

1 **Title**

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

4 *Oreochromis niloticus*

5

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Accepted refereed manuscript of:

Aanyu M, Betancor M & Monroig O (2020) The effects of combined phytochemicals 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>.

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23 **Abstract**

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

39

40 **Keywords**

41 Growth promoters, limonene, Nile tilapia, nutritional physiology, phytogetic compounds,
42 thymol

43

44 **1. Introduction**

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

58 When mixtures of phytogetic compounds are used in animal feed, they can either have
59 synergistic, additive, indifferent or antagonistic effects on growth and other response
60 indicators in monogastric animals (Bassole and Juliani, 2012; Costa et al., 2013; Abd El-
61 hack et al., 2016; Valenzuela-Grijalva et al., 2017; Amer et al., 2018; Youssefi et al., 2019).

62 Synergistic or additive effects of phytogetic compounds on growth performance lead to
63 enhanced growth of animals above the levels attained when the compounds are supplied
64 individually (Windisch et al., 2008; Yang et al., 2015). In fish, the combination of thymol
65 and carvacrol is arguably the most investigated blend of phytogetic compounds for its
66 beneficial effects on growth (Zheng et al., 2009; Ahmadifar et al., 2011; Hyldgaard et al.,
67 2012; Chakraborty et al., 2013; Ahmadifar et al., 2014; Peterson et al., 2014; Perez-
68 Sanchez et al., 2015). For instance, Peterson et al. (2014) reported that channel catfish
69 (*Ictalurus punctatus*) fed on a diet supplemented with a combination of limonene, thymol,
70 carvacrol and anethol gained 44% more weight than the control attributed to synergistic or
71 additive effects of the phytogetic compounds.

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

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

88 The different responses to phytogetics mentioned above highlights the importance of
89 identifying combinations and doses of phytogetic compounds resulting in additive and
90 synergistic effects on fish growth. In our previous study (Aanyu et al., 2018), two
91 phytogetic compounds, namely limonene and thymol, classified as monoterpene and
92 diterpene, respectively, were found to have growth-promoting tendencies in Nile tilapia
93 (*Oreochromis niloticus*). We hypothesised that combinations of limonene and thymol can
94 potentially have additive or synergistic effects on the growth of Nile tilapia. Consequently,
95 this study aimed to investigate the effects of a blend of limonene and thymol, compared
96 with each of the compounds individually, on the growth, feed efficiency and nutritional
97 physiology of Nile tilapia. The study followed a candidate gene approach to investigate
98 physiological pathways underpinning the response of fish to the phytogetic compounds. A
99 selection of marker genes within the pathways of somatotropic axis-mediated growth,
100 nutrient absorption and transport, lipid metabolism and antioxidant enzyme status that
101 showed potential to be regulated by limonene and/or thymol were analysed (Aanyu et al.,
102 2018).

103

104

105 **2. Materials and Methods**

106 *2.1 Ethical statement*

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

111

112 *2.2 Experimental design*

113 The feeding trial was carried out at the Aquaculture Research and Development Center
114 (ARDC), Uganda between March and May 2015. Nile tilapia juveniles from the same
115 cohort were obtained from the ARDC fish farm, acclimatized and size graded to 1.54 ± 0.0
116 g (mean \pm standard error). Thirty eight (38) fish were stocked in each of the 16
117 experimental tanks. Each tank had a water holding capacity of 60 L, in a flow through
118 system with a flow rate of 1-2 L min⁻¹. The water in each tank was aerated using air stones
119 and heated using aquaria water heaters to 25.0 - 26.6 °C. A photoperiod of 12h light-12h
120 dark was maintained.

