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Dietary administration of ferula (Ferula asafoetida) powder as a feed additive in diet of koi carp, Cyprinus carpio koi: effects on hemato-immunological parameters, mucosal antibacterial activity, digestive enzymes, and growth performance

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Abstract This study investigated the impacts of various levels (0, 5, 10, 15, 20, and 25 g kg^{-1}) of dietary ferula (*Ferula asafoetida*) powder (FP) on the hemato-immunological indices, antibacterial properties of skin mucus, survival rate, and growth performance as well as digestive enzyme activity of Koi carp, *Cyprinus carpio* koi, fingerlings. Following 63 days of feeding trail, WBCs, RBCs, Hb, hematocrit, MCV, MCH, MCHC, and lymphocyte levels increased coincident with an increasing FP level in experimental diets compared with control diet ($P < 0.05$). Dietary FP significantly increased total protein content and the activities of total immunoglobulins, lysozyme, and alternative hemolytic complement in a concentration-dependent manner in the serum of koi fish ($P < 0.05$). The elevation of the FP level in experimental diets resulted in an increase in SGR value and a reduction in FCR value ($P < 0.05$). The survival rate also increased significantly coincident with the increasing dietary FP level ($P < 0.05$). The results revealed that dietary ferula powder especially at levels of 20 and 25 g kg^{-1} could be used as an effective herbal dietary supplement in the enhancement of humoral innate immune responses and growth of koi carp.

Keywords *Ferula asafoetida* · Koi carp · Innate immune response · Antibacterial activity · Growth · Digestive enzymes

Highlights •The 25 g kg^{-1} FP diet had the highest intestinal LAB/TVC ratio.
•The 25 g kg^{-1} FP diet showed the highest immunological responses.
•Feeding koi fish with FP diets showed higher mucosal antibacterial activities.

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Introduction

Nowadays, aquaculture industry continues to grow rapidly than other animal feed-producing sectors (Mehana et al. 2015). The use of intensive and super intensive systems has been rapidly developed in aquaculture industry, which can accelerate the disease outbreaks and lead to heavy economic losses (Sirimanapong et al. 2015). During the last decade, the evaluation and introduction of environment-friendly immunostimulants to maintain fish health and promote growth performance have been increased (Carbone and Faggio 2016; Vallejos-Vidal et al. 2016). An immunostimulant can boost both adaptive and innate immune mechanisms to protect fish from infectious diseases (Mehana et al. 2014; Vallejos-Vidal et al. 2016). Because of the popularity of organic fish rearing, the administration of natural

immunostimulants has more recently received attention (Bilen et al. 2011). The beneficial impacts of natural immunostimulants have been reported to improve the immune system in fish (Dügenci et al. 2003; Divyagnaneswari et al. 2007; Yin et al. 2009). Recently, the use of feed additives such as probiotics, prebiotics, synbiotics, parabiotics, organic acids (acidifiers), nucleotides, and phyto-products (phytogenics) in aquafeed production industry is considered a safe dietary option with least concerns with the emphasis on organic aquaculture (Safari and Paolucci 2017a, b).

The genus *Ferula* (Apiaceae) with about 170 species has been used in traditional medicine, and their pharmacological effects have been well documented in both the human and animal studies (Akaberi et al. 2015). Asafetida (*Ferula asafoetida*) is a species of ferula that grows in Iran, Afghanistan, and Kashmir (Khederzadeh et al. 2017). Today, it is grown chiefly in these areas and exported to other parts of the world (Mahendra and Bisht 2012). Numerous compounds including coumarins, sesquiterpene lactones, sesquiterpene coumarins, monoterpenes, and sulfur-containing compounds have been recognized in this genus (Iranshahi et al. 2009; Kasaian et al. 2014; Hadavand Mirzaei and Hasanloo 2014). Various studies have indicated the antiviral (Lee et al. 2009), antibacterial (Al-Yahya et al. 1998), antifungal (Singh 2007; Angelini et al. 2009), antioxidant (Dehpour et al. 2009), and anticancer (Saleem et al. 2001) properties of ferula. Safari et al. (2016) revealed that dietary ferula (*F. asafoetida*) up-regulated the growth and health-related gene expression and increased lysozyme activity of skin mucus in common carp (*Cyprinus carpio*). Shademani et al. (2015) also reported the positive effects of dietary *F. asafoetida* on growth performance, immune response, and population of *Lactobacillus* spp. in broiler chickens.

