxidant enzyme activity and somatotropic axis that regulate growth in Nile tilapia were investigated. Each Trial was carried out for 63 days. A commercial fish diet (INICIO Plus, BioMar Ltd., UK) was used as a standard diet to which limonene and thymol were supplemented at increasing concentrations. Limonene and thymol were obtained from Sigma Aldrich Ltd., UK. In Trial IV, limonene (97 % purity) was supplemented with 0 (control), 200 (L1), 400 (L2) and 600 (L3) mg per kilogram (ppm) of feed. In Trial V, the diet was supplemented with 0 (control), 250 (T1), and 500 (T2) ppm of thymol (99.5 % purity). In order to add the above concentrations of phytogenic compounds in the feed, the phytogenic compounds were dissolved in 100 ml of absolute ethanol and sprayed evenly onto 1 kg of feed. The same quantity of ethanol was added to the control to ensure similar conditions with the other diets and the diets were air-dried for one day before feeding the fish. Supplementation levels of thymol above 500 ppm investigated in Trial III (Chapter 3) were not included in this study (Trial V) because of ethical restrictions by the British Home Office. In both Trials IV and V, the fish were fed to apparent satiation by hand twice a day between 9:00 - 10:00 am and 4:00 - 5:00 pm. The amount of feed eaten was recorded daily. The proximate analysis of the standard diet is presented in Table 4.1.

**Table 4.1:** Proximate composition of the commercial diet used in Trials IV and V.

| **Analysis** | **Quantity** |
|---|---|
| Dry matter (%) | 92.3 |
| Moisture (%) | 7.7 |
| Crude protein (%) | 51.0 |
| Crude fat (%) | 20.9 |
| Crude ash (%) | 7.5 |
| Crude fibre (%) | 1.1 |
| Gross energy (Kj $g^{-1}$) | 22.6 |

The proximate nutritional composition (dry matter, moisture, protein, lipid, fibre, ash and gross energy) of the standard diet was determined according to the methods of the Association of Official Analytical Chemists (AOAC, 1990) and the joint technical committee of the International Organisation for Standardisation and International Electrotechnical Commission (ISO/IEC 17025) as described in section 2.4.1 (Chapter 2). Briefly, dry matter content was estimated by drying a sample in an oven at 105 ˚C to a constant weight and the percentage retained weight from the original sample was the amount of dry matter whereas the percentage loss in weight of the sample was the moisture content. Crude protein content was determined using the Kjeldahl method and crude lipid by petroleum ether extraction using the Soxhlet method. Crude fibre content was analysed by acid / alkaline hydrolysis of a sample and the amount of insoluble residues resistant to hydrolysis was fibre content. Crude ash was determined by combustion of a sample in a furnace at 600 ˚C for 24 h and the gross energy using the bomb calorimetry method.

### 4.2.2 Experimental fish and study design

Nile tilapia juveniles from the same spawning batch were obtained from the tropical aquarium at the Institute of Aquaculture, University of Stirling. The fish were size graded by measuring the weight of each fish to select those with similar weight. Before and during size grading, the fish were anaesthetised with a low dose of benzocaine of 0.05 g $L^{-1}$ for 3 - 5 min and kept in aerated water to minimise stress. After size grading, they were transferred to the rearing tanks. The initial weights of the fish were 1.5 ± 0.0 g for Trial IV and 1.5 ± 0.0 g for Trial V (mean ± standard error). Both trials were conducted in 60 L plastic tanks in a recirculating aquaculture system. Each concentration of phytogenic compound in the diet comprised a treatment and each treatment was assessed in three replicate tanks. The treatments were allocated using a complete randomised design to minimise confounding effects and variability between treatments. In Trial IV (limonene diets), each tank was stocked with 37 fish, whereas 25 fish were stocked in Trial V (thymol diets). The number of fish stocked was based on the numbers of similar-sized fish that were available / accessible when each of the trials was conducted.

In order to maintain the water quality in the experimental system within the conditions for the growth of Nile tilapia, the water quality was monitored and regulated every week. An oxygen meter (OaKton DO 6+, Eutech Instruments Pte Ltd, The Netherlands) was used to determine dissolved oxygen levels and water temperature. The pH and ammonia-nitrogen levels were measured using a fresh water test kit from Tropic Marin Company Ltd. (Germany) following the instructions from the manufacturer. The water temperature ranged from 26.0 to 27.0 $^{o}$C, pH was 6.8 ± 0.5, dissolved oxygen 6.8 ± 0.6 mg $L^{-1}$ and ammonia-nitrogen 0.8 ± 1.0 mg $L^{-1}$ (mean ± standard deviation). Every two or three weeks, the weight (0.1 g) and total length (0.1 cm) of each fish were measured using a digital weighing scale (Signum 1, Sartorius Company Ltd, Germany) and a calibrated metre ruler, respectively. During this exercise, the fish were anesthetised using

0.05 g $L^{-1}$ benzocaine for 3-5 min and thereafter returned to the rearing tank. The number of fish in each tank was recorded. At the end of the growth trials, fish were killed humanely with an overdose of benzocaine. Tissues of liver, fore intestine, and brain were dissected from three fish per replicate (N = 9 per treatment) and placed in 1.5 mL tubes containing RNAlater to preserve RNA integrity. The samples were kept at 4 ºC overnight and transferred to a -20 ºC freezer until RNA extraction.

### 4.2.3 Molecular analyses

#### *4.2.3.1 RNA extraction*

Total RNA from the tissue samples of brain, liver and fore intestine was extracted by homogenising the samples in TRI Reagent (Sigma Aldrich, Dorset, UK), following the protocol described in section 2.4.2.1 (Chapter 2). The quantity and quality of the RNA was assessed by spectrophotometry using an ND-1000 Nanodrop (Nanodrop 1000, Thermo Scientific, Glasgow, UK) at absorbance wavelength ratios of 260/280 and 260/230, respectively. The integrity of the RNA was verified by agarose gel electrophoresis using 200 ng RNA in 1 % agarose gel and 0.5 x TAE buffer containing 0.3 µl ethidium bromide (10 mg $mL^{-1}$). Intact RNA was considered to have clear 28S and 18S rRNA bands with the 28S rRNA band about twice as intense as the 18S rRNA band (Dundar *et al*., 2015).

#### *4.2.3.2 Complementary DNA (cDNA) synthesis*

Complementary DNA (cDNA) was synthesised from each RNA tissue sample (N = 9 per treatment) using a high capacity reverse transcription kit without RNase inhibitor from AB Applied Biosystems (Warrington, UK) following the procedure described in section 2.4.2.2 (Chapter 2).

#### ***4.2.3.3 Quantitative real-time Polymerase Chain Reaction (qPCR)***

Table 4.2 shows the nucleotide sequence of the primers for each of the analysed genes. The expression of the genes was analysed in either liver, fore intestine or brain tissue depending on the key organ where they perform their functions. For instance, the liver is a major site for various physiological processes including lipid metabolism, antioxidant enzyme defence and somatotropic axis growth mediation whereas the fore intestine is a focal location for digestion, absorption and transport of dietary nutrients. Brain, plays an important role in the regulation of fish appetite through the endocrine system.

Efficiency of the primers was assessed using the standard curve dilution method, melting curves and cycle threshold (Ct) values. Primers with efficiency values between 0.80 - 1.10, a single melting peak, Ct value below 30 and one clear band under 1 % agarose gel electrophoresis were considered for this study (Larionov *et al*., 2005). The relative expression of each gene was determined (N = 9 replicates per treatment) using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates. Each sample was analysed in duplicates of 20 µl reaction volumes comprising 5 µl of 20-fold diluted cDNA, 3 µl nuclease-free water, 1 µl (10 pmol) each for the forward and reverse primer of the candidate gene and 10 µl of Luminaris color higreen qPCR Mix (Thermo Scientific, Hemel Hempstead, UK). The same reaction volume was used for the reference genes but 6 µl of nuclease-free water and 2 µl of 20-fold diluted cDNA of each sample were used. In each qPCR, a calibrator sample (20-fold dilution of pool cDNA from all the samples) and a negative control (non template control-NTC) with no cDNA were included. This was to compare the gene expression among the different samples and control any genomic DNA contamination, respectively.

**Table 4.2:** Nucleotide sequences of primers used for quantitative real-time PCR analysis.

| Functional group | Gene symbol | Oligonucleotide sequences (5'- 3') | Size (bp) | Accession number* |
|---|---|---|---|---|
| Nutrient digestion, absorption and transport | *muc* | F: TGCCCAGGAGGTAGATATGC<br>R: TACAGCATGAGCAGGAATGC | 101 | XM_005466350.2 |
| | *pept1* | F: CAAAGCACTGGTGAAGGTCC<br>R: CACTGCGTCAAACATGGTGA | 196 | XM_013271589 |
| | *alp* | F: CTTGGAGATGGGATGGGTGT<br>R: TTGGCCTTAACCCCGCATAG | 200 | XM_005469634.2 |
| | *ctra* | F: AGTGCCGAGAACATCCAGAC<br>R: GAAGTCTCGGCCACACAAAC | 155 | XM_003437588.3 |
| | *pla2* | F: CTCCAAACTCAAAGTGGGCC<br>R: CCGAGCATCACCTTTTCTCG | 177 | XM_005451846 |
| | *glut2* | F: TCTAAAGGGGCCGCATGATC<br>R: GAAAGGTGCATCATGAGGGC | 153 | FJ914656 |
| | *ap* | F: TTACCACTCCGAACCAGACC<br>R: GAGTAGTTCCCTCCTGCCTC | 238 | XM_005449270 |
| | *p-amy* | F: TGGAGGCCCTGGTATCAAAG<br>R: TCCTGTTCCACCACCAGATC | 168 | XM_003448471.2 |
| Lipid metabolism | *lpl* | F: TGCTAATGTGATTGTGGTGGAC<br>R: GCTGATTTTGTGGTTGGTAAGG | 217 | NM_001279753.1 |
| | *pparα* | F: CTGATAAAGCTTCGGGCTTCCA<br>R: CGCTCACACTTATCATACTCCAGCT | 106 | NM_001290066.1 |
| | *srebf1* | F: TGCAGCAGAGAGACTGTATCCGA<br>R: ACTGCCCTGAATGTGTTCAGACA | 102 | XM_005457771.2 |
| | *fas* | F: TGAAACTGAAGCCTTGTGTGCC<br>R: TCCCTGTGAGCGGAGGTGATTA | 144 | GU433188 |
| Appetite regulation | *lepr* | F: TCTGTTCCTTTGGGCATAG<br>R: CCCTCCTGATGTCTTTTCCA | 143 | XM_005460446.1 |
| | *npy* | F: CCTCTTCCTTACGCATCAGC<br>R: CCCCTCCTCCACTTTACGAT | 115 | XM_003448854.2 |
| Antioxidant enzyme activity | *gpx* | F: ACAAGTGACATCGAGGCAGA<br>R: CAAACCCAGGCCTGCTATAA | 186 | NM_001279711.1 |
| | *cat* | F: TCCTGGAGCCTCAGCCAT<br>R: ACAGTTATCACACAGGTGCATCTTT | 79 | JF801726 |
| | *sod2* | F: CTCCAGCCTGCCCTCAA<br>R: TCCAGAAGATGGTGTGGTTAATGTG | 58 | XM_003449940.3 |
| Somato-tropic axis growth mediation | *gh* | F: TCGGTTGTGTGTTTGGGCGTCTC<br>R: GTGCAGGTGCGTGACTCTGTTGA | 90 | XM_003442542 |
| | *ghr-I* | F: ATGGCTCTCTCGCCCTCCTCTAA<br>R: ATGTCGTGTGGTCCCAGTCAGTGA | 109 | NM_001279601 |
| | *igf-I* | F: GTCTGTGGAGAGCGAGGCTTT<br>R: CACGTGACCGCCTTGCA | 70 | NM_001279503 |
| Reference genes | *ef-1α* | F: GCACGCTCTGCTGGCCTTT<br>R: GCGCTCAATCTTCCATCCC | 250 | NM_001279647 |
| | *ß-actin* | F: TGGTGGGTATGGGTCAGAAAG<br>R: CTGTTGGCTTTGGGGTTCA | 217 | XM_003443127.3 |

*muc* mucin-like protein, *pept1* oligo peptide transporter 1, *alp* alkaline phosphatase, *ctra* chymotrypsinogen A-like, *pla2* phospholipase A2, *glut2* glucose Transporter 2, *ap* aminopeptidase N-like, *p-amy* pancreatic alpha-amylase, *lpl* lipoprotein lipase, *pparα* peroxisome proliferator-activated receptor alpha, *srebf1* sterol regulatory element binding transcription factor 1, *fas* fatty acid synthase, *lepr* leptin receptor variant X1, *npy* pro-neuropeptide Y-like , *gpx* glutathione peroxidase, *cat* catalase, *sod2* superoxide dismutase 2, *gh* growth hormone, *ghr-I1* growth hormone receptor I, *igf-I* insulin growth factor I, *ef-1α* elongation factor 1a, *ß-actin* beta-actin.

*GenBank (http://www.ncbi.nlm.nih.gov/); bp, base pairs

Each qPCR cycle comprised pre-heating samples at 50 °C for 2 min followed by 35 cycles: initial denaturing at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 30 s. Using the equation of Pfaffl (2001), the relative expression of each gene among the different treatments was calculated from the PCR efficiency and Ct 20 values of each sample versus the control, and the data were normalised against the genomic mean expression of the references genes, *β-actin* and *ef-1α*.

### 4.2.4 Data computations

When the Trials (IV and V) were terminated, final average fish weight (FW), growth rate (GR), daily growth coefficient (DGC), weight gain (WG), percentage (%) weight gain (% WG), condition factor (CF), % fish survival, feed intake as percentage (% FI) of body weight per day, feed conversion ratio (FCR) and protein efficiency ratio (PER) of the fish in each treatment were determined using the following formulae:

FW (g) = total fish biomass at end of the trial (g) / number of fish at the end of the trial;

GR (g $d^{-1}$) = (final average fish weight (g) - initial average fish weight (g)) / duration of trial (d);

DGC = ((final average fish weight $^{(1/3)}$ (g) - initial average fish weight $^{(1/3)}$ (g)) / d**)** x 100;

WG (g $fish^{-1}$) = final average fish weight (g) - initial average fish weight (g)

% WG = (final average fish weight (g) - initial average fish weight (g)) / initial average fish weight (g) × 100;

CF = (final average fish weight / final average total $length^3$) × 100;

% survival = (number of fish at end of the trial / initial number of fish) × 100;

% FI = (100 × (average feed intake per fish / ((initial average body weight ± final average body weight)/2))) / duration of trial (d);

FCR = average feed intake per fish / average weight gain;

FCE = ((final average fish weight - initial average fish weight) / average feed intake per fish) x 100.

PER = weight gain / protein intake;

### 4.2.5 Statistical data analysis

The Statistical Package for the Social Sciences (SPSS) version 19 (Chicago, USA) was used to perform the statistical analyses. For each treatment, data on performance indicators and gene expression are presented as means ± standard error. Normality of distribution of the data was assessed using Kolmogorov-Smirnov. Data not normally distributed were subjected to square-root (FW), natural logarithm (qPCR data) and arcsin square-root (GR, DGC, % WG, CF, % fish survival, % FI, FCR, FCE and PER) transformation. Differences among treatments were analysed by one-way ANOVA followed by Tukey's test. If heterogeneity of variances existed Welch's test was used with Game-Howell's test to establish differences among treatments. Significant differences were considered at P value < 0.05. The interaction between the duration of the growth trial (days) and supplementation level of limonene or thymol in the diet was analysed using a two-way ANOVA.

## 4.3 Results

### 4.3.1 Growth performance

#### *4.3.1.1 Performance of Nile tilapia fed on limonene supplemented diets (Trial IV)*

The growth performance of Nile tilapia fed on diets supplemented with limonene for 63 days (Trial IV) is presented in Table 4.3.

**Table 4.3:** Performance and survival rates of Nile tilapia fed on diets with 0 (control), 200 (L1), 400 (L2), and 600 (L3) ppm of limonene after 63 days (Trial IV).

| Parameter | Experimental diets | | | | |
|---|---|---|---|---|---|
| | **Control** | **L1** | **L2** | **L3** | **P value** |
| Initial mean weight (g) | 1.5 ± 0.0 | 1.5 ± 0.0 | 1.5 ± 0.0 | 1.5 ± 0.0 | NS |
| Final mean weight (g) | 40.6 ± 1.4$^{a}$ | 44.0 ± 1.4$^{ab}$ | 46.4 ± 1.5$^{b}$ | 47.2 ± 1.9$^{b}$ | 0.012 |
| GR (g $d^{-1}$) | 0.6 ± 0.0 | 0.7 ± 0.0 | 0.7 ± 0.0 | 0.7 ± 0.0 | NS |
| DGC (% $d^{-1}$) | 3.6 ± 0.0$^{a}$ | 3.8 ± 0.0$^{ab}$ | 3.9 ± 0.0$^{b}$ | 3.9 ± 0.1$^{b}$ | 0.043 |
| WG (g $fish^{-1}$) | 39.1 ± 0.7$^{a}$ | 42.6 ± 0.5$^{ab}$ | 44.9 ± 0.6$^{b}$ | 45.8 ± 2.2$^{b}$ | 0.006 |
| % WG | 2657.3 ± 44.7$^{a}$ | 2917.0 ± 33.5$^{ab}$ | 3055.6 ± 38.1$^{b}$ | 3133.0 ± 149.1$^{b}$ | 0.004 |
| CF | 2.0 ± 0.0 | 2.0 ± 0.0 | 2.0 ± 0.0 | 2.0 ± 0.0 | NS |
| % Survival | 90.1 ± 5.0 | 95.5 ± 4.5 | 96.4 ± 1.8 | 97.3 ± 0.0 | NS |
| % FI (% body weight $d^{-1}$) | 3.2 ± 0.1 | 3.0 ± 0.1 | 2.9 ± 0.0 | 2.8 ± 0.1 | NS |
| FCR | 1.1 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | NS |
| FCE | 91.3 ± 2.3 | 99.1 ± 1.8 | 103.2 ± 0.5 | 104.9 ± 3.95 | NS |
| PER | 1.8 ± 0.1 | 1.9 ± 0.0 | 2.0 ± 0.0 | 2.1 ± 0.1 | NS |

*GR, growth rate; DCG, daily growth coefficient; WG, weight gain; % WG, percentage (%) weight gain; CF, condition factor; % FI, feed intake as percentage of body weight per day; FCR, feed conversion ratio; FCE, feed conversion efficiency, and PER, protein efficiency ratio. All values are means of treatments ± standard error. Mean values with different superscript in the same row are significantly different from each other ($P < 0.05$). NS, not significantly different values. For each treatment, N = 111 for initial fish weight; for final fish weight, N = number of alive fish at the end the trial; N = 3 replicates for GR, DGC, % WG, CF, % survival, % FI, FCR, FCE and PER.*

A significant increase in final mean weight (FW), growth rate (GR), daily growth coefficient (DGC), weight gain (WG) and percentage (%) WG (% WG) were found with fish fed on the diets with 400 (L2) and 600 (L3) ppm limonene compared to the control. CF and fish survival were not significantly different among treatments. The mean weight of the fish assessed at different cumulative days (1, 14, 28, 42 and 63 d) of the trial is presented in Figure 4.1. There were no significant differences in fish weight between the treatments on day 1, 14 and 28 but significantly higher fish weights compared to the control were found with diet L2 and L3 on day 42 ($P = 0.008$) and 63 ($P = 0.012$) of the feeding Trial. A two-way ANOVA on the influence of limonene inclusion level (0 (control), 200, 400, 600 ppm limonene) in the diet and cumulative fish rearing days (1, 14, 28, 42 and 63 d) on fish weight showed a significant interaction $F (12, 1771) = 2.17$ ($P < 0.05$; Table 4.4). An increase in cumulative fish rearing days and concentration of limonene in the diet increased the weight of the fish. An increase in the number of days (time) of the growth trial had a bigger contribution ($P < 0.0001$) than the concentration of limonene in the diet ($P < 0.005$) in influencing the somatic growth (fish weight) suggesting that the effectiveness of limonene in enhancing growth was largely time dependent.

No significant differences were found in % feed intake (% FI) per fish body weight, feed conversion ratio (FCR), feed conversion efficiency (FCE) and protein efficiency ratio (PER) between the treatments but there was a strong tendency towards a significantly lower % FI ($P = 0.09$) and higher FCE ($P = 0.072$) and PER ($P = 0.07$) with diet L2 compared with the control.

