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Influence of three spice powders on the survival and histopathology of *Oreochromis mossambicus* before and after *Streptococcus iniae* infection.

Mert Gurkan[1], Sevdan Yilmaz[2*], Hasan Kaya[3], Sebahattin Ergun[2], Samet Alkan[2]

[1]*Department of Biology, Faculty of Arts and Sciences, Canakkale Onsekiz Mart University, Canakkale, Turkey*

[2]*Department of Aquaculture, Faculty of Marine Sciences and Technology, Canakkale Onsekiz Mart University, Canakkale, Turkey*

[3]*Department of Basic Science, Faculty of Marine Sciences and Technology, Canakkale Onsekiz Mart University, Canakkale, Turkey*

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ABSTRACT

The aim of this study was to determine the effects of thyme, rosemary and fenugreek powders on disease resistance and histopathological changes in intestine and liver tissues of *Oreochromis mossambicus* before and after exposure to *Streptococcus iniae*. Four isonitrogenic (37% crude protein) and isolipidic (10% crude lipid) diets were formulated containing 0% (control) or 1.0% thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), or fenugreek (*Trigonella foenum graecum*). Fish were stocked at 20 fish (0.51±0.01 g), each, in twelve 21-L plastic tanks, fed for 60 days, and then infected with *S. iniae*. At the end of the challenge experiment the survival rates in the thyme, rosemary and fenugreek supplemented diets and unsupplemented control diet were 83.3%, 81.7%, 81.7% and 48.3%, respectively. The present study suggested the protective potential of thyme, rosemary and fenugreek in alleviating the intestinal and hepatic damage that can occur after infection with *S. iniae*. It was concluded that 1.0% thyme, rosemary or fenugreek can enhance disease resistance of *O. mossambicus*, suggesting that thyme, rosemary or fenugreek may be an alternative to antibiotics in controlling streptococcal disease in tilapia culture.

Introduction

Streptococcosis is a subacute but often chronic disease (Kitao, 1993) that causes serious problems for some fish species in intensive production systems, particularly in the case of intensively cultured tilapia *Tilapia* spp. (Plumb 1999). *Streptococcus* spp. are gram-positive cocci that are nonmotile, catalase negative, fermentative in glucose, and non-spore-forming (Stoffregen et al. 1996; Darwish et al. 2002; El-Sayed, 2006). Streptococcosis is characterized by ascites and, less frequently, obvious abdominal swelling, petechiae around the anus, mouth, and proximal margins of pectoral fins, both unilateral and bilateral exophthalmia, corneal opacity in some specimens, and congestion (folding) of the dorsal and pectoral fins (Perera et al. 1998). Internally, the liver, spleen, and kidneys are pale and swollen, and infected animals circle listlessly at the water surface and show various signs of disorientation (Perera et al. 1998).

Streptococcal disease is mainly controlled by antibiotics (Darwish et al. 2002; Abutbul et al. 2004; Chen and Bowser 2005). An order to control disease outbreaks, antibiotics and chemotherapeutics have been used indiscriminately, which in turn leads to residual problems in the surrounding environment affecting higher animals as well as humans (Caruso et al. 2013). Moreover, the overuse of antibiotics in aquaculture results in increased antibiotic resistance in fish pathogens (Zilberg et al. 2010). Dietary administrations of medicinal herbs or spices are used as an alternative to pharmacologically active agents, such as antibiotics, chemotherapeutics, vaccines, hormone, and other synthetic

*Corresponding author
E-mail address: sevdanyilmaz@comu.edu.tr (S. Yilmaz)
Tel:+: +90 286 218 00 18 fax: +90 286 218 05 43

compounds (Vaseeharan and Thaya, 2014). Previous studies have shown that herbs or spices such as *Astragalus radix* (Yin et al. 2006), *Eclipta alba* (Christybapita et al. 2007), *Solanum trilobatum* (Divyagnaneswari et al. 2007), *Allium sativum* (Aly et al. 2008a), *Nigella sativa* (Diab et al. 2008), *Syzygium aromaticum* (Rattanachaikunsopon and Phumkhachorn, 2009) and *Cuminum cyminum* (Yılmaz et al. 2012) are successfully used to replace antibiotics in tilapia culture. In addition, several studies reported that oral administration of fenugreek in *Labeo rohita* and *O. mossambicus* (Paul et al. 2004; Mostafa et al. 2009), rosemary in *O. niloticus* (Zilberg et al. 2010), thyme, rosemary and fenugreek in *O. mossambicus* (Yılmaz et al. 2013a) and *Dicentrarchus labrax* (Yılmaz et al. 2013b) improved their growth performance, organ's status, disease resistance and immunity. The histopathological effects of these plants are still unclear. The aim of this study was to determine the effects of thyme, rosemary and fenugreek on disease resistance and histopathological changes in intestine and liver tissues of *O. mossambicus* before and after exposure to *S. iniae*.