121 Water quality was monitored routinely to ensure that it was within the requirements
122 for Nile tilapia growth (Lim and Webster, 2006). A multi-parameter meter (HQ40D model,
123 Hach Ltd Germany) was used to measure dissolved oxygen, pH and water temperature.
124 The level of ammonia-nitrogen was assessed using a fresh water test kit from API
125 Company Ltd UK following the user guide from the manufacturer. Water flowing into the
126 fish rearing tanks had 6.6 ± 0.6 mg L⁻¹ of dissolved oxygen, pH 6.8 ± 0.3 , undetectable
127 levels (< 0.05 mg L⁻¹) of ammonia-nitrogen and water temperature ranging from 23.3 -
128 24.3 °C before heating with aquaria water heaters.

129

130 *2.3 Experimental diets*

131 A standard commercial feed (CP35%, Kampala, Uganda) for juvenile Nile tilapia
132 produced at the ARDC was supplemented with limonene (97 % purity) and/or thymol
133 (95 % purity) from Sigma Aldrich, Kampala, Uganda using concentrations found to have
134 growth-promoting potential in Nile tilapia (Aanyu et al., 2018). The diets included: 0 ppm
135 limonene and thymol (Control); 400 ppm limonene (L); 500 ppm thymol (T); and a
136 combination of 400 ppm limonene and 500 ppm thymol (LT). In order to supply the above
137 concentrations of phytogetic compounds to the feed, each concentration of phytogetic
138 compounds was prepared in 100 mL of absolute ethanol and sprayed onto 1 kg of feed.
139 The control was also coated with a similar amount of ethanol but no phytogetic compound
140 was added. All diets were air-dried for one day, packed in airtight polythene bags and
141 stored at room temperature until use.

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

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

159

160 *2.3 Fish measurements and sample collection*

161 Growth of the fish was estimated by measuring the weight (accuracy of 0.1 g) and total
162 length (0.1 cm) of all fish in each tank every three weeks and at the end of the experiment
163 (63 days). This procedure was carried out while fish were anaesthetised using 0.02 g L⁻¹ of
164 clove oil for 3-5 min, after which the fish were placed in aerated water and taken back to
165 the experimental tanks. At the end of the experiment, the number of live fish in each tank
166 was recorded for survival estimation, and sections of liver and fore intestine were dissected
167 from 16 fish per treatment (n=4 per tank) and placed in 1.5 mL tubes containing
168 RNAlater[®] (Sigma Aldrich, Kampala, Uganda). The liver and fore intestine samples were
169 kept at 4 °C overnight, shipped to the UK and transferred to a -20 °C freezer until RNA
170 was extracted.

171

172 *2.4 Fish performance calculations*

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

175

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

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

180 Condition factor (CF) = (final average fish weight / final average total length³) × 100;
181 Fish survival rate (SR, %) = (number of alive fish at end of trial / initial number of fish
182 stocked) × 100 %;
183 Feed intake (FI, % body weight per day) = (100 × (average feed intake fish⁻¹ / ((initial
184 average body weight ± final average body weight)/2))) / duration of trial (d);
185 Feed conversion ratio (FCR) = average feed intake fish⁻¹ / weight gain;
186 Protein efficiency ratio (PER) = weight gain / protein intake.

187

188 2.5 Molecular analyses

189 2.5.1 RNA extraction and complementary DNA (cDNA) synthesis

190 Tissue samples from the liver and fore intestine were homogenised in TRI Reagent (Sigma
191 Aldrich, Dorset, UK) with a mini bead-beater 16 (Biospec Bartlesville, OK, USA), and
192 total RNA was extracted from the samples (n=16 per tissue and treatment). The
193 concentration and purity of the RNA was measured by spectrophotometry with an ND-
194 1000 Nanodrop (Nanodrop 1000, Thermo Scientific, Glasgow, UK). The integrity of the
195 RNA (aliquots fo 200 ng total RNA) from each sample was further assessed by agarose gel
196 (1 %, v/v) electrophoresis.

197 From a total of 16 RNA samples extracted per tissue and treatment, eight RNA samples
198 were derived by pooling together two samples from the same tank and treatment, taking
199 equal quantity (2.5 µg µl⁻¹) of RNA from each of the two samples being pooled (adapted
200 from Glencross et al., 2015). Thus the final mixture (vortex mixed and centrifuged) had a
201 50 % contribution of each of the two samples that were pooled.