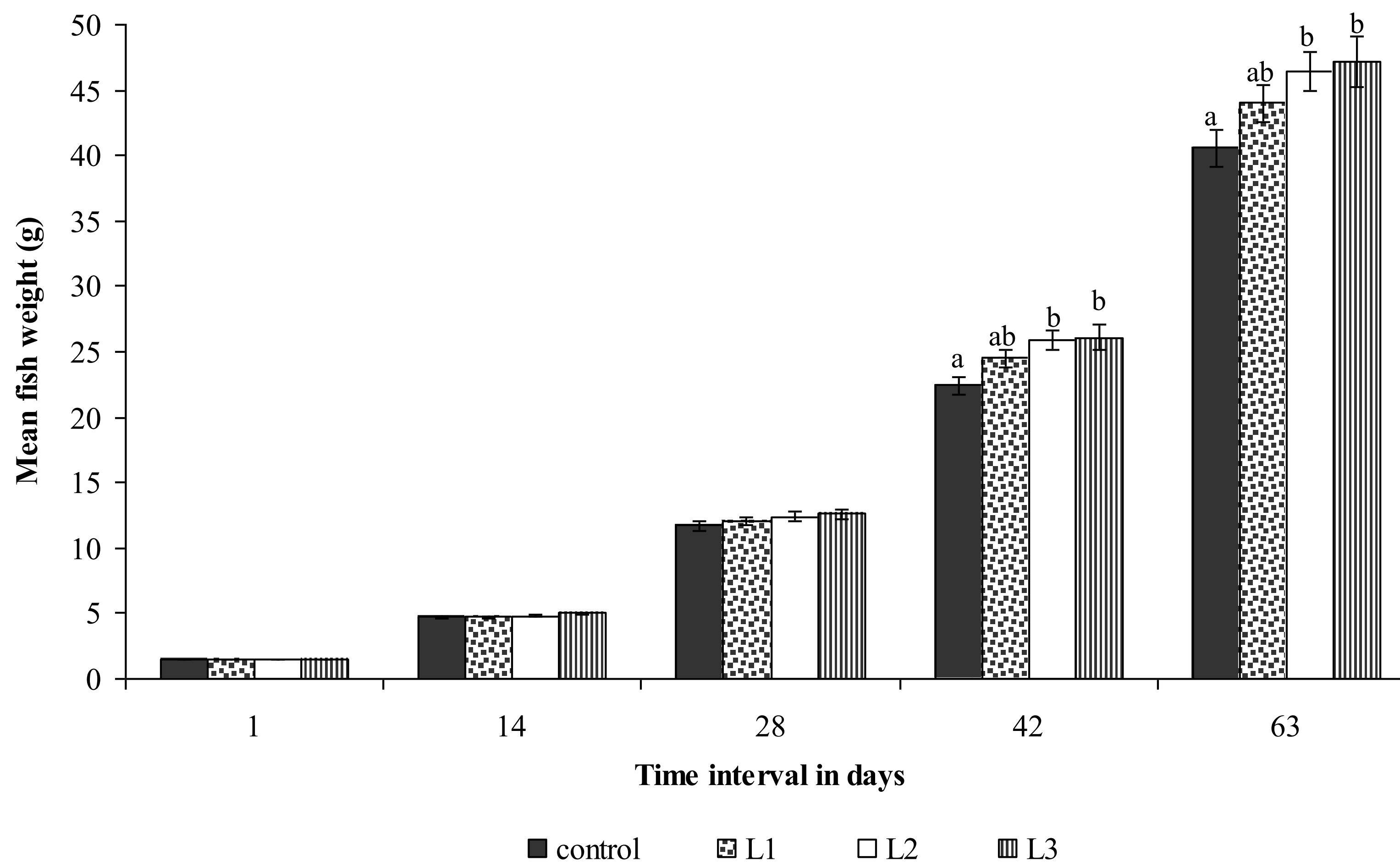



**Figure 4.1:** Mean weight of fish fed on diets supplemented with 0 (control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene on day 1, 14, 28, 42 and 63 of Trial IV. All values are means of treatments ± standard error. Mean values with different superscript in the same time interval are significantly different from each other ($P < 0.05$).

**Table 4.4:** Two-way ANOVA on the effects of diet type and growth duration on the weight of Nile tilapia fed on limonene supplemented diets for 63 days (Trial IV).

| Variable | Fish weight |
|---|---|
| Growth duration (days) | * |
| Diet type | ** |
| Growth duration × diet type | *** |

* $P < 0.0001$, ** $P < 0.0005$, *** $P < 0.05$

#### *4.3.1.2 Performance of Nile tilapia fed on thymol supplemented diets (Trial V)*

Though not significantly different, there was a consistent dose-dependent increase in the absolute values for final mean fish weight, DGC, WG and % WG (Table 4.5) with thymol supplemented diets.

**Table 4.5:** Performance and survival rates of Nile tilapia fed on diets with 0 (control), 250 (T1), and 500 (T2) ppm of thymol after 63 days (Trial V).

| **Parameter** | **Experimental diets** | | | |
|---|---|---|---|---|
| | **Control** | **T1** | **T2** | **P value** |
| Initial mean weight (g) | 1.5 ± 0.1 | 1.5 ± 0.1 | 1.5 ± 0.1 | NS |
| Final mean weight (g) | 37.9 ± 1.3 | 40.1 ± 1.5 | 41.0 ± 1.4 | NS |
| GR (g $d^{-1}$) | 0.6 ± 0.0 | 0.6 ± 0.0 | 0.6 ± 0.2 | NS |
| DGC (% $d^{-1}$) | 3.5 ± 0.0 | 3.6 ± 0.1 | 3.6 ± 0.1 | NS |
| WG (g fish $^{-1}$) | 36.4 ± 0.5 | 38.4 ± 2.2 | 39.4 ± 1.4 | NS |
| % WG | 2361.0 ± 32.0 | 2507.4 ± 140.7 | 2555.8 ± 87.9 | NS |
| CF | 1.9 ± 0.0 | 1.9 ± 0.0 | 1.9 ± 0.0 | NS |
| % Survival | 96.0 ± 2.3 | 93.3 ± 2.7 | 90.7 ± 3.5 | NS |
| % FI (% body weight $d^{-1}$) | 3.5 ± 0.0 | 3.2 ± 0.1 | 3.2 ± 0.01 | NS |
| FCR | 1.1 ± 0.0 | 1.0 ± 0.1 | 1.0 ± 0.0 | NS |
| FCE | 94.3 ± 1.1 | 103.7 ± 4.7 | 103.7 ± 3.3 | NS |
| PER | 1.9 ± 0.0 | 2.0 ± 0.1 | 2.0 ± 0.1 | NS |

*GR, growth rate; DGC, daily growth coefficient; WG, weight gain; % WG, percentage (%) weight gain; CF, condition factor; % survival, % FI, feed intake as percentage of body weight per day; FCR, feed conversion ratio; FCE, feed conversion efficiency and PER, protein efficiency ratio. All values are means of treatments ± standard error. NS, not significantly different values (P > 0.05). For each treatment, N = 75 for intial fish weight, for final fish weight, N = number of alive fish at end of the trial, N = 3 replicates for GR, DGC, WG, CF, % survival, % FI, FCR, FCE and PER.*

Fish weights measured during day 1, 14, 28, 42 and 63 of the trial did not show significant differences between the treatments (Figure 4.2).

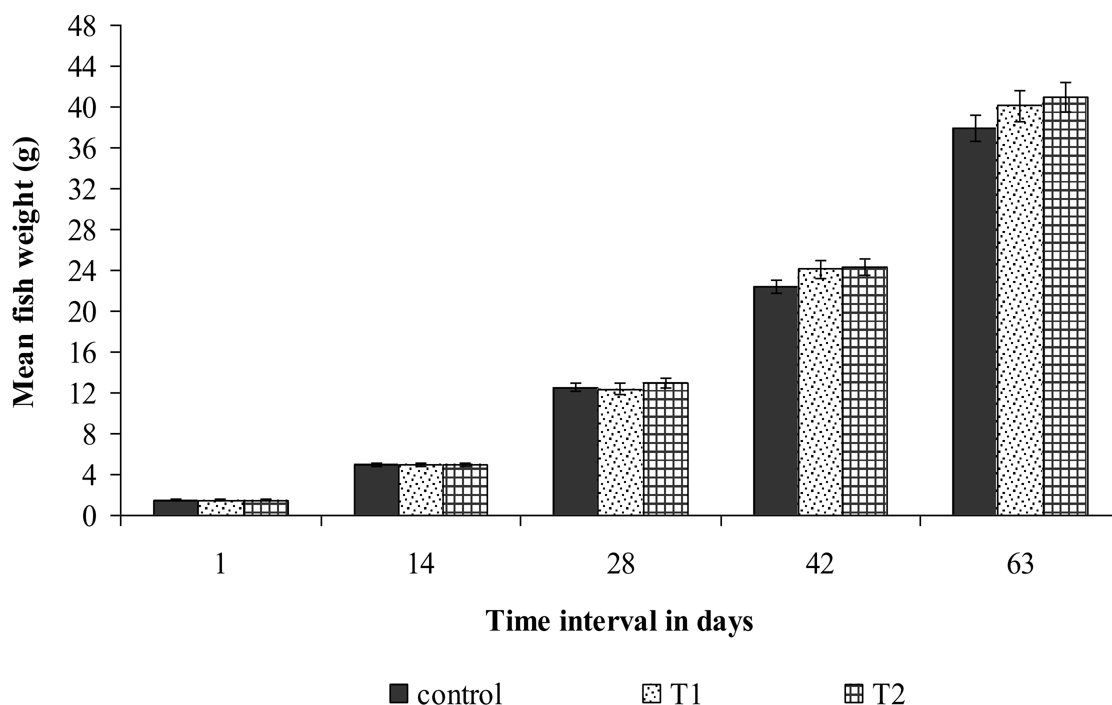



**Figure 4.2:** Mean weight of fish fed on diets supplemented with 0 (control), 250 (T1), and 500 (T2) ppm thymol on day 1, 14, 28, 42 and 63 of Trial V. All values are means of treatments ± standard error.

A two-way ANOVA on the impact of diets containing thymol (0, 250 and 500 ppm) and the duration of the growth trial (1, 14, 28, 42 and 63 d) on the fish weight found no significant interaction of thymol inclusion level and growth duration, $F (8, 927) = 0.71$, $P > 0.05$ (Table 4.6). Only growth duration (time) had a significant effect on the weight of the fish $F (4, 927) = 938.0$, $P < 0.0001$. Fish mortality did not differ significantly between treatments with increasing concentration of thymol in the diets ($P = 0.404$). There were also no significant differences in % FI and the feed utilisation efficiency indicators (FCR, FCE and PER) among treatments (Table 4.5).

**Table 4.6:** Two-way ANOVA on the effects of diet type and growth duration on the weight of Nile tilapia fed on thymol supplemented diets for 63 days (Trial V).

| Variable | Fish weight |
|---|---|
| Growth duration (days) | * |
| Diet type | NS |
| Growth duration × diet type | NS |

** P < 0.0001, NS, not significantly different (P < 0.05).*

### 4.3.2 Relative expression level for candidate genes

Figures 4.3 and 4.4 show heat maps representing relative expression patterns, not based on statistical significance of the marker genes involved in the pathways of somatotropic axis growth-mediation (a), lipid and energy metabolism (b), and antioxidant enzyme defence (c) in the liver, nutrient digestion, absorption and transport (d) in the fore intestine and appetite regulation (e) in the brain of Nile tilapia fed on limonene and thymol supplemented diets, respectively. The fish fed on limonene supplemented diets had the highest number of genes activated with higher relative expression patterns (red).

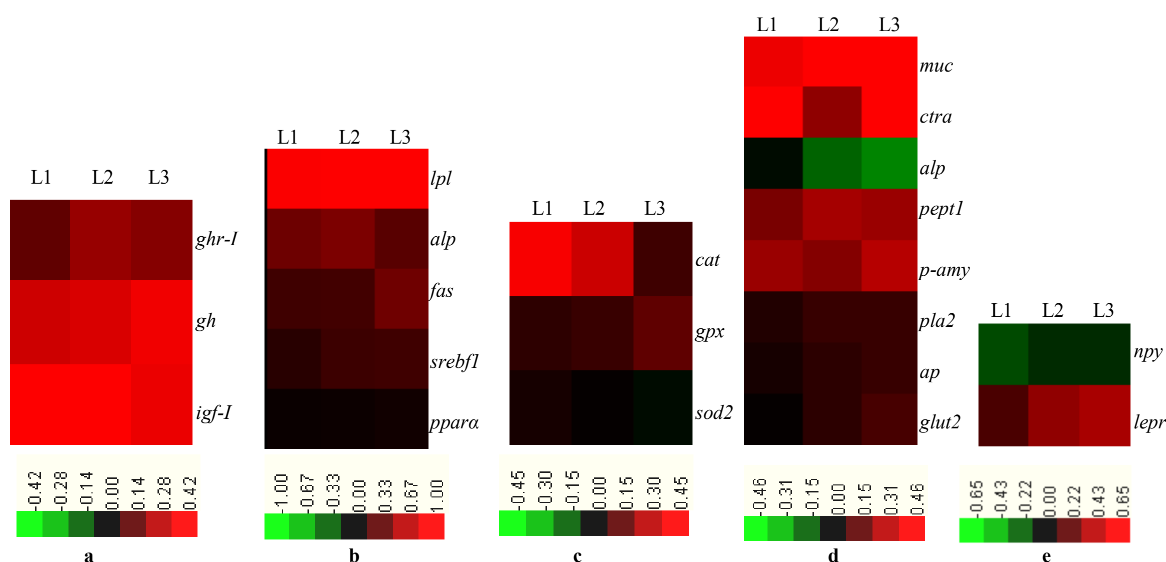



**Figure 4.3:** Heat map indicating expression patterns of twenty selected genes analysed using qPCR data from Nile tilapia fed limonene supplemented diets.

The columns represent mean data values of three dietary treatments L1, L2 and L3 (200, 400 and 600 ppm limonene, respectively). The rows indicate each of the analysed genes in the pathways of somatotropic axis growth-mediation (a), lipid and energy metabolism (b), and antioxidant enzyme defence (c) in the liver, nutrient digestion, absorption and transport (d) in the fore intestine and appetite regulation (e) in the brain of Nile tilapia. Expression level of each gene was natural log transformed and normalised against two reference genes. The colour bars at the bottom represent the mean relative expression levels as low (green), neutral (black) or high (red). The black colour represents genes that had similar relative expression level with the control. *lpl*, lipoprotein lipase; *gpx*, glutathione peroxidase; *pparα*, peroxisome proliferator activated receptor alpha; *cat*, catalase; *fas*, fatty acid synthase; *gh*, growth hormone; *ghr-I*, growth hormone receptor I; *srebf1*, sterol regulatory element-binding transcription factor 1; *pla2*, phospholipase A2; *igf-I*, insulin growth factor I; *alp*, alkaline phosphatase; *muc*, mucin-like protein; *ctra*, chymotrypsin A-like; *sod2*, superoxide dismutase 2; *ap*, aminopeptidase N-like; *glut2*, glucose transporter 2; *pept1*, oligo-peptide transporter 1; *p-amy*, pancreatic alpha-amylase; *npy*, pro-neuropeptide Y-like; *lepr*, leptin receptor variant XI.

The fish fed on thymol supplemented diets (Figure 4.4) exhibited more stable (black to marron) or low (green) gene expression patterns.

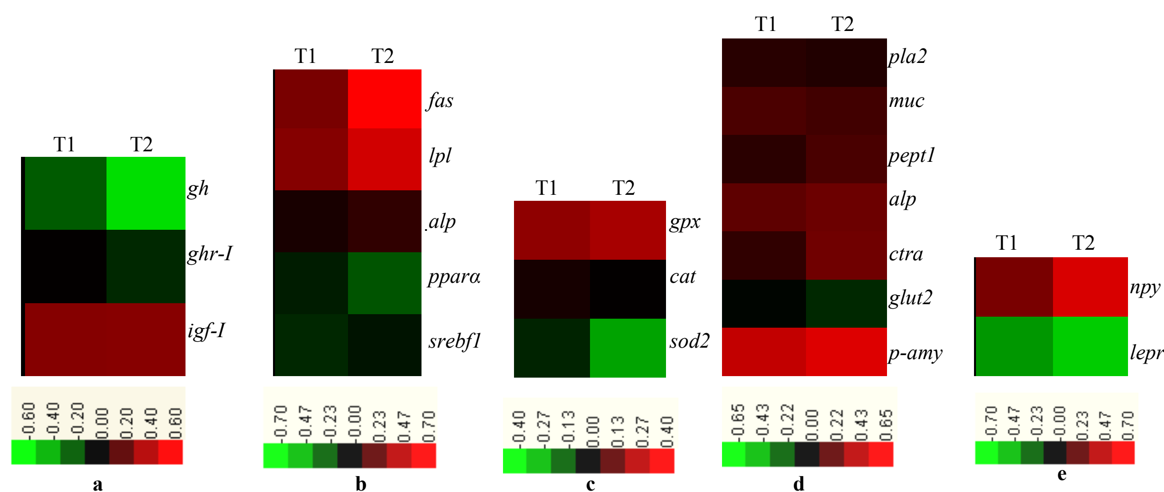



**Figure 4.4:** Heat map indicating expression patterns of twenty selected genes analysed using qPCR data from Nile tilapia fed thymol supplemented diets.

The columns represent mean data values of two dietary treatments T1 and T2 (250 and 500 ppm thymol respectively). The rows indicate each of the analysed genes in the pathways of somatotropic axis growth-mediation (a), lipid and energy metabolism (b), and antioxidant enzyme defence (c) in the liver, nutrient digestion, absorption and transport (d) in the fore intestine and appetite regulation (e) in the brain of Nile tilapia. Expression level of each gene was natural log transformed and normalised against two reference genes. The colour bars at the bottom represent the mean relative expression levels as low (green), neutral (black) or high (red). The black colour represents genes that had similar relative expression level with the control. *lpl*, lipoprotein lipase; *gpx*, glutathione peroxidase; *pparα*, peroxisome proliferator activated receptor alpha; *cat*, catalase; *fas*, fatty acid synthase; *gh*, growth hormone; *ghr-I*, growth hormone receptor I; *srebf1*, sterol regulatory element-binding transcription factor 1; *pla2*, phospholipase A2; *igf-I*, insulin growth factor I; *alp*, alkaline phosphatase; *muc*, mucin-like protein; *ctra*, chymotrypsin A-like; *sod2*, superoxide dismutase 2; *ap*, aminopeptidase N-like; *glut2*, glucose transporter 2; *pept1*, oligo-peptide transporter 1; *p-amy*, pancreatic alpha-amylase; *npy*, pro-neuropeptide Y-like; *lepr*, leptin receptor variant XI.

#### *4.3.2.1 Expression of somatotropic axis genes in the liver of Nile tilapia fed on diets supplemented with limonene and thymol*

Insulin growth factor I (*igf-I*) expression was significantly ($P < 0.01$) up-regulated in the liver of fish fed diets supplemented with limonene compared to the control-fed fish (Figure 4.5). On the other hand, there was no significant difference in the expression of *gh* ($P = 0.519$) and *ghr-I* ($P = 0.311$) in the livers of fish fed on limonene supplemented diets (L1, L2, L3) and the control-fed fish.

