1 **Title**

2

3 The effects of combined phytogenics on growth and nutritional physiology of Nile tilapia

4 *Oreochromis niloticus*

5

6 **Authors**

7 Margaret Aanyu[1,2], Mónica B. Betancor[1], Óscar Monroig[3]*

8

9 **Addresses**

10 [1] Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9

11 4LA, Scotland, United Kingdom

12 [2] National Fisheries Resources Research Institute, Aquaculture Research and Development

13 Center, P. O. Box 530, Kampala, Uganda

14 [3] Instituto de Acuicultura Torre de la Sal (IATS-CSIC), Ribera de Cabanes 12595,

15 Castellón, Spain

16

17 ***Corresponding author**

18 Óscar Monroig

19 Tel: +34 964 319500; Fax: +34 964 319509; E-mail: oscar.monroig@csic.es

20

21

22

Accepted refereed manuscript of:
Aanyu M, Betancor M & Monroig O (2020) The effects of combined phytogenics on growth and nutritional physiology of Nile tilapia Oreochromis niloticus. Aquaculture, 519, Art. No.: 734867. DOI: https://doi.org/10.1016/j.aquaculture.2019.734867.

Abstract

This study investigated whether dietary supplementation of phytogenic compounds limonene and thymol had synergistic or additive effects on growth and selected nutritional physiology pathways in Nile tilapia. A 63-day feeding experiment was conducted using fish of 1.5 ± 0.0 g (± standard error) fed on a commercial diet coated with either 0 ppm limonene and thymol (control), 400 ppm limonene (L), 500 ppm thymol, (T) or a combination of 400 ppm limonene and 500 ppm thymol (LT). Final fish weight (FW) was significantly improved to similar extents by diet LT (16.7 ± 0.3 g) and L (16.6 ± 0.4 g). Dietary thymol alone and the control did not enhance FW (15.0 ± 0.4 g and 13.7 ± 0.4 g respectively). Dietary thymol had shown a strong tendency to improve somatic growth (P=0.052). The analysed candidate genes involved in the pathways of nutrient digestion, absorption and transport (*muc*), lipid metabolism (*lpl*), antioxidant enzymes (*cat*) and somatotropic axis growth (*igf-I*) were also up-regulated to similar extents in Nile tilapia by diet L and LT (P<0.05), above the regulation observed with the diet supplemented exclusively with thymol. This suggests lack of synergistic or additive effects on growth and nutritional physiology pathways when limonene and thymol are supplied in the diet.

Keywords

Growth promoters, limonene, Nile tilapia, nutritional physiology, phytogenic compounds, thymol

1. Introduction

Phytogenic compounds are natural bioactive compounds derived from herbs, shrubs and spices with essential oils extracted from the plant parts being the major source of phytogenic compounds (Yang et al., 2015; Sutili et al., 2017; Upadhaya and Kim, 2017). Each phytogenic compound contains several bioactive components or molecules in different proportions with bioactive components present in higher proportions largely determining the biological properties of the essential oils (Santos *et al*., 2011; Chakraborty *et al*., 2014; Yitbarek, 2015). Bioactive components in the plants are mainly hydrocarbons (e.g. terpenes, sesquiterpenes), oxygenated compounds (e.g. alcohol, aldehydes, ketones) and a small percentage of non-volatile residues (e.g. paraffin, wax) (Losa, 2000). Some active compounds such as thymol, carvacrol, limonene, cinnamaldehyde and eugenol from the plants thyme, oregano, citrus, cinnamon and clove respectively have been noted to exert positive effects on nutrition, performance or health of monogastric animals (Wallace et al., 2010; Chakraborty *et al*., 2014; Sutili et al., 2017; Upadhaya and Kim, 2017).

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 in monogastric animals (Bassole and Juliani, 2012; Costa et al., 2013; Abd El-hack et al., 2016; Valenzuela-Grijalva et al., 2017; Amer et al., 2018; Youssefi et al., 2019).

Synergistic or additive effects of phytogenic compounds on growth performance lead to enhanced growth of animals above 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 its beneficial effects on growth (Zheng et al., 2009; Ahmadifar et al., 2011; Hyldgaard et al., 2012; Chakraborty et al., 2013; Ahmadifar et al., 2014; Peterson et al., 2014; Perez-Sanchez et al., 2015). For instance, Peterson et al. (2014) reported that channel catfish (*Ictalurus punctatus*) fed on a diet supplemented with a combination of limonene, thymol, carvacrol and anethol gained 44% more weight than the control attributed to synergistic or additive effects of the phytogenic compounds.

Often though indifferent effects can be observed when combination of phytogenic compounds show no differences compared to treatments consisting of their individually supplied compounds or the controls (Bassole and Juliani, 2012). Indifferent effects have also been noted when combination of phytogenic compounds exerts significantly higher effects to similar extents with only some treatments composed of their individually supplied phytogenic compounds. Zheng et al. (2009) supplemented diets with 500 ppm of either carvacrol, thymol or a mixture of carvacrol and thymol, and found a significantly

higher weight gain of channel catfish with the dietary mixture of carvacrol and thymol compared to the control and diet with thymol alone, but not with the diet supplemented only with carvacrol. In addition, antagonistic effects occur when individual phytogenic compounds might have positive effects but their combination results in negative effects compared to the controls (Bassole and Juliani, 2012). Such antagonistic effects derived from phytogenic blends have been often attributed to high concentrations of these compounds that potentially result in unpleasant taste and smell and thereby retarding feed intake and consequently growth (Windisch et al., 2008; Steiner, 2009; Costa et al., 2013; Colombo et al., 2014).

The different responses to phytogenics mentioned above highlights the importance of identifying combinations and doses of phytogenic compounds resulting in additive and synergistic effects on fish growth. In our previous study (Aanyu et al., 2018), two phytogenic compounds, namely limonene and thymol, classified as monoterpene and diterpene, respectively, were found to have growth-promoting tendencies in Nile tilapia (*Oreochromis niloticus*). We hypothesised that combinations of limonene and thymol can potentially have additive or synergistic effects on the growth of Nile tilapia. Consequently, this study aimed to investigate the effects of a blend of limonene and thymol, compared with each of the compounds individually, on the growth, feed efficiency and nutritional physiology of Nile tilapia. The study followed a candidate gene approach to investigate physiological pathways underpinning the response of fish to the phytogenic compounds. A selection of marker genes within the pathways of somatotropic axis-mediated growth, nutrient absorption and transport, lipid metabolism and antioxidant enzyme status that showed potential to be regulated by limonene and/or thymol were analysed (Aanyu et al., 2018).

2. Materials and Methods

2.1 Ethical statement

All experiments were subjected to ethical review and approved by the University of Stirling through the Animal and Welfare Ethical Review Body. The project was conducted under the UK Home Office in accordance with the amended Animals Scientific Procedures Act implementing EU Directive 2010/63.

