



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

A THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

By

MARGARET AANYU

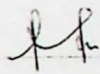
INSTITUTE OF AQUACULTURE, SCHOOL OF NATURAL SCIENCES,
UNIVERSITY OF STIRLING, STIRLING, SCOTLAND, UNITED KINGDOM

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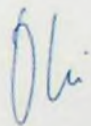
Declaration

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.

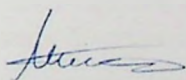
Name of Candidate: Margaret Aanyu

Signature: 

Principle Supervisor: Dr. Oscar Monroig

Signature: 

Additional Supervisor: Dr. Mónica Betancor

Signature: 

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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. Phytochemicals are among the products being investigated as potential naturally derived growth promoters. The aim of this study was to identify phytochemical compounds and doses with growth-promoting effects in Nile tilapia and investigate relevant pathways underlying their growth promotion effects. The phytochemical 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 phytochemical 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 phytochemical 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 somatotrophic 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-

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

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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 its 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 °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 °C, oxygen ≥ 3 mg L⁻¹ and ammonia-nitrogen ≤ 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.

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).

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

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

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

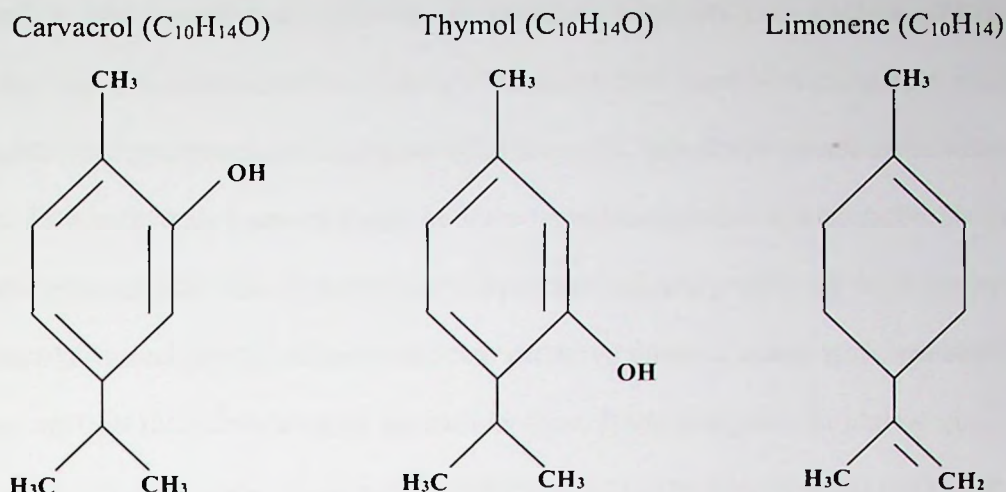


Figure 1.3: Chemical structure of carvacrol, thymol and limonene (Lee *et al.*, 2003b; Turek and Stintzing, 2013).

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 °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,

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 phytogetic compounds such as histological abnormalities, nephropathy, cytotoxicity, alterations in haematological and biochemical parameters (Sun, 2007; Gad, 2012).

1.3 Phytogetics 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 phytogetics, enzymes, probiotics, prebiotics, acidifiers, minerals, organic acids, anti-microbial peptides and vitamins (Lalles *et al.*, 2009; Steiner, 2009; Yang *et al.*, 2015). Phytogetics are being investigated as potential

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 phytonics 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 phytonic products before they became effective in enhancing growth. Besides, some phytonic 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

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 (<i>Allium sativum</i>) 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 <i>et al.</i> , 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 <i>et al.</i> , 2008
	Sterlet sturgeon (<i>Acipenser ruthenus</i>)	500 and 1,000	70	FW, SGR, WG and FCR improved with dietary garlic	Lec <i>et al.</i> , 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 (<i>Foeniculum vulgare</i>) powder	Nile tilapia	1,000	90	Improved FW, mean WG, FI, FCR and PER with dietary fennel powder	Soltan and El-Laithy, 2008
Thyme (<i>Thymus vulgaris</i>) 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 <i>et al.</i> , 2010
Thyme, fenugreek (<i>Trigonella foenum graecum</i>) or neem (<i>Azadirachta indica</i>)	Nile tilapia	1,000	42	Highest FW was with the diet containing thyme	Antache <i>et al.</i> , 2013

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

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 phytogetic product. Therefore, there is need to use a consistent quality of phytogetic products. This could be achieved by using purified bioactive compounds of EOs. Recent research on phytogetic 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 phytogetic compounds on fish growth

There is scanty literature on the effects of phytogetic 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 phytogetic 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

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

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

The effects of phytogetics 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 phytogetic 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 phytogetic compounds exert growth promotion. Although it is suggested that the mode of action by phytogetics 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 phytogetic 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 phytogetic compounds. Therefore, there is need to explore the effect of different phytogetic compounds on the nutritional physiology of different fish species. Given that there is scant information on the effects of phytogetic 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.

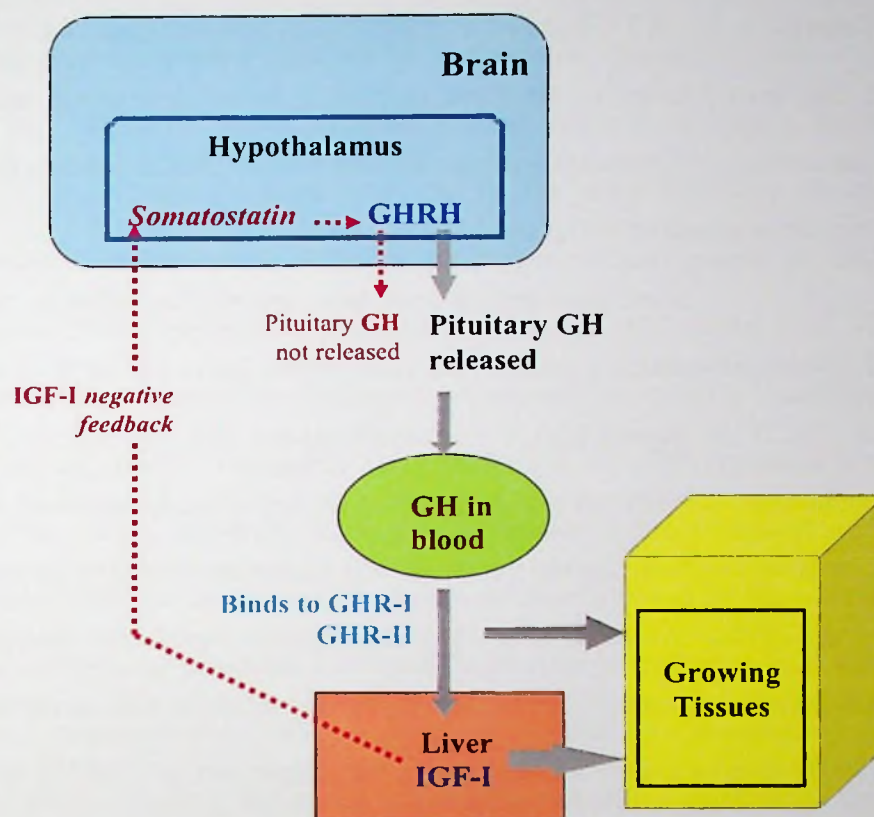


Figure 1.4: Schematic representation of growth hormone (GH) - insulin growth factor-I (IGF-I) regulation in fish (Modified from Moriyama *et al.*, 2000; Reinecke, 2010).

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

1.4.2 Influence of phytogetic 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 phytogetics 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 phytogetic-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 phytogetic-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 phytogetic 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 phytogetic compounds (Bravo *et al.*, 2014). Conversely, some phytogetic 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,

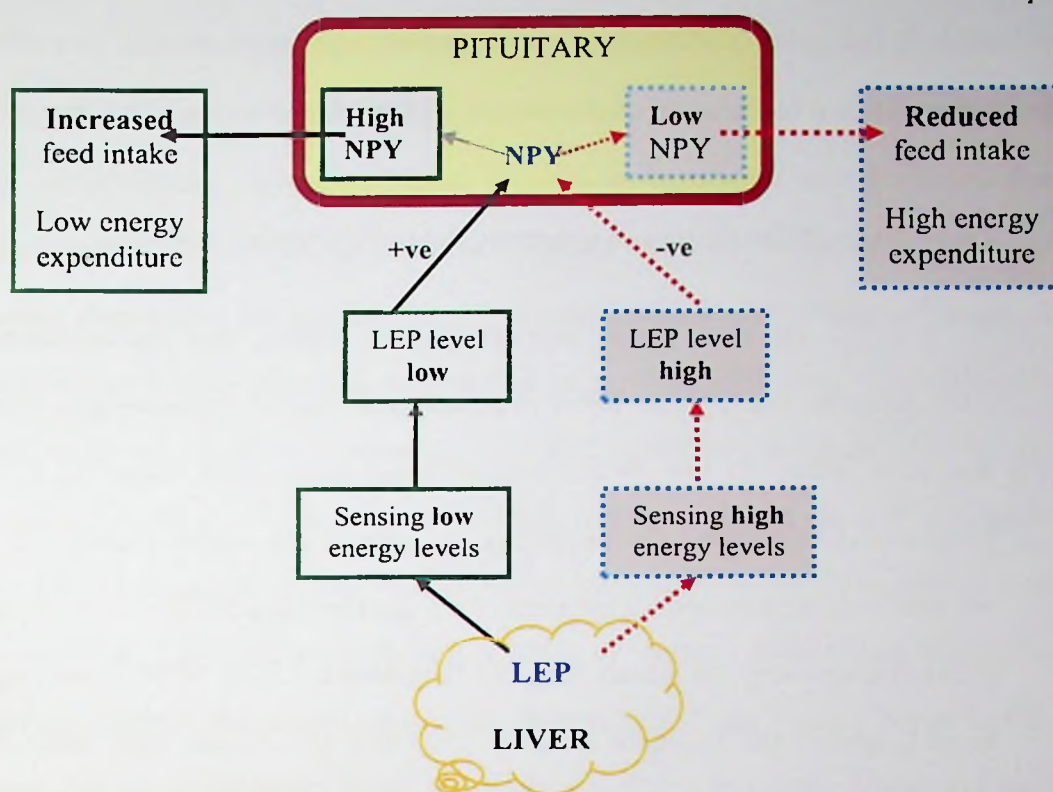


Figure 1.5: Diagram illustrating regulation of fish appetite and feed intake by leptin (LEP) and neuropeptide Y (NPY) hormone in the brain (Modified from Volkoff *et al.*, 2005; Kulczykowska and Vazquez, 2010).

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 *npv* 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*

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 phytogetic 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, phytogetics 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 phytogetic 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 (Panaserat and Kaushik, 2010). For instance, phytogetic 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 (Panaserat and Kaushik, 2010). The stomach and intestine are key locations where phytogetics are metabolised along the GIT (Michiels

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; Dezfali *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-

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 (Kroghdahl *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

the levels of free fatty acids in the tissue (Skiba-Cassy *et al.*, 2012). This means *ppars* 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

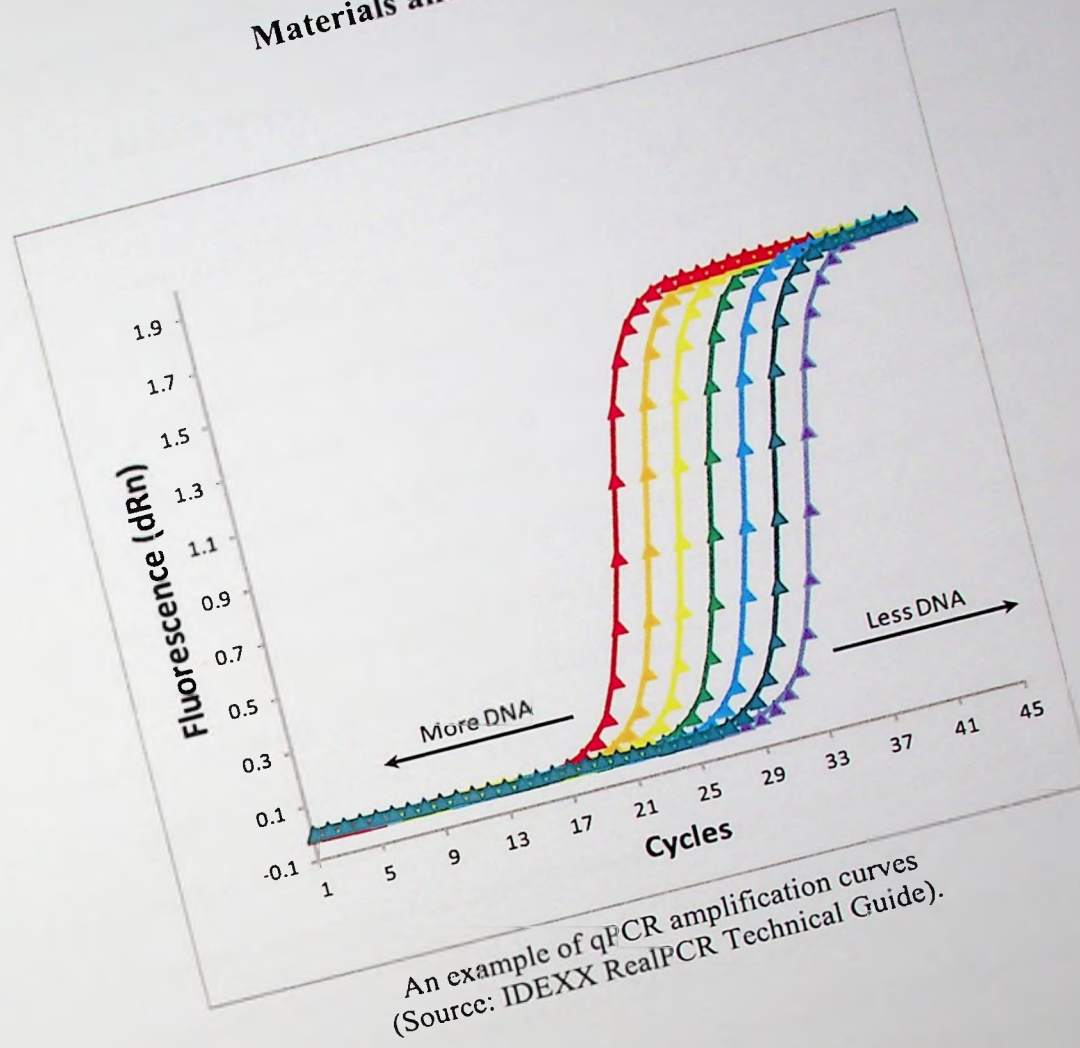
PUFAs in the cell membrane, which can ultimately affect growth (Tocher, 2003; Rajat and Panchali, 2014). The antioxidant defence response in fish is triggered when the fish encounter stressful conditions that increase metabolic processes such as rough handling, poor water quality, toxic substances, poor 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 phytogetic components have potential antioxidant effects (see Table 1.3) (Steiner, 2009). However, most of the studies evaluating antioxidant properties of phytoGENIES 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 phytogetic 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

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 somatotrophic-axis growth-mediation, appetite regulation, nutrient digestion, absorption and transport, lipid metabolism and antioxidant enzyme defence system, to enhance the growth of fish.