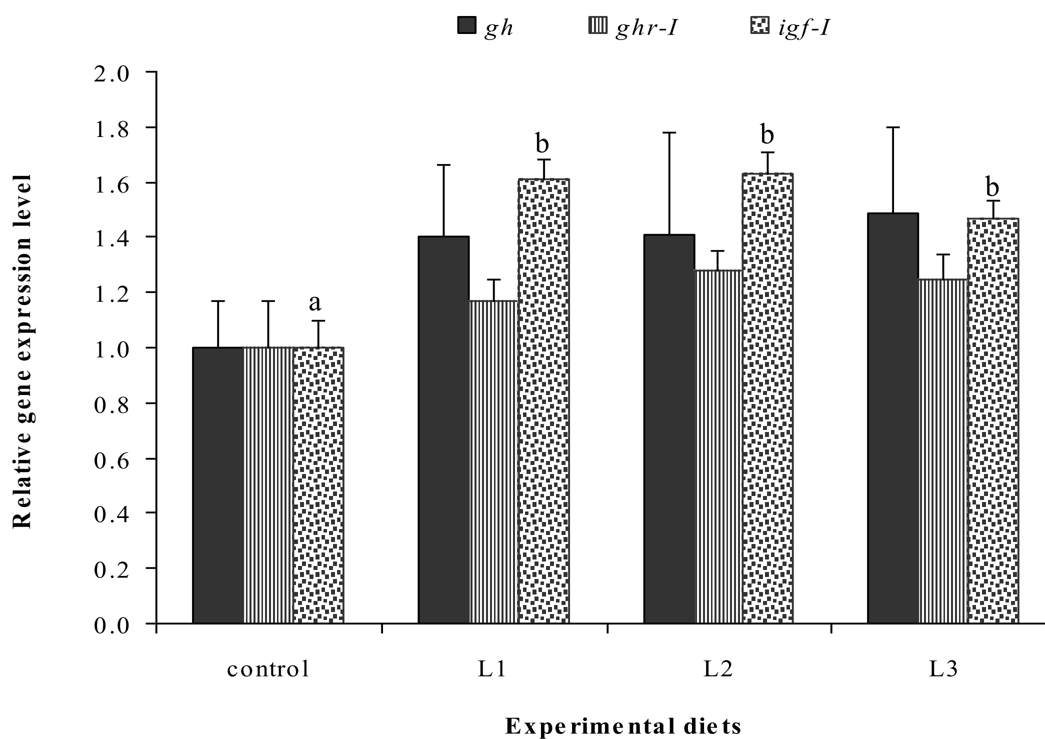



**Figure 4.5:** Expression of growth hormone (*gh*), growth hormone receptor I (*ghr-I*) and insulin growth factor I (*igf-I*) in the liver of Nile tilapia fed on diets with 0 (control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene. Different superscript letters denote significant differences among treatments.

The mRNA levels of *gh*, *ghr-I* and *igf-I* were not significantly different between treatments among livers of fish-fed the thymol supplemented diets compared to the control (Figure 4.6).

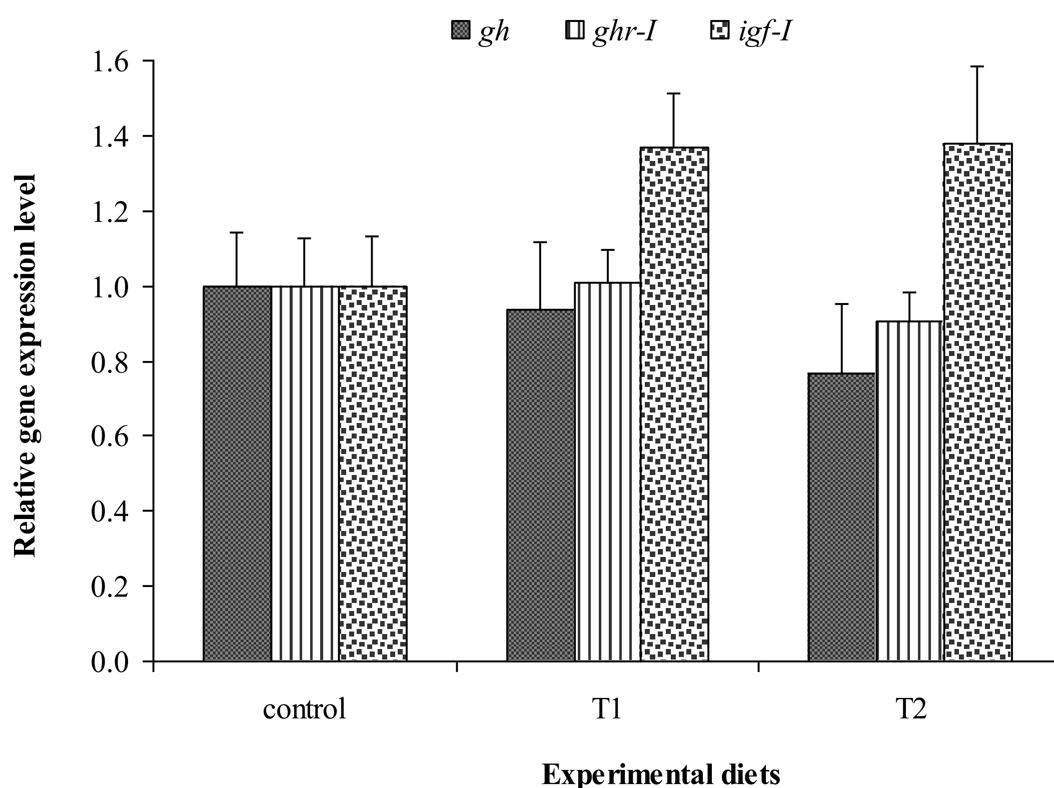



**Figure 4.6:** Expression of growth hormone (*gh*), growth hormone receptor I (*ghr-I*) and insulin growth factor I (*igf-I*) in the liver of Nile tilapia fed on diets with 0 (control), 250 (T1), and 500 (T2) ppm thymol.

#### *4.3.2.2 Expression of genes involved in regulating lipid metabolism in the liver of Nile tilapia fed on diets supplemented with limonene and thymol*

Significantly higher expressions of *lpl* (P = 0.002) and *alp* (P = 0.038) were found in the liver of fish fed on the limonene supplemented diets compared to the control (Figure 4.7). The differences in the expression of *lpl* were between the treatments L1, L2, L3 and the control while *alp* was higher with diet L2 compared to the control. No significant effect of dietary limonene was found in the expression of *fas*, *srebf1* and *pparα* in the liver of the fish.

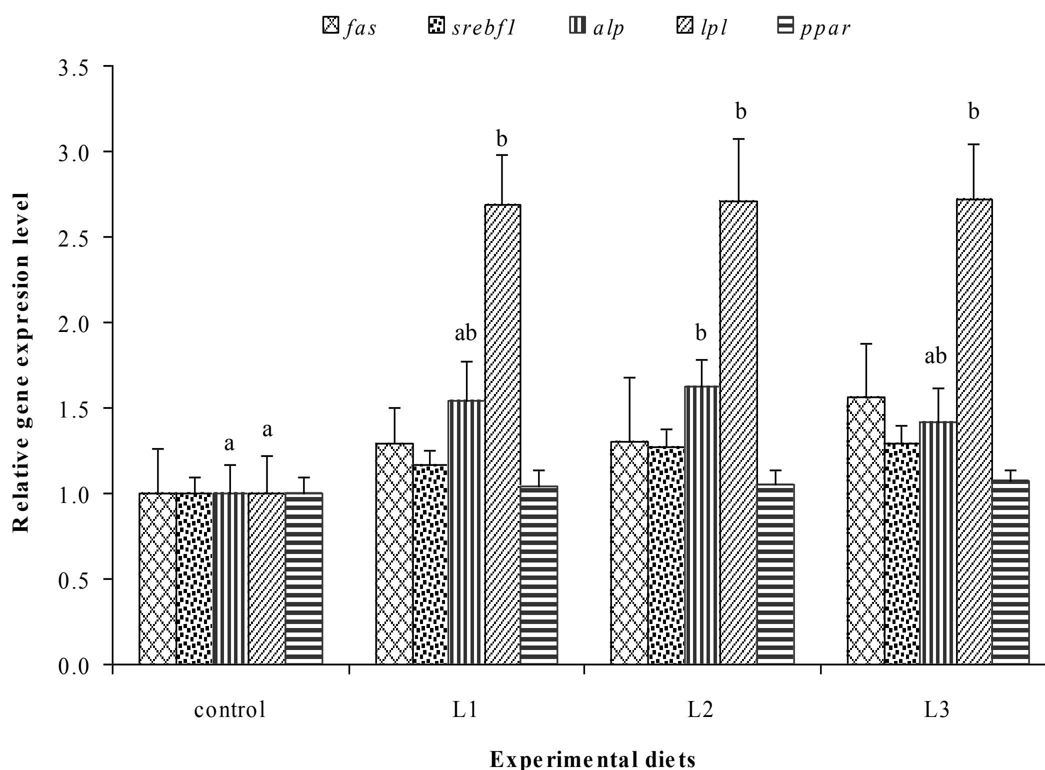



**Figure 4.7:** Expression of fatty acid synthase (*fas*), sterol regulatory element binding transcription factor 1 (*srebf1*), alkaline phosphatase (*alp*), lipoprotein lipase (*lpl*), and peroxisome proliferator-activated receptor (*ppar*) alpha in the liver of Nile tilapia fed on diets with 0 (control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene. Different superscript letters denote significant differences among treatments.

The expression of *fas*, *srebf1*, *alp*, *lpl* and *pparα* genes did not significantly differ between the diets supplemented with thymol and the control (Figure 4.8).

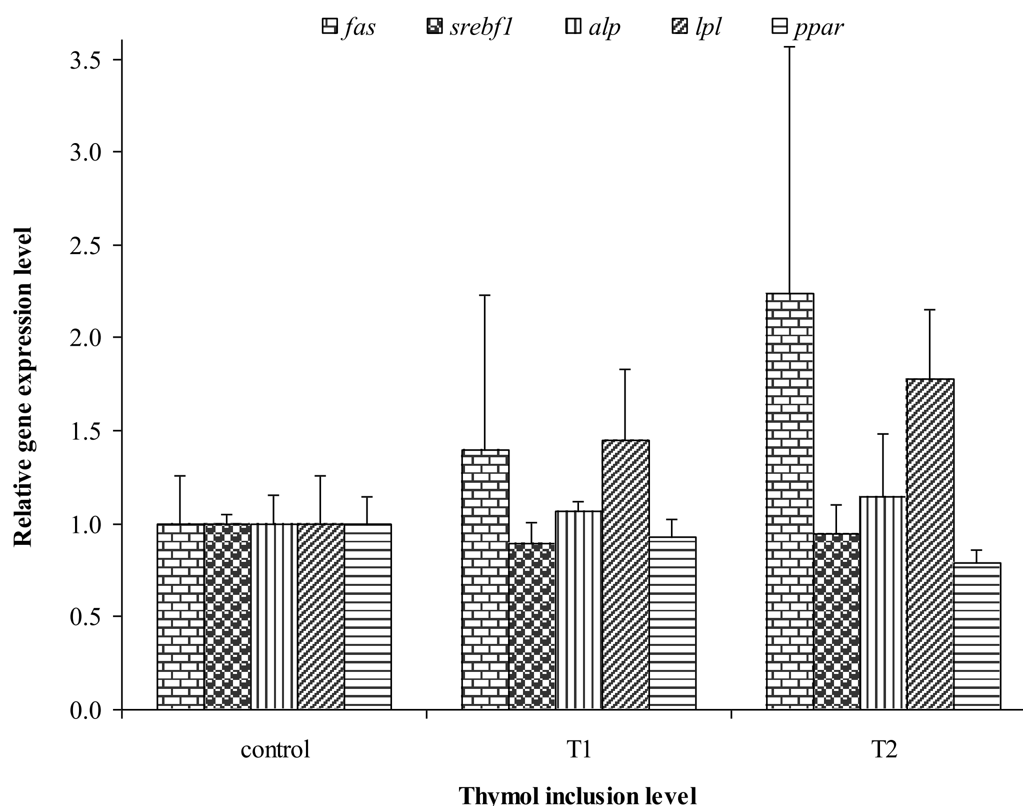



**Figure 4.8:** Expression of fatty acid synthase (*fas*), sterol regulatory element binding transcription factor 1 (*srebf1*), alkaline phosphatase (*alp*), lipoprotein lipase (*lpl*), and peroxisome proliferator-activated receptor (*ppar*) alpha in the liver of Nile tilapia fed on diets with 0 (control), 250 (T1), and 500 (T2) ppm thymol.

#### *4.3.2.3 Expression of genes regulating nutrient digestion, absorption and transport in the fore intestine of Nile tilapia fed on diets supplemented with limonene and thymol*

The mRNA levels of *muc* and *pept1* were significantly higher (P = 0.026 and P = 0.015, respectively) in the fore intestine of fish fed on diets L2 (*pept1*) and L3 (muc and *pept1*) compared to the control (Figure 4.9). No significant differences in the expression of intestinal *alp* (P = 0.575), *ap* (P = 0.858), *pla2* (P = 0.266), *glut2* (P = 0.657), *p-amy* (P = 0.951), and *ctra* (P = 0.322) were observed with increasing concentrations of limonene in the diet (Figure 4.9).

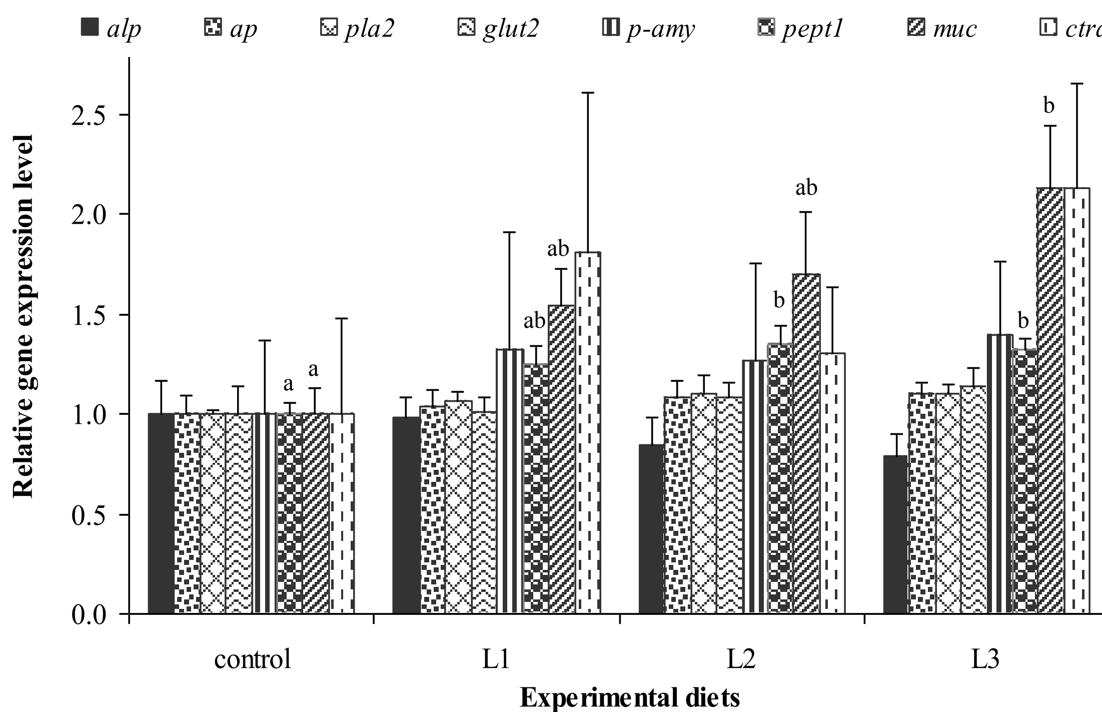



**Figure 4.9:** Expression of alkaline phosphatase (*alp*), amino peptidase (*ap*), phospholipase A2 (pla2), glucose transporter 2 (*glut2*), pancreatic alpha-amylase (*p-amy*) oligo-peptide transporter 1 (*pept1*), mucin-like protein (*muc*), and chymotrypsin A-like (*ctra*) genes in the fore intestine of Nile tilapia fed on diets with 0 (control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene. Different superscript letters denote significant differences among treatments.

In the trial with dietary thymol, the average expression levels of *alp*, *pla2*, *p-amy*, *pept1*, *muc* and *ctra* in the fore intestine of the fish were statistically similar among treatments (Figure 4.10).

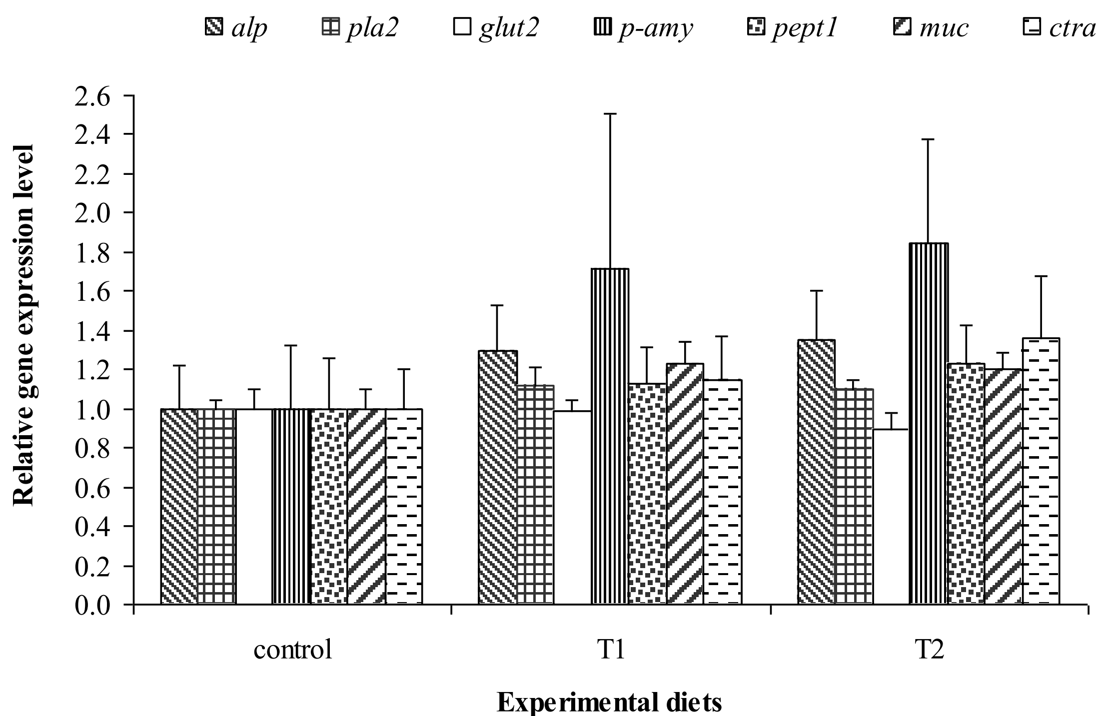



**Figure 4.10:** Expression of alkaline phosphatase (*alp*), phospholipase A2 (pla2), glucose transporter 2 (*glut2*), pancreatic alpha-amylase (*p-amy*) oligo-peptide transporter 1 (*pept1*), mucin-like protein (*muc*), and chymotrypsin A-like (*ctra*) genes in the fore intestine of Nile tilapia fed on diets with 0 (control), 250 (T1), and 500 (T2) ppm thymol.

#### *4.3.2.4 Expression of antioxidant enzymes in Nile tilapia fed on diets supplemented with limonene and thymol*

Catalase (*cat*) was significantly up-regulated ($P < 0.01$) in the liver of fish fed diets supplemented with 200 and 400 ppm limonene compared to the control (Figure 4.11). The relative expression of *gpx* in the liver of fish fed on diets L1, L2 and L3 did not differ significantly from the control ($P = 0.716$). Similarly, *sod2* gene was not significantly regulated in the fore intestine by the experimental treatments.