2.2 Experimental design

The feeding trial was carried out at the Aquaculture Research and Development Center (ARDC), Uganda between March and May 2015. Nile tilapia juveniles from the same cohort were obtained from the ARDC fish farm, acclimatized and size graded to 1.54 ± 0.0 g (mean ± standard error). Thirty eight (38) fish were stocked in each of the 16 experimental tanks. Each tank had 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 to 25.0 - 26.6 ^{o}C. A photoperiod of 12h light-12h dark was maintained.

Water quality was monitored routinely to ensure that it was within the requirements for Nile tilapia growth (Lim and Webster, 2006). A multi-parameter meter (HQ40D model, Hach Ltd Germany) was used to measure dissolved oxygen, pH and water temperature. The level of ammonia-nitrogen was assessed using a fresh water test kit from API Company Ltd UK following the user guide from the manufacturer. Water flowing into the fish rearing tanks had 6.6 ± 0.6 mg L^{-1} of dissolved oxygen, pH 6.8 ± 0.3, undetectable levels (< 0.05 mg L^{-1}) of ammonia-nitrogen and water temperature ranging from 23.3 - 24.3 ^{o}C before heating with aquaria water heaters.

2.3 Experimental diets

A standard commercial feed (CP35%, Kampala, Uganda) for juvenile Nile tilapia produced at the ARDC was supplemented with limonene (97 % purity) and/or thymol (95 % purity) from Sigma Aldrich, Kampala, Uganda using concentrations found to have growth-promoting potential in Nile tilapia (Aanyu et al., 2018). The diets included: 0 ppm limonene and thymol (Control); 400 ppm limonene (L); 500 ppm thymol (T); and a combination of 400 ppm limonene and 500 ppm thymol (LT). In order to supply the above concentrations of phytogenic compounds to the feed, each concentration of phytogenic compounds was prepared in 100 mL of absolute ethanol and sprayed onto 1 kg of feed. The control was also coated with a similar amount of ethanol but no phytogenic compound was added. All diets were air-dried for one day, packed in airtight polythene bags and stored at room temperature until use.

Each experimental diet was tested in quadruplicate tanks and the treatments were distributed randomly. The fish were fed the experimental diets twice a day to apparent satiation and the feed intake was recorded.

The proximate nutritional composition (dry matter, moisture, protein, lipid, fibre, ash and gross energy) of the standard diet was determined according to the methods of the Association of Official Analytical Chemists (AOAC, 1990) and the joint technical committee of the International Organisation for Standardisation and International Electrotechnical Commission (ISO/IEC 17025). Briefly, dry matter content was estimated by drying a sample of feed in an oven at 105-110˚C to a constant weight and the percentage retained weight from the original sample was the amount of dry matter whereas the percentage loss in weight of the sample was the moisture content. Crude protein content was determined using the Kjeldahl method and crude lipid by petroleum ether extraction using the Soxhlet method. Crude fibre content was analysed by acid / alkaline hydrolysis of a sample and the amount of insoluble residues resistant to hydrolysis was the fibre content. Crude ash was determined by combustion of a sample in a furnace at 600 ˚C for 24 h. The gross energy using determined using the bomb calorimetry method. Proximate composition of the standard diet used in this study is shown in Table 1.

2.3 Fish measurements and sample collection

Growth of the fish was estimated by measuring the weight (accuracy of 0.1 g) and total length (0.1 cm) of all fish in each tank every three weeks and at the end of the experiment (63 days). This procedure was carried out while fish were anesthetised using 0.02 g L^{-1} of clove oil for 3-5 min, after which the fish were placed in aerated water and taken back to the experimental tanks. At the end of the experiment, the number of live fish in each tank was recorded for survival estimation, and sections of liver and fore intestine were dissected from 16 fish per treatment (n=4 per tank) and placed in 1.5 mL tubes containing RNAlater® (Sigma Aldrich, Kampala, Uganda). The liver and fore intestine samples were kept at 4 ºC overnight, shipped to the UK and transferred to a -20 ^{o}C freezer until RNA was extracted.

2.4 Fish performance calculations

The effects of the experimental diets on fish performance were assessed by calculating the performance indicators below using the following formulae:

Final average fish weight (FW, g) = total fish biomass at end of the trial (g) / number of fish at end of the experiment;

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

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

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

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

Feed conversion ratio (FCR) = average feed intake fish^{-1} / weight gain;

Protein efficiency ratio (PER) = weight gain / protein intake.

2.5 Molecular analyses

2.5.1 RNA extraction and complementary DNA (cDNA) synthesis

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 was extracted from the samples (n=16 per tissue and treatment). The concentration and purity of the RNA was measured by spectrophotometry with an ND-1000 Nanodrop (Nanodrop 1000, Thermo Scientific, Glasgow, UK). The integrity of the RNA (aliquots fo 200 ng total RNA) from each sample was further assessed by agarose gel (1 %, v/v) electrophoresis.

From a total of 16 RNA samples extracted per tissue and treatment, eight RNA samples were derived by pooling together two samples from the same tank and treatment, taking equal quantity (2.5 µg µl^{-1}) 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 2 µg µl^{-1} RNA from each pool sample (n = 8 per treatment) to cDNA following the protocol provided by the manufacturer.

2.5.2 Quantitative real-time Polymerase Chain Reaction (qPCR)

The mRNA expression levels of selected genes of interest in the pathways of somatotropic axis-mediated growth, nutrient absorption and transport, lipid metabolism and antioxidant enzyme status were analysed by quantitative real-time polymerase chain reaction (qPCR) in tissue samples (liver or fore intestine) in which they perform their major biological functions. The selected target genes included mucin-like protein (*muc*), oligo-peptide transporter 1 (*pept1*), lipoprotein lipase (*lpl*), sterol regulatory element binding transcription factor 1 (*srebf1*), alkaline phosphatise (*alp*), phospholipase A2 (*pla2*),

catalase (*cat*), growth hormone (*gh*), and insulin growth factor I (*igf-I*). Efficiency of the primers was first tested by generating standard dilution curves, assessing the melting curves and cycle threshold (Ct) values (Larionov et al., 2005). Efficient primers were considered to have values between 0.80 - 1.10, a single melting peak, Ct value below 30 and one clear band under 1 % agarose gel electrophoresis. The details of the primers used for qPCR analyses are provided in Table 2. Each qPCR contained duplicate samples (total volume 20 µl each) containing 5 µl of 20-fold diluted cDNA, 3 µl nuclease-free water, 1 µl (10 pmol) each for the forward and reverse primers, and 10 µl of Luminaris color higreen qPCR Mix (Thermo Scientific, Hemel Hempstead, UK). All the reactions were run in 96 well-plates using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany). A calibrator sample (20-fold dilution of all samples pooled cDNA) and a negative control with no cDNA (non-template control-NTC) were included in each plate. The qPCR thermocycling program involved pre-heating samples at 50 °C for 2 min followed by 35 cycles, initial denaturing at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 30 s.