The production and trade of ornamental fish as a growing sector is an effective activity in aquaculture industry (Jaleel et al. 2015). Koi fish is known as an ornamental strain of common carp, *Cyprinus carpio*, and is cultured widely in the world (Tripathi et al. 2003). The use of intensive systems for culture of koi carp can reduce water quality and the outbreaks of diseases, so it is essential to improve the health and immune status of fish using dietary supplements. Therefore, the purpose of the present study was to evaluate the effects of dietary *F. asafoetida* powder on humoral immune status, hematological parameters, and bactericidal activity of koi fish, *C. carpio* koi, fingerlings.

Materials and methods

Preparation of *Ferula asafoetida*

Ferula asafoetida was collected from the desert of Khorasan Razavi Province, Iran. After identification, the aerial parts (flowers, leaves, and stems) of plant were placed in a dark room with proper ventilation for 5 days, air-dried at ambient temperature (Díaz-Maroto et al. 2003), ground into powder, and then added to the diets.

Experimental diets

A basal diet which included 340 g kg^{-1} crude protein, 50 g kg^{-1} crude lipid, and 15.80 MJ kg^{-1} gross energy was formulated by WUFFDA (Windows-Based User-Friendly Feed Formulation, Done Again; University of Georgia, Georgia, USA) software (NRC) and served as the control diet (Table 1). Six isonitrogenous and isoenergetic experimental diets were prepared by adding 0 (control), 5, 10, 15, 20, and 25 g kg^{-1} ferula powder to a basal diet. Ferula powder (FP) was replaced with car`ymethyl cellulose (CMC). All feed ingredients were finely ground in a miller. Water was added to the mixed ingredients until a uniform paste was obtained. Then, the dough was pelleted wet using a meat grinder with a mesh sieve of 2 mm. The prepared pellets were dried at 30 °C for 24 h and kept at 4 °C for further use.

Feeding and culture system

Koi fish (*Cyprinus carpio* koi) fingerlings used in the present study were purchased from Toos Koi Co. (Mashhad, Khorasan Razavi Province, Iran). Before the nutritional experiment, fish were reared in a 1000-L fiberglass tanks, acclimatize to the laboratory situations, and fed on the basal diet (SFT, Saramad Co., Khorasan Razavi Province, Iran) for 2 weeks. Then, koi carp fingerlings (average body weight of 3.92 ± 0.39 g) were stocked randomly into an 18-glass aquarium (180 L) at a density of 25 fish per aquarium at triplicate for each treatment. During the feeding trail (63 days), the fish were fed with prepared diets thrice a day (8:00, 12:00, and 16:00) to apparent satiation. Water of each glass aquarium was exchanged at a rate of 20% and uneaten feeds were siphoned daily. Dissolved oxygen, water temperature, and pH were 26.3 ± 1.4 °C, 7.62 ± 0.18, and 6.4 ± 0.65 mg L^{-1}, respectively, during

Table 1 Proximate analysis of koi fish (*Cyprinus carpio* koi) fingerling basal diet supplemented with ferula (*F. asafoetida*) powder (FP) (g kg^{-1} dry weight)

Ingredients	*F. asafoetida* powder	g kg^{-1} (dry weight basis)
Fish meal		190
Wheat flour		255
Soybean meal		250
Corn gluten		155
Soybean oil		25
Fish oil		25
Mineral premix		35
Vitamin premix		35
CMC		25
Antifungi		15
BHT		15
Vit C		5
Chemical composition		
Dry matter	976.3	967.3
Crude protein	81	340
Crude fat	97	50
Crude fiber	286	380
Ash	172	680
Cross energy (Mj kg^{-1})	8.91	15.80

the experiment. FUM animal ethic rights were implemented for koi fish in all experiments.