Chapter 2: Materials and methods



"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 phytogetic 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 phytogetic 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 phytogetic 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 phytogetic compounds limonene (97 % purity), thymol (99.5 % purity) and carvacrol (98 % purity) were obtained from



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.



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 anaesthetised 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

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:

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

2.5.1.2 Dry matter

The dry matter content was calculated as:

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

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 °C for 20-28 hours (h). The samples were cooled at room temperature in a desiccator and reweighed. The ash content was calculated as:

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 Kjelttec 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:

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

$$\% \text{ protein} = N \times F$$

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).

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 fibre cap capsules, placed in a small holding carousel and de-fatted by agitating the fibre caps 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:

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

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

2.5.2 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 μ l 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 $20,000 \times g$ for 15 min at 4 °C. 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 $20,000 \times g$ 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 $20,000 \times g$ for 5 min at room temperature. Thereafter each supernatant was removed using a separate pipette. The samples were spun for 3-5 s and

water in 0.2 mL tubes, vortex mixed, centrifuged and incubated on ice. To each sample, 10 μ l of the reverse transcription mixture, containing 2 μ l of 10 $\times$ reverse transcription (RT) buffer, 0.8 μ l of 25 $\times$ dNTP mix, 2 μ l of 10 $\times$ random primer in the molar ratio of 1.5:0.5 for the random to oligo-dT primer, respectively, 1.0 μ l of reverse transcriptase and 4.2 μ l of nuclease free water was added. The 20 μ l 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 °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.

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	<i>muc</i>	F: TGCCCAGGAGGTAGATATGC R: TACAGCATGAGCAGGAATGC	101	XM_005466350.2
	<i>pept1</i>	F: CAAAGCACTGGTGAAGGTCC R: CACTGCGTCAAACATGGTGA	196	XM_013271589
	<i>Alp</i>	F: CTTGGAGATGGGATGGGTGT R: TTGGCCTTAACCCCGCATAG	200	XM_005469634.2
	<i>Ctra</i>	F: AGTGCCGAGAACATCCAGAC R: GAAGTCTCGGCCACACAAAC	155	XM_003437588.3
	<i>pla2</i>	F: CTCCAAACTCAAAAGTGGGCC R: CCGAGCATCACCTTTTCTCG	177	XM_005451846
	<i>glut2</i>	F: TCTAAAGGGGCCGCATGATC R: GAAAGGTGCATCATGAGGGC	153	FJ914656
	<i>Ap</i>	F: TTACCACTCCGAACCAGACC R: GAGTAGTTCCCTCCTGCCTC	238	XM_005449270
	<i>p-amy</i>	F: TGGAGGCCCTGGTATCAAAAG R: TCCTGTTCCACCACCAGATC	168	XM_003448471.2
	<i>Lpl</i>	F: TGCTAATGTGATTGTGGTGGAC R: GCTGATTTTGTGGTTGGTAAGG	217	NM_001279753.1
	<i>Ppara</i>	F: CTGATAAAGCTTCGGGCTTCCA R: CGCTCACACTTATCATCTCCAGCT	106	NM_001290066.1
Lipid metabolism	<i>srebf1</i>	F: TGCAGCAGAGAGACTGTATCCGA R: ACTGCCCTGAATGTGTTTCAGACA	102	XM_005457771.2
	<i>Fas</i>	F: TGAAACTGAAGCCTTGTGTGCC R: TCCCTGTGAGCGGAGGTGATTA	144	GU433188
	<i>Lepr</i>	F: TCTGTTCTTTTGGGCATAG R: CCTCCTGATGTCTTTTCCA	143	XM_005460446.1
	<i>Npy</i>	F: CCTCTTCTTACGCATCAGC R: CCCCTCCTCCACTTTACGAT	115	XM_003448854.2
Antioxidant enzyme activity	<i>Gpx</i>	F: ACAAGTGACATCGAGGCAGA R: CAAACCCAGGCCCTGCTATAA	186	NM_001279711.1
	<i>Cat</i>	F: TCCTGGAGCCTCAGCCAT R: ACAGTTATCACACAGGTGCATCTTT	79	JF801726
	<i>sod2</i>	F: CTCCAGCCTGCCCTCAA R: TCCAGAAAGATGGTGTGGTTAATGTG	58	XM_003449940.3
Somato-tropic axis growth mediation	<i>Gh</i>	F: TCGGTTGTGTGTTTGGGCGTCTC R: GTGCAGGTGCGTGA CTCTGTTGA	90	XM_003442542
	<i>ghr-I</i>	F: ATGGCTCTCTGCCCTCCTCTAA R: ATGTCGTGTGGTCCCAAGTCAGTGA	109	NM_001279601
	<i>igf-I</i>	F: GTCTGTGGAGAGCGAGGCTTT R: CACGTGACCGCCTTGCA	70	NM_001279503
Reference genes	<i>ef-1α</i>	F: GCACGCTCTGCTGGCCTTT R: GCGCTCAATCTTCCATCCC	250	NM_001279647
	<i>β-actin</i>	F: TGGTGGGTATGGGTCAGAAAG 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, *ppara* peroxisome proliferator-activated receptor alpha, *srebf1* sterol regulatory element binding transcription factor 1, *fas* fatty acid synthase, *lepr* leptin receptor variant X1, *npv* 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-1 α* elongation factor 1 α , *β -actin* beta-actin.

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

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

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

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

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

$$GR_{MBW} (g Kg^{-0.8} d^{-1}) = (W_t - W_o) / MBW / d$$

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

$$CF = \frac{W_t}{L^3} \times 100$$

W_t = final mean fish weight at end of the trial

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

$$\% survival = \frac{N_f}{N_o} \times 100 \%$$

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

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

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

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

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

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

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

$$PI = F \times p$$

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

p is the fraction of crude protein in the feed

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

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

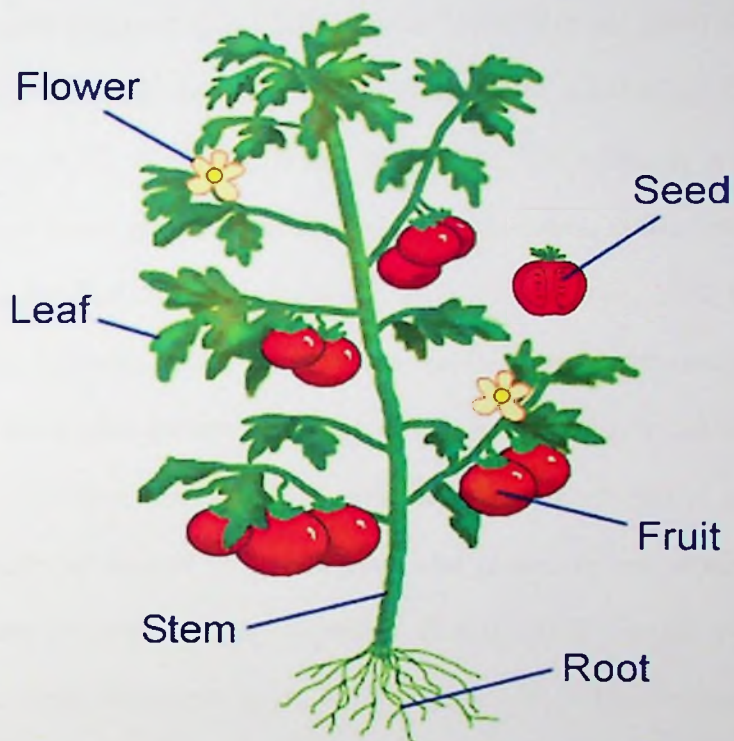
W_o is the average weight (g) of fish at the beginning of the trial

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

p is the fraction of crude protein in the feed

Chapter 3:

Effects of selected phytochemical compounds on growth performance of Nile tilapia



Plant showing parts from which phytochemicals 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 phytochemicals 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 others found no enhanced growth (Mesalhy *et al.*, 2008; Zakes, 2008; Kulthanaparames *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 phytochemicals in an effort to standardise the dose of active compounds.

Presently, few authors have investigated the effects of phytochemical 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

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 phytogetic 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 phytogetic 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

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)
<i>Trial I</i>	7.6 ± 0.3	7.3 ± 0.2	27.3 ± 0.9	< 0.2
<i>Trial II</i>	7.3 ± 0.2	7.0 ± 0.1	27.0 ± 1.0	< 0.2
<i>Trial III</i>	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 anaesthetised 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

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 $\pm$ 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 .

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 $\pm$ 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_{ABW}, % WG, % survival, % FI, FCR and PER.

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					P value
	C0	C250	C500	C750	C1000	
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.

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 superscripts 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.

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 victorinus*) 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 °C) of the water in which the fish were reared. It is argued that although Nile tilapia can thrive at temperatures ranging from 22 - 28 °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.*

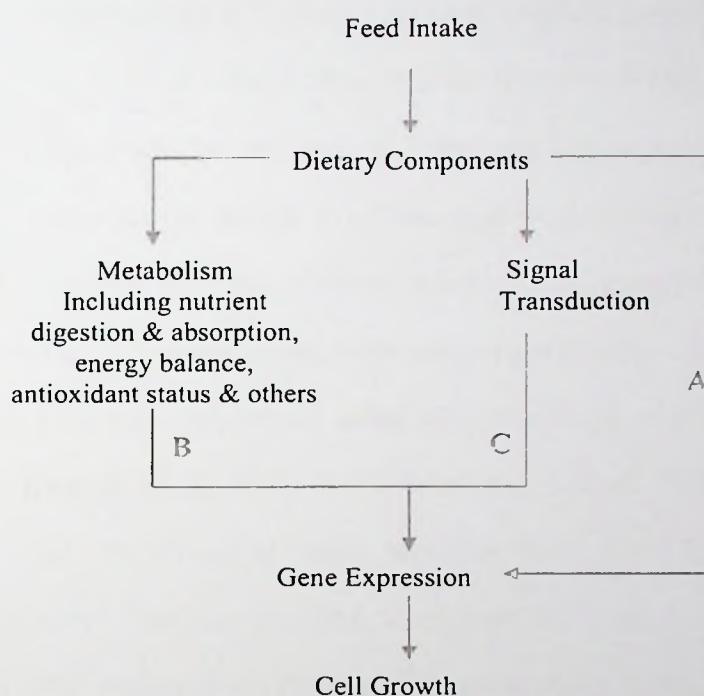
nutrient transportation and absorption, lipid and energy metabolism and antioxidant activity among others (Hashemi and Davoodi, 2010; Muthusamy and Sankar, 2015; Steiner and Syed, 2015; Yitbarek, 2015; Zeng *et al.*, 2015; Abd El-Hack, *et al.*, 2016). It is reported that phytonutrients may use more 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 phytochemical 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 phytogetic 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 somatotrophic axis-mediated growth among other processes (Rust, 2003; Qiang *et al.*, 2012), there is little literature on the pathways activated by phytogetic compounds. In addition, different phytogetic 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 phytogetic compounds in fish, pigs and chicken have been carried out using diets containing combinations of phytogetic 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 phytogetic 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 phytogetic 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

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 somatotrophic 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.

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 $1.5 \pm 0.0 \text{ g}$ for Trial IV and $1.5 \pm 0.0 \text{ g}$ for Trial V (mean $\pm$ standard error). Both trials were conducted in 60 L plastic tanks in a recirculating aquaculture system. Each concentration of phytogetic 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 °C, pH was 6.8 ± 0.5 , dissolved oxygen $6.8 \pm 0.6 \text{ mg L}^{-1}$ and ammonia-nitrogen $0.8 \pm 1.0 \text{ mg L}^{-1}$ (mean $\pm$ 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 anaesthetised using

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 somatotrophic 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 hgreen 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.