Material and methods

Experimental herb and diets

Thyme, rosemary, and fenugreek powders were obtained from a local market. The spices were added to the basal (control) diet at 1%. The diets contained 9% moisture, 37% crude protein, 10% crude lipid and 10% ash. The feed components (g kg^{-1}) of the total mixed diet were 300 g kg^{-1} fish meal, 330 g kg^{-1} soybean meal, 162 g kg^{-1} wheat flour, 65 g kg^{-1} fish oil, 40 g kg^{-1} vitamin–mineral mix, and 103 g kg $starch^{-1}$. The diet was modified by replacing starch with the amount of thyme, rosemary, and fenugreek to give 1%. Feed ingredients were mixed in a blender, the feed was pressed through a 2-mm die in a pelleting machine, and the pellets were dried in a drying cabinet (40°C) until moisture dropped to around 10%, crushed into desirable particle sizes, and stored at -20°C until use. Proximate analysis of the diets was performed using standard methods (AOAC, 1998). Dry matter was analyzed by drying at 105°C in an oven to a constant weight, crude fat by ether extraction, crude protein by the Kjeldahl method, and crude ash by incineration at 525°C in a muffle furnace for 12 h.

Fish and experimental conditions

Healthy cultured *O. mossambicus* (0.51±0.01 g) were produced in Çanakkale Onsekiz Mart University, Faculty of Marine Sciences and Technology. The experiment consisted of triplicate groups for each diet. Twelve 21 L plastic tanks were stocked with 240 fish at 20 fish/tank. Fish were fed a diet containing 37.0% protein and 10.0% lipid before the start of the experiment. Tanks were provided with sponge filters connected via an airline to a Resun Lp-100 air pump. Water was exchanged daily at approximately 10% of the total volume. Temperature ranged 28±0.1°C, pH 7.6±0.1, dissolved oxygen 7.15±0.4 mg/l, and conductivity 610±10 uS.

Bacteria

The bacterium (*S. iniae*) was previously isolated from diseased tilapia specimens collected aseptically from brain and anterior kidney during the post-mortem examination (Yılmaz et al., 2013a). Specimens were cultured directly onto sheep blood agar at 28°C for 24–48 h. Gram-stained positive, beta-hemolytic, catalase negative coccus colonies were subcultured onto blood agar and then identified by APIStrep (Biomerieux). The isolated *S. iniae* were kept frozen in 15% glycerol, 85% Brain Heart Infusion (BHI) broth, in aliquots, at -70°C until used.

Bacterial culture preparation

Five mL of overnight bacterial culture with *S. iniae* was transferred into a 1 L Erlenmeyer flask containing 500 mL of the BHI broth medium. After 24 h of incubation at 28°C, the bacterial culture was centrifuged at 400 *g* at 15°C for 10 min, and the pelleted bacteria were resuspended with 200 mL of PBS to achieve a concentration of $9.0x10^9$ colony-forming units (CFU)/mL (Yılmaz et al. 2013a). This suspension was used for the immersion challenge.

Immersion challenge experiment

The immersion challenge experiment was performed according to the protocol described by Yılmaz et al. (2013c) with some minor modifications. Briefly, after 60 days, fish (20 fish/tank) were stocked in 21 L tank kept at 28°C throughout the challenge experiment. Each tank contained 19.8 L of water and 200 mL of the bacterial suspension and immersion-challenged with $9.0x10^8$ CFU/mL of *S. iniae*. Dead fish were removed from the aquaria daily and mortality was recorded daily for 20 days. The bacterium was re-isolated from the dead fish.

Histological experiments

At the end of the experiments, 10 fishes of each group were selected randomly and tissues from the liver and intestine were removed. Afterwards these tissues fixed in Bouin's solutions then transferred to 70% ethyl alcohol. These tissues were processed with alcohol, xylene, and paraffin series and then paraffin blocks were prepared. Cross sections of 5 µm in thickness were obtained from these blocks, stained with hematoxylin and eosin (H&E), and examined histopathologically under a light microscope. Finally, histological imaging of the preparations were carried out using a camera mounted on an Olympus BX51 light microscope and analyzed using DP2-BSW software.

Statistics

The survival of fish in each challenge treatment group was estimated using Kaplan-Meier analysis and differences between the groups were assessed with the Log-Rank (Mantel–Cox) test for pairwise comparisons.

Results and discussion

At the end of the challenge experiment the survival rates in the thyme, rosemary and fenugreek supplemented diets and unsupplemented control were 83.3%, 81.7 %, 81.7 % and 48.3%, respectively (Figure 1). The survival curves of control fish were statistically different from all spice supplemented fish ($P < 0.05$ by log-rank test, Table 1).