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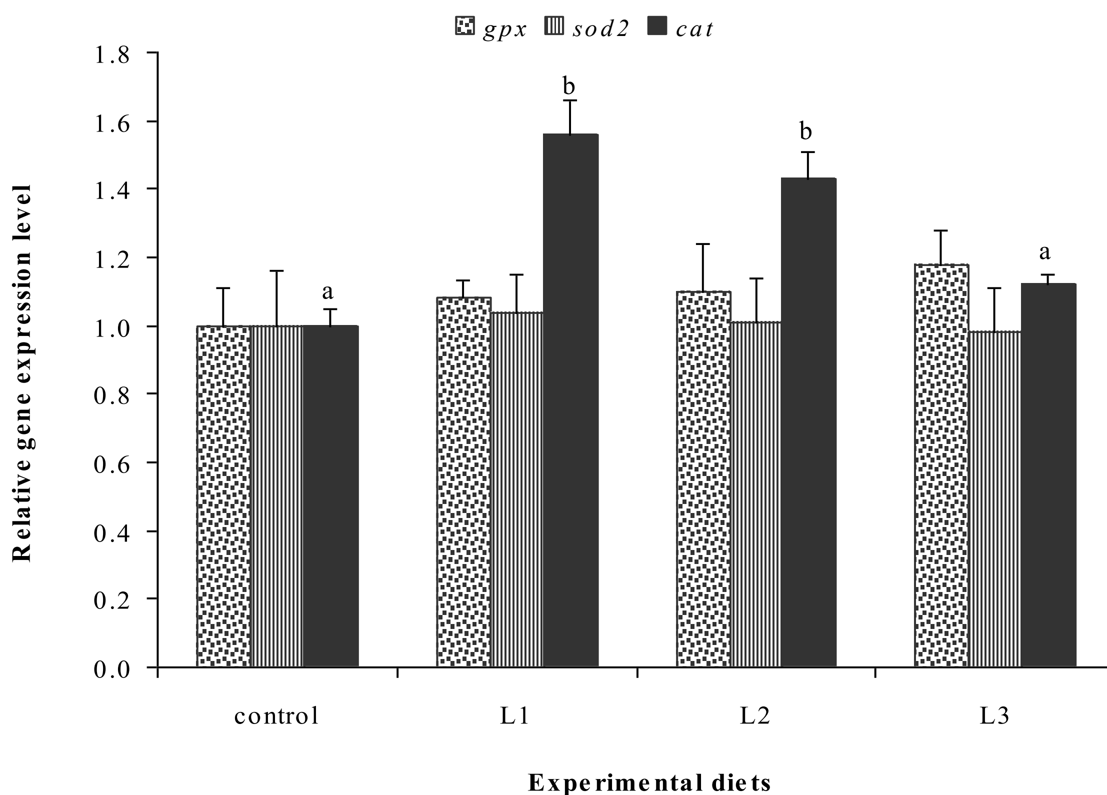



**Figure 4.11:** Expression of antioxidant enzymes glutathione peroxidase (*gpx*) and catalase (*cat*) in the liver, and superoxide dismutase 2 (*sod2*) in the fore intestine of Nile tilapia fed on diets with 0 (control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene. Different superscript letters denote significant differences among treatments.

None of the analysed antioxidant enzymes (*gpx*, *sod2* and *cat*) was significantly up-regulated in the liver by thymol compared with the control (Figure 4.12).

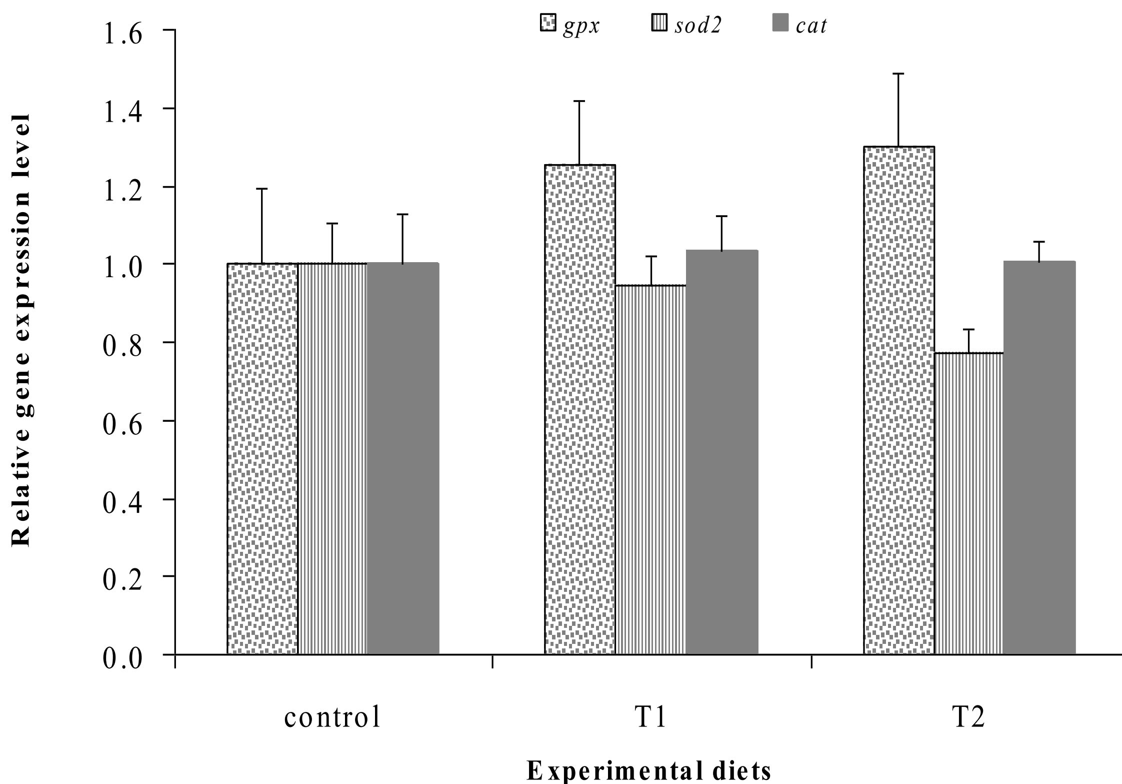



**Figure 4.12:** Expression of the antioxidant enzymes glutathione peroxidase (*gpx*), superoxide dismutase 2 (*sod2*) and catalase (*cat*) in the liver of Nile tilapia fed on diets with 0 (control), 250 (T1), and 500 (T2) ppm thymol.

#### *4.3.2.5 Expression of genes regulating appetite in the brain of Nile tilapia fed on diets supplemented with limonene and thymol*

There was no significant difference in the expression of *npy* (P = 0.918) and *lepr* (P = 0.329) in the brain of Nile tilapia with increasing dietary inclusion of limonene (Figure 4.13).

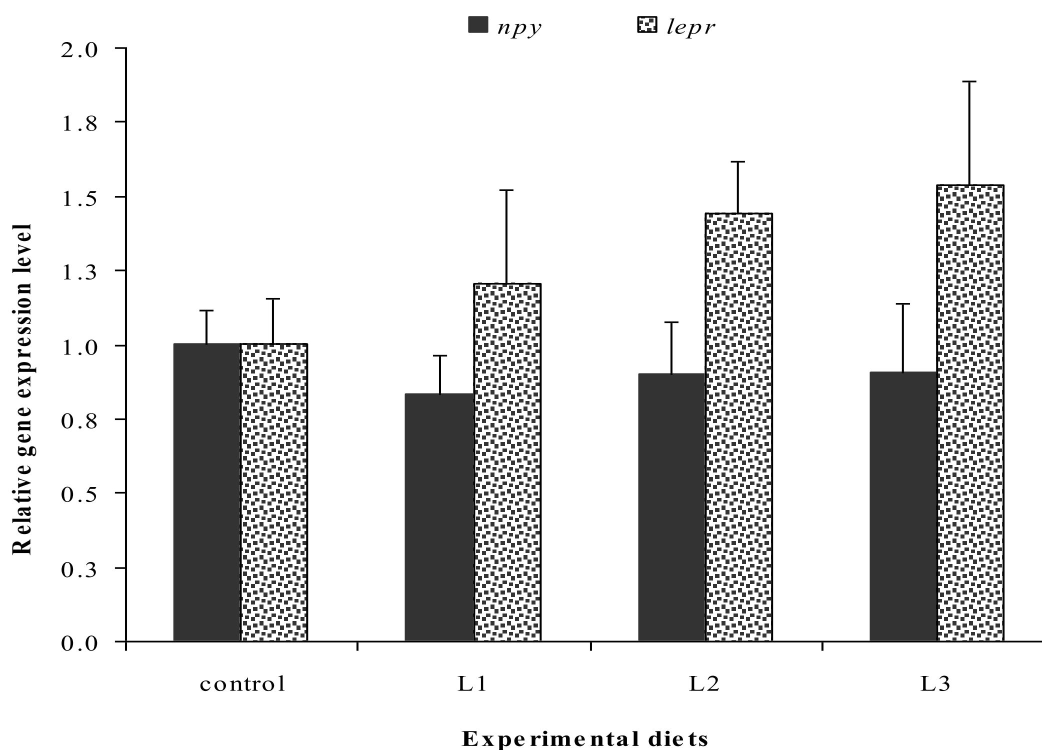



**Figure 4.13:** Expression of pro-neuropeptide y-like (*npy*) and leptin receptor variant XI (*lepr*) genes in the brain of Nile tilapia fed on diets with 0 (control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene.

The fish fed on thymol supplemented diets also had statistically similar expression levels of *npy* (P = 0.227) and *lepr* (P = 0.111) compared with the control (Figure 4.14).

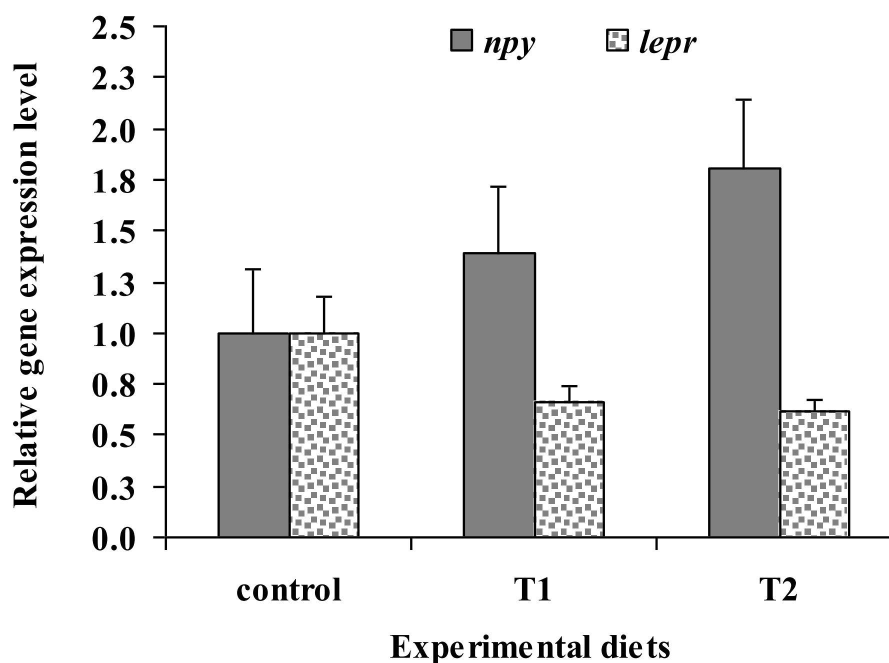



**Figure 4.14:** Expression of pro-neuropeptide Y-like (*npy*) and leptin receptor variant XI (*lepr*) genes in the brain of Nile tilapia fed on diets with 0 (control), 250 (T1), and 500 (T2) ppm thymol.

## 4.4 Discussion

This study investigated pathways in the somatic growth and nutritional physiology of Nile tilapia that could be affected by limonene and thymol, two phytogenic compounds that have shown to promote growth in monogastric animals (Khaled and Megahad, 2014; Acar *et al.*, 2015; Ngugi *et al.*, 2016; Hafeez *et al.*, 2016), as confirmed herein including Nile tilapia (Chapter 3). Given that it remained unclear which pathways are involved in promoting growth, this study focused on genes regulating appetite, nutrient digestion, absorption and transport, lipid metabolism, antioxidant enzymes and growth in Nile tilapia. The expression of selected genes involved in the above pathways was assessed in major tissues where they exert their action, namely brain, fore intestine and liver. More specifically, the brain is a key site for stimulating appetite through the action of appetite regulating peptides (Kiris *et al.*, 2007; Pierce *et al.*, 2012). Moreover, the fore intestine is a pivotal site for digestion, absorption and transport of nutrients, as well as elimination of

microbes (Bakke *et al*., 2010; Kim and Ho, 2010; Fascina *et al*., 2012). On the other hand, liver carries out many metabolic processes including lipid metabolism, antioxidant enzyme status and somatotropic axis peptide activities (Picha *et al*., 2008). This study found that specific genes involved in the somatotropic axis growth (*igf-I*), nutrient digestion, absorption and transport (*muc* and *pept1*), lipid metabolism (*lpl* and *alp*) and antioxidant enzyme activity (*cat*) were significantly regulated by dietary limonene, with largely similar trends observed for thymol although no statistical differences could be established. Moreover, the somatic growth of the fish for both feeding trials (Trials IV and V) largely supported the results of Chapter 3 (Trials I and III). In particular that inclusion of these phytogenics in the diet has growth-promoting effects in Nile tilapia.

In the somatotropic axis, insulin growth factor I (*igf-I*) plays a core role in regulating and controlling somatic growth (Dyer *et al*., 2004; Fox *et al*., 2010; Qiang *et al*., 2012). Generally, with fish of the same species, age and rearing conditions under optimal feeding regimes, higher fish weights correlate with high mRNA expression of *igf-I* (Picha *et al*., 2008; Reindl and Sheridan, 2012). Consistently, the results from this study showed that fish fed on diets supplemented with 400 and 600 ppm limonene exhibited increased mRNA levels of *igf-I*, final weights, growth rates and percentage weight gain (% WG) compared to the control, implying that *igf-I* accounted for improved growth of the fish. Li *et al*. (2012b) also studied the effect of feeding pigs on a diet with a blend of cinnamaldehyde and thymol and found significantly higher weights and *igf-I* mRNA levels with the phytogenic compounds compared to the control, although it remained uncertain whether one or both compounds activated this pathway. Consistently with the results observed here, women administered limonene showed enhanced levels of *igf-I* (Miller *et al*., 2013). With curcumin, an active ingredient of turmeric (*Curcuma longa*), the expression of *igf-I* was also increased in the muscle of Mozambique tilapia (*O. mossambicus*) (Midhun *et al*., 2016). In the present study, the final weight of the fish was

influenced by an interaction between increasing number of days of the growth trial and concentration of limonene. This suggests that the effectiveness of limonene to enhance somatic growth at concentrations of 400 and 600 ppm was time dependent and probably *igf-I* up-regulation followed the same trend given that it is a growth indicator. In agreement, Steiner (2009) reported that the effects of dietary phytogenics on improving growth of animals do occur after a period of time as shown with studies that monitored cumulative performance.

Nutrition is one of the regulators of *igf-I* levels with efficient utilisation of energy and protein playing a key role in activating *igf-I* (Perez-Sanchez and Le Bail, 1999; Picha *et al*., 2008; Reindl and Sheridan, 2012). The pathways of lipid metabolism involving anabolic (lipogenesis) and catabolic (lipolysis) processes are key in meeting the energy requirements of fish (Tocher, 2003). The present results showed that limonene influenced lipid metabolism in liver as noted by the significant increased expression of alkaline phosphatase (*alp*) with diet L2 and lipoprotein lipase (*lpl*) with diets L1, L2 and L3 compared to the control. Lipoprotein lipase is the key enzyme involved in the breakdown of plasma lipids releasing fatty acids that are subsequently transported from the blood stream to tissues for energy production (Nakamura *et al*., 2004; Georgladi and Kersten, 2012). This suggests that limonene increased energy availability thereby meeting the energy requirement of the fish leading to a protein sparing effect of lipids that could have potentially contributed to the enhanced somatic growth. Fish efficiently utilise lipids as energy sources thereby conserving protein for somatic growth (Li *et al*., 2012b). Additionally, *alp* plays a role in maintaining an energy or lipid balance by participating in carbohydrate metabolism, which also contributes to the protein sparing effect (Olagunju *et al*., 2000). Despite the lipid content of the feed used in this particular experiment (20 %) was unusually higher compared to standard Nile tilapia diets (5-7 %) (He *et al*., 2015), it appears that Nile tilapia can feed on diets supplemented with up to 20 % lipid without

impaired growth occuring. Moreover, the observed regulation of *lpl* by dietary limonene in this study (Chapter 4) does not appear to be related to the dietary lipid content (20 %) given that, in Trial VI (Chapter 5) *lpl* was also up-regulated in the liver of fish fed a diet with 3.3 % lipid, supplemented with a combination of limonene and thymol

Enhanced digestion, absorption and transport of nutrients is vital for efficient utilisation of dietary nutrients that ultimately contributes to enhanced growth. In this study, the genes mucin-like protein (*muc*) and oligo-peptide transporter 1 (*pept1*), involved in these processes, were activated by limonene supplemented at 600 ppm, a treatment that attained significantly higher fish weight compared to the control. Muc plays a key role in the secretion of mucus (Neuhaus *et al.,* 2007; Perez-Sanchez *et al*., 2015) and in the fore intestine, it lubricates the gut facilitating nutrient transportation as well as protecting the lumen from acidic chyme, harm by digestive enzymes and pathogens (Kamali *et al*., 2014). Therefore, the high expression of *muc* found in fish fed on the limonene supplemented diet (600 ppm) indicates that limonene triggered mucus secretion, which in turn contributed to improving the efficiency of nutrient transportation and ultimately resulting in enhanced growth. Although previous studies with fish could not be found, in rats and humans, limonene stimulated high mucus secretion in the GIT but no specific genes were analysed/identified (Moraes *et al*. 2009; Rozza *et al*. 2011).

Oligo-peptide transporter 1 is one among the nutrient transporters that use mucus in the intestine as a medium for active transport (Rust, 2003; Bakke *et al*., 2010). The *pept1* is involved in the transport of di/tri peptides from enterocytes in the fore intestine into the blood stream for absorption (Rønnestad *et al*., 2007; Verri *et al*., 2011). The activation of *pept1* by dietary limonene inclusion reported here strongly suggests that this phytogenic compound can facilitate protein absorption, which could partly account for the enhanced growth found in fish fed limonene supplemented diets. In agreement, it has been often argued that dietary di/tripeptides enhance growth of fish in a more efficient

manner compared to individual amino acids (Tengjaroenkul *et al*., 2000; Verri *et al*., 2011). Other accessory genes to *muc* and *pept1* that were assessed in this current study included *p-amy* (pancreatic alpha-amylase), *ctra* (chymotrypsin A-like) and *pla2* (phospholipase A2) involved in digestion of nutrients. The lack of significant differences in the expression level of these genes suggested that limonene and thymol did not improve digestive efficiency of the diets, consistently with the fact that FCRs were not different among treatments.

Metabolic processes including the pathways under investigation here result into production of reactive oxygen intermediates (ROIs), whose levels are controlled by antioxidants to prevent damage to cells and tissues. The antioxidant defence in fish comprises both enzymatic and non-enzymatic systems (Abele and Puntarulo, 2004; Patnaik *et al*., 2013). Limonene supplemented up to 400 ppm enhanced the antioxidant defence capacity as evidenced by up-regulation of *cat* that is involved in the breakdown of ROI hydrogen peroxide into oxygen and water. This action correlated with improved growth of the fish. In agreement, catalase, among other antioxidant enzymes, has been often shown to have a high ability to control ROIs from causing oxidative stress in other vertebrates (Patnaik *et al*., 2013). However, the potential of limonene to activate *cat* occured up to a dietary level of 400 ppm, since higher concentrations of limonene in the diet (600 ppm) did not show any regulation compared to the control.

