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RESEARCH PAPER

Influence of Adding Garlic (*Allium sativum*), Ginger (*Zingiber officinale*), Thyme (*Thymus vulgaris*) and Their Combination on the Growth Performance, Haemato-Immunological Parameters and Disease Resistance to *Photobacterium damselae* in Sobaity Sea Bream (*Sparidentex hasta*) Fry

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Abstract

The present study was aimed at determining the effects of medicinal herbs adjuvants on growth, hematoebiochemical parameters, some non-specific immunity and disease resistance to *Photobacterium damselae* in Sobaity sea bream *(Sparidentex hasta).* The fish (mean body weight 3.08±0.31 g) were fed fishmeal diets supplemented with either 1% of Garlic *(Allium sativum)*, 1% supplemented of Ginger *(Zingiber officinale*), 1% supplemented of thyme (*Thymus vulgaris*), a mixture of these herbs (1% Garlic + 1% Ginger + 1% thyme), and control diet without medicinal herbs, for 8 weeks. The highest weight gains (WG %) and the specific growth rate (SGR) were in fish that fed diets with the mixture of three medicinal herbs ($P<0.05$). The number of WBC and RBC showed a significant increase in ginger and the mixture of three medicinal herbs compared to the control ($P<0.05$). The highest total protein and the lowest cholesterol levels were observed in treated groups with ginger diet ($P<0.05$). The highest albumin and the lowest triglyceride levels were achieved in mixture of these three herbsgroup. The analysis of AST and ALT were significantly decreased in the mixture of these three herbs and ginger groups ($P<0.05$). The lowest ALP level were showed in mixture of these three herbs group. Furthermore, serum lysozyme activity, total immunoglobulin (Ig) and complement activity (ACH50) were significantly increased in the mixture of three medicinal herbs fed fish and ginger groups ($P<0.05$). After feeding experiment, fish were infected with *Photobacterium damselae* strain FN1 and mortalities were recorded. The results of this study demonstrated that dietary mixture of three medicinal herbs and ginger groups could be an improvement in the growth and some non-specific immunity of Sobaity sea bream fry.

***Keywords*:** Growth, haemato-immunological parameters, medicinal herbs, Sobaity sea bream, *Photobacterium damselae.*

Introduction

Sobaity sea bream *(Sparidentex hasta)* is the only species in the Sparidentex genus of sparidae family and its area of distribution includes the Western Indian Ocean, the Persian Gulf and the coasts of India (Carpenter, Krupp, & Jones, 1997). It is carnivorous and feeds on a variety of fish and invertebrates (Tahery Kondor, Sajjadi, Sorynezhad, Daryaee, & Mirzade, 2013). The Regional Aquaculture Information System for the Persian Gulf area (RAIS, 2009) reports for this country an annual production of about 42 mtn in 2006 and 2007. In recent years, there has been an increasing interest for Sobaity sea bream production due to its consumer popularity and a new candidate species in marine culture in the southern seashores of Iran and Arabian countries coastlines (Hussain, Akatsu, & El-Zahr, 1981). The use of dietary additives in fish farms is one of the methods commonly used to improve weight gain, feed efficiency, and/or disease resistance in cultured fish (Akrami, Gharaei, Razeghi Mansour, & Galeshi 2015). Immunostimulants increase resistance to infectious diseases, not only stimulating the acquired immune response, but also enhancing innate immune mechanisms (Galindo-Villegas & Hosokawa, 2004). Garlic (*A. sativum*) is an herb found virtually throughout the world, has been used in humans to treat rheumatoid arthritis, the common cold, diabetes, malaria, and tuberculosis for over 4000 years (Sasaki, 2006). Garlic contains an odorless sulfur-containing compound known as S-allyl-cysteine sulfoxide, which stimulates the hypoglycemic activity (Martins, Tavares-Dias, Fujimoto, Onaka, & Nomura, 2004). Earlier studies have reported that garlic, as a feed additive in fish feed, may stimulate growth, improve antioxidant status, and enhance immunological, hematological and serum biochemical parameters (Yılmaz & Ergün, 2012). Ginger *(Zingiber officinale Roscoe, Zingiberacae)* is widely used around the world in food as a spice. Ginger is generally considered as a