Hemato-immunological analysis

Six 24-h starved fish were randomly sampled from each glass aquarium to collect blood samples through the caudal vein one by one without pooling (at three replicates; 18 fish per treatment) in the 63rd day. They were anesthetized using clove powder (500 mg L^{-1}), and a fraction of obtained blood samples were transferred to heparinized tubes to measure hematological indices. For serum isolation, the rest of blood samples were kept in non-heparinized centrifuge tubes and centrifuged at 1000×*g* for 5 min at 4 °C. The serum samples were kept at − 20 °C for further analysis of lysozyme (LYZ), total immunoglobulin (IG), and alternative hemolytic complement activity (ACH_{50}).

RBCs and WBCs of blood samples were determined based on the method described by Natt and Herrick (1952) using a Neubauer hemocytometer. The hemoglobin (Hb) level was determined based on the cyanmethemoglobin method described by Blaxhall and Daisley (1973) using a spectrophotometer (HACH DR/4000, USA) at 540 nm. Hematocrit (Ht) was measured based on the standard microhematocrit method as described by Brown (1988). For this purpose, blood samples were filled in heparinized microhematocrit capillary tubes and centrifuged at 7000×*g* for 10 min, and Ht values were reported as packed cell volume percentage. Mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) were measured according to the total RBC count, Ht, and Hb concentrations (Lee et al. 1998). Monocyte, neutrophil, and lymphocyte cells were determined by May-Grunwald-Giemsa blood smears (Russia et al. 1992). Blood smears were examined using light microscopy to determine blood cell counts.

Serum total protein was measured based on the Biuret method (Lawrence 1986) using a biochemistry kit by a spectrophotometer (HACH DR/4000, USA) at 540 nm and recorded as micrograms per milliliter.

Serum lysozyme (LYZ) activity was determined based on the procedure explained by Kumari et al. (2006) using *Micrococcus lysodeikticus* (Sigma) as a lysozyme-sensitive Gram-positive bacterium. Briefly, the serum sample (15 μL) was transferred into a 96-well plate in three replicates. Then, sodium acetate buffer (0.02 M) with pH 5.8 (0.02 mg L^{-1}) was used to prepared 150 μL of *M. lysodeikticus* suspension and distributed to each well. The changes in absorbance were recorded at room temperature for 1 h using a spectrophotometer (HACH DR/4000, USA) at 450 nm. The lysozyme activity was calculated based on a standard curve drawn using the lysozyme of hen egg (Sigma) with dilutions of 0 to 20 U mL^{-1} in the same buffer and defined as units per milliliter.

Total serum immunoglobulins (IG) were evaluated based on the method described by Siwicki et al. (1994). Briefly, the immunoglobulin molecules were precipitate by mixing 100 μL of the serum sample with an equal amount of 12% polyethylene glycol and incubation for 2 h under constant agitation at room temperature. Then, the supernatant was removed by centrifuging at 3000×*g* for 15 min, and the protein content was measured. The difference between the total serum protein and measured protein was known as the total IG.

Serum ACH_{50} was measured based on the method explained by Yano (1992) using rabbit red blood cells

(RaRBC). Briefly, the serum samples were diluted at a range of 50–250 μL and allotted into test tubes, and total volume was made up to 250 μL with the addition of ethylene glycol tetraacetic acid–magnesium–gelatin veronal buffer (EGTA–Mg–GVB). After dispersion of 100 mL of RaRBC to each tube test and incubation at 20 °C for 90 min, 3.15 mL of NaCl was allotted to each tube and centrifuged at 1600×*g* for 5 min. The optical density (OD) of the supernatant was determined at 414 nm. The number of ACH_{50} units per milliliter was calculated based on the serum volume which produces 50% hemolysis (ACH_{50}).