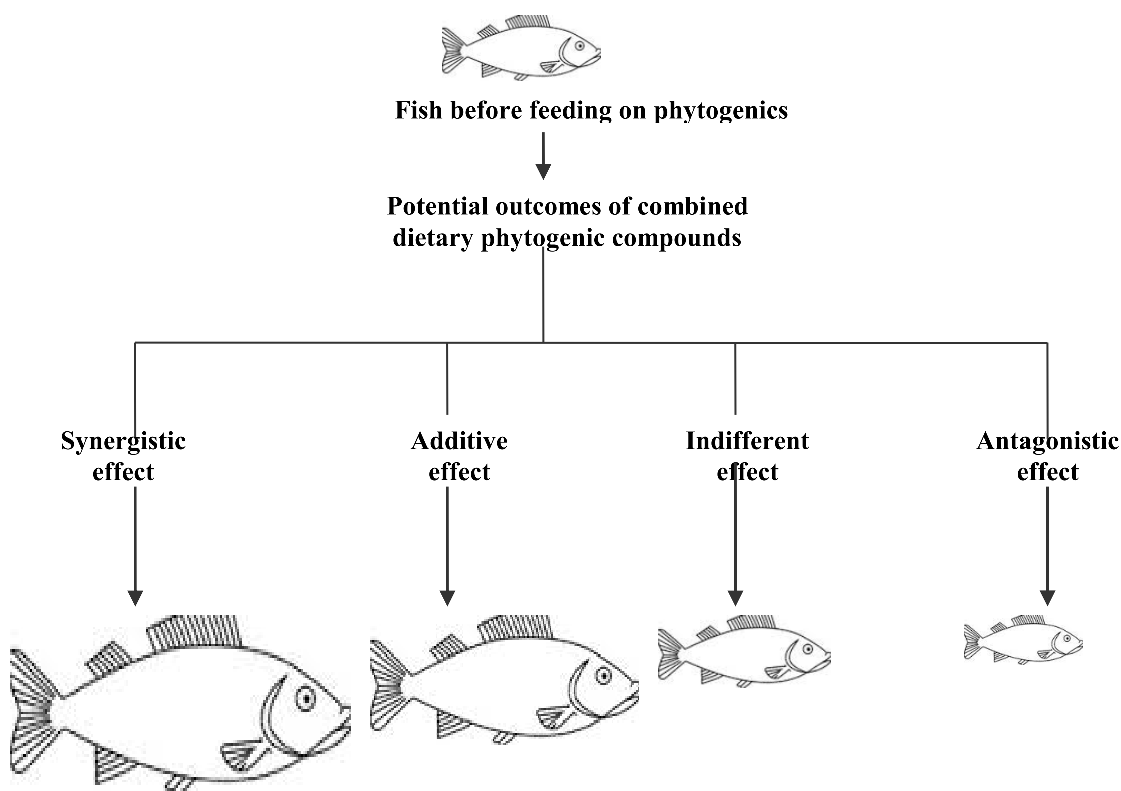
The present study also examined whether the growth-promoting effects of limonene and thymol in Nile tilapia were linked to improvement in the appetite of the fish. Appetite has often been estimated in terms of feed intake but, in order to complement feed intake observations made in this study (Trial IV and V), we further assessed the mRNA levels of neuropeptide Y-like (*npy*) and leptin receptor variant XI (*lepr*), key players in appetite regulation in the brain. The present study found that limonene and thymol did not significantly stimulate the expression of either *npy* or *lepr*, or increased feed intake

suggesting that the growth enhancing effect was not directly associated with an increased appetite of the fish. Despite the present study showing no improvement in fish appetite, Steiner (2009) and Yang *et al*. (2015) in their reviews reported that some phytogenics may stimulate the appetite of animals; probably the responses are different between different animal and fish species or they occur at higher dietary levels of the phytogenics. Thus, future studies could be conducted using higher dietary concentrations of limonene and thymol.

In conclusion, this study demonstrated that limonene enhanced the growth of Nile tilapia and its mode of action involves activation of key genes within the somatotropic axis-mediated growth (*igf-I*), nutrient absorption and transport (*muc* and *pept1*), lipid assimilation (*lpl* and *alp*) and antioxidant enzyme defence (*cat*). The growth enhancing effect of limonene was not associated with an increased appetite and feed intake of the fish. On the contrary, dietary thymol upto 500 ppm, did not significantly improve the growth of Nile tilapia or regulate the selected marker genes involved in somatotropic axis-mediated growth (*igf-I*, *ghr-I*, *gh*), nutrient digestion, absorption and transport (*muc*, *pept1*, *glut2*, *p-amy*, *alp*, *ctra*, and *pla2*) lipid and energy metabolism (*lpl*, *alp*, *fas*, *pparα*, *srebf1*), antioxidant enzyme defence (*cat*, *sod2*, *gpx*) and appetite regulation (*npy* and *lepr*).

# Chapter 5:

# Effects of combined phytogenic compounds on growth and nutritional physiology of Nile tilapia




Diagrammatic illustration of potential effect of combined phytogenic compounds on fish growth
(Original schematic diagram but picture of fish obtained from http://www.clipartpanda.com/categories/fish-clip-art-vector)

"*I can do things you cannot, you can do things I cannot, together we can do great things*"

**Mother Teresa**

## 5.1 Introduction

Phytogenic compounds are bioactive constituents of plants classified as terpenes belonging to various categories such as phenols, aldehydes, ketones, alcohols, esters and ethers (Windisch *et al*., 2008; Steiner, 2009). When mixtures of phytogenic compounds are used in animal feed, they can either have synergistic, additive, indifferent or antagonistic effects on growth and other response indicators (Bassole and Juliani, 2012; Costa *et al*., 2013). These effects can be investigated with an experimental design that includes diets with individually supplemented compounds and their combinations.

Synergistic effects of phytogenic compounds on growth performance lead to enhanced growth of animals above the sum of the levels attained when the compounds are supplied individually (Windisch *et al*., 2008; Yang *et al*., 2015). In fish, the combination of thymol and carvacrol is arguably the most investigated blend of phytogenic compounds for their effects on growth (Zheng *et al*., 2009; Ahmadifar *et al*., 2011; Ahmadifar *et al*., 2014; Perez-Sanchez *et al*., 2015). Zheng *et al*. (2009) supplemented diets with 500 ppm of either carvacrol, thymol, a mixture of carvacrol and thymol, or a commercial product containing carvacrol and thymol (78.3 %) and minor compounds γ-terpinene and p-cymene (12.9 %). Synergistic effects of the compounds in the commercial phytogenic product led to significantly higher weight gain, protein efficiency ratio, condition factor and improved feed conversion ratio in channel catfish compared with fish fed diets containing either carvacrol, thymol, or a mixture of carvacrol and thymol. In contrast to synergism, additive effects of phytogenic compounds are characterised by no significant difference in the effects of combined phytogenic compounds and the sum of the effects of the individual compounds (Hyldgaard *et al*., 2012).

In addition to synergistic and additive effects, combinations of phytogenic compounds have also been shown to have indifferent or antagonistic effects. Indifferent

effects are observed where individual phytogenic compounds might have positive effects but their combination has similar effects compared to the control or either of the phytogenic compounds (Bassole and Juliani, 2012). In this sense, indifferent effects of a diet supplemented with a mixture of carvacrol and thymol were observed in weight gain of channel catfish. The weight gain of the fish was significantly higher with diets supplemented with only carvacrol and the mixture carvacrol and thymol compared to the control and diet with thymol alone with no significant difference in weight gain between the dietary treatment with only carvacrol and the mixture of carvacrol and thymol (Zheng *et al*., 2009). Rattanachaikunsopon and Phumkhachorn (2010) also found an indifferent effect of diets containing carvacrol, cymene and the combination of carvacrol and cymene on the weight gain of Nile tilapia. Carvacrol and cymene are phenols, with cymene being a precursor of carvacrol. However, indifferent effects on growth are not exclusive to chemically similar phytogenic compounds as this has also been observed in experiments testing the effects of combinations of phytogenic compounds of rather different chemical nature such as limonene (a monoterpene), anethol (ether), carvacrol and thymol (both phenols classified as monoterpenoids) (Peterson *et al*., 2015).

Finally, antagonistic effects occur when individual phytogenic compounds might have positive effects but their combination results in negative effects compared to controls (Bassole and Juliani, 2012). For instance, significantly reduced weight gain was found when female broiler were fed on a diet with a blend of carvacrol and cinamaldehyde compared to their individual compounds and the control, although the mechanism accounting for such negative effect could not be established (Lee *et al*., 2004b). In other studies, antagonistic effects derived from phytogenic blends have been attributed to high concentrations potentially resulting in unpleasant taste and smell and thereby retarding feed intake and consequently growth (Windisch *et al*., 2008; Steiner, 2009; Costa *et al*., 2013; Colombo *et al*., 2014).

The studies above highlight the importance of identifying combinations and doses of phytogenic compounds resulting in additive and synergistic effects on fish growth. In previous experiments (Chapters 3 and 4), two phytogenic compounds, namely limonene and thymol, were proven to have growth-promoting effects in Nile tilapia. Gene expression analyses (Chapter 4) further suggested that limonene and thymol appeared to exert complementary actions on similar metabolic pathways, and thus it was hypothesised that combinations of limonene and thymol could potentially have additive or synergistic effects on the growth of Nile tilapia. This study aimed to investigate the effects of a blend of limonene and thymol, compared with the compounds individually, on the growth and nutritional physiology of Nile tilapia. Similar to Chapter 4, this study followed a candidate gene approach to investigate physiological pathways underpinning the response of fish to phytogenic compounds. A selection of genes that are markers within the pathways of somatotropic axis-mediated growth, nutrient absorption and transport, lipid metabolism and antioxidant enzyme status were analysed.

## 5.2 Materials and methods

### 5.2.1 Experimental diets and fish feeding

A standard commercial feed for juvenile Nile tilapia (CP35) produced at the Aquaculture Research and Development Center (ARDC) in Uganda was supplemented with limonene (97 % purity) and/or thymol (95 % purity) from Sigma Aldrich, Uganda using concentrations found to have growth-promoting effects in Nile tilapia in the previous experiments (Chapters 3 and 4). The diets included: 0 ppm limonene and thymol (control); 400 ppm limonene (L); 500 ppm thymol (T); and a combination of 400 ppm limonene and 500 ppm thymol (LT). In order to supply the above concentrations of phytogenic compounds to the standard feed, each concentration of phytogenic compounds was

prepared in 100 ml of absolute ethanol and sprayed onto 1 kg of feed. The control was also coated with a similar amount of ethanol but no phytogenic compound was added. All diets were air-dried for one day, packed in airtight polythene bags and stored at room temperature until use. The fish were fed daily to apparent satiation and the feed intake was recorded.

#### *5.2.1.1 Proximate analysis of the standard diet*

The proximate composition of the standard diet used in Trial VI (Chapter 5) is indicated in Table 5.1.

**Table 5.1:** Proximate analysis of the nutritional composition of the commercial diet (CP35, ARDC - Uganda) used in Trial VI (Chapter 5).

| Analysis | Quantity |
|---|---|
| Dry matter (%) | 89.1 |
| Moisture (%) | 10.9 |
| Crude protein (%) | 33.1 |
| Crude fat (%) | 3.3 |
| Crude ash (%) | 10.9 |
| Crude fibre (%) | 9.9 |
| Gross energy (Kj $g^{-1}$) | 16.9 |

The proximate analysis of moisture, dry matter, protein, lipid, fibre, ash, and gross energy in the standard diet was carried out following the methods of the Association of Official Analytical Chemists (AOAC, 1990) and the joint technical committee of the International Organisation for Standardisation and International Electrotechnical Commission (ISO/IEC 17025) as described in section 2.4.1 (Chapter 2). In summary, crude protein was estimated by measuring the nitrogen content using the

micro-Kjeldahl method and calculating the crude protein level by multiplying the nitrogen content by 6.25. Lipid content was determined by carrying out acid hydrolysis on the feed followed by gravimetric analysis using soxhlet apparatus. Ash content was measured by placing a sample of feed of known weight in a furnace of 600 $^{o}$C overnight and the remaining weight was considered the ash. Moisture content was determined by placing a feed sample of known weight in an oven set at 105-110 $^{o}$C until the sample attained a constant weight. The lost weight from the sample was considered the moisture content and the remaining weight dry matter. Fibre analysis involved defatting feed samples of known weight using petroleum ether and removing digestible material by sequential boiling in 1.25 % sulphuric acid, and sodium hydroxide and washing in ionised water. The samples were dried and incinerated in a furnace and then fibre content was calculated. Finally, gross energy was measured using an adiabatic bomb calorimeter.

### 5.2.2 Fish and experimental design

Nile tilapia fingerlings from the same cohort were size-graded by measuring the weight of each fish to select those with no significant difference in initial weight, 1.6 ± 0.0 g (mean ± standard error). The fish were anaesthetised during this procedure with a low dose of clove oil at 0.02 g $L^{-1}$ for 3-5 min and held in aerated water to limit stress. The fish that were selected were then transferred to the rearing tanks (N = 38 per tank) and grown for 63 days at the ARDC facility. Sixteen tanks were used each with a water holding capacity of 60 L in a flow through system with a flow rate of 1-2 L $min^{-1}$. The water in each tank was aerated using air stones and heated using aquaria water heaters. Each experimental diet was tested in quadruplicate tanks. The treatments were distributed following a complete randomised design. Water quality was monitored routinely to ensure that it was within the requirements for Nile tilapia growth (Webster and Lim, 2006). A multi-parameter meter (HQ40D model, Hach Ltd Germany) was used to measure dissolved

oxygen, pH and water temperature. The level of ammonia-nitrogen was assessed using a fresh water test kit from API Company Ltd UK following the user guide from the manufacturer. Water flowing into the fish rearing tanks had 6.6 ± 0.6 mg $L^{-1}$ of dissolved oxygen, pH 6.8 ± 0.3 and undetectable levels (< 0.05 mg $L^{-1}$) of ammonia-nitrogen. The temperature of the flowing water ranged from 23.3 - 24.3 ºC, and 25.0 - 26.6 ºC in the fish rearing tanks. The growth of fish in the different treatments was assessed every two or three weeks and at the end of the trial by measuring the weight (accuracy of 0.1 g) and total length (0.1 cm) of each fish. This was done while the fish were anesthetised using 0.02 g $L^{-1}$ of clove oil for 3-5 min, after which the fish were taken back to the experimental tanks. At the end of the trial, the number of live fish in each tank was recorded and sections of liver and fore intestine were dissected from 12 fish per treatment (N = 3 per tank) and placed in 1.5 mL tubes containing RNAlater solution obtained from Sigma Aldrich in Uganda. Unlike in Chapter 4, the brain, which is the main organ where appetite and feed intake are regulated, was not extracted in this experiment because the results in Chapter 4 indicated that dietary limonene and thymol do not improve appetite and feed intake. The liver and fore intestine samples were kept at 4 ºC overnight, shipped to UK and transferred to a -20 ºC freezer until RNA was extracted.

### 5.2.3 Molecular analyses

#### *5.2.3.1 RNA extraction*

Tissue samples from the liver and fore intestine were homogenised in TRI Reagent (Sigma Aldrich, Dorset, UK) with a mini bead-beater 16 (Biospec Bartlesville, OK, USA) and total RNA (N = 12 per tissue and treatment) samples were extracted according to the procedure detailed in section 2.4.2.1 (Chapter 2). The concentration and purity of the RNA was measured by spectrophotometry with an ND-1000 Nanodrop (Nanodrop 1000, Thermo Scientific, Glasgow, UK) at absorbance wavelength ratios of 260/280 and 260/230, respectively. The intactness of the RNA from each sample was further assessed by agarose gel electrophoresis using 200 ng RNA in 1 % agarose gel and 0.5 x TAE buffer stained with 0.3 µl ethidium bromide (10 mg $mL^{-1}$).

#### *5.2.3.2 Complementary DNA (cDNA) synthesis*

From a total of 12 RNA samples extracted per tissue and treatment, six RNA samples were derived by pooling together two samples from the same treatment, taking equal quantity (2.5 µg/µl) of RNA from each of the two samples being pooled (adapted from Glencross *et al*., 2015). Thus the final mixture (vortex mixed and centrifuged) had a 50 % contribution of each of the two samples that were pooled.

A high capacity reverse transcription kit without RNase inhibitor from AB Applied Biosystems (Warrington, UK) was used to reverse transcribe RNA from each pool sample (N = 6 per treatment) to cDNA using a PCR thermocycler following the procedure detailed in section 2.4.2.2 (Chapter 2). Briefly, to 2 µg/µl of each RNA pooled sample, nuclease-free water was added to make up a volume of 10 µl, which was incubated in 10 µl of reverse transcription (RT) reaction mixture comprising 2 µl of 10 x RT buffer, 0.8 µl of

25 × dNTP mix, 2 µl of 10 × random primer in the molar ratio of 1.5:0.5 for the random to oligo-dT primer, respectively, 1.0 µl of reverse transcriptase and 4.2 µl of nuclease free water. The thermocyling temperature for cDNA synthesis was: 25 ºC for 10 min, 37 ºC for 120 min and 4 ºC for 15 s.

#### *5.2.3.3 Quantitative real-time Polymerase Chain Reaction (qPCR)*

The expression of genes that showed to be potentially regulated or appeared regulated by limonene and/or thymol in Chapter 4 was analysed by qPCR. More specifically, the selected target genes included mucin-like protein (*muc*), oligo-peptide transporter 1 (*pept1*), lipoprotein lipase (*lpl*), sterol regulatory element binding transcription factor 1 (*srebf1*), alkaline phosphatase (*alp*), phospholipase A2 (*pla2*), catalase (*cat*), growth hormone (*gh*), and insulin growth factor I (*igf-I*). The details of the primers used for qPCR analyses are provided in Table 5.2. Each qPCR reaction (total volume 20 µl) contained 5 µl of 20-fold diluted cDNA, 3 µl nuclease-free water, 1 µl (10 pmol) each for the forward and reverse primers, and 10 µl of Luminaris color higreen qPCR Mix (Thermo Scientific, Hemel Hempstead, UK). All the reactions were run in 96 well-plates using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany). A calibrator sample (20-fold dilution of all samples pooled cDNA) and a negative control with no cDNA (non template control-NTC) were included in each plate.

**Table 5.2:** Details of the primers used for quantitative real-time PCR analyses.

| Functional group | Gene symbol | Primer / oligonucleotide sequences (5'- 3') | Size (base pairs) | Accession number* |
|---|---|---|---|---|
| Nutrient digestion, absorption and transport | *Muc* | F: TGCCCAGGAGGTAGATATGC<br>R: TACAGCATGAGCAGGAATGC | 101 | XM_005466350 |
| | *pept1* | F: CAAAGCACTGGTGAAGGTCC<br>R: CACTGCGTCAAACATGGTGA | 196 | XM_013271589 |
| Lipid metabolism | *Lpl* | F: TGCTAATGTGATTGTGGTGGAC<br>R: GCTGATTTTGTGGTTGGTAAGG | 217 | NM_001279753 |
| | *srebf1* | F: TGCAGCAGAGAGACTGTATCCGA<br>R: ACTGCCCTGAATGTGTTCAGACA | 102 | XM_005457771 |
| | *Alp* | F: CTTGGAGATGGGATGGGTGT<br>R: TTGGCCTTAACCCCGCATAG | 200 | XM_005469634 |
| | *pla2* | F: CTCCAAACTCAAAGTGGGCC<br>R: CCGAGCATCACCTTTTCTCG | 177 | XM_005451846 |
| Antioxidant activity | *Cat* | F: TCCTGGAGCCTCAGCCAT<br>R: ACAGTTATCACACAGGTGCATCTTT | 79 | JF801726 |
| Somatotropic axis-aided growth | *Gh* | F: TCGGTTGTGTGTTTGGGCGTCTC<br>R: GTGCAGGTGCGTGACTCTGTTGA | 90 | XM_003442542 |
| | *igf-I* | F: GTCTGTGGAGAGCGAGGCTTT<br>R: CACGTGACCGCCTTGCA | 70 | NM_001279503 |
| Reference genes | *ef-1α* | F: GCACGCTCTGCTGGCCTTT<br>R: GCGCTCAATCTTCCATCCC | 250 | NM_001279647 |
| | *ß-actin* | F: TGGTGGGTATGGGTCAGAAAG<br>R: CTGTTGGCTTTGGGGTTCA | 217 | XM_003443127 |

*muc* mucin-like protein, *pept1* oligo-peptide transporter 1, *lpl* lipoprotein lipase, *srebf1* sterol regulatory element binding transcription factor 1, *alp* alkaline phosphatase, *pla2* phospholipase A2, *cat* catalase, *gh* growth hormone, *igf-I* insulin growth factor I, *ef-1α* elongation factor 1α, *ß-actin* beta-actin.

*GenBank (http://www.ncbi.nlm.nih.gov/)

The qPCR thermocycling program involved pre-heating samples at 50 °C for 2 min followed by 35 cycles, initial denaturing at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 30 s. Cycle threshold (Ct) values for the duplicate runs of each sample were averaged and the relative expression of each gene was calculated according to the equation of Pfaffl (2001) as detailed in section 2.6.2 (Chapter 2).