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) × 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 length³) × 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) × 100.

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

GR, growth rate; DGC, 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 of the trial; $N = 3$ replicates for GR, DGC, % WG, CF, % survival, % FI, FCR, FCE and PER.

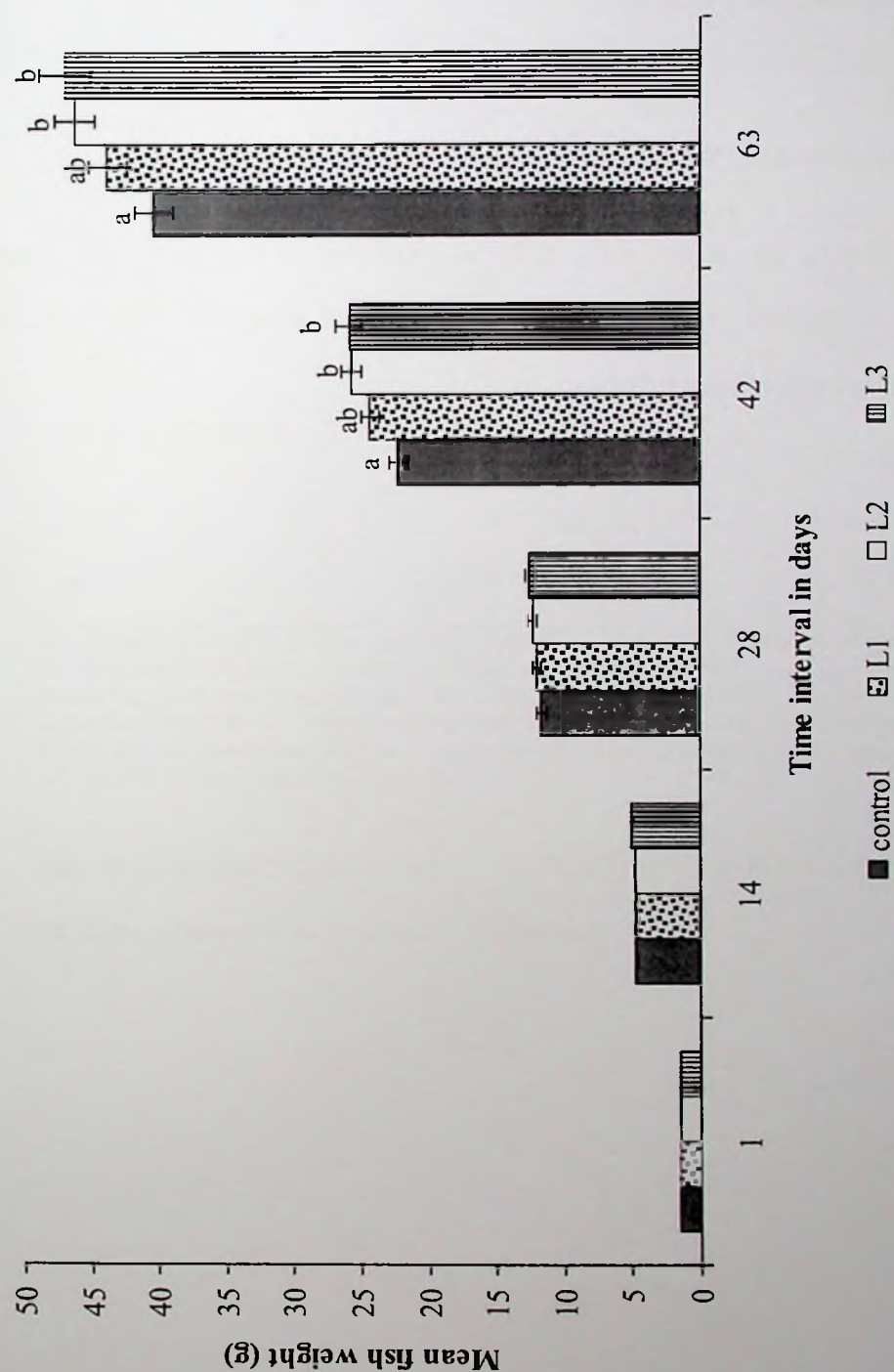


Figure 4.1: Mean weight of fish fed on diets supplemented with 0 (control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene on day 1, 14, 28, 42 and 63 of Trial IV. All values are means of treatments $\pm$ standard error. Mean values with different superscript in the same time interval are significantly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

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			P value
	Control	T1	T2	
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	3.5 ± 0.0	3.2 ± 0.1	3.2 ± 0.01	NS
(% body weight d ⁻¹)				
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 initial 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).

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 somatotrophic 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).

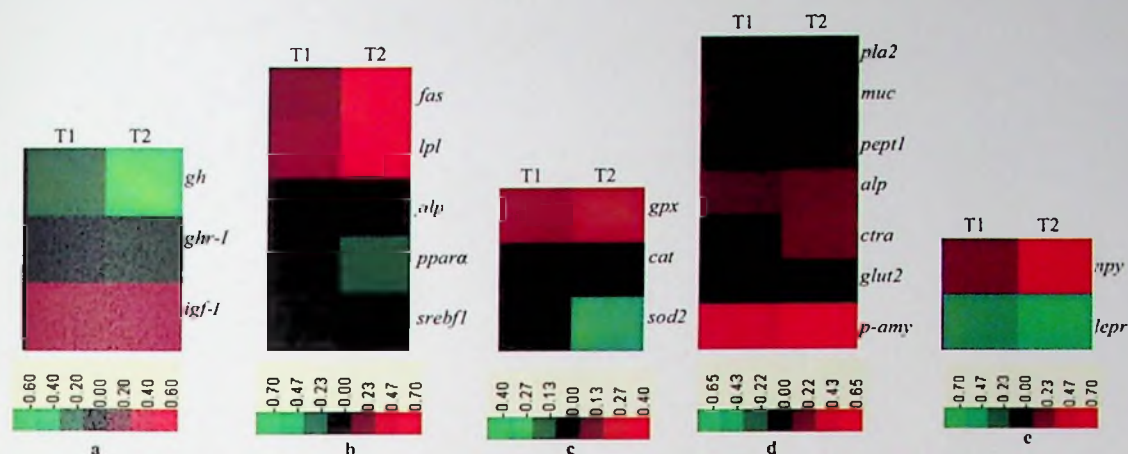


Figure 4.4: Heat map indicating expression patterns of twenty selected genes analysed using qPCR data from Nile tilapia fed thymol supplemented diets.

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 somatotrophic 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; *ppara*, 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; *npv*, pro-neuropeptide Y-like; *lepr*, leptin receptor variant XI.

4.3.2.1 Expression of somatotrophic 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.

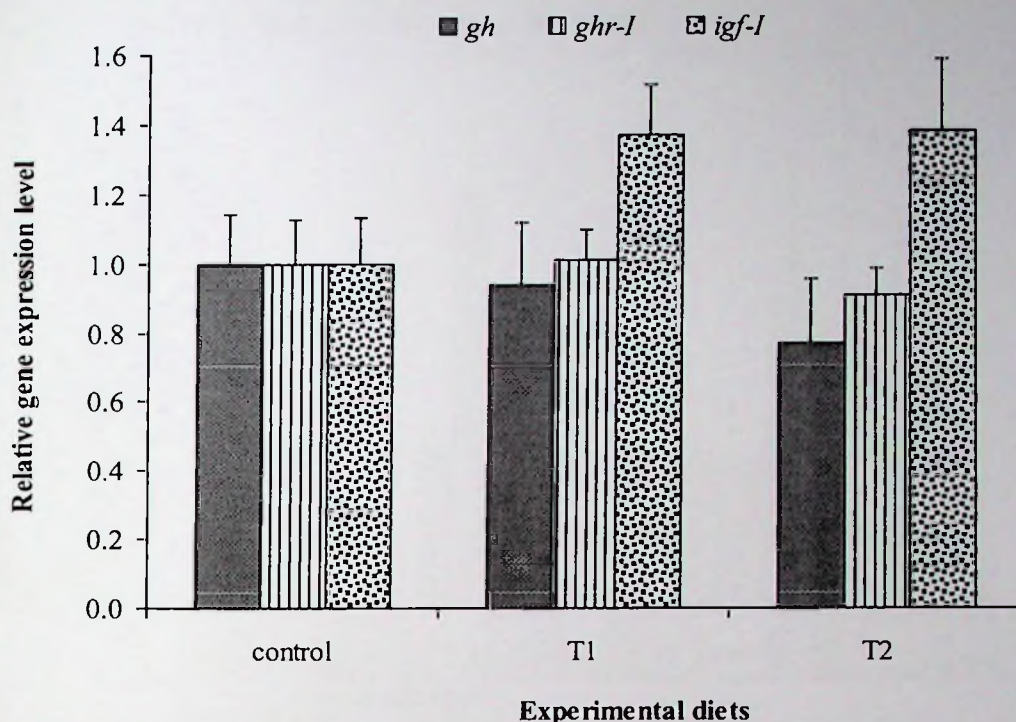


Figure 4.6: Expression of growth hormone (*gh*), growth hormone receptor I (*ghr-I*) and insulin growth factor I (*igf-I*) in the liver of Nile tilapia fed on diets with 0 (control), 250 (T1), and 500 (T2) ppm thymol.

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 *ppara* in the liver of the fish.

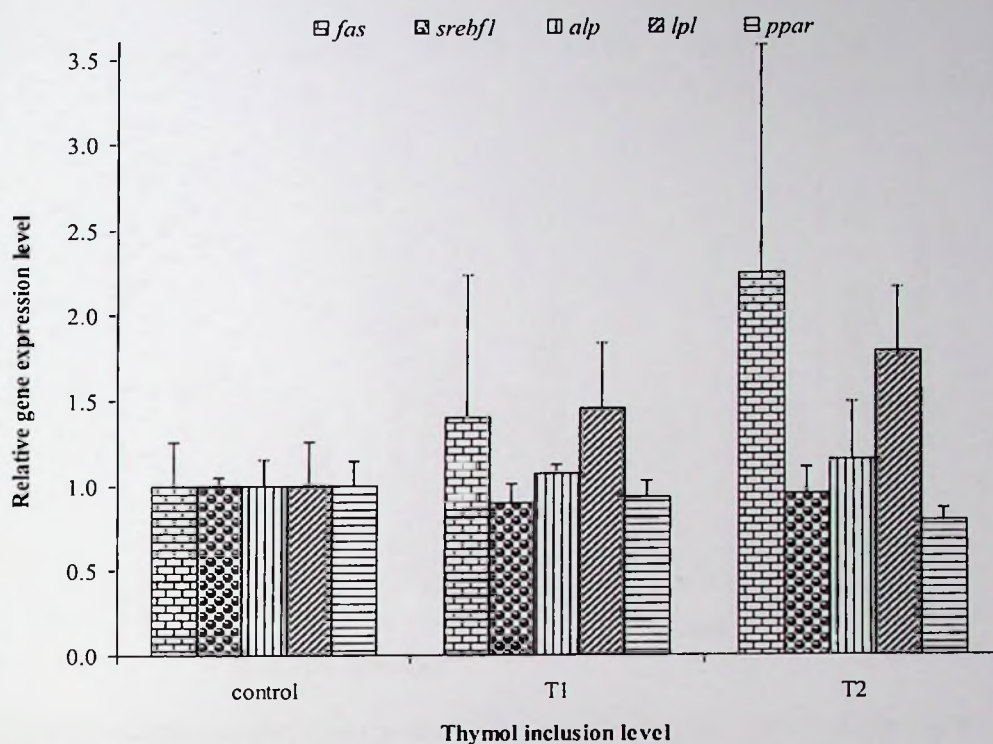


Figure 4.8: Expression of fatty acid synthase (*fas*), sterol regulatory element binding transcription factor 1 (*srebf1*), alkaline phosphatase (*alp*), lipoprotein lipase (*lpl*), and peroxisome proliferator-activated receptor (*ppar*) alpha in the liver of Nile tilapia fed on diets with 0 (control),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