safe herbal medicine (Weidner & Sigwart, 2000); contains alkaloids, flavonoids, polyphenols, saponin, steroids, tannin, fiber, carbohydrate, vitamins, carotenoids and minerals (Otunola, Oloyede, Oladiji, & Afolayan, 2010; Shirin & Prakash, 2010); natural antioxidants as gingerols, shogaols and Zingerone (Hori *et al.*, 2003); essential oils which has potent anti-inflammatory effects and oleoresin (Zarate & Yeoman, 1996). Previously, studies have indicated that ginger is effective for the controling of a range of bacterial, fungal and parasitic conditions (Chrubasik, Pittler, & Roufogalis, 2005). Thyme *(Thymus vulgaris* L.) is an herbaceous perennial plant belonging to the Lamiaceae family. Thyme has a strong antimicrobial and antioxidant activity due to its very high contents of thymol, p-cymene, carvacrol, eugenol, and 4-allylphenol. (Gültepe, Bilen, Yılmaz, Güroy, & Aydın, 2014) Thymol, a major component of thyme-essential oils, has been widely studied for its antimicrobial properties (Dorman & Deans, 2000). Carvacrol, an isomer of thymol, is found in essential oils isolated from oregano and thyme. Like thymol, carvacrol also displays antimicrobial activity (Helander, Alakomi, Latva-Kala, Mattila-Sandholm, & Pol, 1998). Several researches have reported the beneficial effects of dietary garlic, ginger and thyme as dietary additives (Talpur, Ikhwanuddin, & Bolong, 2013; Al-Salahy, 2002; Nya & Austin, 2009; Haghighi & Sharif Rohani, 2013; Shalaby, Khattab, & Abdel Rahman, 2006; Chrubasik *et al.*, 2005; Şahan, Özütok, & Kurutaş, 2016; McDevitt, Hillman, Acamovic, & Cross, 2007) in fish, but there is no documented evidence about the effect of these medicinal herbs or other herbal plants on sobaity sea bream. Pasteurellosis or photobacteriosis is a type of bacterial septicemia in marine fish caused by *Photobacterium damselae* (Naseri *et al.*, 2014). *P. damselae* is an opportunistic pathogen responsible for causing disease in a wide variety of natural hosts (Austin & Austin, 1993) including neritic sharks (Grimes *et al*., 1984), dolphins (Fujioka, Greco, Cates, & Schroeder, 1988), and shrimps, as well as wild and cultivated fish (Romalde, 2002) and humans (Morris *et al*., 1982). The phenotypic characters of the strain appear very diversely with *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*. This strain (FN1) may be pathogenic for other marine animals and humans (Naseri *et al.*, 2014). The aim of this experiment was to determine the effect of feeding sobaity sea bream commercial feed supplemented with medicinal herbs (garlic and/or ginger) on growth, hematological, biochemical and immunological parameters and to examine the protection levels against a challenge by *P. damsella.*

Material and Methods

Experimental Conditions

Healthy sobaity sea bream fingerling was obtained from Persian Gulf aquatic animal reproduction and propagation Center (Kolahi, Bandar Abbas, Iran) and transferred to off-shore Fisheries Research Center (Chabahar, Iran). The specimens were allowed to acclimatize for 2 weeks before the beginning of the treatment regime. During the acclimatization period, fish were fed twice a day with commercial food (Biomar, France) (Table 1). Thereafter, 300 fish with mean weight of 3.08±0.31 g were randomly stocked in 15 fiberglass tanks (300 L), 20 fish in each tank with three replicates per diet. The control group recived no garlic or ginger powder. Continuous aeration was provided to each tank through air stone connected to a central air compressor. During the experimental period, water temperature, dissolved oxygen and pH were recorded 27.14±1.4ºC, 7.1±0.31 mgl^{-1} and 7.98±0.15, respectively.

Diet Preparation

The plants were dried in the shade. The dried plants were crushed into powdered form mechanically and were sieved using a household sifter. Dietary treatment included (T1) basal diet only (control group), (T2) fed basal diet with 1% garlic, (T3) fed basal diet with 1% ginger, (T4) fed basal diet with 1% thyme and (T5) fed basal diet with mixture 1% garlic, 1% ginger and 1% thyme. To prepare the diets, basal diet was mixed with the appropriate powders concentration and water, and sprayed on the pellets, which were allowed to dry for 18h at 45°C by air circulation and stored at 4°C until use. Control diet was prepared adding only water without herbal supplementation (Cerezuela, Cuesta, Meseguer, & Esteban, 2008; Akrami, Iri, Khoshbavar Rostami, & Razeghi Mansour, 2013). Fries were fed the experimental diet for 8 weeks in rate of 3-5% of the body weight per day, spread across three feeding times (06:00, 14:00 and 19:00).

Table 1. Proximity analysis of Biomar formulated food

Chemical composition	% (on DM basis)
Crude Protein (%)	54
Crude fat (%)	18
Fibre (%)	1
Ash (%)	10

Growth Performance

In order to analyze the growth indices of the fish fry, all of fish from each tank were biometried and weighted once every 15 days during the experiment, at least 24-h after the last feeding (Akrami *et al.*, 2013). At the end of the feeding trial, weight gain (WG%), specific growth rate (SGR; % day^{-1}) and feed conversion ratio (FCR) were calculated according to the following formulas (Bekcan, Dogankaya, & Cakirogullari, 2006):

$WG\ (\%) = 100 \times (W_f – W_i)/ W_i$

FCR = dry feed intake (g)/wet WG (g)

$SGR\ (\%\ day^{-1}) = (Ln\ W_f - Ln\ W_i) \times 100/t$

Where W_f and W_i are final and initial body weights, respectively and t is time in days.

Blood sample collection

At the end of the rearing trial, 10 fish were randomly removed from each diet group, and blood was collected from the cutted caudal fin. Blood samples were introduced to both heparinized and non-heparinized tubes, respectively. Then, blood in heparinized tubes was shaken gently and kept in the refrigerator at 4°C, for performing haematological studies. Blood sera were obtained by centrifuging blood samples at 3000 rpm (15,609 g) for 10 mins using a Heraeus Labofuge 400, and the sera were removed with a disposable transfer pipette (Shahsavani, Mohri, & Gholipour Kanani, 2010) and stored at -20°C until analysing for biochemical and immunological studies.