Intestinal microbiota assay

After 63 days of feeding experiment, three fish were randomly sampled from each glass aquarium and transported alive to the laboratory to collect intestine samples. Fish were anesthetized with ice and rinsed with benzalkonium chloride (0.1%) for 60 min. Thereafter, fish were killed and the intestinal tract was removed. Samples were homogenized with sodium chloride (0.9 *w*/*v*) using a homogenizer (DI 18 Disperser) and then centrifuged at 5000×*g* for 5 min at 4 °C. Lactic acid bacteria (LAB) and total viable counts (TVC) of heterotrophic aerobic bacteria were determined through spreading of the prepared samples on the surface of plate de Man, Rogosa, and Sharpe media (MRS; Merck Co., Germany) and plate count agar (PCA; Merck Co.), respectively, in triplicate. Then, the plates were incubated for 5 days at 25 °C. The plates that contain 30–300 colonies were used to calculate colony-forming units (CFU) per gram (Hoseinifar et al. 2017; Safari and Sarkheil 2018). The LAB to TVC value was expressed as percent.

Mucus antibacterial activity

Skin mucus collection

After 63 days of feeding trail, fish were not fed for 24 h, and then three fish were sampled from each glass aquarium (9 fish per treatment) for skin mucus collection (Safari and Sarkheil 2018; Subramanian et al. 2007). Briefly, the sampled fish were anesthetized using 500 mg L^{-1} of clove powder and then transferred into polyethylene bags that contain NaCl solution (50 mM; 5 mL g^{-1} fish; Merck, Germany) for 1 min. Skin mucus was collected through slowly shaking the fish inside the plastic bag. Thereafter, the collected samples were immediately placed in sterile tubes (15 mL) and centrifuged at 1500×*g* for 10 min at 4 °C. The obtained supernatants were stored in 2-mL tubes at − 80 °C for future analysis.

Mucus antibacterial test

The antibacterial activity of the collected mucus were determined against *Streptococcus iniae* (ATCC 29178), *Aeromonas hydrophila* (ATCC 7966), *Micrococcus luteus* (MTCC 3911), *Streptococcus faecium* (ATCC 12755), and *Yersinia ruckeri* (ATCC 29473) as pathogenic bacterial strains according to a standardized single-disc method (Bauer et al. 1966). For this purpose, nutrient broth medium (Merck, Germany) was used to culture the bacterial cells. The cultured cells were incubated in a shaking incubator at 200 rpm (JSSI-200CL; JSR, Gongju City, Korea) for 24 h at 37 °C. Then, 0.1 mL of each broth culture medium that contains 1.5×10^8 CFU mL^{-1} of the bacterial cells was poured on nutrient agar (Merck, Germany). Inoculated paper disc (6 mm diameter) in mucus sample for 20 min was placed on solidified agar gel and incubated for 24 h at 37 °C. The antibacterial activity of each mucus sample was examined through the diameter of the growth inhibition zones created around the paper disc.

Growth performance and survival rate

After 63 days of rearing period, all of the fish were weighted and counted for calculation of growth performance parameters and survival rate. Survival rate and growth performance of koi carp were determined using following equations:

$$\text{Weight gain (WG)} = \text{final weight (g)} - \text{initial weight (g)}$$
$$\text{Specific growth rate (SGR; \%body weight day}^{-1}) = [(\ln W_f - \ln W_i)/t] \times 100$$
$$\text{Feed conversion ratio (FCR)} = (\text{feed consumed}/W_{gain})$$
$$\text{Condition factor (CF)} = W_f/L_f^3 \times 100$$
$$\text{Survival rate (\%)} = (N_f/N_i) \times 100$$

where W_i, W_f, and W_{gain} are initial weight, final weight, and weight increment (g), respectively. L_f is final length (cm), N_i and N_f are initial number and final number of fish, and t is time period (day).