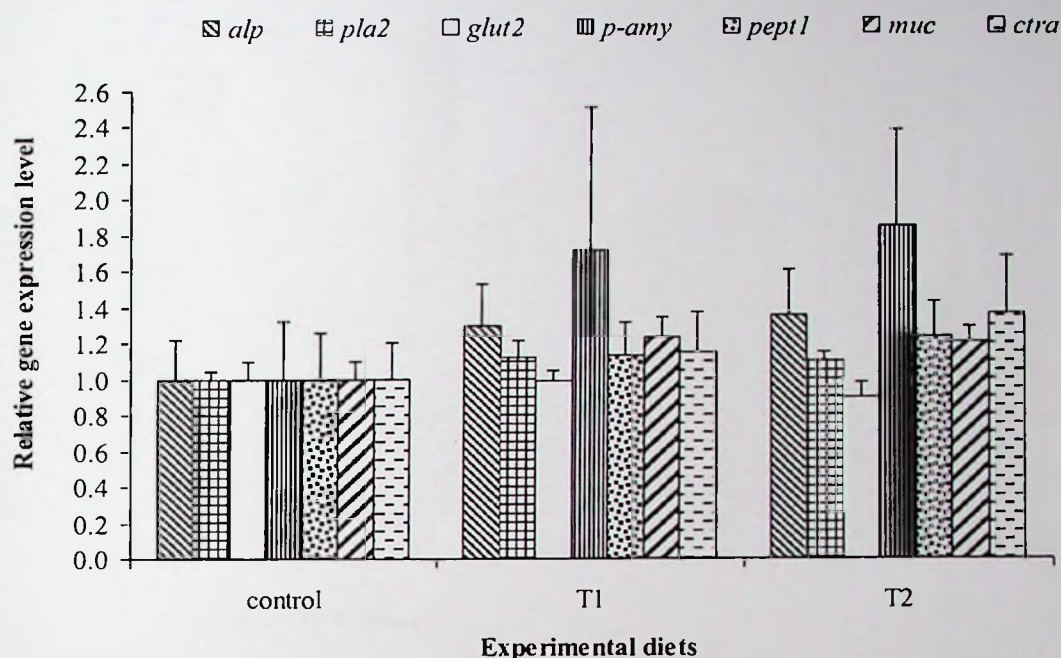


Figure 4.10: Expression of alkaline phosphatase (*alp*), phospholipase A2 (*pla2*), glucose transporter 2 (*glut2*), pancreatic alpha-amylase (*p-amy*), oligo-peptide transporter 1 (*pept1*), mucin-like protein (*muc*), and chymotrypsin A-like (*ctra*) genes in the fore intestine of Nile tilapia fed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

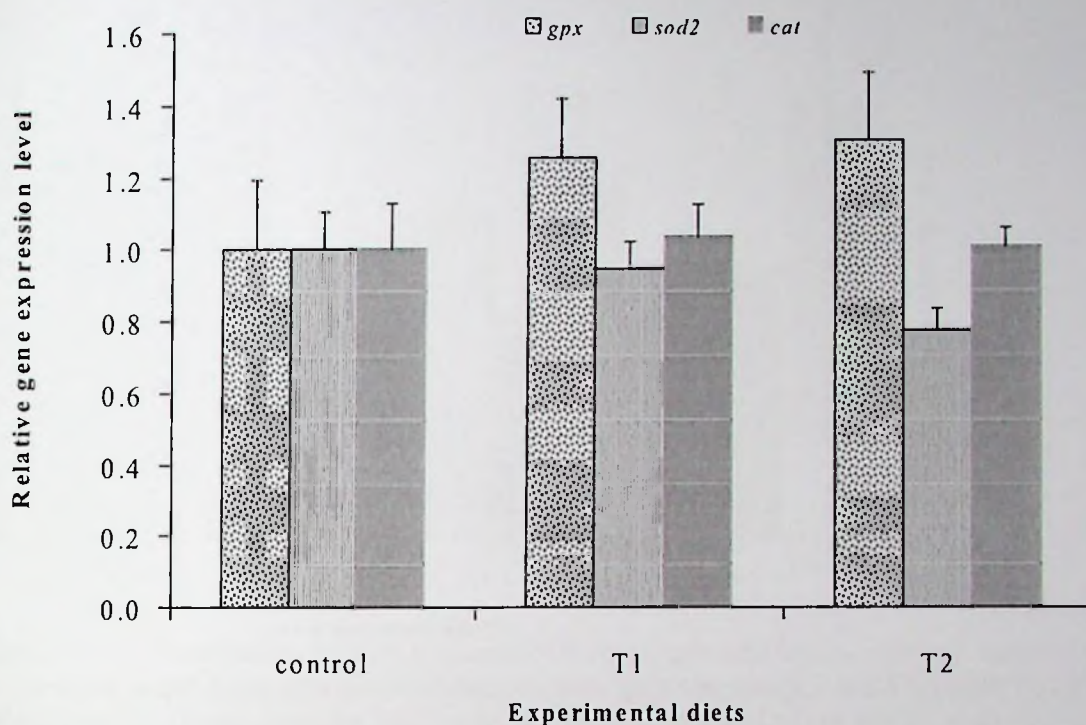


Figure 4.12: Expression of the antioxidant enzymes glutathione peroxidase (*gpx*), superoxide dismutase 2 (*sod2*) and catalase (*cat*) in the liver of Nile tilapia fed on diets with 0 (control), 250 (T1), and 500 (T2) ppm thymol.

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 *npv* ($P = 0.918$) and *lepr* ($P = 0.329$) in the brain of Nile tilapia with increasing dietary inclusion of limonene (Figure 4.13).

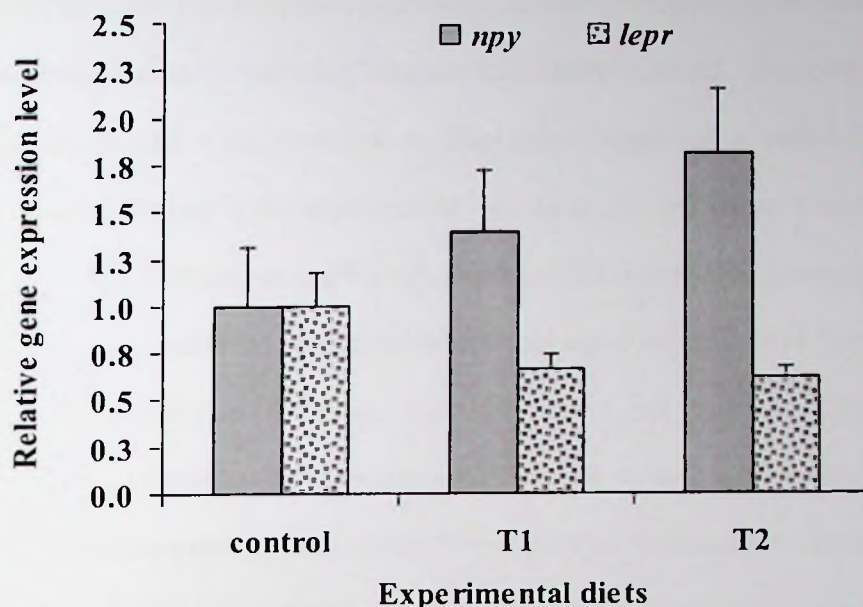


Figure 4.14: Expression of pro-neuropeptide Y-like (*npy*) and leptin receptor variant XI (*lepr*) genes in the brain of Nile tilapia fed on diets with 0 (control), 250 (T1), and 500 (T2) ppm thymol.

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 phytochemical compounds that have shown to promote growth in monogastric animals (Khaled and Megahad, 2014; Acar *et al.*, 2015; Ngugi *et al.*, 2016; Hafecz *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

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 phytonutrients 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; Georgiadi 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

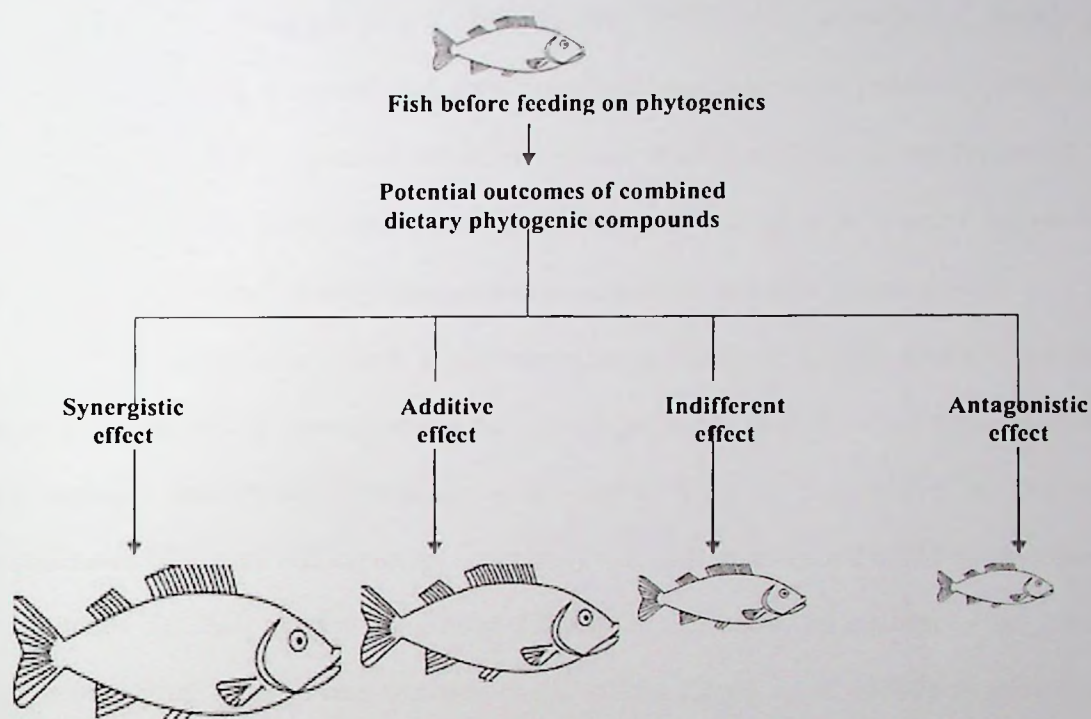
manner compared to individual amino acids (Tengjaroenkul *et al.*, 2000; Verri *et al.*, 2011). Other accessory genes to *muc* and *pept1* that were assessed in this current study included *p-amy* (pancreatic alpha-amylase), *ctra* (chymotrypsin A-like) and *pla2* (phospholipase A2) involved in digestion 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* occurred 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 (*npv*) 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 *npv* or *lepr*, or increased feed intake

Chapter 5:

Effects of combined phytogetic compounds on growth and nutritional physiology of Nile tilapia



Diagrammatic illustration of potential effect of combined phytogetic 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 (Hyltdgaard *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

The studies above highlight the importance of identifying combinations and doses of phytogetic compounds resulting in additive and synergistic effects on fish growth. In previous experiments (Chapters 3 and 4), two phytogetic 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 phytogetic compounds. A selection of genes that are markers within the pathways of somatotrophic 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 phytogetic compounds to the standard feed, each concentration of phytogetic compounds was

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 °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 °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 $\pm$ 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

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 μ l 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 μ g/ μ l) 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 μ g/ μ l of each RNA pooled sample, nuclease-free water was added to make up a volume of 10 μ l, which was incubated in 10 μ l of reverse transcription (RT) reaction mixture comprising 2 μ l of 10 x RT buffer, 0.8 μ l of

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	<i>Muc</i>	F: TGCCCCAGGAGGTAGATATGC R: TACAGCATGAGCAGGAATGC	101	XM_005466350
	<i>pept1</i>	F: CAAAGCACTGGTGAAGTCC R: CACTGCGTCAACACATGGTGA	196	XM_013271589
Lipid metabolism	<i>Lpl</i>	F: TGCTAATGTGATTGTGGTGGAC R: GCTGATTTTGTGGTTGGTAAGG	217	NM_001279753
	<i>srebf1</i>	F: TGCAGCAGAGAGACTGTATCCGA R: ACTGCCCTGAATGTGTTTCAGACA	102	XM_005457771
	<i>Alp</i>	F: CTGGAGATGGGATGGGTGT R: TTGGCCTTAACCCCGCATAG	200	XM_005469634
	<i>pla2</i>	F: CTCCAAACTCAAAAGTGGGCC R: CCGAGCATCACCTTTTCTCG	177	XM_005451846
Antioxidant activity	<i>Cat</i>	F: TCCTGGAGCCTCAGCCAT R: ACAGTTATCACACAGGTGCATCTTT	79	JF801726
Somatotrophic axis-aided growth	<i>Gh</i>	F: TCGGTGTGTGTTGGGCGTCTC R: GTGCAGGTGCGTGACTCTGTGA	90	XM_003442542
	<i>igf-1</i>	F: GTCTGTGGAGAGCGAGGCTTT R: CACGTGACCCGCTTGCA	70	NM_001279503
	<i>ef-1a</i>	F: GCACGCTCTGCTGGCCTTT R: GCGCTCAATCTTCCATCCC	250	NM_001279647
Reference genes	<i>β-actin</i>	F: TGGTGGGTATGGGTCAGAAAG 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-1* insulin growth factor 1, *ef-1a* elongation factor 1 α , *β -actin* beta-actin.

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

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

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

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

$\text{Protein intake} = \text{feed intake} \times (\% \text{ crude protein in the diet} / 100)$

$\text{PER} = \text{weight gain} / \text{protein intake}.$

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 $\pm$ 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

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.