Hematological Assays

The erythrocyte (RBC) and leukocyte (WBC) counts were determined using a Neubaeur hemocytometer according to Martins *et al*. (2004). Hemoglobin levels (Hb) were obtained by the cyanomethemoglobin spectrophotometry method. Hematocrit (Hct) was measured using the standard microhematocrit method (Collier, 1944). Differential leukocyte counts (monocyte, lymphocyte, neutrophil and eozinophil) were determined using Giemsa staining method and detected blood smears under light microscope (Ghiasi, Mirzargar, Badakhshan, & Shamsi, 2010).

Biochemical Assays

Total protein (Biuret method), triglycerides using lipase (lipase/GPO-PAP), cholesterol (cholesterol oxidase), albumin (Bromocresol Green method) (Borges, Scotti, Siqueira, Jurinitz, & Wassermann, 2004) were performed.

Liver Enzymes

At the end, 3 fish were randomly separated and autopsied from each diet group. The liver tissue of each fish was separately isolated and washed with physiological saline solution. In order to provide extract of the liver tissue, a solution of Triton X-100 and PBS pH7/5was used with the volume ratio of 1 to 10. After tissue homogenates, for creating tissue extract, the solution was placed in a Centrifuges at a speed of 15,000 rpm for 15 minutes and a constant temperature of 4°C. The supernatant was separated to measure liver enzymes levels (Banaee, Keyhani, & Ahmadi, 2013). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes were determined colorimetrically and alkaline phosphatase (ALP) was determined using an enzymatic method (Borges *et al.*, 2004).

Immunological Assays

At the end of the experiments to assess immunological parameters, 5 fish were washed three times with sterile PBS. Afterwards, they were homogenized in 10 ml of PBS 1× per gram of tissue using the Ultra-Turrax T25 homogenizer and centrifuged at 5000 × g for 15 min at 4°C. The supernatants were collected and stored at -70°C (Banaee *et al.*, 2013).

Lysozyme Activity

The lysozyme activity was measured using a method based on the ability of lysozyme to lyse the bacterium *Micrococcus lysodeikticus* (Ellis, 1990). In a 96-well microtray, 100 ml of each sample (fry homogenate) in four twofold serial dilutions in 0.05 M phosphate buffer pH 6.2, were mixed with 100 ml of a 0.4 mg ml^{-1} suspension of M. Lysodeikticus (Sigma) in phosphate buffer. The microtray was incubated at 22°C and the optical density (OD) was read at 450 nm at 0, 15, 30 and 60 mins. Lysozyme concentration was calculated using a hen egg white lysozyme (Sigma, USA) standard curve made by twofold serial dilutions starting at 10 mg ml^{-1}. As negative control, buffer was used instead of fry homogenate. A unit of lysozyme activity was defined as the amount of sample causing a decrease in the OD reading of 0.001 min^{-1}. Lysozyme activity was expressed as units per gram of tissue (U g^{-1}).

Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD) activity was measured spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals (Ai *et al*., 2011). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA,

0.1 mM xanthine, 0.013 mM cytochrome C and 0.024 IU ml^{-1} xanthine oxidase. The reaction was triggered after the addition of the xanthine oxidase. One activity unit was defined as the amount of enzyme necessary to produce 50% inhibition of the ferricytochrome C reduction rate that measured at 550 nm (McLord & Fridovich, 1969). Enzyme activity was expressed as units per ml serum (U ml^{-1}).

Total Immunoglobulin (Ig)

Total immunoglobulin (Ig) levels were determined according to the method described by Siwicki and Anderson (1993). Briefly, serum total protein content was measured based on microprote in determination method (C-690; Sigma), prior to and after precipitating down the immunoglobulin molecules, using a 12%solution of polyethylene glycol (Sigma).

Alternative Complement Pathway (ACH50) Activity

Alternative complement pathway activity (ACH50) levels was measured by the method cited by Yano, Hatayama, Matsuyama, and Nakao (1988) using a rabbit red blood cells. The reaction was conducted at 20°C in the presence of 10 mM ethylene glycol-bis (2-aminoethylether) tetraacetic acid and 10 mM $MgCl_2$, and changes in absorbance of the reaction mixture at 414 nm were recorded. One unit of ACH50 activity was defined as 50% hemolytic activity of 4×10^7 rabbit red blood cells under the reaction conditions.

Challenge Tests with *Photobacterium damselae*

After feeding experiment, fish were infected with *Photobacterium damselae* strain FN1 and mortalities were recorded. The bacterial strains were streaked on tryptic soy agar (TSA:Merck), supplemented with 2% Nacl and thiosulfate citrate bile salt sucrose agar (TCBS: Merck) media and incubated for 48 h at 26°C (Snieszko, Bullock, Hollis, & Boone, 1964). The isolated strain was characterized and identified according to standard morphological, physiological and biochemical techniques (Gauthier, Gauthier, & Christen, 1995). Brifly, after the growth trial (60 day),12 fry in each group were removed and placed in 15 small tanks of 10 L capacity with aerated sea water (4 fish in each tank with three replicates per diet). Fish from 4 groups (12 tanks) were submitted to an immersion bath in a solution containing 1.0×10^5 CFU of *P. damsella* /mL for 15 minutes. Fish from the other three tanks received the same procedure without bacteria (Martins *et al*., 2010). The fry was monitored for another 6 days until mortalities had ceased in a challenge trial. The bacterium was re-isolated from the dead fish.