2.5.3 Gene expression computations

Ct values for the duplicate runs of each sample were averaged. The data were normalised using the geometric mean expression of the references genes (*β-actin* and *ef-1α*) and the relative expression of each gene was calculated according to the equation of Pfaffl (2001). Heat maps enabling cluster analysis and visualisation of the expression patterns of the analysed genes were generated but not based on statistical differences. Java Tree View Software was used to plot the data and perform cluster analysis based on Euclidean distance. Expression level of each gene was natural *log* transformed and normalised against two reference genes (*β-actin* and *ef-1α*).

2.6 Statistical analysis

The data were analysed using the Statistical Package for the Social Sciences (SPSS) version 19 (Chicago, USA) (Landau and Everitt, 2004). The fish performance and qPCR results are expressed as means ± standard error. Normality of distribution of the data was assessed using Kolmogorov-Smirnov's tests. Data not normally distributed were subjected to natural logarithm *ln* (qPCR data) and *arcsin* square-root (WG, CF, SR, FI, FCR, and PER) transformation. Differences among dietary treatments were analysed by one-way ANOVA followed by Tukey's test. When heterogeneity of variances occurred, Welch's test was performed with Game-Howell's test to determine differences between treatments.

Significant differences were considered at P value < 0.05. In addition, Pearson's correlation analysis was performed to indicate the relationship and degree of correlation between FI and FCR, FW and SR, FW and PER. The significance level of correlation was set to $P < 0.05$.

3. Results

3.1 Fish performance

Table 3 shows the performance of Nile tilapia fed on diets supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and the combination of 400 ppm limonene with 500 ppm thymol (LT) for 63 days (9 weeks). There was a significant increase ($P < 0.01$) in the final weight of fish fed on the diets supplemented with limonene, that is, diets L and LT, compared to the control. No significant increases in fish weight were observed with diet L and LT during day 1, 21, and 42 of the feeding experiment (data not shown). The diet supplemented exclusively with thymol (T) did not significantly improve the final weight of the fish ($P = 0.052$). There was a significantly higher percentage weight gain (% WG; $P = 0.01$) of fish fed on diets L and LT compared to the control, whilst fish fed diet T did not show significant differences compared with the control. Despite there were no significant differences in the final survival of the fish among treatments, there was a strong significant positive correlation ($r = 0.967$, $P = 0.033$) between the survival rate and final weight of the fish (Table 3). Condition factor (CF) was not significantly different among treatments.

The protein efficiency ratio (PER) was significantly higher in fish fed the diets L and LT compared to the control. While 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) among the treatments supplemented with L, T and LT, but fish fed diets L and LT had significantly lower FCR ($P = 0.006$) than the control-fed fish (Table 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.

283 *3.2 Relative mRNA gene expression*

284 The heat map in Figure 1 represents the relative expression patterns (not based on
285 statistical differences) of genes analysed in the liver (a) and fore intestine (b) of Nile tilapia
286 fed on the experimental feeds. There were more genes with patterns of higher relative
287 expression levels (red) among the fish fed on diets L and LT compared with diet T, when
288 all dietary treatments were compared to the control.

289

290 3.2.1 Expression of genes involved in somatotropic axis in liver

291 Insulin growth factor I (*igf-I*) was significantly (P = 0.025) up-regulated in the liver of fish
292 fed on diets L and LT compared with control fish (Figure 2). However, the expression of
293 *igf-I* did not differ significantly between the fish fed on diets L, T and LT (Figure 2). In
294 addition, the relative expression levels of *gh* was not significantly different in the livers of
295 fish fed on diets L, T, LT and the control.

296

297 3.2.2 Expression of genes involved in lipid metabolism in liver

298 The expression of *lpl*, *alp* and *srebf1* in the liver of Nile tilapia fed on the experimental
299 diets is shown in Figure 3. Levels of *lpl* mRNA were significantly (P = 0.003) higher in
300 fish fed on diet LT compared with the control. The expression of *lpl* in the fish fed on diets
301 L and T was not significantly different from the control. Similarly, no significant
302 differences in the relative expression of *alp* and *srebf1* were found among the experimental
303 treatments (Figure 3).

304

305 3.2.3 Expression of genes regulating nutrient digestion, absorption and transport in the fore
306 intestine

307 The mRNA levels of *muc* were significantly higher (P = 0.025) in the fore intestine of fish
308 fed on diet LT compared with the control (Figure 4). Besides, the expression of *muc* in the
309 fish fed on diets L and T did not differ significantly from the control (P = 0.097). The
310 expression of *pla2* also did not statistically differ among the dietary treatments (P = 0.086).
311 Oligo-peptide transporter 1 (*pept1*) expression was significantly (P = 0.047) up-regulated
312 in the fish fed on diet LT compared with the control, although expression levels in fish fed
313 diets L and T did not differ statistically compared with the control (Figure 4).

314

315 3.2.4 Expression of antioxidant enzymes in liver

316 The expression of *cat* was significantly higher (P = 0.006) in the liver of fish fed on diets L
317 and LT compared with the control (Figure 5).

318

319

4. Discussion

The present study investigated the effects of diets containing limonene and thymol, supplemented both individually and in combination, on growth and nutritional physiology of Nile tilapia. The goal was to establish whether blends of limonene and thymol had synergistic and/or additive effects on the growth of Nile tilapia. A selection of gene markers regulating nutrient digestion, absorption and transport, lipid metabolism, antioxidant enzymes and somatotropic axis growth were investigated.

Fish fed diets supplemented with limonene alone (L) and the blend of limonene and thymol (LT) had significantly higher FW, % WG, PER and lower FI and FCR than the control. On the contrary, fish fed on the diet supplemented with only thymol (T) did not show any statistical difference in such parameters compared with the control. Similarly, Zheng et al. (2009) found no synergistic or additive effects of a combination of carvacrol and thymol on the weight gain of channel catfish. The fish fed on the diet supplemented with only carvacrol, and the blend of carvacrol and thymol attained statistically higher weight gain compared with the diet with only thymol and the control, but the dietary mixture of carvacrol and thymol did statistically increase weight gain to the same extent as the diet with only carvacrol.

Among the feed utilisation parameters, the present study found enhanced feed efficiency (i.e., lower FCR) in fish fed diets L and LT, and a strong significant positive correlation between PER and FW. The correlation between PER and FW showed that, as the utilisation of protein from the feed was enhanced (high PER), FW of the fish was increased. This could have contributed to the significantly improved somatic growth of the fish fed on diets L and LT, both treatments with increased PER compared to the control. The increased WG and lowered FI levels observed with diet L and LT fed fish are in agreement with Hashemipour et al. (2013) who found lower FI corresponding with the highest WG and feed efficiency in broiler chicken fed on a diet with a mixture of 200 ppm of thymol and carvacrol compared to the control. It is known that efficient growth in fish does not necessarily coincide with maximum or higher FI because fish adjust their FI according to their energy requirements (Ali and Jauncey, 2004), with better feed efficiency occurring below maximum FI (Rad et al., 2003; Sawhney, 2014). Conversely, some studies with phytogenic compounds (thymol and carvacrol) in pig diets found low FI corresponding with low WG (Lee et al., 2003a; Lee et al., 2003b; Zhai et al., 2018). 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 are complementary to each other (Hashemi and Davoodi, 2010; Steiner and Syed, 2015). In this study, insulin growth factor I (*igf-I*), which plays a core role in regulating growth in the somatotropic axis, was up-regulated to a similar extent in the liver of fish fed diets L and LT, corresponding also to higher final FW and feed utilisation efficiency (FCR) than the control. This observation implies that *igf-I* was largely activated by limonene suggesting that there was no synergistic or additive effect of limonene and thymol in influencing somatotropic axis-mediated growth.