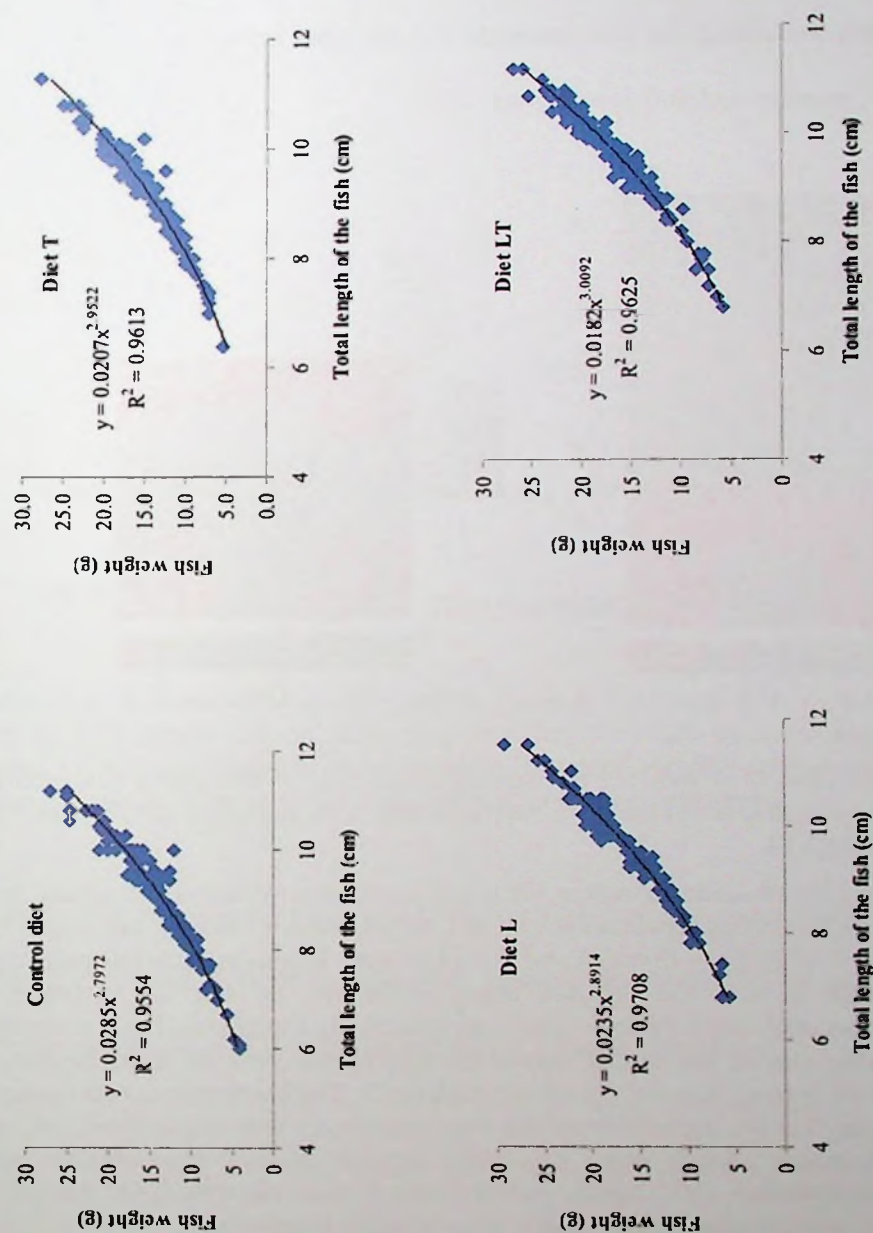


Figure 5.1: Length-weight relationship of Nile tilapia fed on diets T, L, LT and the control for 63 days.

5.3.2.1 Expression of somatotrophic 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).

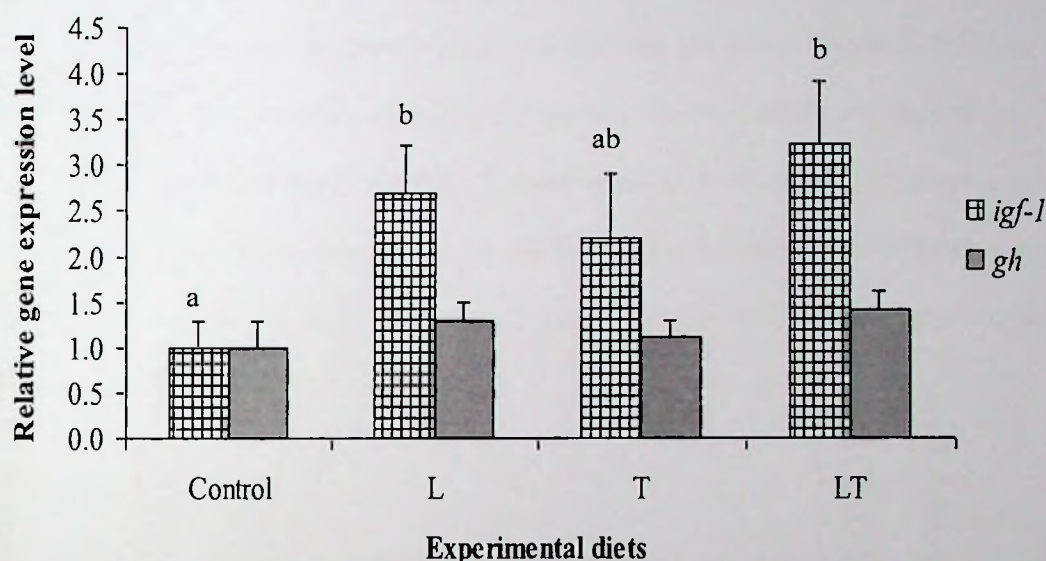


Figure 5.3: Expression of insulin growth factor I (*igf-I*) and growth hormone (*gh*) in the liver of Nile tilapia fed on diets supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and the combination of 400 ppm limonene and 500 ppm thymol (LT). Different superscript letters denote significant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

5.3.2.3 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).

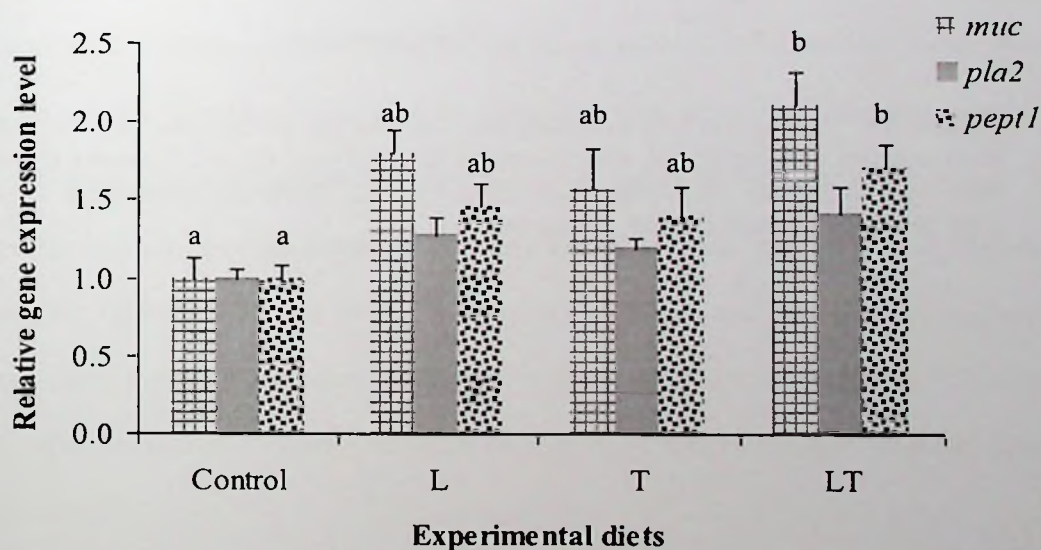


Figure 5.5: Expression of mucin-like protein (*muc*), phospholipase A2 (*pla2*), and oligo-peptide transporter 1 (*pept1*) genes in the fore intestine of Nile tilapia fed on diets supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and a combination of 400 ppm limonene and 500 ppm thymol (LT).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

5.4 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 somatotrophic 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 somatotrophic axis-mediated growth (*igf-1*), nutrient absorption and transport (*muc* and *pept1*), lipid metabolism (*lp1*) 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

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 phytochemical 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 somatotrophic 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 somatotrophic 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).

such metabolic response in fish fed diet LT. Given that *lpI* 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 *lpI* 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 *lpI* and a corresponding somatic growth enhancement further confirmed the results obtained in Chapter 4.

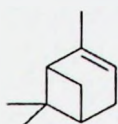
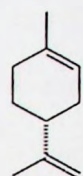
Metabolic processes in the body result into production of reactive oxygen intermediates (ROIs), which can induce damage to cells and tissues if their levels are not maintained low (Covarrubias *et al.*, 2008; Costa *et al.*, 2013). This can ultimately impair adequate physiological function and subsequently 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 phytochemical 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*.

Chapter 6:

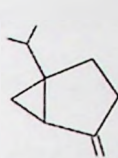
General discussion, conclusions and future perspectives

Terpenes

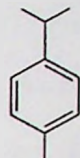
Monoterpenes

 α -Pinene

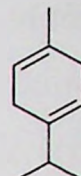
Limonene



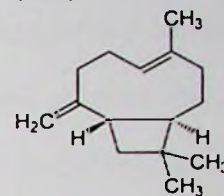
Sabinene



p-Cymene

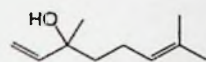
 γ -Terpinene

Sesquiterpenes

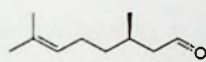
 β -Caryophyllene

Terpenoids

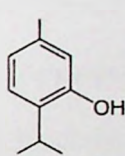
Monoterpenoids



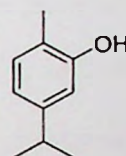
Linalool



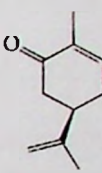
Citronellal



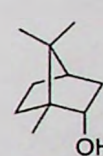
Thymol



Carvacrol



Carvone



Borneol

Chemical structure of some phytogetic 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 phytochemicals as potential natural growth-promoting products. Phytochemicals 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 phytochemical compounds (Applegate *et al.*, 2010). The overall aim of this present research was to investigate the effects of selected phytochemical 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 phytochemical 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 phytochemical 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 phytochemical 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

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

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

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 phytogetic 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 phytogetics in exerting their actions, there exists a cause-effect relationship between phytogetic inclusion and growth enhancement. On the other hand,

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.

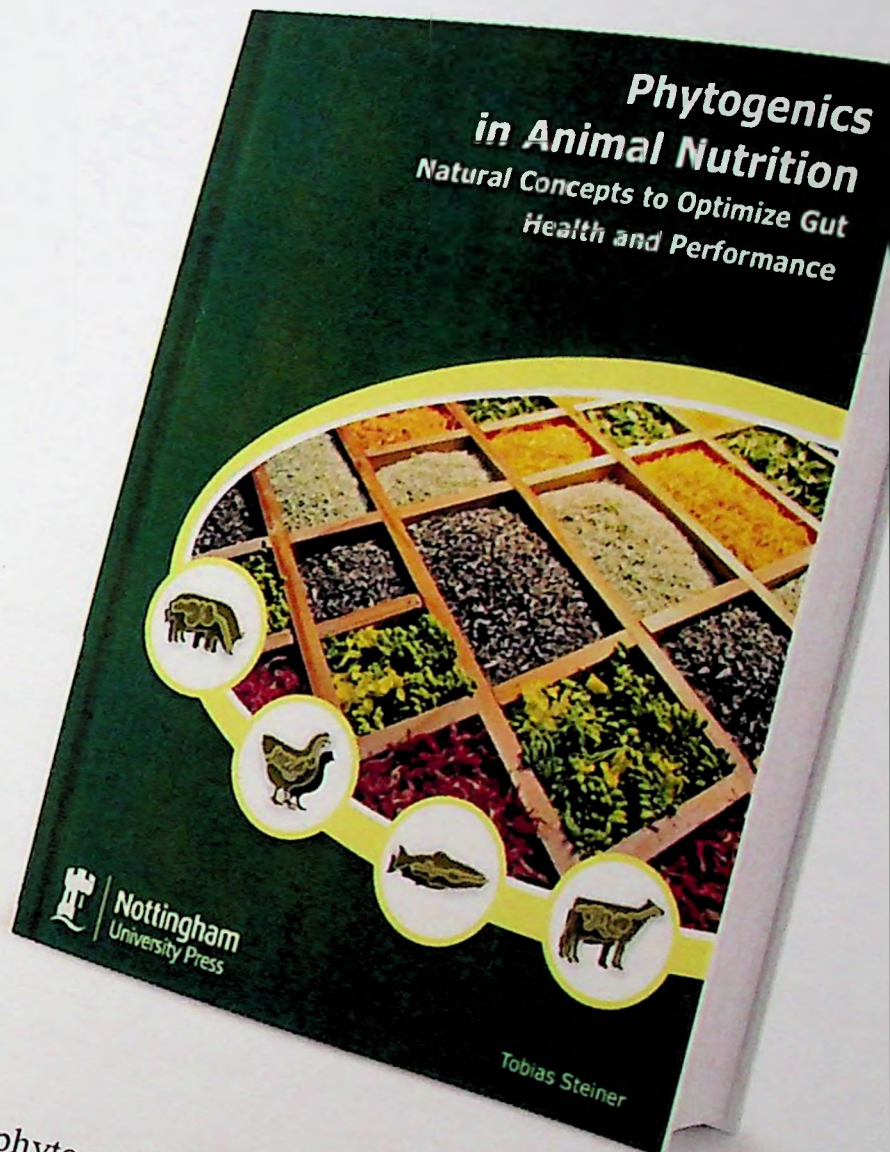
6.3 Conclusions

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 intestine 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*, *ppara*, *fas*, *srebf1*, *sod2*, *gpx*, *cat*, *lepr*. and *npv* 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

and nutritional physiology of different gender of fish. In this study, phytogetic compounds were sprayed onto the pellets and the fish were fed little portions of feed at a time to maximise pellet consumption and phytogetic uptake by fish. In order to further minimise potential leaching of phytogetics out from the pellet, future studies need to explore alternative inclusion strategies to prevent such a potential leaching and thus optimise delivery of phytogetic 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 phytogetics in the diets. An information gap also exists on where phytogetic 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 phytogetic 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.