Statistical Analysis

Values were expressed as Mean ± standard error (SE). Growth performance, haematological, biochemical and immunological parameters were tested using one-way ANOVA and Duncan's multiple range test was used for comparing of the mean values at the 5% level of significance using SPSS software (Version 19.0).

Results

The comparison of the given data on the weight gain, specific growth rate (SGR) and food conversion ratio (FCR) of the treatment groups are shown in Table 2. According to the tests, there were difference in the mean values of weight gain (WG%), SGR and FCR among the treatment groups ($P<0.05$). The highest weight gain, the specific growth rate was recorded on treatment group fed with a mixture of these three herbs adjuvant ($P<0.05$). Also lower level of feed conversion ratio was recorded on treatment group fed with a mixture of these three herbs adjuvant and ginger group ($P<0.05$) (Table 2).

After 8 weeks of treatments, statistical analysis of data showed that there were differences on the RBC, WBC count, Hct and Hb among the treatment groups ($P<0.05$). Highest WBC count were recorded on ginger group, highest RBC were showed on treatment group fed with the mixture of these three herbs, but highest Hct and Hb were recorded on treatment groups fed with the mixture of these three herbs and ginger group ($P>0.05$). There were significant differences of differential leukocyte counts ($P<0.05$) (Table 3).

The effects of dietary garlic, ginger, thyme and the mixture of these three herbs on some biochemical parameters in Sobaity sea bream are shown in Table 4. The highest total protein and the lowest cholesterol levels were observed in treated groups with ginger diet ($P<0.05$). The higher albumin level was achieved on the mixture of these three herbs and ginger groups

Table 2. Growth parameters of sobaity sea bream fed the test diets with medicinal herb adjuvants for 8 weeks

Parameter	T_1	T_2	T_3	T_4	T_5
WG (%)	131.80 ± 7.11^{c}	152.94 ± 14.42^{abc}	195.66 ± 58.52^{ab}	141.04 ± 23.08^{bc}	210.66 ± 21.92^{a}
SGR (% day^{-1})	1.40 ± 0.05^{d}	1.54 ± 0.09^{abc}	1.78 ± 0.35^{ab}	1.46 ± 0.16^{bc}	1.89 ± 0.11^{a}
FCR	1.72 ± 0.09^{a}	1.75 ± 0.16^{a}	1.28 ± 0.25^{b}	1.92 ± 0.28^{a}	1.08 ± 0.02^{b}

but the lower triglyceride was showed on treatment group fed with the mixture of these three herbs ($P<0.05$).

The effect of dietary garlic, ginger and the mixture of three the herbs on some Immunological parameters are shown in Table 5. In the fish fed herbal supplemented diets, lysozyme activity, superoxide dismutase (SOD) activity, total immunoglobulin (Ig) and ACH50 levels showed a significant increase ($P<0.05$) when compared with other groups at the end of experiment. Serum lysozyme activity, total immunoglobulin (Ig) and alternative complement activity (ACH50) were significantly increased on the mixture of three medicinal herbs fed fish and ginger groups ($P<0.05$), in turn, the SOD activity had a significant change on treatment group fed with a mixture of these three herbs adjuvant ($P<0.05$).

The results from the virulent pathogen challenges are shown in Figure 1. The highest mortality was observed in control group (59%), fish fed with garlic powder (52%) and fish fed with thyme powder (50%), while 40% of fish died in the group fed with ginger powdered and the lowest mortality (38%) was observed when fish were fed with a combination of three herbs.

Discussion

In aquaculture, the application of dietary medicinal herbs as immunostimulants can elevate the innate defense mechanisms of fish against pathogens during stress periods, such as, intensive farming practices, grading, sea transfer, vaccination and reproduction (Dineshkumar, Rajakumar, & Mani, 2014). Some of medicinal herbs and their mixture in diets induced higher growth performance than the fish fed the control diet. In greasy grouper *Epinephelus tauvina* (Sivaram *et al*., 2004) and shrimp *Penaes indicus* (Immanuel, Vicncybai, Sivaram, Palavesam, & Marian, 2004), herbs in diets promoted growth and feed efficiency. In current study, the highest weight

Table 3. Haematological parameters of sobaity sea bream fed the test diets with medicinal herb adjuvants (garlic/ginger/thyme) for 8 weeks

