Aquaculture 488 (2018) 217–226

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Effects of dietary limonene and thymol on the growth and nutritional physiology of Nile tilapia (*Oreochromis niloticus*)

Margaret Aanyu[a,b], Mónica B. Betancor[a], Oscar Monroig[a,*]

[a] Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, United Kingdom
[b] National Fisheries Resources Research Institute, Aquaculture Research and Development Center, P. O. Box 530, Kampala, Uganda

ARTICLE INFO

Keywords:
Essential oil
Gene regulation
Growth promoters
Nile tilapia
Phytochemical
Phytogenics

ABSTRACT

Phytogenic compounds such as limonene and thymol have been shown to have growth-promoting properties in farmed animals but studies in fish are scarce. Two experiments (Experiments I and II) were carried out to investigate the individual effects of dietary limonene and thymol on the growth and nutritional physiology of juvenile Nile tilapia (*Oreochromis niloticus*). In Experiment I, the fish were fed on a commercial diet coated with limonene at 0 (Control), 200, 400, and 600 mg kg^{-1} (ppm), while in Experiment II thymol was supplemented in the diet at 0 (Control), 250 and 500 ppm. Our results showed a significant increase in fish weight and weight gain with diets supplemented with 400 and 600 ppm limonene compared to the control. Moreover, the expression of insulin growth factor I (*igf-I*), mucin-like protein (*muc*), oligo-peptide transporter I (*pept1*), lipoprotein lipase (*lpl*), alkaline phosphatase (*alp*) and catalase (*cat*) was up-regulated by dietary limonene. Our results confirm that dietary limonene can enhance the growth of Nile tilapia juveniles through the activation of key genes involved in somatotropic axis-mediated growth, nutrient digestion and antioxidant enzyme defence. Dietary thymol did not seem to influence growth or regulate the same pathways activated by limonene in Nile tilapia juveniles at inclusion levels up to 500 ppm. Overall, the present results suggest that potential growth-promoting effects are dependent upon the phytogenetic itself and its inclusion level.

1. Introduction

Limonene and thymol are major phytogenic compounds of the essential oils (EOs) from citrus fruits and thyme herbs, respectively (Gad, 2012; Sun, 2007). Currently phytogenics are being widely investigated as naturally derived growth-promoters for use in animal production (Hashemi and Davoodi, 2010; Hernandez et al., 2004; Peric et al., 2009). Phytogenics are reported to be safer and healthier than antibiotics and hormone growth-promoters, compounds that have been discouraged from use in animal feeds by the World Health Organisation since 2006 (Brenes and Roura, 2010; Windisch et al., 2008). Thus, antibiotic growth-promoters have the risk of creating resistance to bacteria pathogenic to animals and humans, while hormones might deposit in animal tissue and negatively affect human health (Jeong et al., 2010; Mathivanan and Edwin, 2012; Sicuro et al., 2010; Syahidah et al., 2015).

Some studies have shown that dietary supply of limonene and thymol have demonstrated growth-promoting effects on growth of poultry and some fish species (Acar et al., 2015; Ahmadifar et al., 2011, 2014; Dalkilic et al., 2015; Ngugi et al., 2017; Pérez-Sánchez et al., 2015; Shad et al., 2016; Zidan et al., 2016). The effects of these compounds are largely dose-dependent and appear to be species-specific. Among fish species, weight gain of Mozambique tilapia (*Oreochromis mossambicus*) was significantly improved with a diet formulated with 1000 ppm of an EO containing 83.0% limonene but not with 3000 and 5000 ppm (Acar et al., 2015). The growth of Ningu (*Labeo victorianus*) was improved with diets supplemented with 1000, 2000, 5000 and 8000 ppm of an EO containing 81.4% limonene (Ngugi et al., 2017). However, in both studies it remained uncertain whether the enhanced fish growth was a result of synergistic effects between limonene and the compounds present at lower proportions in the EO. With regards to thymol, a dietary combination of thymol and carvacrol at 1000, 2000 and 3000 ppm enhanced the growth of rainbow trout (*Oncorhynchus mykiss*) and European sturgeon (*Huso huso*) (Ahmadifar et al., 2011, 2014). Conversely, 500 ppm of dietary thymol did not stimulate growth of channel catfish (*Ictalurus punctatus*) (Zheng et al., 2009). Giannenas et al. (2012) did not find significant increase in weight of rainbow trout fed a diet with 1000 ppm of a phytogenic product with 6000 ppm thymol. Overall, the studies above highlight the importance of identifying the right phytogenic compounds and doses that result in growth

* Corresponding author.
E-mail address: oscar.monroig@stir.ac.uk (O. Monroig).

https://doi.org/10.1016/j.aquaculture.2018.01.036
Received 6 December 2017; Received in revised form 22 January 2018; Accepted 23 January 2018

improvement in different fish species. Presently, there is scarce information on the impact of limonene or thymol on the growth of Nile tilapia (*Oreochromis niloticus*).