### 5.2.4 Data computations

Final average fish weight (FW), daily growth coefficient (DGC), mean metabolic body weight (MBW), growth rate per metabolic body weight ($GR_{MBW}$), weight gain (WG), percentage (%) weight gain (% WG), condition factor (CF), percentage (%) fish survival, feed intake as % body weight per day (% FI), feed conversion ratio (FCR), feed conversion efficiency (FCE), protein intake (PI) and protein efficiency ratio (PER) were computed using the following formulae:

FW (g) = total fish biomass at end of the trial (g) / number of fish at end of the trial;

DGC = ((final average fish weight $^{(1/3)}$ (g) - initial average fish weight $^{(1/3)}$ (g)**) /** duration of trial (d) x 100;

MBW = ((initial average fish weight/1000)$^{0.8}$ + (final average fish weight/1000)$^{0.8}$) / 2;

$GR_{MBW}$ = (body weight gain / ((initial average fish weight/1000)$^{0.8}$ + (final average fish weight/1000)$^{0.8}$) / 2) / duration of trial (d);

WG = final average fish weight - initial average fish weight;

% WG = ((final average fish weight (g) - initial average fish weight (g)) / initial average fish weight (g)) × 100;

CF = (final average fish weight / final average total length$^3$) × 100;

% fish survival = (number of alive fish at end of trial / initial number of fish stocked) × 100;

% FI = (100 × (average feed intake fish$^{-1}$ / ((initial average body weight ± final average body weight)/2))) / duration of trial (d);

FCR = average feed intake fish$^{-1}$ / weight gain;

FCE = ((final average fish body weight-initial average fish body weight) / average feed intake fish$^{-1}$) x 100.

Protein intake = feed intake x (% crude protein in the diet / 100)

PER = weight gain / protein intake.

Moreover, the length-weight relationship of the fish was established to determine the structure of the body of the fish in different treatments using scatter plot diagrams and the regression line equation $W = aL^b$ (Froese, 2006) whereby:

W is the fish weight (g)

L is the fish`s total length (cm)

a is the intercept which shows the rate at which the weight changes with total length

b is the slope of the power regression line of weight and total length that predicts the weight at unit length

Based on the cube law, isometric fish growth (body form) is associated with a critical value of the exponent "b" equal to 3 (Froese, 2006). It is an average value derived from "b" values ranging from 2.6 to 3.32, obtained from different fish species.

### 5.2.5 Statistical data analysis

The data were analysed using the Statistical Package for the Social Sciences (SPSS) version 19 (Chicago, USA). The fish performance and qPCR results for each treatment are expressed as means ± standard error. Normality of distribution of the data was assessed using Kolmogorov-Smirnov's tests. Data not normally distributed were subjected to natural logarithm (qPCR data) and arcsin square-root (DGC, $GR_{MBW}$, % WG, CF, % fish survival, % FI, FCR, FCE, and PER) transformation. Differences between

treatments were analysed by one-way ANOVA followed by Tukey's test. When heterogeneity of variances occurred, Welch's test was performed with Game-Howell's test to determine differences between treatments. Significant differences were considered at P value < 0.05. In addition, Pearson's correlation analysis was performed to indicate the relationship and degree of correlation between FI and FCR, FW and % fish survival, FW and PER. The significance level of correlation was set to P < 0.05.

## 5.3 Results

### 5.3.1 Growth performance

Table 5.3 shows the growth performance of Nile tilapia fed on diets with 400 ppm limonene (L), 500 ppm thymol (T) and the combination of 400 ppm limonene with 500 ppm thymol (LT). There was a significant (P < 0.01) increase in the final weight of fish fed on the diets supplemented with limonene, that is, diets L and LT, compared to the control. The diet supplemented exclusively with thymol (T) did not significantly improve the final weight of the fish (P = 0.052). There was a significantly higher daily growth coefficient (DCG; P = 0.007), growth rate per metabolic body weight ($GR_{MBW}$; P = 0.007), weight gain (WG; P = 0.007) and percentage (%) weight gain (% WG; P = 0.01) of fish fed on diets L and LT compared with the control, whilst fish fed diet T did not show significant differences compared with the control. Though there were no significant differences in the final survival of the fish among treatments, there was a strong significant positive correlation (r = 0.967, P = 0.033) between the survival rate and final weight of the fish (Table 5.3). Condition factor (CF) was not significantly different among treatments. On the other hand, the length-weight relationship of the fish established using the regression coefficient 'b' indicated that all dietary treatments had a high degree of association between the total length and fish weight with coefficient of determination ($R^2$) ranging

from 0.9554 - 0.9625 (Figure 5.1). The control had the lowest 'b' value of 2.7972 ($R^2$ = 0.9554), followed by diet L with 2.8914 ($R^2$ = 0.9708), then diet T with 2.9522 ($R^2$ 0.9613) and finally diet LT with the highest value of 3.0092 ($R^2$ = 0.9625).

No significant differences were found in protein intake (g $fish^{-1}$) between treatments but protein efficiency ratio (PER) was significantly higher with the diets L and LT compared with the control. Though no significant differences in PER were observed among fish fed on diets L, T and LT, PER had a strong significant positive correlation (r = 0.974, P = 0.026) with final fish weight and thus higher PER corresponded with higher final fish weight. This study found no significant differences in feed conversion ratio (FCR) and feed conversion efficiency (FCE) among the treatments supplemented with L, T and LT, but fish fed diets L and LT had significantly lower FCR (P = 0.006) and higher FCE (P = 0.011) than the control - fed fish (Table 5.3). Despite % feed intake (% FI) not being significantly different among fish fed diets L, T and LT, significantly lower (P = 0.019) % FI was obtained with the fish fed on diets L and LT compared with the control. In addition, there was a strong positive significant correlation (r = 0.996, P = 0.004) between FI and FCR, and lower FI corresponded with low FCR and therefore better feed utilisation efficiency.

**Table 5.3:** Initial mean weight, final mean weight, daily growth coefficient (DGC), growth rate per metabolic body weight ($GR_{MBW}$), weight gain (WG), percentage weight gain (% WG), condition factor (CF), percentage (%) survival, feed intake as % body weight per day (% FI), feed conversion ratio (FCR), feed conversion efficiency (FCE), protein intake (PI) and protein efficiency ratio (PER) of Nile tilapia fed on diets with 400 ppm limonene (L), 500 ppm thymol (T) and a combination of 400 ppm limonene and 500 ppm thymol (LT) for 63 days.

| Parameter | Experimental diet | | | | |
|---|---|---|---|---|---|
| | Control | L | T | LT | P value |
| Initial mean weight (g) | 1.5 ± 0.0 | 1.6 ± 0.0 | 1.5 ± 0.0 | 1.6 ± 0.0 | NS |
| Final mean weight (g) | 13.7 ± 0.4$^{a}$ | 16.6 ± 0.4$^{b}$ | 15.0 ± 0.4$^{a}$ | 16.7 ± 0.3$^{b}$ | < 0.01 |
| DGC (% $d^{-1}$) | 2.0 ± 0.0$^{a}$ | 2.2 ± 0.1$^{b}$ | 2.1 ± 0.0$^{ab}$ | 2.2 ± 0.1$^{b}$ | 0.007 |
| $GR_{MBW}$ (g $kg^{-0.8}$ $d^{-1}$) | 10.2 ± 0.2$^{a}$ | 11.0 ± 0.2$^{b}$ | 10.6 ± 0.1$^{ab}$ | 11.1 ± 0.2$^{b}$ | 0.007 |
| WG (g $fish^{-1}$) | 12.2 ± 0.5$^{a}$ | 15.0 ± 0.8$^{b}$ | 13.5 ± 0.2$^{ab}$ | 15.2 ± 0.6$^{b}$ | 0.007 |
| % WG | 793.2 ± 29.1$^{a}$ | 957.3 ± 51.9$^{b}$ | 887.0 ± 16.1$^{ab}$ | 980.0 ± 41.3$^{b}$ | 0.01 |
| CF | 1.8 ± 0.0 | 1.8 ± 0.0 | 1.9 ± 0.0 | 1.9 ± 0.0 | NS |
| % Survival | 94.1 ± 3.5 | 97.4 ± 1.5 | 94.8 ± 3.0 | 98.1 ± 0.7 | NS |
| % FI (% body weight $d^{-1}$) | 4.5 ± 0.1$^{b}$ | 3.9 ± 0.1$^{a}$ | 4.3 ± 0.2$^{ab}$ | 4.0 ± 0.1$^{a}$ | 0.019 |
| FCR | 1.8 ± 0.1$^{b}$ | 1.5 ± 0.0$^{a}$ | 1.7 ± 0.1$^{ab}$ | 1.5 ± 0.0$^{a}$ | 0.006 |
| FCE (%) | 56 2 ± 2.0$^{a}$ | 67.0 ± 1.5$^{b}$ | 61.2 ± 2.4$^{ab}$ | 66.2 ± 1.8$^{b}$ | 0.011 |
| PI (g $fish^{-1}$) | 7.2 ± 0.2 | 7.4 ± 0.3 | 7.3 ± 0.3 | 7.6 ± 0.2 | NS |
| PER | 1.7 ± 0.1$^{a}$ | 2.0 ± 0.1$^{b}$ | 1.9 ± 0.1$^{ab}$ | 2.0 ± 0.1$^{b}$ | 0.009 |

*All values are means of treatments ± standard error. Mean values with different superscript in the same row are significantly different from each other at P < 0.05. NS, refers to not significantly different values. For each treatment, N =152 for initial fish weight, for final fish weight, N = number of alive fish at the end of the trial and N = 4 replicates for DGC, $GR_{MBW}$, WG, % WG, CF, % survival, % FI, FCR, FCE, PI and PER.*

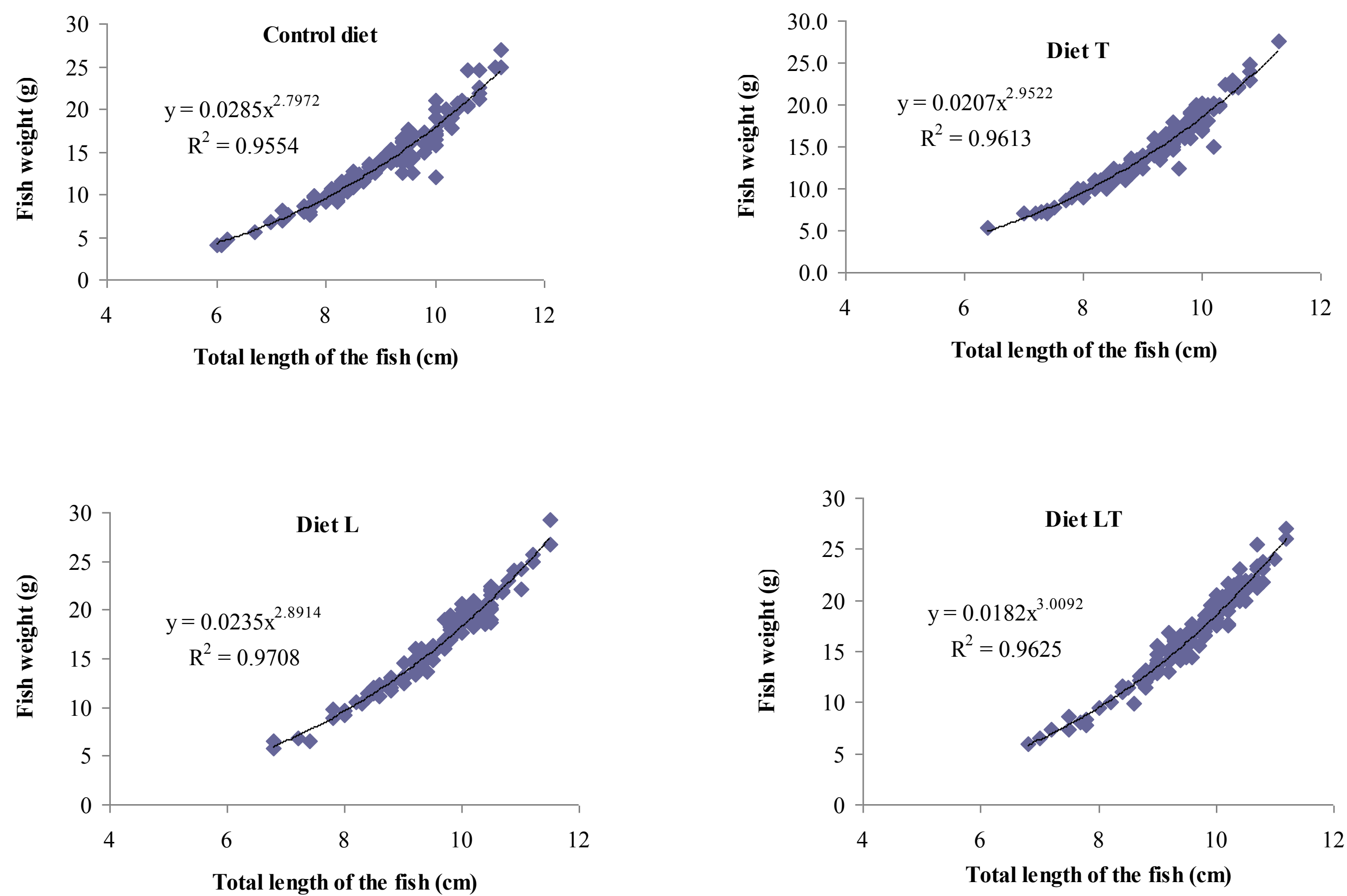



**Figure 5.1:** Length-weight relationship of Nile tilapia fed on diets T, L, LT and the control for 63 days.

### 5.3.2 Relative mRNA gene expression

The heat map in Figure 5.2 represents the relative expression patterns , not statistical differences of genes analysed in the liver (a) and fore intestine (b) of Nile tilapia fed on diets containing 400 ppm limonene (L), 500 ppm thymol (T) and the combination of 400 ppm limonene and 500 ppm thymol (LT).

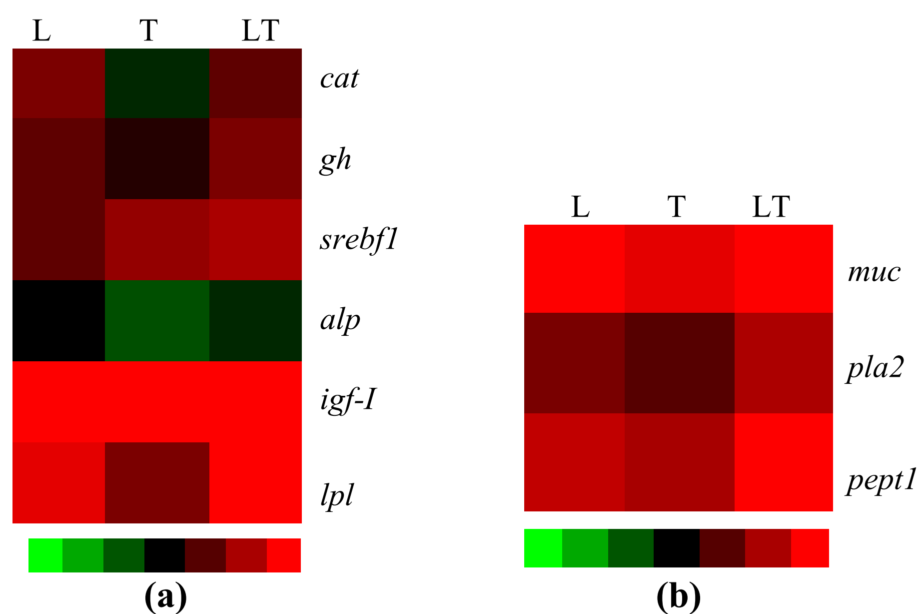



**Figure 5.2:** Heat map showing the expression patterns of nine genes analysed using qPCR data from Nile tilapia fed on diets supplemented with limonene (L), thymol (T) and their combination (LT).

Data were plotted using java tree view and rows were clustered according to Euclidean distance. The columns represent mean data values of three dietary treatments L (400 ppm limonene), T (500 ppm thymol) and LT (400 ppm limonene and 500 ppm thymol). The rows indicate each of the analysed genes in the liver (a) and fore intestine (b) of Nile tilapia. Expression level of each gene was natural log transformed and normalised against two reference genes. The colour bars at the bottom represent the mean relative expression levels as low (green), neutral (black) or high (red). The black colour represents the control to which the relative expression of the other treatments was determined. *cat*, catalase; *gh*, growth hormone; *srebf1*, sterol regulatory element binding transcription factor 1; *alp*, alkaline phosphatase; *igf-I*, insulin growth factor I; *pla2*, phospholipase A2; *lpl*, lipoprotein lipase; *muc*, mucin-like protein; *pept1*, oligo-peptide transporter 1.

There were more genes with patterns of higher relative expression levels (red) compared to the control among the fish fed on diets L and LT compared with diet T.

### *5.3.2.1 Expression of somatotropic axis genes in the liver of Nile tilapia fed on diets supplemented with limonene, thymol and their combination*

Insulin growth factor I (*igf-I*) was significantly (P = 0.025) up-regulated in the liver of fish fed on diets L and LT compared with the control fish (Figure 5.3).

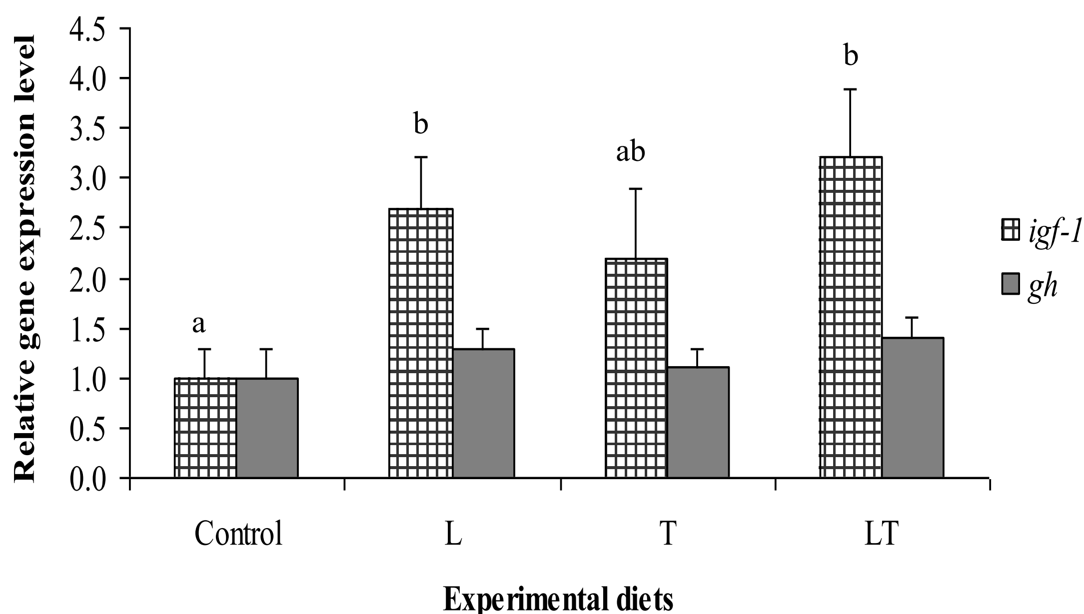



**Figure 5.3:** Expression of insulin growth factor I *(igf-I*) and growth hormone (*gh*) in the liver of Nile tilapia fed on diets supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and the combination of 400 ppm limonene and 500 ppm thymol (LT). Different superscript letters denote significant differences among treatments.

However, the expression of *igf-I* did not differ significantly between the fish fed on diets L, T and LT (Figure 5.3). In addition, the relative expression of *gh* was not significantly different in the livers of fish fed on diets L, T, LT and the control.