202 A high capacity reverse transcription kit without RNase inhibitor from AB Applied
203 Biosystems (Warrington, UK) was used to reverse transcribe 2 µg µl⁻¹ RNA from each
204 pool sample (n = 8 per treatment) to cDNA following the protocol provided by the
205 manufacturer.

206

207 2.5.2 Quantitative real-time Polymerase Chain Reaction (qPCR)

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

215 catalase (*cat*), growth hormone (*gh*), and insulin growth factor I (*igf-I*). Efficiency of the
216 primers was first tested by generating standard dilution curves, assessing the melting
217 curves and cycle threshold (Ct) values (Larionov et al., 2005). Efficient primers were
218 considered to have values between 0.80 - 1.10, a single melting peak, Ct value below 30
219 and one clear band under 1 % agarose gel electrophoresis. The details of the primers used
220 for qPCR analyses are provided in Table 2. Each qPCR contained duplicate samples (total
221 volume 20 μ l each) containing 5 μ l of 20-fold diluted cDNA, 3 μ l nuclease-free water, 1 μ l
222 (10 pmol) each for the forward and reverse primers, and 10 μ l of Luminaris color hgreen
223 qPCR Mix (Thermo Scientific, Hemel Hempstead, UK). All the reactions were run in 96
224 well-plates using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen,
225 Germany). A calibrator sample (20-fold dilution of all samples pooled cDNA) and a
226 negative control with no cDNA (non-template control-NTC) were included in each plate.
227 The qPCR thermocycling program involved pre-heating samples at 50 °C for 2 min
228 followed by 35 cycles, initial denaturing at 95 °C for 10 min, denaturation at 95 °C for 15 s,
229 annealing at 60 °C for 30 s, and an extension at 72 °C for 30 s.

230

231 2.5.3 Gene expression computations

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

240

241 2.6 Statistical analysis

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

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

254

255 **3. Results**

256 *3.1 Fish performance*

257 Table 3 shows the performance of Nile tilapia fed on diets supplemented with 400 ppm
258 limonene (L), 500 ppm thymol (T) and the combination of 400 ppm limonene with 500
259 ppm thymol (LT) for 63 days (9 weeks). There was a significant increase (P < 0.01) in the
260 final weight of fish fed on the diets supplemented with limonene, that is, diets L and LT,
261 compared to the control. No significant increases in fish weight were observed with diet L
262 and LT during day 1, 21, and 42 of the feeding experiment (data not shown). The diet
263 supplemented exclusively with thymol (T) did not significantly improve the final weight of
264 the fish (P = 0.052). There was a significantly higher percentage weight gain (% WG; P =
265 0.01) of fish fed on diets L and LT compared to the control, whilst fish fed diet T did not
266 show significant differences compared with the control. Despite there were no significant
267 differences in the final survival of the fish among treatments, there was a strong significant
268 positive correlation (r = 0.967, P = 0.033) between the survival rate and final weight of the
269 fish (Table 3). Condition factor (CF) was not significantly different among treatments.

270 The protein efficiency ratio (PER) was significantly higher in fish fed the diets L and LT
271 compared to the control. While no significant differences in PER were observed among
272 fish fed on diets L, T and LT, PER had a strong significant positive correlation (r = 0.974,
273 P = 0.026) with final fish weight and thus higher PER corresponded with higher final fish
274 weight. This study found no significant differences in feed conversion ratio (FCR) among
275 the treatments supplemented with L, T and LT, but fish fed diets L and LT had
276 significantly lower FCR (P = 0.006) than the control-fed fish (Table 3). Despite % feed
277 intake (% FI) not being significantly different among fish fed diets L, T and LT,
278 significantly lower (P = 0.019) FI was obtained with the fish fed on diets L and LT
279 compared with the control. In addition, there was a strong positive significant correlation (r
280 = 0.996, P = 0.004) between FI and FCR, and lower FI corresponded with low FCR and
281 therefore better feed utilisation efficiency.