Digestive enzyme activity assay

After feeding fish for 63 days, three starved fish for 24 h were sampled from each glass aquarium (9 fish

per treatment) for enzymatic analysis. Fish were anesthetized using 500 mg L^{-1} of ground clove extract, and then the entire intestine of each fish was removed and rinsed with distilled water cooled to 4 °C (Huang et al. 1999). Thereafter, the intestine content of each fish was separately homogenized in phosphate-buffered saline (pH 7.5; 30 g/70 mL PBS) using DI 18 Disperser homogenizer. The supernatant obtained through centrifuging at 15000×*g* for 15 min at 4 °C was kept in liquid nitrogen for further assays.

Protease activity measurement was performed based on the casein-hydrolysis method explained by Hidalgo et al. (1999). Briefly, the homogenate supernatant (0.05 mL) was mixed with casein (1% *w*/*v*; 0.125 mL) and buffer (0.1 M Tris-HCl, pH 9.0; 0.125 mL) and incubated for 1 h at 37 °C. Then, trichloroacetic acid (TCA) (8% *w*/*v*) solution (0.3 mL) was added to stop the reaction. The samples were kept for 1 h at 4 °C and were then centrifuged at 1800×*g* for 10 min. After keeping the samples at 4 °C for 1 h, they were centrifuged at 1800×*g* for 10 min. Finally, the absorbance of the supernatant was measured at 280 nm.

Lipase activity was measured using 4-nitropheny lmyristate (0.4 mM) as a substrate at 25 °C. The optical density (OD) was recorded at 405 nm (Gawlicka et al. 2000).

α-Amylase activity was determined using 1% starch (diluted in a buffer at pH 6.9, 0.02 M Na_2HPO_4, and 0.006 M NaCl) as substrate based on the 3,5-dinitrosalicylic acid method explained by Worthington (1991) at an OD of 540 nm. A microplate scanning spectrophotometer (HACH DR/4000, USA) was used to measure the activity of all digestive enzymes and expressed in terms of units per milligram protein per minute.

Statistical analysis

Data were recorded as mean ± standard deviation (SD). The arcsine method was used to transform the percentage data. Data were processed using SPSS software (Version, 19). Kolmogorov-Smirnov test was run to determine normality assumption of data. One-way analysis of variance (ANOVA) followed by Duncan's new multiple range test was used to evaluate significant differences between the means at the level of $P < 0.05$.

Results

Hematological analysis

The results of hematological indices of koi fish fed FP-supplemented diets are shown in Table 2. The levels of RBCs in all supplemented treatments except 5 g kg^{-1} dietary FP treatment were significantly higher than those of the control diet ($P < 0.05$). WBCs, Hb, hematocrit, MCV, MCH, MCHC, and lymphocyte levels increased significantly in fish fed dietary FP compared with those of the control diet ($P < 0.05$). The levels of these parameters increased coincident with an increasing FP level in the supplemented diets. The highest levels of the abovementioned parameters were observed in 25-g kg^{-1} FP diet ($P < 0.05$). Monocyte count decreased significantly in the plasma of koi fish with increasing FP levels in diets compared with the control diet ($P < 0.05$). The lowest monocyte count was recorded in the plasma of koi carp fed diets supplemented with 20 and 25 g kg^{-1} of FP ($P < 0.05$). Neutrophil count showed no significant difference between fish fed FP-supplemented diets and control ($P > 0.05$).

Immunological analysis

Innate immune responses of koi carp fed diets supplemented with the various levels of FP are shown in Fig. 1a–d. Total protein content, lysozyme, total IG, and ACH_{50} activities increased significantly along with elevation of dietary FP level in the serum of koi fish compared with those of the control diet ($P < 0.05$). The highest activity of these parameters was recorded in koi carp fed 25-g kg^{-1} FP diet ($P < 0.05$).