Parameter	T_1	T_2	T_3	T_4	T_5
WBC($\times10^3$ m^{-1})	121.00 ± 1.00^{d}	120.66 ± 0.57^{d}	138.00 ± 1.00^{a}	125.33 ± 0.57^{c}	134.33 ± 0.57^{b}
RBC ($\times10^6$ m^{-1})	0.64 ± 0.005^{c}	0.64 ± 0.005^{c}	0.67 ± 0.005^{b}	0.59 ± 0.01^{d}	0.76 ± 0.01^{a}
Hb (g / dl)	7.40 ± 0.00^{b}	7.33 ± 0.05^{d}	7.86 ± 0.05^{a}	7.16 ± 0.05^{c}	7.90 ± 0.02^{a}
Hct (%)	19.33 ± 0.57^{b}	19.66 ± 0.57^{b}	21.33 ± 0.57^{a}	18.00 ± 1.00^{c}	21.66 ± 0.57^{a}
Monocyte (%)	1.00 ± 0.00^{a}	1.33 ± 0.57^{a}	1.66 ± 0.57^{a}	1.33 ± 0.57^{a}	1.66 ± 0.57^{a}
Lymphocyte (%)	87.66 ± 1.52^{b}	85.33 ± 1.52^{c}	90.33 ± 0.57^{a}	83.33 ± 1.52^{c}	89.66 ± 0.57^{ab}
Neutrophil (%)	8.33 ± 0.57^{cd}	7.33 ± 0.57^{d}	11.66 ± 0.57^{b}	9.00 ± 1.00^{c}	13.00 ± 0.00^{a}
Eosinophil (%)	1.33 ± 0.57^{a}	1.33 ± 0.57^{a}	1.33 ± 0.57^{a}	1.00 ± 0.00^{a}	2.00 ± 1.00^{a}

Data are represented as mean ± SE. Values in the same column sharing the same superscript letter are not significantly different ($P>0.05$).

Table 4. Biochemical parameters of Sobaity sea bream fed the test diets with medicinal herb adjuvants (garlic/ginger/thyme) for 8 weeks

Parameter	T_1	T_2	T_3	T_4	T_5
Total protein (mg l^{-1})	0.36 ± 0.05^{d}	0.73 ± 0.05^{c}	1.13 ± 0.05^{a}	0.23 ± 0.05^{e}	0.86 ± 0.05^{b}
Triglycerides (mg dl^{-1}	87.33 ± 0.57^{b}	95.00 ± 1.00^{a}	43.33 ± 2.08^{d}	47.66 ± 1.52^{c}	25.33 ± 1.52^{e}
Cholesterol (mg dl^{-1})	3.00 ± 0.00^{b}	4.66 ± 0.57^{a}	1.33 ± 0.57^{c}	5.33 ± 0.57^{a}	2.66 ± 0.57^{b}
Albumin (mg l^{-1})	0.20 ± 0.10^{b}	0.10 ± 0.10^{b}	0.50 ± 0.00^{a}	0.13 ± 0.05^{b}	0.56 ± 0.05^{a}
AST (IU dl^{-1})	302.33 ± 1.00^{b}	334.66 ± 3.51^{a}	292.33 ± 1.52^{c}	337.33 ± 0.57^{a}	171.33 ± 1.00^{d}
ALT (IU dl^{-1})	7.00 ± 1.00^{a}	7.66 ± 0.57^{a}	4.66 ± 0.57^{c}	6.33 ± 0.57^{ab}	5.00 ± 0.57^{bc}
ALP (IU dl^{-1})	647.66 ± 2.51^{a}	348.66 ± 0.57^{c}	301.33 ± 2.51^{d}	487.33 ± 2.08^{b}	298.33 ± 2.51^{d}

Data are represented as mean ± SE. Values in the same column sharing the same superscript letter are not significantly different ($P>0.05$).

Table 5. Immunological parameters of sobaity sea bream fed the test diets with medicinal herb adjuvants (garlic/ginger/thyme) for 8 weeks

Parameter	T_1	T_2	T_3	T_4	T_5
Lysozyme activity(unit ml^{-1})	12.66 ± 1.00^{c}	20.66 ± 1.00^{b}	24.66 ± 0.57^{a}	21.33 ± 1.15^{b}	26.33 ± 0.57^{a}
SOD activity(unit ml^{-1})	21.00 ± 1.00^{c}	24.33 ± 0.01^{b}	24.00 ± 1.00^{b}	22.00 ± 1.00^{c}	26.66 ± 1.52^{a}
Ig (mg l^{-1})	10.33 ± 0.10^{c}	11.33 ± 0.57^{c}	17.66 ± 0.57^{a}	14.66 ± 0.57^{b}	18.00 ± 1.00^{a}
ACH50(unit ml^{-1})	105.00 ± 1.00^{c}	119.66 ± 0.57^{b}	123.66 ± 0.57^{a}	120.33 ± 1.15^{b}	125.33 ± 0.57^{a}

Data are represented as mean ± SE. Values in the same column sharing the same superscript letter are not significantly different ($P>0.05$).