Key mechanisms underlying feed utilisation efficiency include nutrient digestion, absorption and transport, in which mucin-like protein (*muc*) and oligo-peptide transporter 1 (*pept1*) are important components (Verri et al., 2011; Fascina et al., 2012). The present study found a significantly higher expression of *muc* in the fore intestine of fish fed on diet LT compared with the control, with diets L and T showing no differences in expression of *muc* with the control and diet LT. The high expression of *muc* found with diet LT can be associated with an increase in the secretion/quantity of mucus, which then serves as a lubricant aiding absorption of nutrients into the bloodstream through which they are transferred to tissues for various functions including growth (Kamali et al., 2014). Moreover, high expression of *muc* corresponded with enhanced somatic growth of the fish in the LT treatment. Despite that Tsirtsikos et al. (2012) did not specifically investigate *muc* expression, their study on broilers fed on diets containing a blend of limonene, carvacrol and anethol also reported an increase in mucus volume in the fore intestine. Additionally, Jamroz et al. (2006) found higher mucus secretion in the fore intestine of broilers fed diets supplemented with a combination of phytogenics including carvacrol, cinnamaldehyde and capsicum oleoresin. The present results for Nile tilapia are consistent with these terrestrial animal studies, suggesting that the mechanism of action is somewhat conserved across vertebrates.

The movement of nutrients from the lumen of the intestine, aided by mucus, into epithelial cells takes place through diffusion and/or active transport regulated by nutrient transporters (Rust, 2003). The nutrient transporter *pept1* that aids the transport of protein in the form of di/tri peptides through the above process (Verri et al., 2011), was significantly regulated by diet LT compared with the control. Moreover, the higher expression of *pept1* in fish fed diet LT corresponded with significantly improved feed efficiency (lower FCR) and PER compared with the control, with diet L also having enhancing feed efficiency

389 (lower FCR) and PER compared with the control. This suggested that limonene drove the
390 improved protein absorption, which could have contributed to increased growth. Similarly,
391 dietary peppermint and Digestarom P.E.P (Biomin GmbH, Herzogenburg, Austria), a
392 commercial matrix-encapsulated phytogenic mixture, improved protein utilisation in
393 broilers (Upadhaya and Kim, 2017) and gilthead seabream *Sparus aurata* (Goncalves and
394 Santos, 2015).
395 In order to maximise the use of dietary protein for somatic growth, energy for
396 supporting metabolic processes can be derived from non-protein sources, particularly lipids
397 (Nankervis et al., 2000). Lipid metabolism including, among others, processes such as lipid
398 catabolism or fatty acid and triglyceride synthesis occurs along with lipid transport and
399 deposition with the liver as the main active site (He et al., 2015). In the present study, diet
400 LT activated lipid metabolism as reflected by significantly increased expression of
401 lipoprotein lipase (*lpl*) in comparison to the control. Since the expression of *lpl* in the fish
402 fed diet T did not differ from that of the control, it is reasonable to deduce that limonene,
403 not thymol, is the compound that triggers such metabolic response in fish fed diet LT.
404 Given that *lpl* plays a pivotal role in breaking down plasma lipids into free fatty acids and
405 transporting them for use in energy production (Tian et al., 2015), the high gene expression
406 of *lpl* found in this study suggests that dietary limonene increased the energy level of the
407 fish, thereby providing sufficient energy for running metabolic processes and sparing
408 protein, which significantly improved fish growth in the dietary treatments L and LT. Such
409 effect of limonene to regulate *lpl* and a corresponding somatic growth enhancement further
410 confirmed the results obtained by Aanyu et al. (2018) in the same teleost species.
411 Metabolic processes in the body result into production of reactive oxygen intermediates
412 (ROIs), which can induce damage to cells and tissues if their levels are not maintained low
413 (Covarrubias et al., 2008; Costa et al., 2013) This can ultimately impair adequate
414 physiological function and subsequently negatively affect growth. In this study, the
415 expression of catalase (*cat*), a key antioxidant enzyme that breaks down the ROI hydrogen
416 peroxide, was significantly increased by dietary treatment with limonene (i.e., treatments L
417 and LT) to similar extents. These results suggest that the enhanced antioxidant status by
418 *cat* could reduce the hydrogen peroxide levels and thus result in improved somatic growth
419 of the fish fed on diet L and LT. Recent research has shown that, when ROIs are at low
420 concentrations, they are vital molecules mediating physiological processes including
421 somatic growth (Covarrubias et al., 2008; Barbieri and Sestili, 2012). The herein reported
422 action of dietary limonene on *cat* up regulation (catalase enzyme activity) has not been
423 observed for other phytogenic compounds. For instance, Zheng et al. (2009) did not find

enhanced activity of catalase enzyme in channel catfish fed on 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*.

5. Conclusions

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

Acknowledgements

This work was funded by the Schlumberger Foundation PhD Training Program, and the Government of Uganda through the Agricultural Technology and Agribusiness Services (ATAAS) Project of the National Agricultural Research Organization (NARO).

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Aanyu, M., Betancor, M.B., Monroig, O., 2018. Effects of dietary limonene and thymol on the growth and nutritional physiology of Nile tilapia (*Oreochromis niloticus*). Aquaculture 488, 217-226.

Abd El-hack, M.E., Alagawany, M., Farag, M.R., Tiwari, R., Karthik, K., Dhama, K., Zorriehzahra, J., Adel, M., 2016. Beneficial impacts of thymol essential oil on health and production of animals, fish and poultry: a review. J. Essent. Oil Res. 28, 365-382.

Ahmadifar, E., Falahatkar, B., Akrami, R., 2011. Effects of dietary thymol-carvacrol on growth performance, hematological parameters and tissue composition of juvenile rainbow trout, *Oncorhynchus mykiss*. J. Appl. Ichthyol. 27, 1057-1060.

Ahmadifar, E., Mansour, M.R., Amirkolaie, A.K., Rayeni, M.F., 2014. Growth efficiency, survival, and haematological changes in great sturgeon (*Huso huso* Linnaeus, 1758) juveniles fed on diets supplemented with different levels of thymol-carvacrol. Anim. Feed Sci. Technol. 198, 304-308.