282

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 *srebfl* 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 *srebfl* 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

320 **4. Discussion**

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

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

337 Among the feed utilisation parameters, the present study found enhanced feed
338 efficiency (i.e., lower FCR) in fish fed diets L and LT, and a strong significant positive
339 correlation between PER and FW. The correlation between PER and FW showed that, as
340 the utilisation of protein from the feed was enhanced (high PER), FW of the fish was
341 increased. This could have contributed to the significantly improved somatic growth of the
342 fish fed on diets L and LT, both treatments with increased PER compared to the control.
343 The increased WG and lowered FI levels observed with diet L and LT fed fish are in
344 agreement with Hashemipour et al. (2013) who found lower FI corresponding with the
345 highest WG and feed efficiency in broiler chicken fed on a diet with a mixture of 200 ppm
346 of thymol and carvacrol compared to the control. It is known that efficient growth in fish
347 does not necessarily coincide with maximum or higher FI because fish adjust their FI
348 according to their energy requirements (Ali and Jauncey, 2004), with better feed efficiency
349 occurring below maximum FI (Rad et al., 2003; Sawhney, 2014). Conversely, some studies
350 with phytogetic compounds (thymol and carvacrol) in pig diets found low FI
351 corresponding with low WG (Lee et al., 2003a; Lee et al., 2003b; Zhai et al., 2018). While
352 it is difficult to identify the exact causes of such an apparent discrepancy with the present
353 results, one possible reason might stem from the pungent odour of thymol and carvacrol

354 that can affect palatability and ultimately feed intake since, compared to fish, pigs are more
355 sensitive to smell (Michiels et al., 2012; Muthusamy and Sankar, 2015).

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

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

382 The movement of nutrients from the lumen of the intestine, aided by mucus, into
383 epithelial cells takes place through diffusion and/or active transport regulated by nutrient
384 transporters (Rust, 2003). The nutrient transporter *pept1* that aids the transport of protein in
385 the form of di/tri peptides through the above process (Verri et al., 2011), was significantly
386 regulated by diet LT compared with the control. Moreover, the higher expression of *pept1*
387 in fish fed diet LT corresponded with significantly improved feed efficiency (lower FCR)
388 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

424 enhanced activity of catalase enzyme in channel catfish fed on diets containing thymol,
425 carvacrol or their mixture, although the fish attained higher weight with the diet containing
426 both compounds and carvacrol alone. Thymol did not appear to have an obvious role in
427 regulation of antioxidant enzymes such as *cat*, and thus it can be assumed that, as noted
428 above, limonene exerts a major action in up-regulating *cat*.