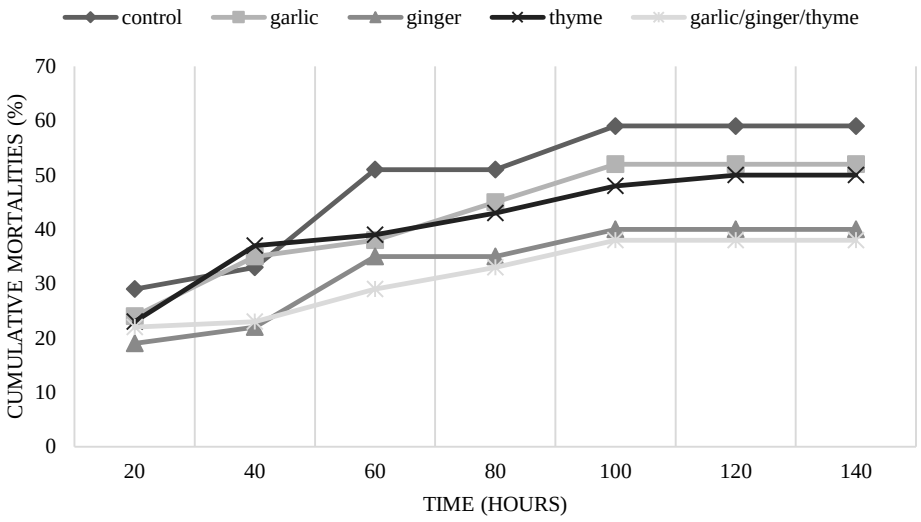

Figure 1. Cumulative mortalities (%) of fish in control group and in groups fed diets with medicinal herb adjuvants (garlic/ginger/thyme) for 8 weeks. The figure shows a six-day period following an artificial infection with *P. damselae* strain FN1.

gain, the specific growth rate was recorded on treatment group fed with a mixture of these three herbs adjuvant ($P<0.05$). Also lower level of feed conversion ratio was recorded on treatment group fed with a mixture of these three herbs adjuvant and ginger group ($P<0.05$). Similar to the present study, the increase of weight gain and specific growth rate were recorded in Red sea bream (*Pagrus major)* fed diet containing a mixture of four medicinal herbs (*Massa medicata*, *Crataegi fructus*, *Artemisia capillaries*, *Cnidium officinale*) for 12 weeks (Ji *et al*., 2007). In addition, traditional Chinese medicines had a beneficial effect on the growth of Common carp (*Cyprinus carpio*) (Jian & Wu, 2004). American ginseng (*Panax quinquefolium*), Green tea (*Camellia sinensis*) and Cinnamon (*Cinnamomum zeylanicum*) enhanced the growth performance and feed utilization of Nile tilapia (*Oreochromis niloticus*) (Abdel-Tawwab, Ahmad, Seden, & Sakr, 2010; Ahamad, El Mesallamy, Samir, & Zshran, 2011). Hosseini Mansoub and Pooryousef Myandoab (2012) reported that the highest amount of daily gain observed in the group fed with the control diet + 2% of the mixture of both herbal plants contains Alfalfa (*Medicago sativa*) and Black cumin (*Nigella sativa*) powder. This improvement of body weight gains maybe found due to the active materials in the herbal plants which increased the efficiency of utilization of feed, resulting in enhanced growth. Similarly, dietary supplementation of two levels of Black cumin seeds and Turmeric (*Curcuma longa*) mixture improved growth performance of *Mugil cephalus* (El-Bahr & Saad, 2008). Abdelwahab and El-Bahr (2012) can concluded that, the mixture of Black cumin seed and Turmeric combination in Asian sea bass *(Lates calcarifer)* diets relatively improved the growth performance. Zakes, Kowalska, Demska-Zakes, Jeney and Jeney (2008) suggested that supplementing diets with the medicinal herbs *Astragalus radix* and *Lonicera japonica* did not increase juvenile Pikeperch (*Sander lucioperca*) growth rate or FCR. Zaki, Labib, Nour, Tonsy and Mahmoud (2012) tested several phytobiotics, in two concentrations, 1% and 2%, at *Oreochromis niloticus* species, with an average initial weight of 0.82±0.3g/fish. The results showed that dietary supplementation with fenugreek, eucalyptus, pepper, chamomile and thyme, in a concentration of 1%, had a positive effect on growth performance parameters, feed conversion ratio, nutrient utilization, protein efficiency and also on physiological parameters. Ginger stimulates the secretion of pancreatic enzymes and the bile from the liver and makes fast food digestion and helps to balance the intestinal bacteria (Platel & Srinivasan, 2004). Ginger root also contains high levels of proteolytic enzymes and lipolytic plants which leads to improved digestion of dietary protein and lipid (Venkataramalingam, Godwin, & Citarasu, 2007). Moreover, previous work suggested that these functions are attributable to the bioactive components of the plant garlic which include allin and allicin. Allicin is the most abundant compound representing almost 70% of all present thiosulfinates (Han, Lawson Han, & Han, 1995). Khalil, Nadia, and Soliman (2001) reported that garlic contains allicin, which improves the performance of intestinal flora, thus improving digestion. This consequently enhances the utilization of energy, bringing about improved growth. These results can be explained by the various effects of the additives used, for example garlic enhances the performances of the

intestinal microbiota while ginger stimulate the appetite (Christine Reinbach, Martinussen, & Møller, 2010; Yeomans, Gray, Mitchell, & True, 1997). Thymol and carvacrol from thyme are active material in the plant, which are considered as appetizer and stimulating of digestion, in addition to their antimicrobial activity against intestinal bacteria resulting of enhancing health status and growth (Jameel, Abed, & Al-Shimmary, 2014). One factor that may affect the effectiveness of the herbal adjuvant as a growth stimulant might also be the period in which the supplemented diet is applied. The immunostimulatory effect is apparent after a 2-4-week treatment (Düğenci, Arda, & Candan, 2003). It should be tested to what extent a species has a particular reaction to this type of adjuvant, or if the size of the studied fish species influences the results. The Sobaity sea bream in this study were fed herbal adjuvant for 8 weeks. Therefore, the application of dietary additives should be carefully considered because their favorable roles in fish is really depended on fish species, targeting activities of additives, administration method, dose (concentration), physiological and nutritional status of fish and/or dietary nutrition composition, etc. The inclusion of herbs mixtures in diets often provides a cooperative action to various physiological functions, in contrast to a single herb dose. This synergistic effect of herbs has also been reported in other fish including juvenile olive flounder (Kim, Moon, Jeong, & Kim, 2000), Nile tilapia (Kim, Noh, Jung, & Jo, 1998) and Rock bream, *Oplegnathus fasciatus* (Kim, Lee, Baek, Cho, & Kim, 2003).