Ali, M., Jauncey, K., 2004. Optimal dietary carbohydrate to lipid ratio in African catfish *Clarias gariepinus* (*Burchell* 1822). Aquac. Int. 12, 169-180.

Amer, S.A., Metwally, A.E. Ahmed, S.A.A., 2018. The influence of dietary supplementation of cinnamaldehyde and thymol on the growth performance, immunity and antioxidant status of monosex Nile tilapia (*Oreochromis niloticus*). Egypt. J. Aquat. Res. 44, 251-256.

AOAC., 1990. Official Methods of Analysis. 15th edition. *Association of Official Agricultural Chemists*, Washington, D.C.

Barbieri, E., Sestili, P., 2012. Reactive oxygen species in skeletal muscle signalling. J. Signal Transduct. 2012, 17 pages.

Bassole, I.H.N., Juliani, H.R., 2012. Essential oils in combination and their antimicrobial properties. Molecules, 17, 3989-4006.

Chakraborty, S.B., Horn, P., Hancz, C., 2013. Application of phytochemicals as growth-promoters and endocrine modulators in fish culture. Rev. Aquacult. 5, 1-19.

477 Colombo, M., Priori, D., Gandolfi, G., Boatto, G., Nieddu, M., Bosi, P., Trevisi, P., 2014.
478 Effect of free thymol on differential gene expression in gastric mucosa of the young pig.
479 Animal 8, 786-791.

480 Costa, L.B., Luciano, F.B., Miyada, V.S., Gois, F.D., 2013. Herbal extracts and organic acids
481 as natural feed additives in pig diets. S. Afr. J. Anim. Sci. 43, 181-193.

482 Covarrubias, L., Hernandez-Garcia, D., Schnabel, D., Salas-Vidal, E., Castro-Obregon, S.,
483 2008. Function of reactive oxygen species during animal development passive or active.
484 Dev. Biol. 320, 1-11.

485 Fascina, V.B., Sartori, J.R., Gonzales, E., Carvalho, F.B., Souza, I.M.G.P., Polycarpo, G.D.V.,
486 Stradiotti, A.C., Cristina, V., 2012. Phytogenic additives and organic acids in broiler
487 chicken diets. R. Bras. Zootec. 4, 2189-2197.

488 Glencross, B.D., De Santis, C., Bicskei, B., Taggart, J.B., Bron, J.E., Betancor, M.B., Tocher,
489 D.R., 2015. A comparative analysis of the response of the hepatic transcriptome to dietary
490 docosahexaenoic acid in Atlantic salmon (*Salmo salar*) post-smolts. BMC Genomics. 16,
491 1-12.

492 Goncalves, R.A., Santos, G.A., 2015. Phytogenic Feed Additive Stimulate Performance,
493 Health Gains in Fish, Shrimp. Experiment Findings. Pages 59-62.

494 Hashemi, S.R., Davoodi, H., 2010. Phytogenics as new class of feed additive in poultry
495 industry. J. Anim. Vet. Adv. 9, 2295-2304.

496 Hashemipour, H., Kermanshahi, H., Golian, A., Veldkamp, T., 2013. Effect of thymol and
497 carvacrol feed supplementation on performance, antioxidant enzyme activities, fatty acid
498 composition, digestive enzyme activities and immune response in broiler chickens. Poult
499 Sci. 92, 2059-2069.

500 He, A., Ning, L., Chen, L., Chen, Y., Xing, Q., Li, J., Qiao, F., Li, D., Zhang, M., Du, Z.,
501 2015. Systemic adaptation of lipid metabolism in response to low-and high-fat diet in Nile
502 tilapia (*Oreochromis niloticus*). Physiol. Rep. 3, 1-8.

503 Hyldgaard, M., Mygind, T., Meyer, R.L., 2012. Essential oils in food preservation: mode of
504 action, synergies and interactions with food matrix components. Front. Microbiol. 3, 1-24.

505 Jamroz, D., Wiliczkiewicz, A., Wertelecki, T., Orda, J., Skorupinska, J., 2006. Use of active
506 substances of plant origin in chicken diets based on maize and locally grown cereals. Br.
507 Poult. Sci. 46, 485-493.

508 Kamali, S.A., Masoudi, A.A., Hosseini, S.A., 2014. The effects of herbal plants on mucin 2
509 gene expression and performance in ascetic broilers. Iran. J. Vet. Med. 8, 47-52.

510 Landau, S., Everitt, B.S., 2004. A handbook of statistical analyses using Spss. Chapman and
511 Hall / A CRC Press Company, London, UK.

512 Larionov, A., Krause, A., Miller, W., 2005. A standard curve based method for relative real
513 time PCR data processing. BMC Bioinformatics, 6, 1-16.

514 Lee, K.W., Everts, H., Kappert, H.J., Frehner, M., Losa, R., Beynen, A.C., 2003a. Effects of
515 dietary essential oil components on growth performance, digestive enzymes and lipid
516 metabolism in female broiler chickens. Br. Poult. Sci. 44, 450-457.

517 Lee, K.W., Everts, H., Kappert, H.J., Yeom, K.H., Beynen, A.C., 2003b. Dietary carvacrol
518 lowers body weight gain but improves feed conversion in female broiler chickens. J. Appl.
519 Poult. Res. 12, 394-399.

520 Lillehoj, H., Liu, Y., Calsamiglia, S., Fernandez-Miyakawa, M.E., Chi, F., Cravens, R.L., Oh,
521 S., Gay, C.G., 2018. Phytochemicals as antibiotic alternatives to promote growth and
522 enhance host health. Vet. Res. 49, 76.

523 Lim, C.E., Webster, C.D., 2006. Nutrient requirements In: Tilapia: biology, culture and
524 nutrition. *The Haworth Press, Inc., Binghamton, New York*. pp 469-501.

525 Michiels, J., Missottenb, J., Ovynb, A., Dierick, N., Fremauta, D., De Smet, S., 2012. Effects
526 of dose of thymol and supplemental flavours or camphor on palatability in a choice feeding
527 study with piglets. Czech. J. Anim. Sci. 57, 65-74.

528 Muthusamy, N., Sankar, V., 2015. Phytogenic compounds used as a feed additives in poultry
529 production. Int. J. Sci. Environ. Technol. 4, 167-171.

530 Nankervis, L., Matthews, S.J., Appleford, P., 2000. Effect of dietary non-protein energy
531 source on growth, nutrient retention and circulating insulin-like growth factor I and
532 triiodothyronine levels in juvenile barramundi, *Lates calcarifer* L. Aquaculture 191, 323-
533 335.

534 Pérez-Sánchez, J., Benedito-Palos, L., Estensoro, I., Petropoulos, Y., Calduch-Giner, J.A.,
535 Browdy, C.L., Sitjà-Bobadilla, A., 2015. Effects of dietary NEXT ENHANCE®150 on
536 growth performance and expression of immune and intestinal integrity related genes in
537 gilthead sea bream (*Sparus aurata* L.). Fish *Shellfish Immunol.* 44, 117-128.