#### *5.3.2.2 Expression of genes involved in regulating lipid metabolism in the liver of Nile tilapia fed on diets supplemented with limonene, thymol and their combination*

The expression of *lpl*, *alp* and *srebf1* in the liver of Nile tilapia fed on the experimental diets is shown in Figure 5.4. Levels of *lpl* mRNA were significantly (P = 0.003) higher in fish fed on diet LT compared with the control. The expression of *lpl* in the fish fed on diets L and T was not significantly different from the control. Similarly, no significant differences in the relative expression of *alp* and *srebf1* were found among the experimental treatments (Figure 5.4)

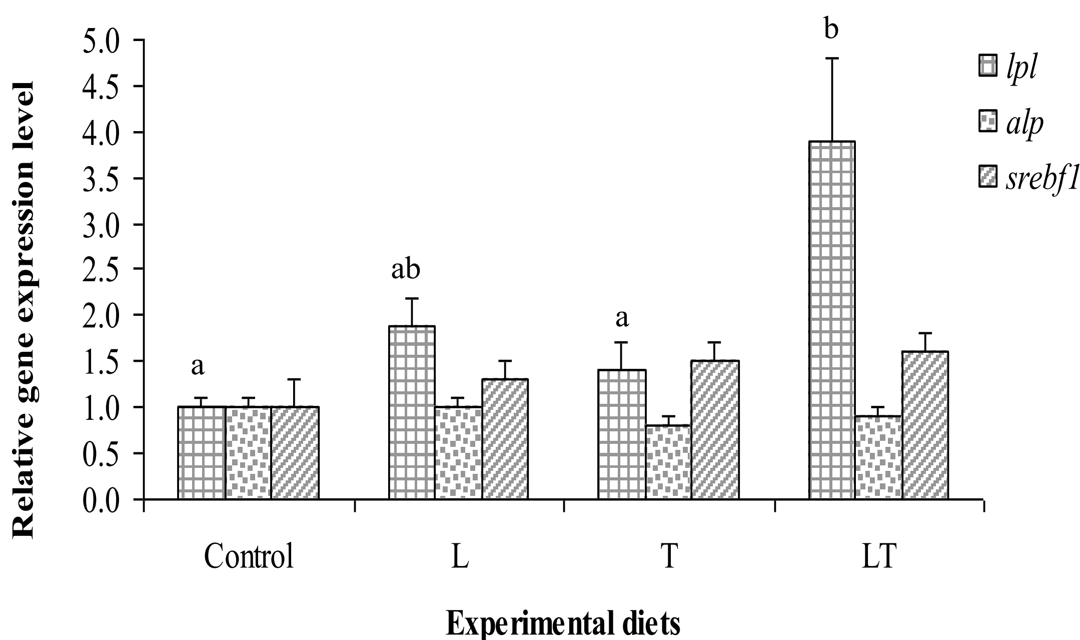



**Figure 5.4:** Expression of lipoprotein lipase (*lpl*), alkaline phosphatase (*alp*), and sterol regulatory element binding transcription factor 1 (*srebf1*) in the liver of Nile tilapia fed on diets supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and a combination of 400 ppm limonene and 500 ppm thymol (LT). Different superscript letters denote significant differences among treatments.

### *5.3.2.3 Expression of genes regulating nutrient digestion, absorption and transport in the fore intestine of Nile tilapia fed on diets supplemented with limonene, thymol and their combination*

The mRNA levels of *muc* were significantly higher (P = 0.025) in the fore intestine of fish fed on diet LT compared with the control (Figure 5.5). Besides, the expression of *muc* in the fish fed on diets L and T did not differ significantly from the control (P = 0.097). The expression of *pla2* also did not statistically differ among the dietary treatments (P = 0.086). Oligo-peptide transporter 1 (*pept1*) expression was significantly (P = 0.047) up-regulated in the fish fed on diet LT compared with the control, although expression levels in fish fed diets L and T did not differ statistically compared with the control (Figure 5.5).

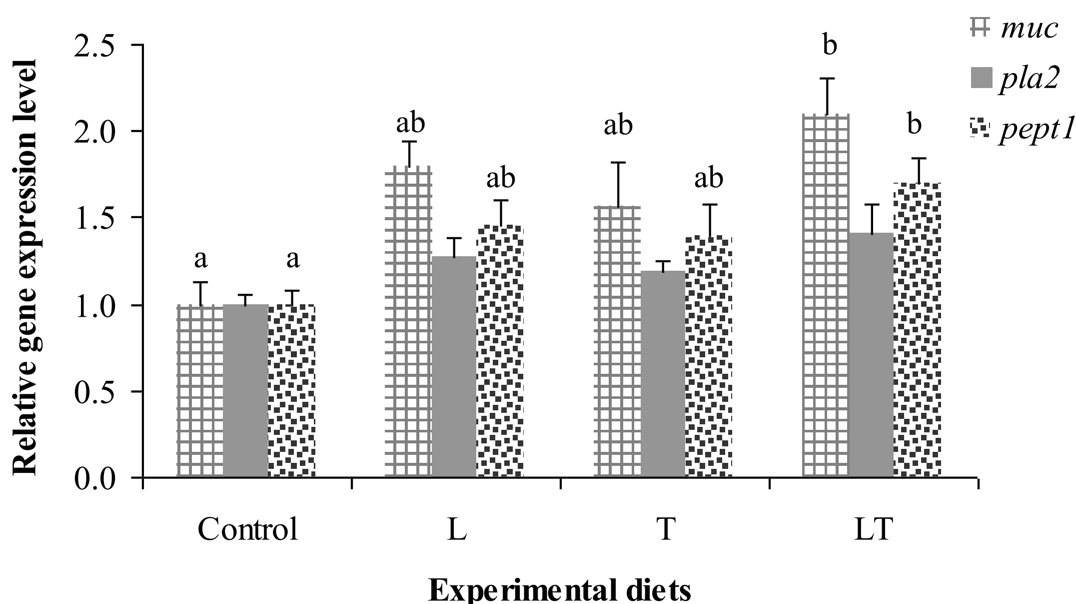



**Figure 5.5:** Expression of mucin-like protein (*muc*), phospholipase A2 (*pla2*), and oligo-peptide transporter 1 (*pept1*) genes in the fore intestine of Nile tilapia fed on diets supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and a combination of 400 ppm limonene and 500 ppm thymol (LT). Different superscript letters denote significant differences among treatments.

### *5.3.2.4 Expression of the antioxidant enzyme catalase in Nile tilapia fed on diets with limonene, thymol and their combination*

The expression of *cat* was significantly higher (P = 0.006) in the liver of fish fed on diets L and LT compared with the control (Figure 5.6).

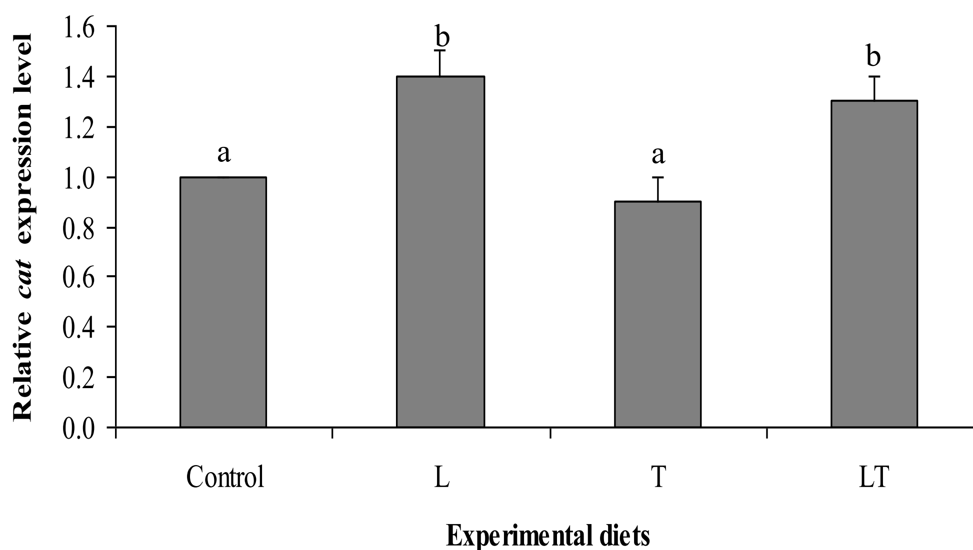



**Figure 5.6:** Expression of antioxidant enzyme catalase in the liver of Nile tilapia fed on diets supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and a combination of 400 ppm limonene and 500 ppm thymol (LT). Different superscript letters denote significant differences among treatments.

## 5.4 Discussion

The present study investigated the effects of diets containing limonene and thymol, supplemented both individually and in combination, on growth and nutritional physiology of Nile tilapia. The goal was to establish whether blends of limonene and thymol had synergistic and/or additive effects on the growth of Nile tilapia. A selection of gene markers regulating nutrient digestion, absorption and transport, lipid metabolism, antioxidant enzymes and somatotropic axis growth, and previously identified to respond to dietary limonene in Nile tilapia (Chapter 4), were investigated. The results of this study (Trial VI), particularly on gene expression, suggest that a combination of 400 ppm limonene and 500 ppm thymol (LT) did not exert synergistic or additive effects on promoting growth in Nile tilapia. The dietary supplementation with both limonene and thymol (LT) significantly increased the expression of key genes involved in somatotropic axis-mediated growth (*igf-I*), nutrient absorption and transport (*muc* and *pept1*), lipid metabolism (*lpl*) and antioxidant enzyme activity (*cat*) in Nile tilapia compared to the control, and LT showing generally no statistical differences with dietary treatments with 400 ppm limonene (L) and 500 ppm thymol (T). Growth and feed utilisation parameters also did not reflect synergistic or additive effects derived from the diet LT, rather it appeared obvious that dietary limonene exerted its effects to a larger extent compared to thymol.

It was observed that fish fed diets L and LT had significantly higher final weights (FW), daily growth coefficient (DGC), growth rate per metabolic body weight ($GR_{MBW}$), weight gain (WG), percentage (%) WG, feed conversion efficiency (FCE), protein efficiency ratio (PER) and lower feed intake (FI) and feed conversion ratio (FCR) than the control. On the contrary, fish fed on diet T did not show any statistical difference in such parameters compared with the control. On the other hand, the regression

coefficients ('b' values) obtained from the length-weight relationship of the fish showed that diets L, T, LT and the control had "b" values within the range of 2.6 to 3.3 reported for most fish species, from which the critical mean value of 3.0 associated with isometric growth / body structure is derived (Richter *et al*., 2000; Froese, 2006; Datta *et al*., 2013; Nehemia *et al*., 2014). Fish associated with isometric growth / body structure are desired by aquaculturists and fish consumers who buy whole fish (Monfort, 2007; Ighwela *et al*., 2011). It is also worth noting that different sexes of Nile tilapia have different values of 'b', which can partly affect the final value in mixed sex Nile tilapia groups (Froese *et al*., 2014; Othieno *et al*., 2014). Overall, the growth results above indicate that dietary supplementation of both limonene and thymol did not have synergistic or additive effects on growth of Nile tilapia as initially hypothesised. Similarly, Zheng *et al*. (2009) found no synergistic or additive effects of a combination of carvacrol and thymol on the weight gain of channel catfish. The fish fed on the diet supplemented with only carvacrol, and the blend of carvacrol and thymol attained statistically higher weight gain compared with the diet with only thymol and the control but the dietary mixture of carvacrol and thymol did statistically increase weight gain to the same extent as the diet with only carvacrol.

Among the feed utilisation parameters, the present study found enhanced feed efficiency (FCR) in fish fed diets L and LT, and a strong significant positive correlation between PER and FW. The correlation between PER and FW showed that, as the utilisation of protein from the feed was enhanced (high PER), FW of the fish was increased. This could have contributed to the significantly improved somatic growth of the fish fed on diets L and LT, both treatments with increased PER compared to the control. The increased WG and lowered FI levels observed with diet L and LT fed fish are in agreement with Hashemipour *et al*. (2013) who found lower FI corresponding with the highest WG and feed efficiency in broiler chicken fed on a diet with a mixture of 200 ppm of thymol and carvacrol compared to the control. It is known that efficient growth in fish

does not necessarily coincide with maximum or higher feed intake because fish adjust their FI according to their energy requirements (Ali and Jauncey, 2004), with better feed efficiency occurring below maximum FI (Rad *et al*., 2003; Sawhney, 2014). Conversely, some studies with phytogenic compounds (thymol and carvacrol) in pig diets found low FI corresponding with low WG (Lee *et al*., 2003b; 2004b). While it is difficult to identify the exact causes of such an apparent discrepancy with the present results, one possible reason might stem from the pungent odour of thymol and carvacrol that can affect palatability and ultimately feed intake since, compared to fish, pigs are more sensitive to smell (Michiels *et al*., 2012; Muthusamy and Sankar, 2015).

The actions of genes regulating growth in the pathways within nutritional physiology do complement each other (Hashemi and Davoodi, 2010; Steiner and Syed, 2015). In this study, insulin growth factor I (*igf-I*), which plays a core role in regulating growth in the somatotropic axis, was up-regulated to a similar extent in the liver of fish fed diets L and LT, corresponding also to higher final FW and feed utilisation efficiency (FCR) than the control. This observation implies that *igf-I* was largely activated by limonene suggesting that there was no synergistic effect of limonene and thymol in influencing somatotropic axis-mediated growth.

Key mechanisms underlying feed utilisation efficiency include nutrient digestion, absorption and transport, in which mucin-like protein (*muc*) and oligo-peptide transporter 1 (*pept1*) are important components (Verri *et al*., 2011; Fascina *et al*., 2012). The present study found a significantly higher expression of *muc* in the fore intestine of fish fed on diet LT compared with the control, with diets L and T showing no differences in expression of *muc* with the control and diet LT. The high expression of *muc* found with diet LT can be associated with an increase in the secretion/quantity of mucus, which then serves as a lubricant aiding absorption of nutrients into the bloodstream through which they are transferred to tissues for various functions including growth (Kamali *et al*., 2014).

Moreover, high expression of *muc* corresponded with enhanced somatic growth of fish in the LT treatment. Despite Tsirtsikos *et al*. (2012) did not specifically investigate *muc* expression, their study on broilers fed on diets containing a blend of limonene, carvacrol and anethol also reported an increase in mucus volume in the fore intestine. Additionally, Jamroz *et al*. (2006) found higher mucus secretion in the fore intestine of broilers fed diets supplemented with a combination of phytogenics including carvacrol, cinnamaldehyde and capsicum oleoresin. The present results for Nile tilapia are consistent with these terrestrial animal studies, indicating the mechanism of action is somewhat conserved across vertebrates.

The movement of nutrients from the lumen of the intestine, aided by mucus, into epithelial cells takes place through diffusion and/or active transport regulated by nutrient transporters (Rust, 2003). The nutrient transporter *pept1* that aids the transport of protein in the form of di/tri peptides through the above process (Verri *et al*., 2011), was significantly regulated by diet LT compared with the control. Moreover, the higher expression of *pept1* by diet LT corresponded with significantly improved FCR and PER compared with the control, with diet L also having enhanced FCR and PER compared with the control. This suggested that limonene drove the improved protein absorption, which could have contributed to increased growth.

In order to maximise the use of dietary protein for somatic growth, energy for supporting metabolic processes can be derived from non-protein sources, particularly lipids (Nankervis *et al*., 2000). Lipid metabolism including, among others, processes such as lipid catabolism, fatty acid and triglyceride synthesis occurs along with lipid transport and deposition with the liver as the main site (He *et al*., 2015). In this study, diet LT activated lipid metabolism as reflected by significantly increased expression of lipoprotein lipase (*lpl*) in comparison to the control. Since the expression of *lpl* in the fish fed diet T did not differ from the control, it can be concluded that it is dietary limonene that triggers

such metabolic response in fish fed diet LT. Given that *lpl* plays a pivotal role in breaking down plasma lipids into free fatty acids and transporting it for use in energy production (Tian *et al*., 2015), the high gene expression of *lpl* found in this study suggests that dietary limonene increased the energy level of the fish, thereby providing sufficient energy for running metabolic processes and sparing protein which significantly improved fish growth in the dietary treatments L and LT. Such effect of limonene to regulate *lpl* and a corresponding somatic growth enhancement further confirmed the results obtained in Chapter 4.

Metabolic processes in the body result into production of reactive oxygen intermediates (ROIs), which can induce damage to cells and tissues if their levels are not maintained low (Covarrubias *et al*., 2008; Costa *et al.*, 2013) This can ultimately impair adequate physiological function and subsequently negatively affect growth. In this study, the expression of catalase (*cat*), a key antioxidant enzyme that breaks down hydrogen peroxide (a ROI), was significantly increased by dietary treatment with limonene (diets L and LT) to similar extents. This suggests that the enhanced antioxidant enzyme status by catalase could reduce the hydrogen peroxide levels and thus result in improved somatic growth of the fish fed on diet L and LT. Recent research has shown that, when ROIs are at low concentrations, they are vital molecules mediating physiological processes including somatic growth (Covarrubias *et al*., 2008; Barbieri and Sestili, 2012). The herein reported action of dietary limonene on catalase has not been observed for other phytogenic compounds. For instance, Zheng *et al*. (2009) did not find enhanced activity of *cat* with diets containing thymol, carvacrol or their mixture although the fish attained higher weight with the diet containing both compounds and carvacrol alone. Thymol did not appear to have an obvious role in regulation of antioxidant enzymes such as *cat*, and thus it can be assumed that, as noted above, limonene exerts a major action in up-regulating *cat*.

Overall, this study confirmed that dietary limonene and the blend of limonene and thymol improved somatic growth and feed utilisation efficiency of Nile tilapia to similar extents, although thymol individually showed no effects on enhancing growth performance. This points to dietary limonene being the sole or at least the major contributor towards the enhanced fish growth observed, suggesting lack of synergistic or additive effects of the combined compounds. The gene expression of biomarkers for nutrient digestion, absorption and transport, lipid metabolism, antioxidant enzymes and somatotropic axis growth also largely indicated lack of synergistic or additive effects of the dietary combination of limonene and thymol in Nile tilapia.

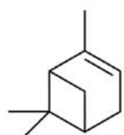
# Chapter 6:

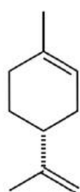
# General discussion, conclusions and future perspectives

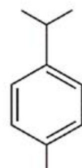
**Terpenes**

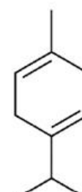
Monoterpenes

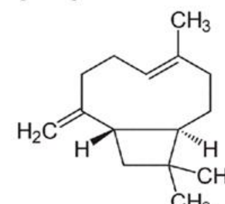
Sesquiterpenes







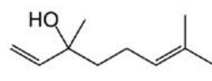




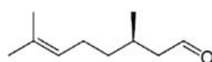

α-Pinene Limonene Sabinene P-cymene γ-Terpinene β-Caryophyllene

**Terpenoids**

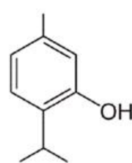
Monoterpenoids

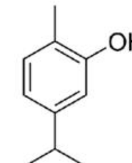


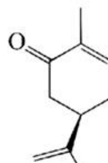

Linalool




Citronellal



Thymol Carvacrol Carvone Borneol

Chemical structure of some phytogenic compounds catergorised as terpenes and terpenoids (from Hyldgaard *et al*., 2012)

"*You have all the reason in the world to achieve your grandest dreams. Imagination plus innovation equals realisation*"

**Denis Waitley**

## 6.1 Overview of the research

In order to meet the growing global demand for fish for human consumption, various alternative strategies are being evaluated to increase fish productivity in aquaculture systems. These include dietary supply of phytogenics as potential natural growth-promoting products. Phytogenics in the form of herbs and essential oils extracted from plants are extensively being investigated (Steiner, 2009; Chakraborty *et al.*, 2014). The results have, however, been somewhat contradictory largely because the concentrations of bioactive compounds in herbs and essential oils vary depending on the type and part of the plant used, species of plant, duration of storage, method of processing and extraction among others (Applegate *et al.*, 2010; Yang *et al.*, 2015). Focus has now shifted to evaluating purified bioactive phytogenic compounds (Applegate *et al.*, 2010). The overall aim of this present research was to investigate the effects of selected phytogenic compounds on the growth and nutritional physiology of Nile tilapia. Six Trials were carried out, precisely, three (Trials I, II and III) in Chapter 3, two (Trials IV and V) in Chapter 4 and one (Trial VI) in Chapter 5. In Chapter 3, the objective was to identify phytogenic compounds and doses with growth-promoting effects in Nile tilapia (Objective 1) with respect to final fish weight, daily growth coefficient, growth rate per metabolic body weight, weight gain, fish survival, feed intake, feed conversion ratio, and protein efficiency ratio. This was accomplished by investigating the effects of the phytogenic compounds limonene, thymol and carvacrol individually on the growth performance and feed utilisation parameters of Nile tilapia. More specifically, Trial I investigated limonene, Trial II focused on carvacrol and Trial III on thymol. The phytogenic compounds were supplemented to two standard Nile tilapia diets (Ugachick Poultry Breeders Ltd (Diet A – CP40) and Aquaculture Research and Development Center - ARDC (Diet B – CP35) produced commercially in Uganda at the time that the Trials were conducted. Limonene

was supplemented at 100, 200, 300, 400 and 500 ppm to Diet A. Carvacrol was supplemented to Diet A at 250, 500, 750 and 1000 ppm while thymol was supplemented to Diet B at 250, 500, 750 and 1000 ppm. Each Trial had a control treatment that was not supplemented with any phytogenic compound. In Chapter 4 selected pathways of nutritional physiology and growth of Nile tilapia activated by individually supplemented phytogenic compounds and potentially responsible for growth enhancement were investigated (Objective 2). More specifically, limonene and thymol were chosen as they were found to have growth- promoting effects in Chapter 3. The compounds were supplemented to a commercial fish diet (INICIO Plus  from BioMar Ltd. UK) and the expression of candidate genes regulating fish appetite, nutrient digestion, absorption and transport, lipid metabolism, antioxidant enzyme activity and somatotropic axis-mediated growth in Nile tilapia investigated. Trial IV assessed the effects of limonene (0, 200, 400 and 600 ppm), while Trial V evaluated thymol (0, 250 and 500 ppm). In Chapter 5 (Trial VI), the objective was to determine the effects of combined phytogenic compounds (limonene and thymol) on growth, nutrient absorption and transport, lipid metabolism, antioxidant enzyme activity and the somatotropic axis in Nile tilapia (Objective 3). This was to assess whether the combined phytogenics have synergistic or additive effects on Nile tilapia growth, as well as complementary effects on the pathways mentioned above. Concentrations of limonene and thymol of 400 and 500 ppm, respectively, were evaluated in Chapter 5 using a commercial Nile tilapia diet, CP35 produced at the ARDC in Uganda. The information obtained from this research project provides insights on the formulation of functional feeds supplemented with growth-promoting phytogenic compounds in Nile tilapia and potentially to other species.