429

430 **5. Conclusions**

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

440

441 **Acknowledgements**

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

445

446 **Data Availability Statement**

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

449

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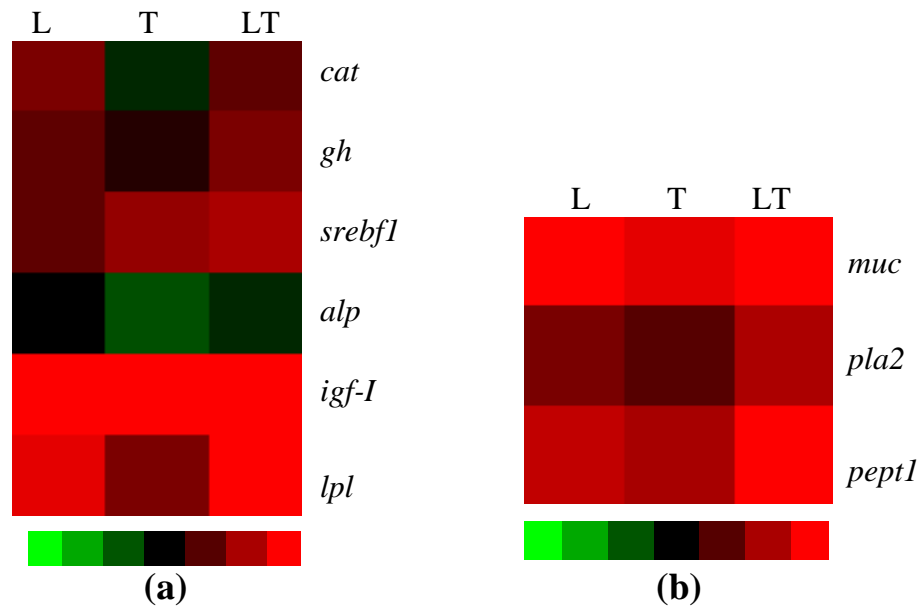
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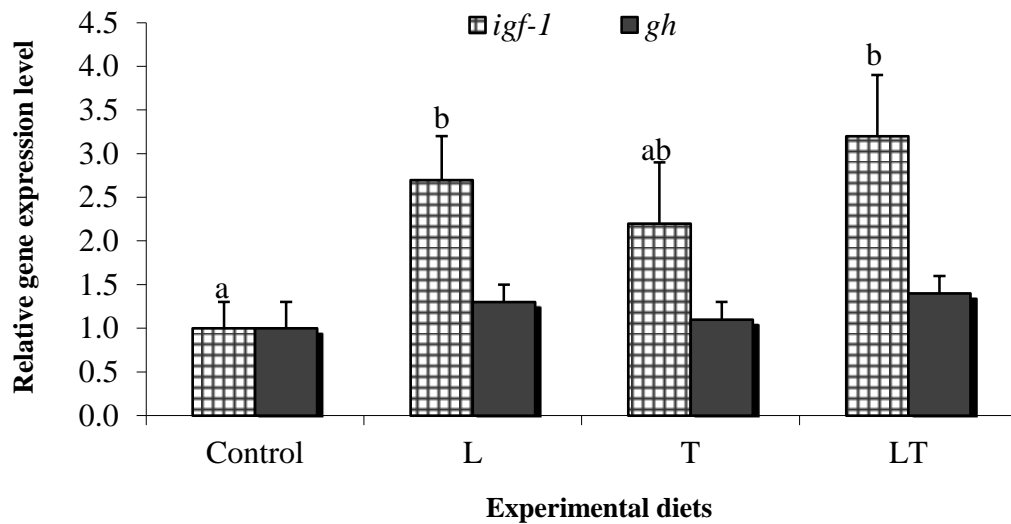
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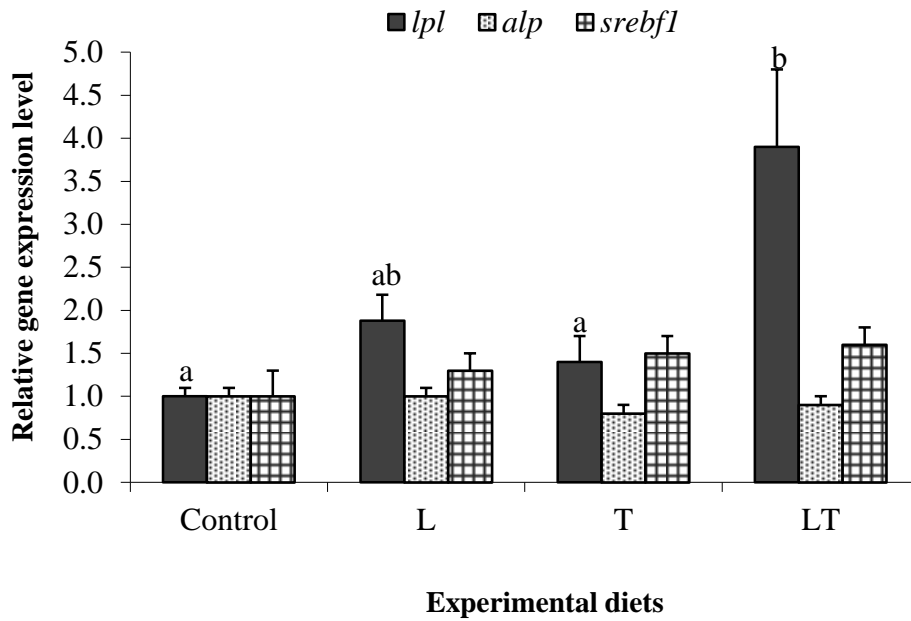
593 **FIGURE 1** Heat map showing the expression patterns of nine genes analysed using qPCR
594 data from Nile tilapia fed on diets supplemented with limonene (L), thymol (T) and their
595 combination (LT). Data were plotted using Java Tree View and rows were clustered
596 according to Euclidean distance. The columns represent mean data values of three dietary
597 treatments L (400 ppm limonene), T (500 ppm thymol) and LT (400 ppm limonene and
598 500 ppm thymol). The rows indicate each of the analysed genes in the liver (a) and fore
599 intestine (b) of Nile tilapia. Expression level of each gene was natural log transformed and
600 normalised against two reference genes. The colour bars at the bottom represent the mean
601 relative expression levels as low (green), neutral (black) or high (red). The black colour
602 represents the control to which the relative expression of the other treatments was
603 determined. *cat*, catalase; *gh*, growth hormone; *srebfl*, sterol regulatory element binding
604 transcription factor 1; *alp*, alkaline phosphatase; *igf-I*, insulin growth factor I; *pla2*,
605 phospholipase A2; *lpl*, lipoprotein lipase; *muc*, mucin-like protein; *pept1*, oligo-peptide
606 transporter 1.