538 Peterson, B.C., Bosworth, B.G., Li, M.H., Jr, R.B., Santos, G.A., 2014. Assessment of
539 phytogenic feed additive (Digestatrom P.E.P MGE) on growth performance, processing
540 yield, fillet composition, and survival of channel catfish. J. World. Aquacult. Soc. 45(2).

541 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-
542 PCR. Nucleic Acids Res. 29, e45.

543 Rad, F., Koksal, G., Kindir, M., 2003. Growth performance and food conversion ratio of
544 Siberian sturgeon (*Acipenser baeri* Brandt) at different daily feeding rates. Turk. J. Vet.
545 Anim. Sci. 27, 1085-1090.

546 Rust, M.B., 2003. Nutrition physiology In: Halver, J.E., & Hardy, R. W (Eds.), Fish Nutrition.
547 Academic Press, New York, pp 368-446.

548 Sawhney, S., 2014. Effect of feeding levels on the growth of *Tor putitora* at fry and fingerling
549 stage. AJST. 5, 348-351.

550 Steiner, T., 2009. Phytogenics in Animal Nutrition: Natural Concepts to optimise gut health
551 and performance (1stedition). Nottingham University Press, Nottingham.

552 Steiner, T., Syed, B., 2015. Phytogenic feed additives in animal nutrition. In: Medicinal and
553 aromatic plants of the world: Scientific, production, commercial and utilisation aspects.
554 Springer, Dordrecht, The Netherlands. Page 403-423.

555 Sutili, F.J., Gatlin, D.M., Heinzmann, B.M., Baldisserotto, B., 2017. Plant essential oils as
556 fish diet additives: benefits on fish health and stability in feed. Rev. Aquacult. 0, 1-11.

557 Tian, J., Wu, F., Yang, C., Jiang, M., Liu, W., Wen, H., 2015. Dietary lipid levels impact
558 lipoprotein lipase, hormone-sensitive lipase, and fatty acid synthetase gene expression in
559 three tissues of adult GIFT strain of Nile tilapia, *Oreochromis niloticus*. Fish. Physiol.
560 Biochem. 41, 1-18.

561 Tsirtsikos, P., Fegeros, K., Kominakis, A., Balaskas, C., Mountzouris, K.C., 2012.
562 Modulation of intestinal mucin composition and mucosal morphology by dietary
563 phytogenic inclusion level in broilers. Animal Consortium, 6, 1049-1057.

564 Upadhaya, S.D., Kim, I.H., 2017. Efficacy of phytogenic feed additive on performance,
565 production and health status of monogastric animals - A reveiw. Ann. Anim. Sci. 17, 929-
566 948.

567 Valenzuela-Grijalva, N.V., Pinelli-Saavedra, A., Muhlia-Almazan, A., Domínguez-Díaz, D.,
568 González-Ríos, H., 2017. Dietary inclusion effects of phytochemicals as growth promoters
569 in animal production. J. Anim. Sci. Technol. 59, 1-17.

570 Verri, T., Terova, G., Dabrowski, K., Saroglia, M., 2011. Peptide transport and animal growth:
571 the fish paradigm. Biol. Lett. 7, 597-600.

572 Wallace, R.J., Oleszek, W., Franz, C., Hahn, I., Baser, K.H.C., Mathe, A., Teichmann, K.,
573 2010. Dietary plant bioactives for poultry health and productivity. Brit. Poultry. Sci. 51,
574 461-487.

575 Windisch, W., Schedle, K., Plitzner, C., Kroismayr, A., 2008. Use of phytogenic products as
576 feed additives for swine and poultry. J. Anim. Sci. 86, 140-148.

577 Yang, C., Chowdhury, M.A.K., Hou, Y., Gong, J., 2015. Phytogenic compounds as
578 alternatives to in-feed antibiotics: Potentials and challenges in application. Pathogens 4,
579 137-156.

580 Youssefi, M.R., Tabari, M.A., Esfandiari, A., Kazemi, S., Moghadamnia, A.A., Sut, S.,
581 Dall'Acqua, S., Benelli, G., Maggi, F., 2019. Efficacy of Two Monoterpenoids, Carvacrol
582 and Thymol, and Their Combinations against Eggs and Larvae of the West Nile Vector
583 *Culex pipiens*. Molecules 24, 1867.

584 Zhai, H., Liu, H., Wang, S., Wu, J., Kluenter, A-M., 2018. Potential of essential oils for
585 poultry and pigs. Anim. Nutr. 4, 179-186.

586 Zheng, Z.L., Tan, J.Y.W., Liu, H.Y., Zhou, X.H., Xiang, X., Wang, K.Y., 2009. Evaluation of
587 oregano essential oil (*Origanum heracleoticum* L.) on growth, antioxidant effect and
588 resistance against *Aeromonas hydrophila* in channel catfish (*Ictalurus punctatus*).
589 Aquaculture 292, 214-218.