References



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

"Treat the past as a school"

Jim Rob

- Abd El-Hack, M. E; Alagawany, M; Farag, M. R; Tiwari, R; Karthik, K; Dhama, K; Zorrichzahra, J. and Adel, M. (2016). Beneficial impacts of thymol essential oil on health and production of animals, fish and poultry: a review. *Journal of Essential Oil Research* 28, 365-382.
- Abdel-Tawwab, M. (2012). The use of American Ginseng (*Panax quinquefolium*) in practical diets for Nile tilapia (*Oreochromis niloticus*): Growth performance and challenge with *Aeromonas hydrophila*. *Journal of Applied Aquaculture* 24, 366-376.
- Abele, D. and Puntarulo, S. (2004). Formulation of reactive species and induction of antioxidant defense systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology, Part A* 138, 405-415.
- Acar, U; Kesbi, O. S; Yilmaz, S; Gultepe, N. and Turke, A. (2015). Evaluation of the effects of essential oil extracted from sweet orange peel (*Citrus sinensis*) on growth rate of tilapia (*Oreochromis mossambicus*) and possible disease resistance against *Streptococcus iniae*. *Aquaculture* 437, 282-286.
- Ahmadifar, E; Falahatkar, B. and Akrami. R. (2011). Effects of dietary thymol-carvacrol on growth performance, hematological parameters and tissue composition of juvenile rainbow trout, *Oncorhynchus mykiss*. *Journal of Applied Ichthyology* 27, 1057-1060.

- Ali, A. E; Mekhamar, M. I; Gadel-Rab, A. G. and Osman A. G. M. (2015). Evaluation of growth performance of Nile Tilapia *Oreochromis niloticus* fed *Piophilacasei* Maggot Meal (Magmeal) diets. *American Journal of Life Sciences* 3, 24-29.
- Ali, M. and Jauncey, K. (2004). Optimal dietary carbohydrate to lipid ratio in African catfish *Clarias gariepinus* (Burchell 1822). *Aquaculture International* 12, 169-180.
- Aliakbarpour, H. R; Chamani, M; Rahimi, G; Sadeghi, A. A. and Qujeq, D. (2012). The *Bacillus subtilis* and lactic acid bacteria probiotics influences intestinal mucin gene expression, histomorphology and growth performance in broilers. *Asian Australasian Journal of Animal Science* 25, 1285-1293.
- Allen, A. L. (2003). The diagnosis of acetaminophen toxicosis in a cat. *The Canadian Veterinary Journal* 44, 509-510.
- Alvarez, A; Garcia, B. G; Jordan, M. J; Martinez-Conesa, C. and Hernandez, M. D. (2012). The effect of diets supplemented with thyme essential oils and rosemary extract on the deterioration of farmed gilthead seabream (*Sparus aurata*) during storage on ice. *Food Chemistry* 132, 1395-1405.
- Amik, B. and Bilkei, G. (2004). Influence of farm application of oregano on performances of sows. *The Canadian Veterinary Journal* 45, 674-677.
- Amooz, M. A. and Dastar, B. (2009). Effects of alcoholic extract of two herbs (garlic and thymus) on the performance and blood lipids of broiler chickens. *Journal of Agricultural Sciences and Natural Resources* 16 (1).

- Ari, M. M; Barde, R. E; Ogah, D. M; Agade, Y. I; Yusuf, N. D; Hassan, I. D. and Muhammed, M. M. (2012). Utilisation of garlic (*Allium sativum*) as a supplementary phytogetic feed additive for broilers fed commercial feeds. *Egypt Poultry Science Journal* 32, 13-21.
- Ashraf, M. A. and Goda, S. (2008). Effect of dietary ginseng herb (Ginsana[®] G115) supplementation on growth, feed utilisation, and hematological indices of Nile tilapia, *Oreochromis niloticus* (L.), fingerlings. *Journal of the World Aquaculture Society* 39, 205-214.
- Awaad, M. H. H; Elmenawey, M. and Ahmed, K. A. (2014). Effect of a specific combination of carvacrol, cinnamaldehyde, and capsicum oleoresin on the growth performance, carcass quality and gut integrity of broiler chickens. *Veterinary World* 7, 284-290.
- Bakke, A. M; Glover, C. and Krogh, A. (2010). Feeding, digestion and absorption of nutrients In: The multifunctional gut of fish. Grosell, M; Farrell, A. P. and Brauner, C. J. (eds). *Fish Physiology* 30, 57-110.
- Bampidis, V. A; Christodoulou, V; Florou-Paneri, P; Christaki, E; Chatzopoulou, P. S; Tsiligianni, T. and Spais, A. B. (2005). Effect of dietary dried oregano leaves on growth performance, carcass characteristics and serum cholesterol of female early maturing turkeys. *British Poultry Science* 46, 595-601.
- Barbieri, E. and Sestili, P. (2012). Reactive oxygen species in skeletal muscle signalling. *Journal of Signal Transduction*, Article ID 982794, 17 pages.
- Bassole, I. H. N. and Juliani, H. R. (2012). Essential oils in combination and their antimicrobial properties. *Molecules* 17, 3989-4006.

- Buyukcapar, H. M; Atalay, A. I. and Kamalak, A. (2011). Growth performance of Nile Tilapia (*Oreochromis niloticus*) fed with diets containing different levels of hydrolysable and condensed tannin. *Journal of Agricultural Science and Technology* 13, 1045-1051.
- Cabuk, M; Bozkurt, M; Alcicek, A; Cathy, A. U. and Baser, K. H. C. (2006). Effect of a dietary essential oil mixture on performance of laying hens in the summer season. *South African Journal of Animal Science* 36, 215-221.
- Cameron, C; Moccia, R; Azevedo, P. A. and Leatherland, J. F. (2007). Effect of diet and ration on the relationship between plasma growth hormone and insulin growth factor I concentrations in Arctic charr, *Salvelinus alpinus* (L.). *Aquaculture Research* 38, 877-886.
- Caruana, S; Yoon, G. H; Freeman, M. A; Mackie, J. A. and Shinn, A. P. (2012). The efficacy of selected plant extracts and bioflavonoids in controlling infections of *Saprolegnia australis* (Saprolegniales; Oomycetes). *Aquaculture* 358-359, 146-54.
- Castro, M. (2005). Use of additives on the feeding of monogastric animals. *Cuban Journal of Agricultural Science* 39, 439-445.
- Cerda-Reverter, J. M. and Larhammar, D. (2000). Neuropeptide Y family of peptides: structure, anatomical expression, function and molecular evolution. *Biochemistry and Cell Biology* 78, 371-392.

- Covarrubias, L; Hernandez-Garcia, D; Schnabel, D; Salas-Vidal, E. and Castro-Obregon, S. (2008). Function of reactive oxygen species during animal development passive or active. *Developmental Biology* 320, 1-11.
- Cullen, S. P; Monahan, F. J; Callan, J. J. and Odoherthy, J. V. (2005). The effect of dietary garlic and rosemary on grower-finisher pig performance and sensory characteristics of pork. *Irish Journal of Agricultural and Food Research* 44; 57-67.
- Dalkilic, B; Simsek, U. G; Ciftci, M. and Baykalir, Y. (2015). Effect of dietary orange peel essential oil on physiological, biochemical and metabolic responses of Japanese quails as affected by early age thermal conditioning and fasting. *Revue Medecine Veterinaire* 166, 154-162.
- Darabighane, B; Zarei, A; Shahneh, A. Z. and Mahdavi, A. (2011). Effects of different levels of *Aloe vera* gel as an alternative to antibiotic on performance and ileum morphology in broilers. *Italian Journal of Animal Science* 10 (3), e36. DOI: 10.4081/ijas.2011.e36.
- Datta, S. N; Kaur, V. I; Dhawan, A. and Jassal, G. (2013). Estimation of length-weight relationship and condition factor of spotted snakehead *Channa punctata* (Bloch) under different feeding regimes. *SpringerPlus* 2 (436), 1-5.
- DeLong, D. P; Losordo, T. M. and Rakocy, J. E. (2009). Tank culture of tilapia. *The Southern Regional Aquaculture Center Publication* 282, 1-8.
- Denbow, D. M. (2000). Gastrointestinal anatomy and physiology In: Sturkie's Avian Physiology. Academic Press, New York, NY USA, pp. 299-325.

- El-Gendy, M. O; El-Komy, H. M. A; El-din, M. T. E. S; Tolan, A. E. and Salem, M. F. I. (2010). Effect of some medical plants on growth parameters and health status of Nile tilapia (*Oreochromis niloticus*). *Journal of Animal and Poultry Production* 1, 11-22.
- El-Sayed, S. A. A; Abd El-Galil, S. Y. and Rashied, N. A. (2014) Effects of some herbal plants as natural feed additives in comparison with antibiotic on growth performance and immune status of Nile tilapia (*Oreochromis niloticus*). *International Journal of Research in Fisheries and Aquaculture* 4(1), 22-30.
- El-sherif, M. S. and El-feky, A. M. (2008). Effect of ammonia on Nile tilapia (*Oreochromis niloticus*) performance and some hematological and histological measures. 8th *International Symposium on Tilapia in Aquaculture*, Page 513-530.
- El-sherif, M. S. and El-feky, A. M. (2009). Performance of Nile tilapia (*Oreochromis niloticus*) fingerlings. II. Influence of different water temperatures. *International Journal of Agriculture and Biology* 11, 301-305.
- European Food Safety Authority (2012). Scientific Opinion on the safety and efficacy of phenol derivatives containing ring-alkyl, ring-alkoxy and side-chains with an oxygenated functional group (chemical group 25) when used as flavourings for all species. *European Food Safety Authority Journal* 10(2), 2573.
- FAO (2009). Global agriculture towards 2050. High level expert forum-How to feed the world in 2050.

- Fox, B. K; Breves, J. P; Davis, L. K; Pierce, A. L; Hirano, T. and Grau, E. G. (2010). Tissue specific regulation of growth hormone/insulin growth factor axis during fasting and re-feeding: Importance of muscle expression of IGF-I and IGF-II in the tilapia. *General and Comparative Endocrinology* 166, 573-580.
- Frankic, T; Voljc, M; Salobir, J. and Rezar, V. (2009). Use of herbs and spices and their extracts in animal nutrition. *Acta Agriculturae Slovenica*, 94, 95-102.
- Franz, C; Baser, K. H. C. and Windisch, W. (2010). Essential oils and aromatic plants in animal feeding - a European perspective. A review. *Flavour and Fragrance Journal* 25, 327-340.
- Freccia, A; Sílvia, M. N. S; Meurer, F; Butzge, A. J; Mewes, J. K. and Bombardelli, R. A. (2014). Essential oils in the initial phase of broodstock diets of Nile tilapia. *Revista Brasileira de Zootecnia* 43, 1-7.
- Froese, R. (2006). Cube law, condition factor and weight-length relationship: history, meta-analysis and recommendations. *Journal of Ichthyology* 22, 241-253.
- Froese, R; Thorson, J. T. and Reyes, R. B. (2014), A Bayesian approach for estimating length-weight relationships in fishes. *Journal of Applied Ichthyology* 30, 78-85.
- Fuentes, E. N; Kling, P; Einarisdottir, I. E; Alvarez, M; Valdes, J. A; Molina, A. and Bjornsson, B. T. (2012). Plasma leptin and growth hormone levels in the fine flounder (*Paralichthys adspersus*) increase gradually during fasting and decline rapidly after refeeding. *General and Comparative Endocrinology* 177, 120-127.

- Gharib, H. B. (2014). Evaluation of using dietary phytochemicals, as growth promoters, on broiler performance, under normal and sub-normal temperature conditions. *Egyptian Journal of Animal Production* 51, 49-59.
- Giannenas, I; Triantafyllou, E; Stavrakakis, S; Margaroni, M; Mavridis, S; Steiner, T. and Karagouni, E. (2012). Assessment of dietary supplementation with carvacrol or thymol containing feed additives on performance, intestinal microbiota and antioxidant status of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 350-353, 26-32.
- Glencross, B. D; De Santis, C; Bicskei, B; Taggart, J. B; Bron, J. E; Betancor, M. B. and Tocher, D. R. (2015). A comparative analysis of the response of the hepatic transcriptome to dietary docosahexaenoic acid in Atlantic salmon (*Salmo salar*) post-smolts. *Biomedical Genomics Center* 16 (684), 1-12.
- Grarup, N; Stender-Petersen, K. L; Andersson, E. A; Jorgensen, T; Borch-Johnsen, K; Sandbaek, A; Lauritzen, T; Schmitz, O; Hansen, T. and Pedersen, O. (2008). Association of variants in the sterol regulatory element-binding factor 1 (SREBF1) gene with type 2 diabetes, glycemia, and insulin resistance. A study of 15,734 Danish subjects. *Diabetes* 57, 1136-1142.
- Guroy, B; Mantoglu, S; Kayali, S. and Sahin, I. (2014). Effect of dietary *Yucca schidigera* extract on growth, total ammonia-nitrogen excretion and haematological parameters of juvenile striped catfish *Pangasianodon hypophthalmus*. *Aquaculture Research* 45, 647-654.