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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 \pm standard error (n=8). Different superscript letters denote
 616 significant differences in the expression of *igf-I* between the treatments.

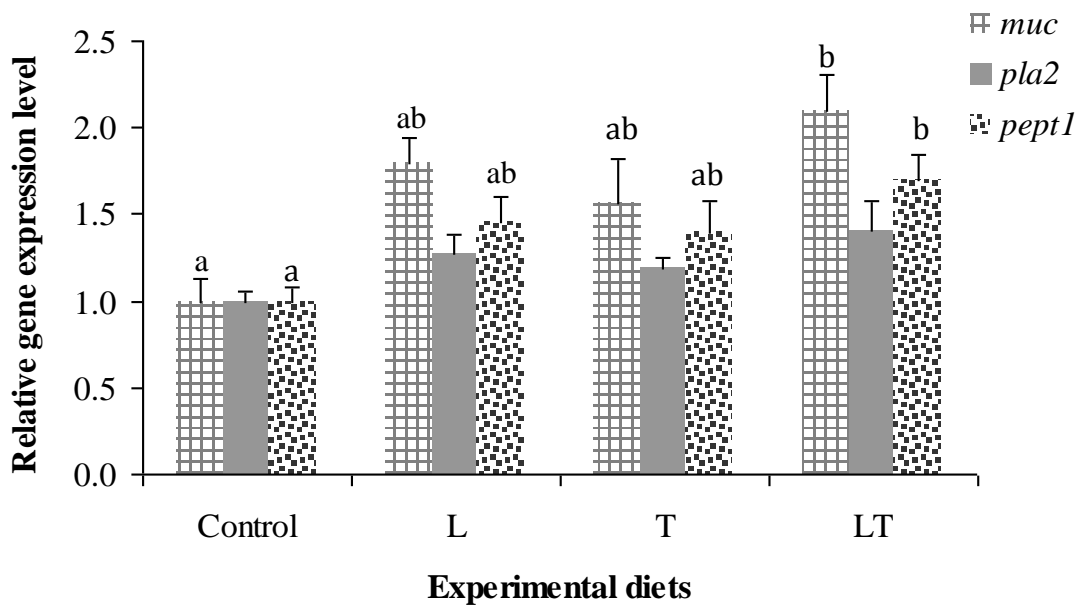


617

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 \pm
 622 standard error (n=8). Different superscript letters denote significant differences in the
 623 expression of *lpl* between the treatments.