FIGURES

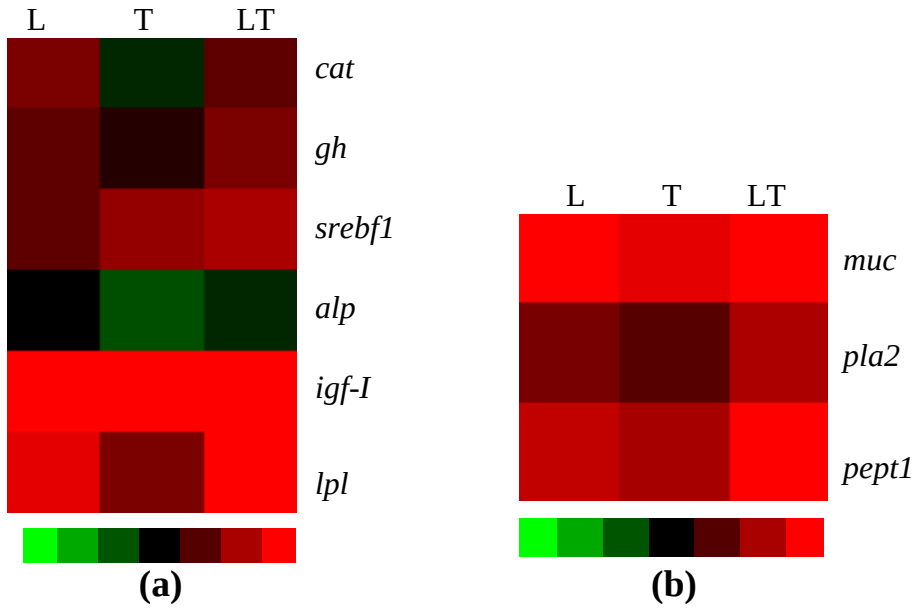
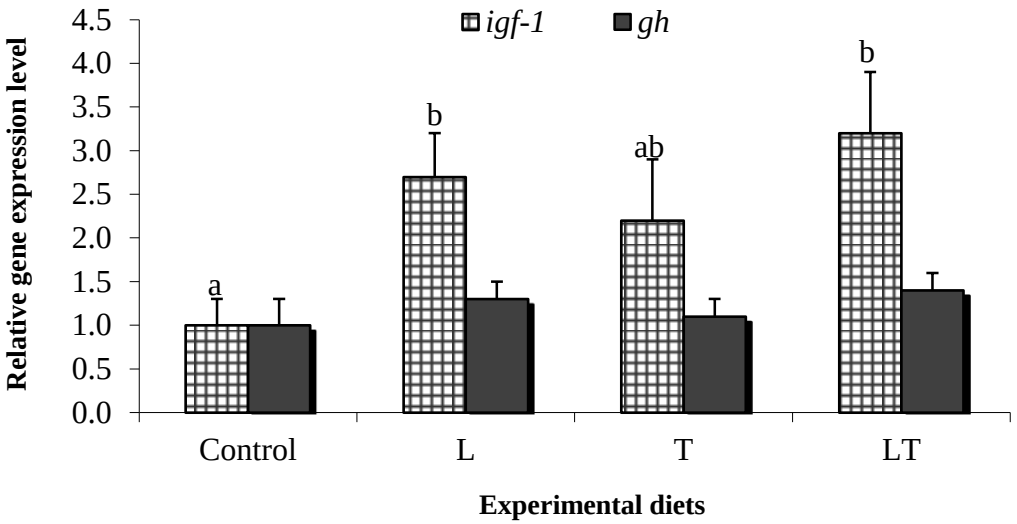
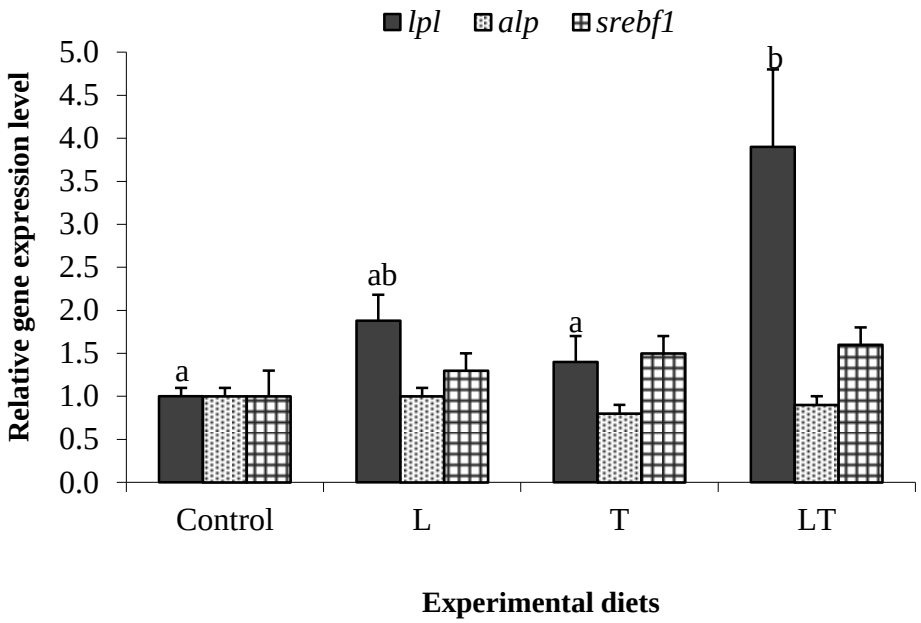


FIGURE 1 Heat map showing the expression patterns of nine genes analysed using qPCR data from Nile tilapia fed on diets supplemented with limonene (L), thymol (T) and their combination (LT). Data were plotted using Java Tree View and rows were clustered according to Euclidean distance. The columns represent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



611

612 **FIGURE 2** Expression of insulin growth factor I *(igf-I*) and growth hormone (*gh*) in the
613 liver of Nile tilapia fed on diets supplemented with 400 ppm limonene (L), 500 ppm
614 thymol (T) and the combination of 400 ppm limonene and 500 ppm thymol (LT). All
615 values are means of treatments ± standard error (n=8). Different superscript letters denote
616 significant differences in the expression of *igf-I* between the treatments.

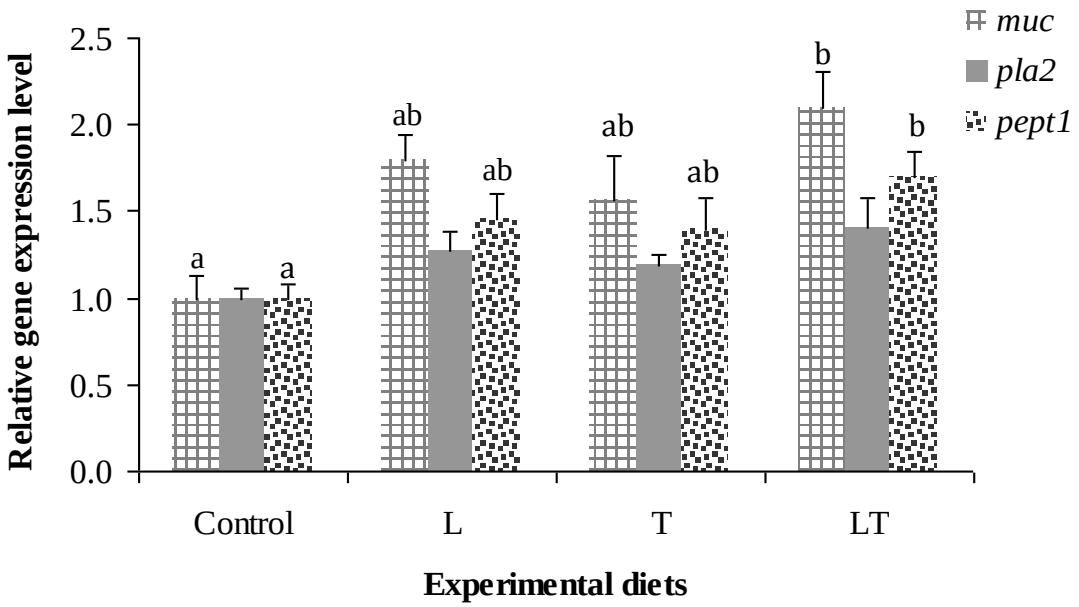


617

618 **FIGURE 3** Expression of lipoprotein lipase (*lpl*), alkaline phosphatase (*alp*), and sterol
619 regulatory element binding transcription factor 1 (*srebf1*) in the liver of Nile tilapia fed on
620 diets supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and a combination of
621 400 ppm limonene and 500 ppm thymol (LT). All values are means of treatments ±
622 standard error (n=8). Different superscript letters denote significant differences in the
623 expression of *lpl* between the treatments.