Nutritional physiology mechanisms underlying the growth-enhancing effects of phytogenic compounds are also not fully understood. Although it is known that diets can influence appetite, nutrient digestion, absorption and transport, lipid metabolism, antioxidant enzyme activity and somatotropic axis-mediated growth among other processes (Qiang et al., 2012; Rust, 2003), there are few reports in the literature on the pathways regulated by phytogenic compounds. In addition, different phytogenic compounds seem to have different effects on the physiology of animals (Lee et al., 2003a, 2003b, 2004; Lillehoj et al., 2011), thus potentially activating different pathways to enhance growth. Presently, most of the studies that have investigated the mode of action of phytogenic compounds in fish, as well as terrestrial monogastric animals such as pigs and poultry, have been carried out using diets containing combinations of several phytogenic compounds (Awaad et al., 2014; Hafeez et al., 2016; Hashemipour et al., 2013; Hashemipour et al., 2016; Jiang et al., 2015; Li et al., 2012; Matysiak et al., 2012; Pérez-Sánchez et al., 2015; Zheng et al., 2009). From these studies, it is difficult to elucidate the specific pathways activated by individual compounds. However, other studies have investigated the effects of phytogenic compounds supplemented individually in the diet (Galeotti et al., 2012; Yilmaz et al., 2014), although there is still limited understanding on pathways that different phytogenic compounds influence in nutritional physiology, particularly for fish.

Some nutritional physiology processes through which phytogenics exert their alluded to above growth-promoting effects include feed intake, digestion, absorption and lipid metabolism among other functions (Hashemi and Davoodi, 2010; NRC, 2011). The aim of the present study was to investigate the individual effects of dietary limonene or thymol on the growth and nutritional physiology of Nile tilapia. Specifically, the study investigated the expression of genes regulating food intake, nutrient digestion and transport, lipid metabolism, antioxidant enzyme status and somatotropic axis-mediated growth in Nile tilapia fed on diets with either limonene or thymol along with control diets.

2. Materials and methods

2.1. Ethics statement

All experiments were subjected to ethical reviewed 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

Two feeding experiments (Experiments I and II) were carried out at the Institute of Aquaculture, University of Stirling, UK. In each experiment, the pathways in the nutritional physiology of Nile tilapia potentially influenced by limonene or thymol were investigated by analysing the expression of genes regulating food intake, nutrient digestion and transport, lipid metabolism, antioxidant enzyme status and somatotropic axis-mediated growth. Both feeding experiments were run for 63 days.

2.3. Diets and fish feeding

A commercial fish diet (INICIO Plus, BioMar Ltd., Stirling, UK) (see proximate composition in Table 1) was used as a standard diet to which limonene or thymol was supplemented at increasing concentrations. Limonene (97% purity) and thymol (99.5% purity) were obtained from Sigma Aldrich Ltd., England, UK. In Experiment I, the diets were supplemented with 0 (control), 200 (L1), 400 (L2) and 600 (L3) mg kg^{-1} (ppm) of limonene. In Experiment II, the diets were supplemented with 0 (Control), 250 (T1), and 500 (T2) ppm of thymol. In order to achieve the above concentrations in the experimental diets, the phytogenic compounds were first dissolved in 100 ml of absolute ethanol and subsequently sprayed evenly onto 1 kg of feed. The same volume of ethanol was added to the control diet to ensure similar conditions with phytogenic compound-supplemented diets. The diets were air-dried for one day before being used to feed the fish.

Table 1
Proximate composition of the commercial diet used in Experiments I and II.

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

2.4. Experimental fish and husbandry conditions

Nile tilapia juveniles from the same spawning batch were obtained from the Tropical Aquarium, Institute of Aquaculture, University of Stirling, UK. The fish were size graded during which they were anaesthetised with a low dose of benzocaine of 0.05 g l^{-1} for 3–5 min and kept in aerated water to minimise stress. The initial weight of the fish was 1.5 ± 0.0 g for Experiment I (limonene) and 1.5 ± 0.1 g for Experiment II (thymol) (mean ± standard error). Both experiments were conducted in 60 l plastic tanks in a recirculating aquaculture system. All treatments were assessed in three replicate tanks allocated using a complete randomised design. In Experiment I (limonene supplementation), each tank was stocked with 37 fish, whereas 25 fish per tank were stocked in Experiment II (thymol supplementation). In both experiments, fish were fed to apparent satiation by hand between 9:00–10:00 am and 4:00–5:00 pm. The amount of feed eaten was recorded daily.

The water quality in the experimental system was assessed weekly and maintained within the conditions for growth of Nile tilapia. An oxygen meter (OaKton DO 6+, Eutech Instruments Pte Ltd., Landsmeer, The Netherlands) was used to determine dissolved oxygen levels and water temperature. The pH and ammonia-nitrogen levels were measured using a fresh water test kit from Tropic Marin Company Ltd. (Wartenberg, Germany) following the instructions from the manufacturer. The water temperature ranged from 26.0 to 27.0 °C, pH was 6.8 ± 0.5, dissolved oxygen 6.8 ± 0.6 mg l^{-1} and ammonia-nitrogen 0.8 ± 1.0 mg l^{-1} (mean ± standard deviation). A light regime of 12 L:12D was maintained.

2.5. Sampling and measurements

Growth of fish, previously anesthetised with 0.05 g l^{-1} benzocaine for 3–5 min, was monitored every two or three weeks by measuring weight (0.1 g accuracy) and total length (0.1 cm accuracy). The number of fish in each tank was also recorded. At the end of the experiments, fish were humanely killed with an overdose of benzocaine. Samples of liver, fore intestine, and brain were collected from three fish per replicate ($N = 9$ per treatment) and placed in 1.5 ml tubes containing RNAlater (Sigma aldrich, Poole, UK) to preserve RNA integrity. The samples were kept at 4 °C overnight and transferred to a −70 °C freezer until RNA extraction.