The increase in WBC counts and other blood cells, following feeding of ginger and the mixture of three herbs diets, demonstrates the immunostimulatory effects of ginger which is in line with Gholipour Kanani, Nobahar, Kakoolaki, and Jafarian (2014) who obtained increased WBC in juvenile beluga after feeding with ginger diet. Leukocyte functions which can be improved by natural immunostimulants was previously reported in gold fish (*Carassius auratus*) that were supplemented with a mixture of chosen herbs (*Punica granatum*, *Chrysanthemum cinerariaefolium* and *Zanthoxylum schinifolium*) incorporated in shrimp and fish diet (Harikrishnan, Balasundaramb, & Heo, 2010). This is in contrary with Abdelwahab and El-Bahr (2012) who reported that hematological parameters were not altered in all treated Asian sea bass fish. The Hb content were significantly increased ($P<0.05$) in the mixture of these three herbs groups compared with the control group. The increment of Hb percentage may be attributed either to increasing the synthesis of enzymes needed for biosynthesis of them (El-Tahir, Ashour, & Al–Harbi, 1993) or increase the size of red blood cells (El-Feki, Tawfek, & Awad, 1993). Lymphocyte and monocyte levels showed an increase in fish fed with ginger but neutrophil and Eosinophil levels showed an increase in fish fed with the mixture of three herbs dites among the experimental groups ($P>0.05$). Similar this, Gholipourkanani, Nobahar, Kakoolaki, and Jafarian (2014) reported an increase in lymphocyte and monocyte levels of juvenile beluga after feeding with ginger diet ($P>0.05$).

In the present study, triglycerides and cholesterol reduced in the mixture of these three herbs and ginger groups, respectively. Recently, ginger has been reported to have lower cholesterol levels and blood-thinning properties (Verma, Singh, & Khamesra, 1993). One of its active components, gingerol, is reported to inhibit platelet aggregation *in vitro* by acting on prostaglandin and thromboxane synthesis (Srivastava, 1984) and inhibiting arachidonic acid-induced platelet aggregation. Serum total protein is an important nonspecific immune variable (Magnadóttir, 2006). Its concentration in fish is less stable than that in mammals, and stress can cause a reduction in the plasma total protein for a few days. For this reason, an increase in total protein level will enable the fish to be stronger and more tolerant of stressful conditions (Satchell, 1991). The increase in total protein content usually supported by elevating the white blood cell counts (WBC) as a major source of serum protein (Misra, Das, Mukherjee, & Meher, 2006) could show the positive effect of dietary ginger on non-specific immunity. The present study's results confirm the positive significant effects of ginger powder diet on elevating the total protein. Some certain herbals have been observed to show positive effects on increasing total proteins and their component as reported for Rohu, *Labeo rohita* (Rao, Das, Jyotyrmayee, & Chakrabarti, 2006) and Common carp (Alishahi, Ranjbar, Ghorbanpour, Peyghan, & Mesbah, 2010), but some other species did not show any such of these effects as reported in rainbow trout (Ispir & Dörücü, 2005).

Our results show that AST and ALT levels significantly decreased in fish fed with the mixture of these three herbs powders and ginger groups, respectively, but ALP level showed a significant decrease in fish fed with the mixture of these three herbs powders and ginger groups ($P<0.05$) when compared with the control group. AST, ALT, and ALP serum Low activities in the group fed by the mixture of these three herbs powders and ginger suggest healthy liver with negligible damage, supporting suppressed hepatic amino acid utilization. It could be explained that the bioactive compounds polyphenols, flavonoids, tannins and saponins found in three these herbs prevented fish from infection by triggering immune system and its administration might prevent lipid peroxidation of cell membranes and inhibit the release of foresaid enzymes into the plasma. This liver damage was not observed when both plants administered together with the present dose which introduces an evidence that one of the plants perhaps prevents or inhibits the drawbacks of the other one when administered together at the examined dose. Reduction or no increase in enzyme

levels in plasma of fish may be due to the effect of flavonoids and antioxidants in the mixture of medicinal herbs powder on the physiological function of cell membranes in different tissues, especially the liver. In fact, the presence of flavonoids and antioxidants in the mixture of powdered herbs may increase the antioxidant capacity of cells and increase the stability of cell membranes, hence, the release of intracellular enzymes will prevent its secretion into the blood.