624

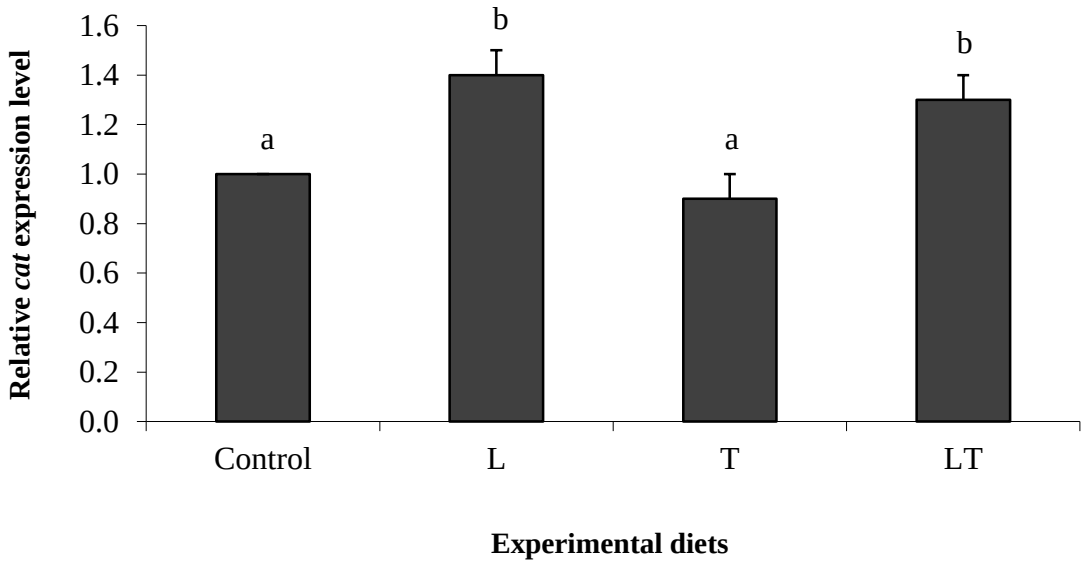
625



626

627 **FIGURE 4** Expression of mucin-like protein (*muc*), phospholipase A2 (*pla2*), and oligo-
628 peptide transporter 1 (*pept1*) genes in the fore intestine of Nile tilapia fed on diets
629 supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and a combination of 400
630 ppm limonene and 500 ppm thymol (LT). All values are means of treatments ± standard
631 error (n=8). For each gene (*muc* or *pept1*), different superscript letters denote significant
632 differences in the expression of each gene between the treatments.

633
634



635
636 **FIGURE 5** Expression of the antioxidant enzyme catalase (*cat*) in the liver of Nile tilapia
637 fed on diets supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and a
638 combination of 400 ppm limonene and 500 ppm thymol (LT). All values are means of
639 treatments ± standard error (n=8). Different superscript letters denote significant
640 differences in the expression of *cat* between the treatments.

641

642

643 **TABLES**

644 **TABLE 1** Proximate analysis of the nutritional composition of the diet (CP35, ARDC -
645 Uganda) used in the present trial to feed Nile tilapia (*Oreochromis niloticus*) for 63 days.

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

646
647
648

TABLE 2 Details of the primers used for quantitative real-time PCR analyses.

Functional group	Gene symbol	Primer / oligonucleotide sequences (5'-3')	Size (base pairs)	Accession number*
Nutrient digestion, absorption and transport	*muc*	F: TGCCCAGGAGGTAGATATGC R: TACAGCATGAGCAGGAATGC	101	XM_005466350
	pept1	F: CAAAGCACTGGTGAAGGTCC R: CACTGCGTCAAACATGGTGA	196	XM_013271589
Lipid metabolism	*lpl*	F: TGCTAATGTGATTGTGGTGGAC R: GCTGATTTTGTGGTTGGTAAGG	217	NM_001279753
	srebf1	F: TGCAGCAGAGAGACTGTATCCGA R: ACTGCCCTGAATGTGTTCAGACA	102	XM_005457771
	alp	F: CTTGGAGATGGGATGGGTGT R: TTGGCCTTAACCCCGCATAG	200	XM_005469634
	pla2	F: CTCCAAACTCAAAGTGGGCC R: CCGAGCATCACCTTTTCTCG	177	XM_005451846
Antioxidant activity	*cat*	F: TCCTGGAGCCTCAGCCAT R: ACAGTTATCACACAGGTGCATCTTT	79	JF801726
Somatotropic axis-aided growth	*gh*	F: TCGGTTGTGTGTTTGGGCGTCTC R: GTGCAGGTGCGTGACTCTGTTGA	90	XM_003442542
	igf-I	F: GTCTGTGGAGAGCGAGGCTTT R: CACGTGACCGCCTTGCA	70	NM_001279503
Reference genes	*ef-1α*	F: GCACGCTCTGCTGGCCTTT R: GCGCTCAATCTTCCATCCC	250	NM_001279647
	ß-actin	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-I*, insulin growth factor I; *ef-1α*, elongation factor 1α; *ß-actin* beta-actin.

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

TABLE 3 Growth, feed utilisation efficiency and survival rate of Nile tilapia fed on diets with 400 ppm limonene (L), 500 ppm thymol (T) and a combination of 400 ppm limonene and 500 ppm thymol (LT) for 63 days.

Parameter	Experimental diet				
	Control	L	T	LT	P value
Initial mean weight (g)	1.5 ± 0.0	1.6 ± 0.0	1.5 ± 0.0	1.6 ± 0.0	NS
Final mean weight (g)	13.7 ± 0.4^{a}	16.6 ± 0.4^{b}	15.0 ± 0.4^{a}	16.7 ± 0.3^{b}	0.001
% WG	793.2 ± 29.1^{a}	957.3 ± 51.9^{b}	887.0 ± 16.1^{ab}	980.0 ± 41.3^{b}	0.011
CF	1.8 ± 0.0	1.8 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	NS
% Survival	94.1 ± 3.5	97.4 ± 1.5	94.8 ± 3.0	98.1 ± 0.7	NS
% FI (% body weight d^{-1})	4.5 ± 0.1^{b}	3.9 ± 0.1^{a}	4.3 ± 0.2^{ab}	4.0 ± 0.1^{a}	0.019
FCR	1.8 ± 0.1^{b}	1.5 ± 0.0^{a}	1.7 ± 0.1^{ab}	1.5 ± 0.0^{a}	0.027
PER	1.7 ± 0.1^{a}	2.0 ± 0.1^{b}	1.9 ± 0.1^{ab}	2.0 ± 0.1^{b}	0.009

All values are means of treatments ± standard error. Mean values with different superscript in the same row are significantly different from each other at $P < 0.05$. NS, refers to not significantly different values. For each treatment, n =152 for initial fish weight, for final fish weight, n = number of alive fish at the end of the trial, and n = 4 for percentage of weight gain (% WG), condition factor (CF), survival rate (% survival), food intake (% FI), feed conversion ratio (FCR) and protein efficiency ratio (PER).