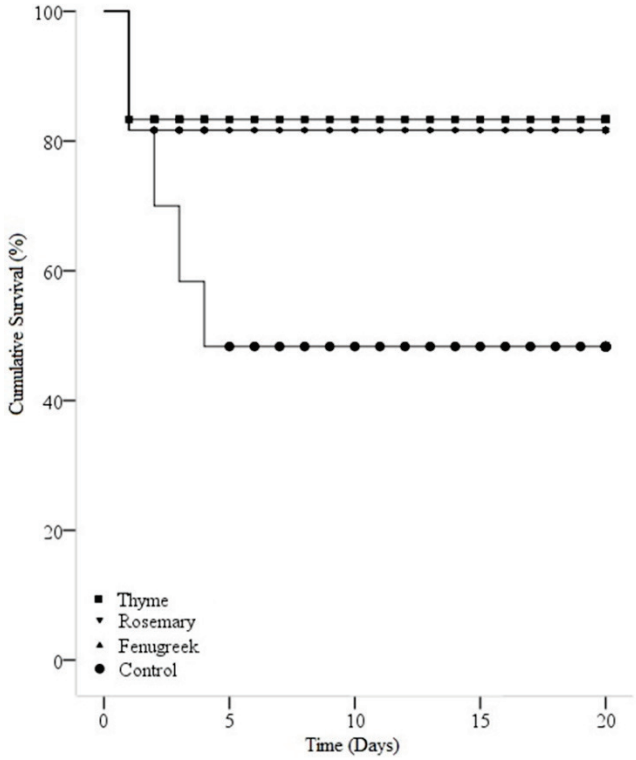

Figure 1. Results of the challenge test exposure of Tilapia to a *S. iniae* suspension containing a concentration of $9.0x10^8$ CFU/mL. The exposure took place after rearing the fish for 60 days under spices feeding regimes with added Thyme, Rosemary and Fenugreek at 1%. Data represent Kaplan-Meier survivorship curves. Sample size = 60 fish per treatment.

Table 1 Pairwise comparisons by Log-Rank (Mantel–Cox)

	Control	Thyme	Rosemary	Fenugreek
Control		X^2 =12.217	X^2=10.886	X^2 =10.886
		P<0.000	P=0.001	P=0.001
Thyme			X^2 =0.057	X^2 =0.057
			P=0.811	P=0.811
Rosemary				X^2 =0.000
				P=1.000
Fenugreek				

Previous study showed that the tilapias which were fed with diets including *O. sanctum* (Logambal et al. 2000), *R. officinalis* (Abutbul et al. 2004; Zilberg et al. 2010), *Cinnamomum verum* (Rattanachaikunsopon and Phumkhachorn, 2010), *Andrographis paniculata* (Rattanachaikunsopon and Phumkhachorn, 2009), *Cuminum cyminum* (Yılmaz et al. 2012), *T. vulgaris, R. officinalis, T. foenum graecum* (Yılmaz et al. 2013a) increased the survival rate against streptococcal challenge. The control fish (without *S. iniae* infection) showed no histopathological abnormalities in the intestine and liver (Figure 2a and 3a) while all the infected with *S. iniae* fishes showed variable histopathological alterations (Figure 2b and 3b). Infected with *S. iniae* fishes showed mucosal deformations, mononuclear cellular infiltrations in intestine, and focal haemorrhages, hepatocellular vacuolation, increase in the numbers of melano-macrophages centres and fatty change in liver. The above histopathological alterations in the intestine and liver were effectively controlled when treated with thyme (Fig. 2c and Fig. 3c), rosemary (Fig. 2d and Fig. 3d), fenugreek groups (Figure 2e and 3e). Only in the liver of fishes which were treated with thyme and rosemary, mononuclear cellular infiltrations were observed.