- He, S; Liang, X; Li, L; Sun, J. and Shen, D. (2013). Differential gut growth, gene expression and digestive enzyme activities in young grass carp (*Ctenopharyngodon idella*) fed with plant and animal diets. *Aquaculture* 410-411, 18-24.
- Hernandez, A. B; Garcia, G. B; Jordan, M. J. and Hernandez, M. D. (2014a). Improved conservation of gilthead seabream (*Sparus aurata*) in ice storage. The influence of doses of rosemary extract added to feed. *Aquaculture* 426-427, 31-40.
- Hernandez, A; Garcia, G. B; Jordan, M. J. and Hernandez, M. D. (2014b). Natural antioxidants in extruded fish feed: Protection at different storage temperatures. *Animal Feed Science and Technology* 195, 112-119.
- Hernandez, F; Madrid, J; Garcia, V; Orengo, J. and Megias, M. D. (2004). Influence of two plant extracts on broilers performance, digestibility and digestive organ. *Poultry Science* 83, 169-174.
- Hernandez-Mosqueira, C; Velez-delValle, C. and Kuri-Harcuch, W. (2015). Tissue alkaline phosphatase is involved in lipid metabolism and gene expression and secretion of adipokines in adipocytes. *Biochimica et Biophysica Acta* 1850, 2485-2496.
- Hippenstiel, F; Abdel-Wareth, A. A. A; Kehraus, S. and Sudekum, K-H. (2011). Effects of selected herbs and essential oils, and their active components on feed intake and performance of broilers - a review. *Arch.Geflügelk* 75, 226-234.

- Jamroz, D; Wiliczekiewicz, A; Wertelecki, T; Orda, J. and Skorupinska, J. (2005). Use of active substances of plant origin in chicken diets based on maize and locally grown cereals. *British Poultry Science* 46, 485-493.
- Janczyk, P; Pieper, R; Urubschurov, V; Wendler, K. R. and Souffrant, W. B. (2009). Investigations on the effects of dietary essential oils and different husbandry conditions on the gut ecology in piglets after weaning. *International Journal of Microbiology* 2009, Article ID 730809, 9 pages, doi:10.1155/2009/730809.
- Jeong, S-H; Kang, D; Lim, M-W; Kang, C. S and Sung, H. J. (2010). Risk assessment of growth hormones and antimicrobial residues in meat. *Toxicology Research* 261, 301-313.
- Ji, S-C; Jeong, G-S; IM, G-S; Lee, S-W; Yoo, J-H. and Takii, K. (2007). Dietary medicinal herbs improve growth performance, fatty acid utilisation, and stress recovery of Japanese flounder. *Fisheries Science* 73, 70-76.
- Jiang, X. R; Awat. A; Agazzi, A; Vitari, F; Ferrari, A; Bento, H; Crestani, M; Domeneghini, C. and Bontempo, V. (2015). Effects of a blend of essential oils and an enzyme combination on nutrient digestibility, ileum histology and expression of inflammatory mediators in weaned piglets. *Animal* 9, 417-426.
- Jobling, M. (1994). Fish bioenergetics. Chapman and Hill, London (UK).
- Juin H; Elgaard, T. and Chicoteau P. (2003). Effect of a citrus extract (NOR-SPICE AB) on broiler performances. *British Poultry Science* 44, 810-811.

- Kim, E; Choi, Y; Jang, J. and Park, T. (2013). Carvacrol protects against hepatic steatosis in mice fed a high-fat diet by enhancing SIRT1-AMPK signaling. *Evidence-Based Complementary and Alternative Medicine* Article ID 290104, 10 pages.
- Kim J. S; Park J. H; Cho H. S; Park J. S. and Hong E. K. (2002). Effects of YGF251 on secretion of IGF-I in human blood. *Korean Journal of Biotechnology and Bioengineering* 17, 403-408.
- Kim, K. T; Jeon, G. H; Cho, S. H; Lim, S. G; Kwon. M. and Yoo, J. (2013). Effects of dietary inclusion of various concentrations of *Scutellaria baicalensis* Georgi extract on growth, body composition, serum chemistry and challenge test of far eastern catfish (*Silurus asotus*). *Aquaculture Research* 44, 1502-1510.
- Kim, Y. S. and Ho, S. B. (2010). Intestinal goblet cells and mucins in health and disease: Recent insights and progress. *Current Gastroenterology Reports* 12, 319-330.
- Kiris, G. A; Kumlu, M. and Dikel, S. (2007). Stimulatory effects of neuropeptide Y on food intake and growth of *Oreochromis niloticus*. *Aquaculture* 264, 383-389.
- Kleveland, E. J; Ruyter, B; Vegusdal, A; Sundvold, H; Berge, R. K. and Gjoen, T. (2006). Effects of 3-thia fatty acids on expression of some lipid related genes in Atlantic salmon (*Salmo salar* L.). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 145, 239-248.
- Kochhar, K. P. (1999). Effects of dietary herbs and spices. *Journal of Orthomolecular Medicine* 14, No. 4.

- Lalles, J. P; Bosi, P; Janczyk, P; Koopmans, S. J. and Torrallardona, D. (2009). Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: a review. *Animal* 3, 1625-1643.
- Landau, S. and Everitt, B. S. (2004). A handbook of statistical analyses using spss. Chapman and Hall / A CRC Press Company, London, UK.
- Larionov, A; Krause, A. and Miller, W. (2005). A standard curve based method for relative real time PCR data processing. *Biomedical Center Bioinformatics* 6, 1-16.
- Lawal, O. A; Ogunwande, I. A; Owolabi, M. S; Giwa-Ajeniya, A. O; Kasali, A. A; Abudu, F. A; Sanni, A. A. and Opoku, A. R. (2014). Comparative analysis of essential oils of *Citrus aurantifolia* Swingle and *Citrus reticulata* Blanco, from two different localities of Lagos State, Nigeria. *American Journal of Essential Oils and Natural Products* 2, 8-12.
- Lee, D; Ra, C; Song, Y; Sung, K. and Kim, J. (2012). Effects of dietary garlic extract on growth, feed utilisation and whole body composition of juvenile sterlet sturgeon (*Acipenser ruthenus*). *Asian-Australian Journal of Animal Science* 25, 577-583.
- Lee, K. W; Everts, H. and Beynen, A. C. (2004b). Essential oils in broiler nutrition. *International Journal of Poultry Science* 3, 738-752.
- Lee, K. W; Everts, H; Kappert, H. J. and Beynen, A. C. (2004a). Growth performance of broiler chickens fed a carboxymethyl cellulose containing diet with supplemental carvacrol and/or cinnamaldehyde. *International Journal of Poultry Science* 3, 619-622.

- Lin, X; Volkoff, H; Narnaware, Y; Bernier, N. J; Peyon, P. and Peter, R. E. (2000). Brain regulation of feeding behavior and food intake in fish. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 126, 415-434.
- Liu, G. M; Wei, Y; Wang, Z. S; Wu, D; Zhou, A. G. and Liu, G. L. (2008). Effects of herbal extract supplementation on growth performance and insulin-like growth factor (IGF-I) system in finishing pigs. *Journal of Animal and Feed Science* 17, 538-547.
- Liu, Y; Song, M; Che, T. M; Bravo, D; Maddox, C. W. and Pettigrew, J. E. (2014). Effects of *capsicum oleoresin*, garlic botanical, and *turmeric oleoresin* on gene expression profile of ileal mucosa in weaned pigs. *Journal of Animal Science* 92, 3426-3440.
- Loum, A; Sagne, M; Fall, J; Ndong, D; Diouf, M; Sarr, A. and Thiaw, O. T. (2013). Effects of dietary protein level on growth performance, carcass composition and survival rate of fry monosex Nile tilapia *Oreochromis niloticus* reared under recirculating system. *Journal of Biology and Life Science* 4, 13-22.
- Mackay, D; Shiu, W. Y; Ma, K. and Lee, S. C. (2006). Hand book of physical-chemical properties and environmental fate for organic chemicals. 2nd edition. Pages 1-919. *CRC Press, Taylor and Francis Group, LLC*.
- Maenner, K; Vahjen, W. and Simon, O. (2011). Studies on the effects of essential-oil-based feed additives on performance, ileal nutrient digestibility, and selected bacterial groups in the gastrointestinal tract of piglets. *Journal of Animal Science* 89, 2106-2112.

- Melanie, G. and Isabelle, P. O. (2008). Immunomodulators as efficient alternatives to in-feed anti-microbials in pig production? *Archiva Zootechnica* 11, 15-32.
- Mesalhy, A. S; Abdel Atti, N. M. and Mohamed, M. F. (2008). Effect of garlic on the survival, growth, resistance and quality of *Oreochromis niloticus* Proceedings of the 8th International Symposium on Tilapia in Aquaculture, Cairo, Egypt, 12-14 October 2008. Page 277-296.
- Michiels, J; Missotten, J; Dierick, N; Fremaut, D; Maene, P. and De Smet, S. (2008). In-vitro degradation and in vivo passage kinetics of carvacrol, thymol, eugenol and trans-cinnaldehyde along the gastrointestinal tract of piglets. *Journal of Science of Food and Agriculture* 88, 2371-2381.
- Michiels, J; Missottenb, J; Ovynd, A; Dierick, N; Fremauta, D. and De Smet, S. (2012). Effects of dose thymol and supplemental flavours or camphor on palatability in a choice feeding study with piglets. *Czech Journal of Animal Science* 57, 65-74.
- Midhun, S. J; Arun, D; Edatt, L; Sruthi, M. V; Thushara, V. V; Oommen, O. V; Kumar, S. V. B. and Divya, L. (2016). Modulation of digestive enzymes, GH, IGF-1 and IGF-2 genes in the teleost, tilapia (*Oreochromis mossambicus*) by dietary curcumin. *Aquaculture International* 24, 1277-1286.
- Miller, E. L. (2004). Protein nutrition requirements of farmed livestock and dietary supply. In: Protein sources for the animal feed industry. Expert Consultation Workshop Bangkok 29th April - 3rd May 2002. Food and Agriculture Organisation of the United Nations (FAO), Animal Production and Health.

- Muhl, A. and Liebert, F. (2007a). Growth and parameters of microflora in intestinal and faecal samples of piglets due to application of a phytogenic feed additive. *Journal of Animal Physiology and Animal Nutrition* 91, 411-418.
- Muhl, A. and Liebert, F. (2007b). No impact of a phytogenic feed additive on digestion and unspecific immune reaction in piglets. *Journal of Animal Physiology and Animal Nutrition* 91, 426-431.
- Murashita, K; Jordal, A-E. O; Nilsen, T. O; Stefansson, S. O; Kurokawa, T; Bjornsson, B. T; Moen, A-G. G. and Ronnestad, I. (2011). Leptin reduces Atlantic salmon growth through the central pro-opiomelanocortin pathway. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 158, 79-86.
- Muthusamy, N. and Sankar, V. (2015). Phytogenic compounds used as a feed additives in poultry production. *International Journal of Science Environment and Technology* 4, 167-171.
- Nakamura, M. T; Cheon, Y; Li, Y. and Nara, T. Y. (2004). Mechanisms of regulation of gene expression by fatty acids. *Lipids* 39, 1077-1083.
- Nankervis, L; Matthews, S. J. and Appleford, P. (2000). Effect of dietary non-protein energy source on growth, nutrient retention and circulating insulin-like growth factor I and triiodothyronine levels in juvenile barramundi, *Lates calcarifer* L. *Aquaculture* 191, 323-335.
- Narisawa, S; Huang, L; Iwasaki, A; Hasegawa, H; Alpers, D. H. and Millan J. L. (2003). Accelerated fat absorption in intestinal alkaline phosphatase knockout mice. *Molecular and Cellular Biology* 23, 7525-7530.

- Ngugi, C. C; Oyoo-Okoth, E. and Muchiri, M. (2016). Effects of dietary levels of essential oil (EO) extract from bitter lemon (*Citrus limon*) fruit peels on growth, biochemical, haemato-immunological parameters and disease resistance in juvenile *Labeo victorianus* fingerlings challenged with *Aeromonas hydrophila*. *Aquaculture Research*. doi:10.1111/arc.13062.
- NRC, 2011. Nutrient requirements of fish and shrimp. Animal Nutrition Series, National Research Council of the National Academies. The National Academies Press, Washington, D.C., USA. 376 pp.
- Nya, E. J. and Austin, B. (2011). Dietary modulation of digestive enzymes by the administration of feed additives to rainbow trout, *Oncorhynchus mykiss* Walbaum. *Aquaculture Nutrition* 17, 459-466.
- Olagunju, J. A; Oladunni, S. O. and Oladimeji, M. S. (2000). Status of phosphatase activities in the liver and kidney of rats treated with isosaline leaf and stem-bark extracts of *Harungana madagascariensis* (L). *Cytobios* 103, 17-24.
- Oluremi, O. I. A; Ngi, J. and Andrew, I. A. (2007). Phytonutrients in citrus fruit peel meal and nutritional implication for livestock production. *Livestock Research for Rural Development* 19 (7).
- Othieno, O. N; Kitaka, N. and Njiru, J. M. (2014). Length-weight relationship, condition factor, length at first maturity and sex ratio of Nile tilapia, *Oreochromis niloticus* in Lake Naivasha, Kenya. *International Journal of Fisheries and Aquatic Studies* 2, 67-72.