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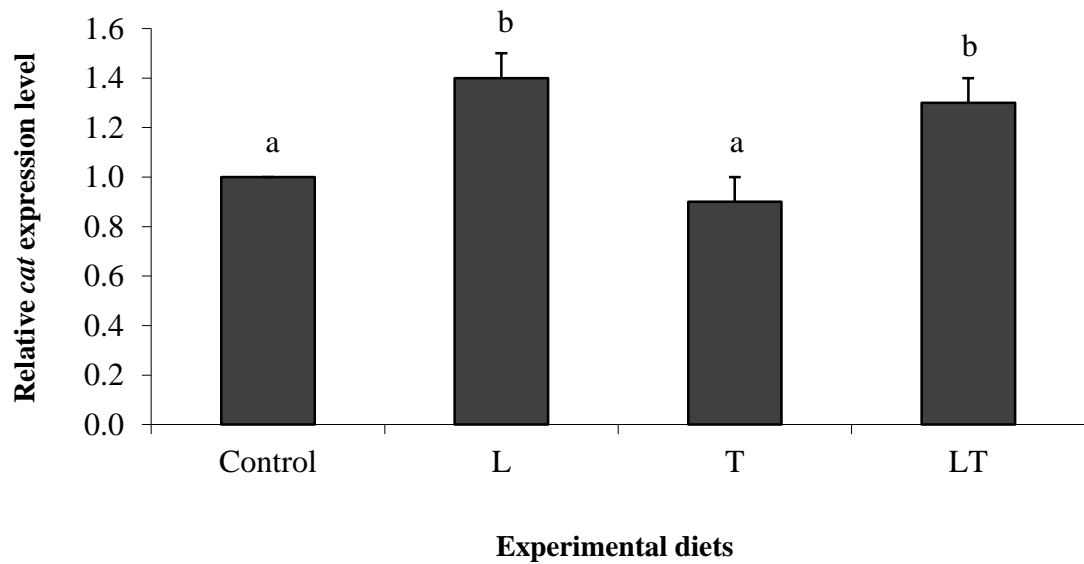


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627 **FIGURE 4** Expression of mucin-like protein (*muc*), phospholipase A2 (*pla2*), and oligo-
 628 peptide transporter 1 (*pept1*) genes in the fore intestine of Nile tilapia fed on diets
 629 supplemented with 400 ppm limonene (L), 500 ppm thymol (T) and a combination of 400
 630 ppm limonene and 500 ppm thymol (LT). All values are means of treatments \pm 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.

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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 \pm standard error (n=8). Different superscript letters denote significant
 640 differences in the expression of *cat* between the treatments.

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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.

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

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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 | <i>muc</i> | F: TGCCCAGGAGGTAGATATGC R: TACAGCATGAGCAGGAATGC | 101 | XM_005466350 |
| | <i>pept1</i> | F: CAAAGCACTGGTGAAGGTCC R: CACTGCGTCAAACATGGTGA | 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: CTTGGAGATGGGATGGGTGT R: TTGGCCTTAACCCCGCATAG | 200 | XM_005469634 |
| | <i>pla2</i> | F: CTCCAAACTCAAAGTGGGCC R: CCGAGCATCACCTTTTCTCG | 177 | XM_005451846 |
| Antioxidant activity | <i>cat</i> | F: TCCTGGAGCCTCAGCCAT R: ACAGTTATCACACAGGTGCATCTTT | 79 | JF801726 |
| Somatotropic axis-aided growth | <i>gh</i> | F: TCGGTTGTGTGTTTGGGCGTCTC R: GTGCAGGTGCGTGACTCTGTTGA | 90 | XM_003442542 |
| | <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 |

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-1 α* , elongation factor 1 α ; *β -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 | | | | P value |
|---------------------------------------|---------------------------|---------------------------|----------------------------|---------------------------|---------|
| | Control | L | T | LT | |
| 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).