## 6.2 General discussion

The results from Objective 1 (Chapter 3) indicated that dietary limonene at 400 ppm and 500 ppm, and thymol at 750 ppm had growth-promoting effects in Nile tilapia juveniles. With these diets, the final fish weights were significantly enhanced compared to controls. Consistent with these results, growth-promoting effects of limonene in Japanese quail and Mozambique tilapia (*O. mossambicus*) were previously reported (Acar *et al*., 2015; Dalkilic *et al*., 2015). With thymol, no growth promoting effects were found with diets supplemented with 200 ppm fed to female broiler chicken (Lee *et al*., 2003b), 500 ppm administered to rainbow trout (Zheng *et al*., 2009) and 1000 ppm fed to channel catfish (Giannenas *et al*., 2012). In the current study, the diets supplemented with carvacrol did not improve growth performance, similarly to the results of Rattanachaikunsopon and Phumkhachom (2010) and Volpatti *et al*. (2012) with Nile tilapia and sea bass, respectively. The observed growth enhancement with limonene and thymol diets appeared not to be directly related to improved feed intake and feed utilisation efficiency as feed intake was similar between treatments and the feed conversion ratio (FCR), and protein efficiency ratio (PER) were not enhanced. Thus, alternative pathways could have accounted for the observed effects, a hypothesis that was tested in Objective 2.

In Objective 2 (Chapter 4), which investigated nutritional physiology and growth pathways activated by limonene (Trial IV) and thymol (Trial V) to enhance somatic growth of Nile tilapia, fish weights and growth rates were significantly increased with dietary limonene at 400 and 600 ppm compared to controls. Dietary thymol did not significantly increase fish weight at 250 and 500 ppm (the concentrations used in Trial V) compared to the control. However, the growth-promoting effects of limonene and thymol in Nile tilapia were observed in Chapter 3 with a dietary concentration of 750 ppm and the somatic growth of the fish was not significantly different from that of fish fed at a dietary level of 500 ppm. Unfortunately, the dietary concentration of 750 ppm thymol (Trial III

conducted in Uganda) was not tested in Trial V (Chapter 4) due to ethical restrictions by the British Home Office. Consistent with the observation from Chapter 3, the improved somatic growth in the present study (Chapter 4) did not appear to be related to improved feed intake or feed efficiency given that feed intake and FCR did not differ between the treatments.

Corresponding with the enhanced fish growth, the candidate genes regulating somatotropic axis growth (*igf-I*), nutrient digestion, absorption and transport (*muc* and *pept1*), lipid metabolism (*lpl*) and antioxidant enzyme activity (*cat*) were up-regulated in Nile tilapia fed limonene supplemented diets. In particular, the fish fed on dietary limonene at 400 and 600 ppm that had higher final live weights, had higher expression levels of the candidate genes compared to controls. Thymol generally showed an apparent activation of similar genes as limonene but differences were not significant. Insulin growth factor I (*igf-I*), core in mediating and controlling somatic growth in the somatotropic axis (Qiang *et al*., 2012; Reindl and Sheridan, 2012), was up-regulated with diets containing 400 and 600 ppm limonene which suggested that *igf-I* contributed to improved somatic growth of the fish. Additionally, limonene influenced lipid metabolism in the liver by up-regulating lipoprotein lipase (*lpl*) that is fundamental in breaking down plasma lipids into free fatty acids used in tissues for energy production (Georgladi and Kersten, 2012). This study found significantly higher levels of *lpl* in all limonene dietary treatments (200, 400 and 600 ppm). These results strongly suggest that limonene enhances energy availability, which could have spared protein for growth resulting into the observed enhanced somatic growth. Moreover, improved dietary energy utilisation (nutritional status) is known to enhance IGF levels leading to growth stimulation (Picha *et al*., 2008; Reindl and Sheridan, 2012). Furthermore, alkaline phosphatase (*alp*), which participates in regulating energy and lipid levels in the body, was also up-regulated by limonene (400 ppm) in the liver, potentially contributing to protein sparing effects and ultimately growth.

*Alp* is involved in adipocyte differentiation and/or gluconeogenesis that are among the processes through which lipid and energy balance are maintained (Olagunju *et al*., 2000; Hernandez-Mosqueira *et al*., 2015). However, the regulation of *alp* by dietary limonene was not observed in Trial VI possibly because the diet in Trial VI had lower lipid content (3.3 %) than the diet used in Trial V (21 %). In agreement, high dietary fat levels have been reported to correlate with high ALP activity and adipocyte differentiation (Narisawa *et al*. 2003; Hernandez-Mosqueira *et al*. 2015). All dietary nutrients required for supporting somatic growth are made available through digestion, absorption and transport, processes at which limonene seems to have a further influence. Thus, the present results showed that fish fed on dietary limonene at 600 ppm had enhanced expression of mucin-like protein (*muc*), a key player in secretion of mucus in the fore intestine (Perez-Sanchez *et al*., 2015). Mucus lubricates the gut enabling nutrient transportation (Kamali *et al*., 2014). As such, the enhanced fish weight and expression of *muc* with dietary limonene (600 ppm) suggested that limonene increased mucus secretion and thereby contributed to enhanced transportation of nutrients that could have resulted into increased growth. Further benefits from mucin are derived from the presence of oligosaccharides (N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and sialic acids) known to be fermented by bacteria in the distal intestine stimulating growth of probiotic bacteria to the detriment of pathogenic bacteria and thereby improving absorption of nutrients (Schroers *et al*., 2009; Aliakbarpour *et al*., 2012). Moreover, increased mucus secretion is also reported to minimise adhesion of pathogens to the intestinal mucosa thereby improving nutrient absorption (Costa *et al*., 2013). In addition, oligo-peptide transporter 1 (*pept1*), which uses active transport to transport protein in form of di/tri peptides from the intestine to the blood stream (Verri *et al*., 2011), was up-regulated in the fish fed on 400 and 600 ppm limonene corresponding with higher growth rates and final fish weights. The

enhanced *pept1* expression suggested that limonene improved protein absorption, which probably contributed to higher somatic growth.

The biological processes discussed in this study and also others produce reactive oxygen intermediates (ROIs), compounds that may damage cells and tissues at high concentrations and thus impairing proper operation of physiological processes (Kelestemur *et al*., 2012; Costa *et al*., 2013). In this study, the gene encoding catalase (*cat*), one of the key antioxidant enzymes that breaks down hydrogen peroxide (a ROI), was up-regulated by dietary limonene at 400 ppm, which probably minimised the concentration of ROIs and accounted for antioxidant protection potentially leading to higher somatic growth of the fish. The increased expression of *cat* found in fish fed diets with 400 ppm limonene was not found with a higher concentration (600 ppm limonene) despite the enhanced fish growth observed in that treatment. It is known that high feed intake may also contribute to increased growth. However, neuropeptide Y-like (*npy*) and leptin receptor variant XI (*lepr*), both involved in regulating fish appetite in the brain, were not up-regulated by either dietary limonene or thymol suggesting that the growth-promoting effects of limonene and thymol were not linked to improved appetite of the fish. Possibly higher inclusion levels of limonene and thymol in the diet could have enhanced feed intake (Franz *et al*., 2010).

Given that in Trial III, thymol at 750 ppm increased the final weight of the fish, it was hypothesised that limonene and thymol may have complementary roles leading to synergistic or additive effects on the growth of the fish. Consequently, effects of dietary combinations of limonene and thymol on growth and nutritional physiology of Nile tilapia were investigated in Chapter 5 (Objective 3). As stated in Chapter 4, a concentration of 500 ppm thymol was also evaluated in Chapter 5 given that no statistical difference was found in the final weight of fish fed on diets incorporated with 500 ppm and 750 ppm in Trial III. The results in Chapter 5 (Objective 3) indicate that the dietary combination of 400

ppm limonene and 500 ppm thymol (Diet LT) did not have either synergistic or additive effects on the somatic growth of Nile tilapia. Clearly, limonene had a stronger influence on growth of Nile tilapia compared to thymol. This was indicated by significantly increased final fish weight, daily growth coefficient (DGC), growth rate per metabolic body weight ($GR_{MBW}$), weight gain (WG), % WG, feed conversion efficiency (FCE), protein efficiency ratio (PER), and feed conversion ratio (FCR) in fish fed on diets LT and L (400 ppm limonene) compared with the control, while no differences were found for fish fed on diet T (500 ppm thymol). The improved growth in fish fed diets L and LT seemed to be related to enhanced feed efficiency (FCR, FCE and PER), which appeared to meet the energy requirement for growth of the fish with a lower feed intake compared with the control. Reduced feed intake and FCR have the benefit of reducing feed costs (Yitbarek, 2015). Interestingly, in Chapter 3 and 4, limonene did not enhance feed efficiency (FCR and PER). It is hypothesised that such an apparent contradiction might be caused by a difference in chemical composition among diets used in trials within this study. In this regard, it has been reported that the efficacy of phytogenic compounds can be affected by the chemical composition of the diet (Jamroz *et al*., 2005; Pirgozliev *et al*., 2015). For instance, Jamroz *et al* (2005) found better FCR compared to the control when a dietary mixture of carvacrol, cinnamaldehyde and capsaicin was added to a maize-based and not wheat-based diet at similar concentrations, this hypothesised to occur because maize contains less antinutritional factors that limit digestibility of nutrients compared to wheat (Krogdahl *et al*., 2010). Despite the diets tested in the trials of this study had different proximate composition, particularly in terms of crude protein and lipid contents, the growth-promoting effects of limonene and thymol existed regardless the diet. These results imply that, whereas the chemical composition of the diets might partly determine the effectiveness of the phytogenics in exerting their actions, there exists a cause-effect relationship between phytogenic inclusion and growth enhancement. On the other hand,

the diet used in Chapter 4 might have been highly digestible as suggested by lower FCR values of 1.0-1.1. It is reported that phytogenics may not improve the digestibility of nutrients in diets with highly digestible ingredients (Lee *et al*., 2003a; Applegate *et al*., 2010; Fascina *et al*., 2012). Possibly that is also why there were no significant differences in the expression of digestive enzymes pancreatic amylase (*p-amy*), chymotrypsin A-like (*ctra*), aminopeptidase (*ap*), alkaline phosphatase (*alp*) and phospholipase A2 (*pla2*) among treatments in trials within Chapter 4.

The genes analysed in Chapter 5 suggested that a combination of limonene and thymol (diet LT) did not enhance the growth of Nile tilapia through either synergistic or additive effects. Compared to the control, Nile tilapia fed on diet LT exhibited up-regulation of key genes in a variety of processes. Specifically, insulin growth factor I (*igf-I*) was up-regulated in the liver of the fish to comparable levels between diets L and LT but diet T had no effect. This suggests that *igf-I* was mainly up-regulated by limonene with no synergistic or additive effect of limonene and thymol in mediating growth through the somatotropic axis. On the other hand, mucin-like protein (*muc*) was significantly higher in the fore intestine of fish fed diet LT, while diets L and T resulted in similar levels of expression of *muc* compared to the control. This regulation of *muc* correlated with enhanced growth of the fish fed diets L and LT, potentially linked to enhanced mucus secretion and absorption of dietary nutrients for growth. Consistent with this, oligo-peptide transporter 1 (*pept1*) had a higher expression in fish fed diet LT and better feed utilisation efficiency (FCR and PER) compared with the control. Given that feed utilisation efficiency was also enhanced with diet L compared with the control, it was suggested that limonene influenced the enhanced absorption of protein and ultimately improved the growth of the fish. Lipoprotein lipase (*lpl*) was activated in the fish fed diet LT compared to the control and diet T. Given the role of *lpl* in lipid catabolism (Tian *et al*., 2015), the results indicated that dietary limonene boosted energy levels of the fish that spared protein for growth and

could thus have accounted for the enhanced fish growth found with diet LT and L. Such an activation of *lpl* by limonene corresponding with improved somatic fish growth is concurring with our earlier results in Chapter 4. Also consistent with Chapter 4, limonene activated the antioxidant defences of Nile tilapia as shown by a significantly higher expression of catalase (*cat*) with dietary treatments L and LT compared to the control. This suggested that *cat* minimised the concentration of ROIs in dietary treatments L and LT thereby allowing bodily functions to run adequately. This may have potentially contributed to the improved somatic growth of the fish fed on diets L and LT.

## 6.3 Conclusions

The results from this research showed that:

1. Limonene supplemented at 400, 500 and 600 ppm and thymol at 750 ppm in the diet of Nile tilapia juveniles had growth-promoting effects. Dietary levels of 250, 500, 750 and 1000 ppm carvacrol did not enhance the growth of Nile tilapia juveniles.
2. The growth-promoting effects of limonene (400 and 600 ppm) occurred after some period of time as reflected by strong interaction / correlation between concentration of limonene and growth duration.
3. The growth-promoting effects of limonene and thymol were not associated with an improvement in feed intake and appetite.
4. Dietary limonene at 400 and 600 ppm has an effect on the expression of *igf-I*, *alp*, *lpl* and *cat* in the liver and *muc* and *pept1* in the fore intetine of juvenile Nile tilapia. Dietary thymol supplied up to 500 ppm did not improve growth of Nile tilapia nor regulate the expression of *igf-I*, *gh*, *ghr-I*, *muc*, *pept1*, *glut2*, *ap*, *p-amy*, *ctra*, *pla2*, *alp*, *lpl*, *pparα*, *fas*, *srebf1*, *sod2*, *gpx*, *cat, lepr, and npy* genes investigated in this study.
5. A combination of limonene (400 ppm) and thymol (500 ppm) in the diet did not have synergistic or additive effects on the growth performance of juvenile Nile tilapia, with

limonene mainly influencing the attained somatic growth. In addition, key genes regulating nutrient digestion, absorption and transport (*muc* and *pept1*), lipid metabolism (*lpl*), antioxidant enzymes (*cat*) and somatotropic axis growth (*igf-I*) did not exhibit synergistic or additive effects of the dietary combination of limonene and thymol in enhancing the growth of Nile tilapia.

## 6.4 Future perspectives

This thesis presents results of a novel study on the effects of phytogenic compounds on growth and nutritional physiology of Nile tilapia. The pathways within the growth and nutritional physiology of Nile tilapia investigated herein were selected from previous studies involving mainly pigs, chicken, rats and humans since studies on fish are scarce. Despite previous studies had reported on the activation of various nutritional physiology pathways, the data existing prior this study were somewhat fragmented and unclear. As discussed above, the present study unveiled some pathways regulated in Nile tilapia by dietary limonene or the combination of limonene and thymol, although it is also fair to acknowledge that further research is still required to expand our findings in this research area.

Given that fish of different life stages utilise dietary nutrients differently, it is reasonable to expect that responses to various phytogenic compounds and doses will vary among fish of different developmental stages. Therefore, it will be worth evaluating effects of limonene and thymol and potentially others phytogenic compounds on Nile tilapia of other life stages (i.e. fry and adults) not investigated in this study. There is also need to investigate whether the growth-promoting effects of the phytogenic compounds are sustained from fry stage until the stage at which fish attain marketable size. Some literature suggested that there can be gender-specific differences in the utilisation of phytogenics. Therefore, it will be interesting to evaluate the effects of phytogenic compounds on growth

and nutritional physiology of different gender of fish. In this study, phytogenic compounds were sprayed onto the pellets and the fish were fed little portions of feed at a time to maximise pellet consumption and phytogenic uptake by fish. In order to further minimise potential leaching of phytogenics out from the pellet, future studies need to explore alternative inclusion strategies to prevent such a potential leaching and thus optimise delivery of phytogenic compounds to the fish. It will also be interesting to investigate the effects of feed processing methods and storage duration of the feed on the quantity of phytogenics in the diets. An information gap also exists on where phytogenic compounds end up in the GIT of fish, whether they are digested and absorbed intact in the gut and what effects they have on microflora in the gut. Moreover, given that the phytogenic compounds are administered in the feed over a period of time, there is need to investigate the effects of the compounds on the taste of the fish and develop approaches to minimise off-flavours incase they exist.

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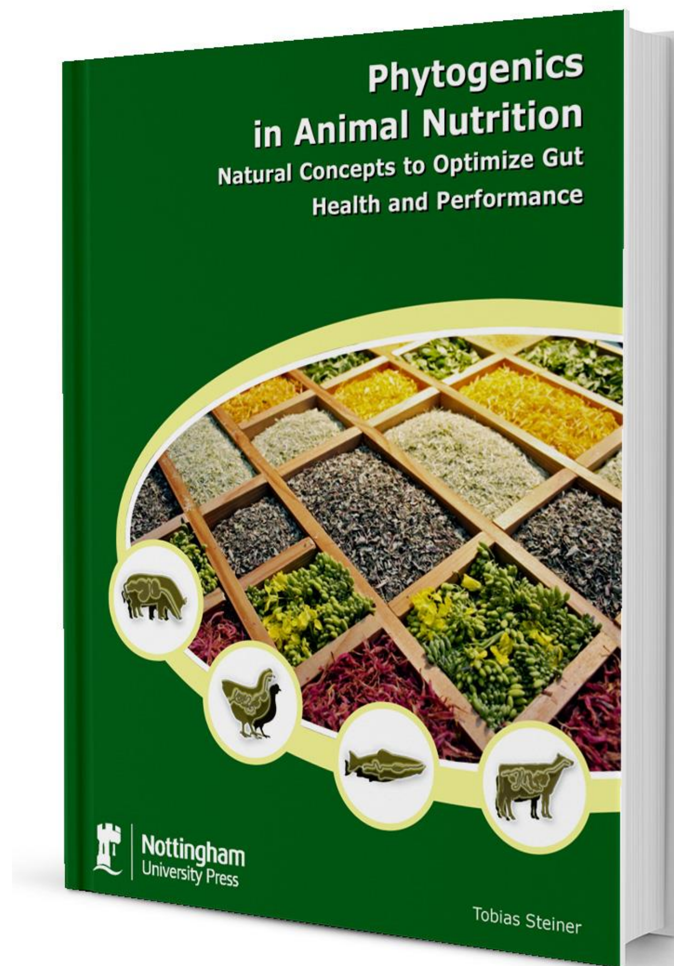


Book on phytogenics in animal nutrition (Source: Steiner, 2009)

"Treat the past as a school"

**Jim Rohn**

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# Outputs from the research

## Conference Presentations

1. Post Graduate Conference, February 2015. Institute of Aquaculture, University of Stirling, United Kingdom. Poster presentation: Aanyu, M. and Monroig, O. Effects of selected phytogenic compounds on the growth performance of Nile tilapia, *Oreochromis niloticus*.
2. Conference of the Aquaculture European Society, October 2015. Rotterdam, The Netherland. Poster presentation: Aanyu, M, Betancor, M. B. and Monroig, O. Effects of limonene supplemented diets on the growth and expression of genes regulating appetite, antioxidant enzyme status, mucus secretion and lipid metabolism in Nile tilapia, *Oreochromis niloticus.*

## Manuscripts in Preparation

1. A review paper on effects of phytogenic compounds on growth and nutritional physiology of monogastric animals.
2. Effects of individually supplied phytogenic compounds on genes regulating growth and nutritional physiology in Nile tilapia.
3. Effects of combined phytogenic compounds on the growth and nutritional physiology of Nile tilapia.