- Perez-Sanchez, J. and Le Bail, P. (1999). Growth hormone axis as marker of nutritional status and growth performance in fish. *Aquaculture* 177, 117-128.
- Peric, L; Zikic, M. and Lukic, M. (2009). Application of alternative growth promoters in broiler production. *Biotechnology in Animal Husbandry* 25, 387-397.
- Peterson, B. C; Peatman, E; Ourth, D. D. and Waldbieser, G. C. (2015). Effects of a phytogenic feed additive on growth performance, susceptibility of channel catfish to *Edwardsiella ictaluri* and levels of mannose binding lectin. *Fish and Shellfish Immunology* 44, 21-25.
- Peterson, B. C. and Bosworth, B. G. (2014). Assessment of a phytogenic feed additive Digestarom P.E.P. MGE) on growth performance, processing yield, fillet composition, and survival of channel catfish. *Journal of the World Aquaculture Society* 45, 206-212.
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* 29(9), e45.
- Picha, M. E; Turano, M. J; Beckman, B. R. and Borski, R. J. (2008). Endocrine biomarkers of growth and applications in aquaculture: A mini-review of growth hormone, insulin growth factor (IGF)-I and IGF-binding proteins as potential growth indicators in fish. *North American Journal of Aquaculture* 70, 196-211.
- Pierce, A. L; Breves, J. P; Moriyama, S; Uchida, K. and Grau, E. G. (2012). Regulation of growth hormone (GH) receptor (GHR-1 and GHR-2) mRNA level by GH and metabolic hormones in primary cultured tilapia hepatocytes. *General and Comparative Endocrinology* 179, 22-29.

- Rainer, A. and Lea, H. (2015). Secondary plant compounds are more than essential oils. *Lohmann Information* 49, (1).
- Rajat, G. and Panchali, D. (2014). A study on antioxidant properties of different bioactive compounds. *Journal of Drug Delivery and Therapeutics* 4, 105-115.
- Ran, C; Hu, J; Liu, W; Liu, Z; He, S; Dan, B. C; Diem, N. N; Ooi, E. L. and Zhou, Z. (2016). Thymol and carvacrol affect hybrid tilapia through the combination of direct stimulation and an intestinal microbiota-mediated effect: insights from a germ-free zebrafish model. *Journal of Nutrition* 146, 1132-1140.
- Rattanachaikunsopon, P. and Phumkhachorn, P. (2010). Assessment of synergistic efficacy of carvacrol and cymene against *Edwardsiella tarda* in vitro and in Tilapia (*Oreochromis niloticus*). *African Journal of Microbiology Research* 4, 420-425.
- Reindl, K. M. and Sheridan, M. A. (2012). Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 163, 231-245.
- Reinecke, M. (2010). Influences of the environment on the endocrine and paracrine fish growth hormone-insulin-like growth factor-I system. *Journal of Fish Biology* 76, 1233-1254.
- Reinecke, M; Bjornsson, B. T; Dickhoff, W. W; McCormick, S. D; Navarro, I; Power, D. M. and Gutierrez, J. (2005). Growth hormone and insulin-like growth factors in fish: where we are and where to go. *General Comparative Endocrinology* 142, 20-24.

- Saccol, E. M. H; Uczay, J; Pes, T. S; Finamor, I. A; Ourique, G. M; Riffel, A. P. K; Schmidt, D; Caron, B. O; Heinzmann, B. M; Llesuy, S. F; Lazzari, R; Baldisserotto, B. and Pavanato, M. A. (2013). Addition of *Lippia alba* (Mill) N. E. brown essential oil to the diet of the silver catfish: An analysis of growth, metabolic and blood parameters and the antioxidant response. *Aquaculture* 416-417, 244-254.
- Samadian, F; Zeinoaldini, S; Towhidi, A; Torshizi, M. A. K; Pirasaraei, Z. A. and Gholamzadeh, P. (2013). Evaluation of some phytogenic feed additives in growing chicks diet. *International Journal of Agriculture: Research and Review* 3. 35-43.
- Santos, M. R. V; Moreira, F. V; Fraga, B. P; De Sousa, D. P; Bonjardim, L. R. and Quintans-Junior, L. J. (2011). Cardiovascular effects of monoterpenes: a review. *Brazilian Journal of Pharmacognosy* 21, 764-771.
- Santos, V. B; Mareco, E. A. and Silva, M. D. P. (2013). Growth curves of Nile tilapia (*Oreochromis niloticus*) strains cultivated at different temperature. *Acta Scientiarum. Animal Sciences* 35, 235-242.
- Sawhney, S. (2014). Effect of feeding levels on the growth of *Tor putitora* at fry and fingerling stage. *Asian Journal of Science and Technology* 5, 348-351.
- Schroers, V; Marel, Mv; Neuhaus, H. and Steinhagen, D. (2009). Changes of intestinal mucus glycoproteins after perioral application of *Aeromonas hydrophila* to common carp (*Cyprinus carpio*). *Aquaculture* 288, 184-189.

- Skiba-Cassy, S; Polakof, S; Seilez, I. and Panserat, S. (2012). The nutritional and hormonal regulation of fish glucose and lipid metabolism. In: Functional genomics in aquaculture. Saroglia, M. and Liu, Z. H. (eds). John Wiley and Sons, Inc. Publishers.
- Smirnor, A; Tako, E; Ferket, P. R. and Uni, Z. (2006). Mucin gene expression and mucin content in the chicken intestinal goblet cells are affected by in ovo feeding of carbohydrates. *Poultry Science* 85, 669-673.
- Soltan, M. A. and El-Laithy, S. M. (2008). Effect of probiotics and some spices as feed additives on the performance and behaviour of the Nile tilapia, *Oreochromis niloticus*. *Egyptian Journal of Aquatic Biology and Fisheries* 12, 63-80.
- Speier, J. S; Yadgary, L; Uni, Z. and Wong, E. A. (2012). Gene expression of nutrient transporters and digestive enzymes in the yolk sac membrane and small intestine of the developing embryonic chick. *Poultry Science* 91, 1941-1949.
- Steiner, T. (2009). PhytoGenics in Animal Nutrition: Natural Concepts to optimise gut health and performance. *Nottingham University Press, Nottingham*. ISBN: 978-1-904761-71-6.
- Steiner, T. (2010). Optimising gut health and performance through a phytogenic feed additive. *Krmiva* 52, Zagreb, 4; 217-227.
- Steiner, T. and Syed, B. (2015). Phytogenic feed additives in animal nutrition. In: Medicinal and aromatic plants of the world: Scientific, production, commercial and utilisation aspects. Springer, Dordrecht, The Netherlands. Page 403-423.

- Takaoka, O; Ji, S-C; Ishimaru, K; Lee, S-W; Jeong, G-S; Biswas, A. and Takii, K. (2016). Dietary medicinal herbs and enzyme treated fish meal improve stress resistances and growth performance at early juvenile stage of red sea bream *Pagrus major*. *Aquaculture Research* 47, 390-397.
- Tengjaroenkul, B; Smith, B. J; Caceci, T. and Smith, S. A. (2000). Distribution of intestinal enzyme activities along the intestinal tract of cultured Nile tilapia, *Oreochromis niloticus* L. *Aquaculture* 182, 317-327.
- Terova, G; Robaina, L; Izquierdo, M; Cattaneo, A; Molinari, S; Bernardini, G. and Terova, M. S. (2013). PepT1 mRNA expression levels in sea bream (*Sparus aurata*) fed different plant protein sources. *SpringerPlus* 2, 17.
- Teugels, G. G. and Thys van den Audenaerde, D. F. E. (2003). Cichlidae. P. 521-600. In: Paugy, D; Leveque, C. and Teugels, G. G. (eds). The fresh and brackish water fishes of West Africa Volume 2. Coll. faune et flore tropicales 40. Institut de recherche de developpement, Paris, France, Museum national d'histoire naturelle. Paris, France and Musee royal de l'Afrique Central, Tervuren, Belgium, 815p.
- Tian, J; Wu, F; Yang, C; Jiang, M; Liu, W. and Wen, H. (2015). Dietary lipid levels impact lipoprotein lipase, hormone-sensitive lipase, and fatty acid synthetase gene expression in three tissues of adult GIFT strain of Nile tilapia, *Oreochromis niloticus*. *Fish Physiology and Biochemistry* 41, 1-18.

- Turek, C. and Stintzing, F. C. (2013). Stability of essential oils: A review. *Comparative Reviews in Food Science and Food Safety* 12, 40-53.
- Velasco, V. and Williams, P. (2011). Improving meat quality through natural antioxidants. *Chilean Journal of Agricultural Research* 71, 313-322.
- Verri, T; Maffia, M; Danieli, A; Herget, M; Wenzel, U; Daniel, H. and Storelli, C. (2000). Characterisation of the h⁺/peptide co-transporter of eel intestinal brush-border membranes. *The Journal of Experimental Biology* 203, 2991-3001.
- Verri, T; Terova, G; Dabrowski, K. and Saroglia, M. (2011). Peptide transport and animal growth: the fish paradigm. *Biology Letters* 7, 597-600.
- Volkoff, H. (2006). The role of neuropeptide Y, orexins, cocaine and amphetamine-related transcript, cholecystokinin, amylin and leptin in the regulation of feeding in fish. *Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology* 144, 325-331.
- Volkoff, H; Canosa, L. F; Unniappan, S; Cerda-Reverter, J. M; Bernier, N. J; Kelly, S. P. and Peter, R. E. (2005). Neuropeptides and the control of food intake in fish. *General Comparative Endocrinology* 15, 142: 3-19.
- Volkoff, H; Xu, M; MacDonald, E. and Hoskins, L. (2009). Aspects of the hormonal regulation of appetite in fish with emphasis on goldfish, Atlantic cod and winter flounder: Notes on actions and responses to nutritional, environmental and reproductive changes *Comparative Biochemistry and Physiology, Part A* 153, 8-12.

- Workagegn, K. B; Ababboa, E. D; Yilmer, G. T. and Amare, T. A. (2014). Growth performance of the Nile tilapia (*Oreochromis niloticus* L.) fed different types of diets formulated from varieties of feed ingredients. *Journal of Aquaculture Research and Development* 5(3), 235 doi:10.4172/2155-9546.1000235.
- Xie, L-L; Cao, J-H; Yang, S-X; Zhao, C-Y. and Ren, L. (2009). The impact of dietary Chinese herbal medicines on growth performance and muscular composition in juvenile tilapia. *Fisheries Science/Shuichan Kexue* 28, 11-14.
- Yan, J; Liao, K; Wang, T; Mai, K; Xu, W. and Ai, Q. (2015). Dietary lipid levels influence lipid deposition in the liver of large yellow croaker (*Larimichthys crocea*) by regulating lipoprotein receptors, fatty acid uptake and triacylglycerol synthesis and catabolism at the transcriptional level. *PLoS One* 10(6), 1-16.
- Yang, C; Chowdhury, M. A. K; Hou, Y. and Gong, J. (2015). Phytogetic compounds as alternatives to in-feed antibiotics: Potentials and challenges in application. *Pathogens* 4, 137-156.
- Yang, Y; Iji, P. A. and Choct, M. (2009). Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. *World Poultry Science Journal* 65, 97-114.
- Yilmaz, S; Ergun, S. and Celik, E. S. (2012). Effects of herbal supplements on growth performance of sea bass (*Dicentrarchus labrax*): Change in body composition and some blood parameters. *Journal of BioScience and Biotechnology* 1, 217-222.

- Zambonino-Infante, J. L. and Cahu, C. L. (2007). Dietary modulation of some digestive enzymes and metabolic processes in developing marine fish: Applications to diet formulation. *Aquaculture* 268, 98-105.
- Zeng, Z; Zhang, S; Wang, H. and Piao. X. (2015). Essential oil and aromatic plants as feed additives in non-ruminant nutrition: a review. *Journal of Animal Science and Biotechnology* 6, 1-10.
- Zeppenfeld, C. C; Hernandez, D. R; Santinon, J. J; Heinzmann, B. M; da Cunha, M. A; Schmidt, D. and Baldisserotto, B. (2015). Essential oil of *Aloysia triphylla* as feed additive promotes growth of silver catfish (*Rhamdia quelen*). *Aquaculture Nutrition* 22, 933-940.
- Zhang, X. H; Lowe, D; Giles, P; Fell, S; Connock, M. J. and Maslin, D. J. (2001). Gender may affect the action of garlic oil on plasma cholesterol and glucose levels of normal subjects. *Journal of Nutrition* 131, 1471-1478.
- Zheng, J; Luo, Z; Hu, W; Pan, Y. and Zhuo, M. (2015). Dietary fenofibrate reduces hepatic lipid deposition by regulating lipid metabolism in yellow catfish *Pelteobagrus fulvidraco* exposed to waterborne Zn. *Lipids* 50, 417-426.
- Zheng, Z. L; Tan, J. Y. W; Liu, H. Y; Zhou, X. H; Xiang, X. and Wang, K. Y. (2009). Evaluation of oregano essential oil (*Origanum heracleoticum* L.) on growth, antioxidant effect and resistance against *Aeromonas hydrophila* in channel catfish (*Ictalurus punctatus*). *Aquaculture* 292, 214-218.
- Zhou, Y; Liang, X; Yuan, X; Li, J; He, Y; Fang, L; Guo, X; Liu, L; Li, B. and Shen, D. (2013). Neuropeptide Y stimulates food intake and regulates metabolism in grass carp, *Ctenopharyngodon idellus*. *Aquaculture* 380-383, 52-61.

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 phytogetic 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 phytogetic compounds on growth and nutritional physiology of monogastric animals.
2. Effects of individually supplied phytogetic compounds on genes regulating growth and nutritional physiology in Nile tilapia.
3. Effects of combined phytogetic compounds on the growth and nutritional physiology of Nile tilapia.