In this study an increasing trend in lysozyme activity has been shown in fish fed with the mixture of these three herbs powders and ginger groups, which is in agreement with several reports indicating the role of herbal immunostimulants in enhancing lysozyme activity (Ispir & Dörücü, 2005; Choi *et al*., 2008). Elevated lysozyme level was measured in Crucian carp (*Carassius auratus gibelio)* (Chen, Wu, Yin, & Li, 2003) and Large yellow croaker (*Pseudosciaena crocea*) (Jian & Wu, 2003) after feeding the fish with various Chinese herbal. However, unlike this study, lysozyme activity was not influenced in juvenile Beluga (*Huso huso*) fed with ginger and garlic (Gholipour Kanani *et al*., 2014) and Nettle *(Urticadioica)* (Binaii *et al*., 2014). The increasing trend could be due to lysozyme, a humoral component of the non-specific defense mechanism, that has the ability to prevent the growth of infectious microorganism by splitting β-1, 4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell walls (Choi *et al*., 2008; Secombes, 1996).

The increase in the complement activity (ACH50) observed in fish fed with the mixture of these three herbs powders and ginger groups, may help to identify and eliminate bacterial agents by phagocytosis. Therefore, the increased total protein levels in plasma of fish treated with ginger powder diet is accompanied by the increased levels of immune parameters which have a protein structure, such as total complements. Several authors reported an increase in complement activity following administration of different immunostimulants such as herbal derivatives (Jian & Wu, 2003; 2004; Christybapita, Divyagnaneswari, & Michael, 2007). Unlike the present study, in juvenile Red sea bream it was observed that ACH50 activity of diet mixture of all the herbs group was significantly higher than in the control diet group (Ji *et al*., 2007).

Our study showed increasing Ig levels in fish fed with the mixture of these three herbs powders and ginger groups after six weeks. Some changes may be related to the size and/or age (Magnadóttir *et al*., 1999), the environmental conditions (Picchietti *et al*., 2001) or disease status (Klesius, 1990).

SOD is metalloenzymes that play major roles in protection of cells against oxidative damage (Metaxa *et al*., 2006). In this study, significant differences in SOD level were observed between experimental groups ($P<0.05$) and the highest SOD level was showed in the mixture of these three herbs group. However, unlike this study, Yuan *et al*. (2007) noticed that there was no significant difference in SOD activity between the 0.5% and 1% herbal immune regulation mixture groups and the control group.

After a challenge with *P. damselae* strain FN1, survival of fish fed with medicinal herb adjuvants (garlic/ginger/thyme) was improved when compared with the control group. The survival showed significant differences among treatment groups. It is possible that this is the result of the enhancement of non-specific immune system of the fish by the combination of ginger and garlic powders. A similar result was reported after feeding *Oreochromis niloticus* with *Astragalus membranaceus, Lonicera japonica* and boron and subsequent infection with *Aeromonas hydrophila* (Ardó *et al*., 2008). In additional, the survival rate in Common caro (*Cyprinus carpio*) juveniles after challenge with *Aeromonas hydrophila* was higher in both experimental groups (garlic + ginger and oregano + Echinacea). Group fed with the mixture of garlic and ginger had no mortalities recorded (Gabor, Şara, Benţea, Creţa, & Baciu, 2012). Yin *et al*. (2009) reported that after a challenge with *A. hydrophila*, mortalities were significantly reduced in all groups compared to controls, with the lowest mortality in vaccinated fish fed with both herbs (*Astragalus radix* and *Ganoderma lucidum*). Disease resistance was also influenced by the combined use of phytoadditives, indicating immuno-stimulating effect of the additives also the possibility to use them as prophylactic agents, to limit the use of antibiotics. It was considered that we would be able to use the herbs without the addition of a specific vaccine for inducing protection in carp against *A. hydrophila*. Mixed medicinal herbs from various herbs such as *Viscum album*, *Urtica dioica* and *Zingiber officinale* (Düğenci, Arda, & Candan, 2003) and *Radix astragalin* and *Radix angelicae* (Jian & Wu, 2004) have also enhanced immunity in fish to bacterial infection. In 2010, it was reported that mixing the herbs (*Azadirachta indica*, *Ocimum sanctum* and *Curcuma longa*) show the haematological and biochemical parameters near to normal values and triggeried the immune system of the specific and innate immunity of Goldfish (*Carassius auratus*) against *A. hydrophila* when treated with 400 mg/kg or 800 mg/kg of the mixed herbal supplementation (Harikrishnan *et al*., 2010). Saad, Abou El-Geit, El-Hammady, and Mona (2013) reported that after a challenge with *Pseudomonas fluorescence*, mortalities were significantly reduced in all groups compared to controls, with the lowest mortality in fish fed with the combined mixture of both plants (Black Cumin Seeds + Turmeric). The superiority of administration of a combined mixture of the plants over the individual administration perhaps has two explanations. The first is postulated that these plants perhaps are of synergistic effect at the examined dose. The second is based on that one of

the plants perhaps remove or inhibits the drawbacks of the other. One the results of this study demonstrated that the dietary mixture of three powder and ginger groups can improve the growth, hematological and biochemical parameters and immune function of Sobaity sea bream fry. Combination of herbs increased the survival of fish after the challenge with *P. damselae* strain FN1. Thus, it can be concluded that the herb powders added to diets act as immunostimulants and appear to improve the immune status and disease resistance of fish. Still, more studies are needed to determine the cost/efficiency ratio of a large-scale use of phytoadditives but also their efficiency against other pathogens.

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