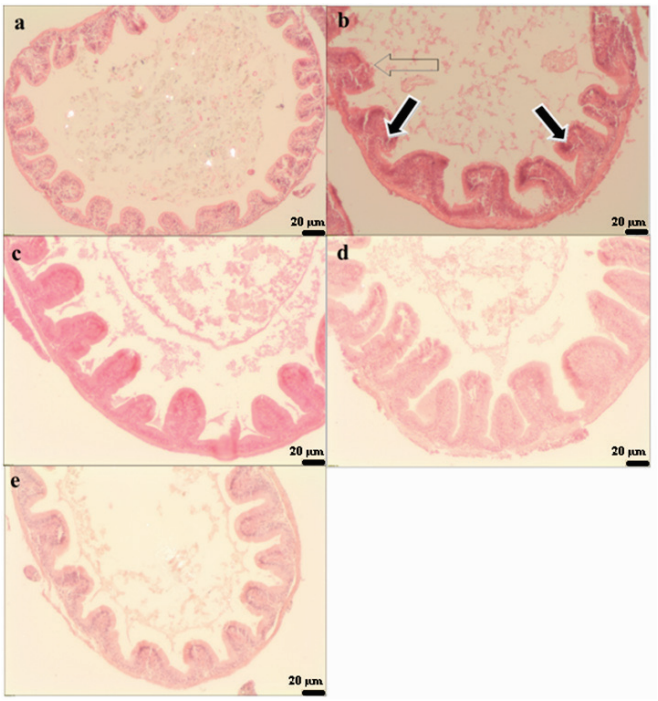

Figure 2. a. Control without *S. iniae* infection, b. Control with *S. iniae* infection, c. 1.0% thyme, d. 1.0% rosemary and e. 1.0% fenugreek following infection with *S. iniae*, *O. mossambicus* intestine. Mononuclear cellular infiltrations (black arrow) and deformations of the villi (transparent arrow). H&E.

Many herbal additives have been reviewed to improve fish immunity and increase disease resistance (Chakraborty and Hancz, 2011). Herb products have been reported to promote various activities like antistress, immunostimulation, and antimicrobial properties in fish culture with many active components (Citarasu, 2010). Thymol are the major active component of *T. vulgaris* and its antibacterial and antioxidant activity has been known (Lee et al. 2005; Rota et al. 2008). Braga et al. (2006) demonstrated that the thymol significantly increased the percentage of elastase and calcium mobilization inhibition in human neutrophils due to the helpful effects in controlling the peripheral immune/inflammatory processes in many infections. Carnosic acid and rosmarinic acid are the main chemical constituents of rosemary, and they have particularly high antioxidant activity (Thorsen and Hildebrandt, 2003; Erkan et al. 2008). Beninca et al. (2011) also reported that *R. officinalis* crude extracts and its different components showed an important anti-inflammatory activity. Fenugreek is rich in apigenin,

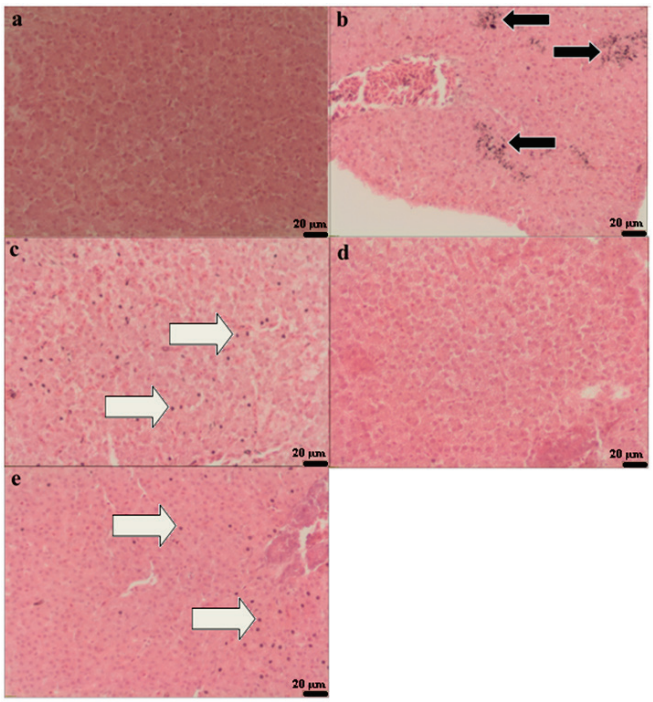

Figure 3. a. Control without *S. iniae* infection, b. Control with *S. iniae* infection, c. 1.0% thyme, d. 1.0% rosemary and e. 1.0% fenugreek following infection with *S. iniae*, *O. mossambicus* liver. Melano-macrophages centres (black arrow) and mononuclear cellular infiltrations (white arrow). H&E.

kaempferol, quercetin, diosgenin and yamogenin, and their characteristic functions have been shown to protected the organisms from oxidative damage (Kaviarasan et al. 2004). Moreover, a previous study has indicated that aqueous extract of *T. foenum graecum* increased some cellular and humoral immune parameters such as delayed type of hypersensitivity, plaque-forming cell, antibody titre, phagocytic index and phagocytic capacity of macrophages (Bin-Hafeez et al. 2003). Anti-inflammatory, analgesic, antimicrobial, antioxidant and/or immunomodulatory are properties that should be most effective in reducing the severity or disabling effects of a variety of infectious or inflammatory conditions in animals and humans (Hart, 2005).

In this study three spice powders (thyme, rosemary or fenugreek) administration resulted in overall improvement in the intestine and liver histology of the fish. They also increased disease resistance of *O. mossambicus* to *S. iniae*. The results of this study suggested the potential of these spices to alleviate the intestinal and hepatic damage caused by infection with *S. iniae*. This suggests that thyme, rosemary or fenugreek may be an alternative to antibiotics in controlling streptococcal disease in tilapia culture.

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