Bacteriological analysis

Intestinal microbiota analysis of koi carp fed experimental diets is shown in Fig. 2. Statistical analysis of data showed that LAB/TVC ratio (%) was higher in koi carp fed FP-supplemented diets compared with control ($P < 0.05$). The maximum LAB/TVC ratio (68.11%) was recorded in koi carp fed 25 g kg^{-1} FP diet ($P < 0.05$).

Antibacterial activity of skin mucus

Fig. 3 shows the results of antibacterial activity of skin mucus obtained from fish fed the experimental diets for

Table 2 The hematological indices of koi fish fed diets supplemented with different levels of ferula (*F. asafoetida*) powder after 63 days of feeding trail (mean ± SD, $n = 3$)

Hematological indices	Dietary ferula powder (FP) levels (g kg^{-1})					
	0 (control)	5	10	15	20	25
RBC (× 10^6/μL)	$0.01^{e} \pm 1.17$	$0.01^{e} \pm 1.21$	$0.05^{d} \pm 1.32$	$0.05^{c} \pm 1.52$	$0.05^{b} \pm 1.79$	$0.05^{a} \pm 1.89$
WBC (× 10^3/μL)	$0.57^{f} \pm 29.33$	$0.57^{e} \pm 31.67$	$0.57^{d} \pm 34.33$	$1.15^{c} \pm 37.33$	$0.57^{b} \pm 40.33$	$0.57^{a} \pm 41.67$
Hb (mmol/L)	$0.05^{d} \pm 1.47$	$0.05^{c} \pm 1.64$	$0.05^{c} \pm 1.74$	$0.05^{b} \pm 1.97$	$0.05^{a} \pm 2.17$	$0.05^{a} \pm 2.27$
Hematocrit (%)	$0.57^{f} \pm 15.33$	$0.57^{e} \pm 17.33$	$0.57^{d} \pm 19.67$	$0.57^{c} \pm 22.67$	$0.57^{b} \pm 24.67$	$0.57^{a} \pm 27.33$
MCV (fL)	$1.52^{f} \pm 103.33$	$2.00^{e} \pm 109.00$	$1.73^{d} \pm 119$	$2.30^{c} \pm 123.33$	$1.52^{b} \pm 138.33$	$1.15^{a} \pm 142.33$
MCH (pg)	25.33 ± 0.57^{f}	$0.57^{e} \pm 27.67$	$0.57^{d} \pm 30.66$	$0.57^{c} \pm 33.33$	$0.57^{b} \pm 35.33$	$0.57^{a} \pm 36.67$
MCHC (mmol/L)	$0.57^{f} \pm 9.33$	$0.57^{e} \pm 10.67$	$1.00^{d} \pm 14.00$	$0.57^{c} \pm 16.33$	$0.57^{b} \pm 18.33$	$0.57^{a} \pm 23.33$
Lymphocyte (%)	$1.00^{e} \pm 65.00$	$0.57^{d} \pm 67.33$	$0.57^{c} \pm 69.67$	$0.57^{b} \pm 71.33$	$0.57^{a} \pm 73.67$	$0.57^{a} \pm 74.67$
Monocyte (%)	$1.15^{a} \pm 17.33$	$0.57^{b} \pm 14.33$	$1.52^{c} \pm 11.33$	$1.00^{d} \pm 10.00$	$1.52^{e} \pm 7.33$	$0.57^{e} \pm 6.67$
Neutrophil (%)	$0.57^{a} \pm 17.67$	$0.57^{a} \pm 18.33$	$1.00^{a} \pm 19.00$	$0.57^{a} \pm 18.67$	$1.00^{a} \pm 19.00$	$0.57^{a} \pm 18.67$

Values with different letters within a row are significantly different (ANOVA, $P < 0.05$)

63 days. The skin mucus of fish fed FP-supplemented diets inhibited significantly the growth of *Streptococcus iniae*, *Aeromonas hydrophila*, *Micrococcus luteus*, *Streptococcus faecium*, and *Yersinia ruckeri* ($P < 0.05$). The skin mucus of koi carp fed 20 g kg^{-1} FP diet had higher antibacterial effects against the five bacterial

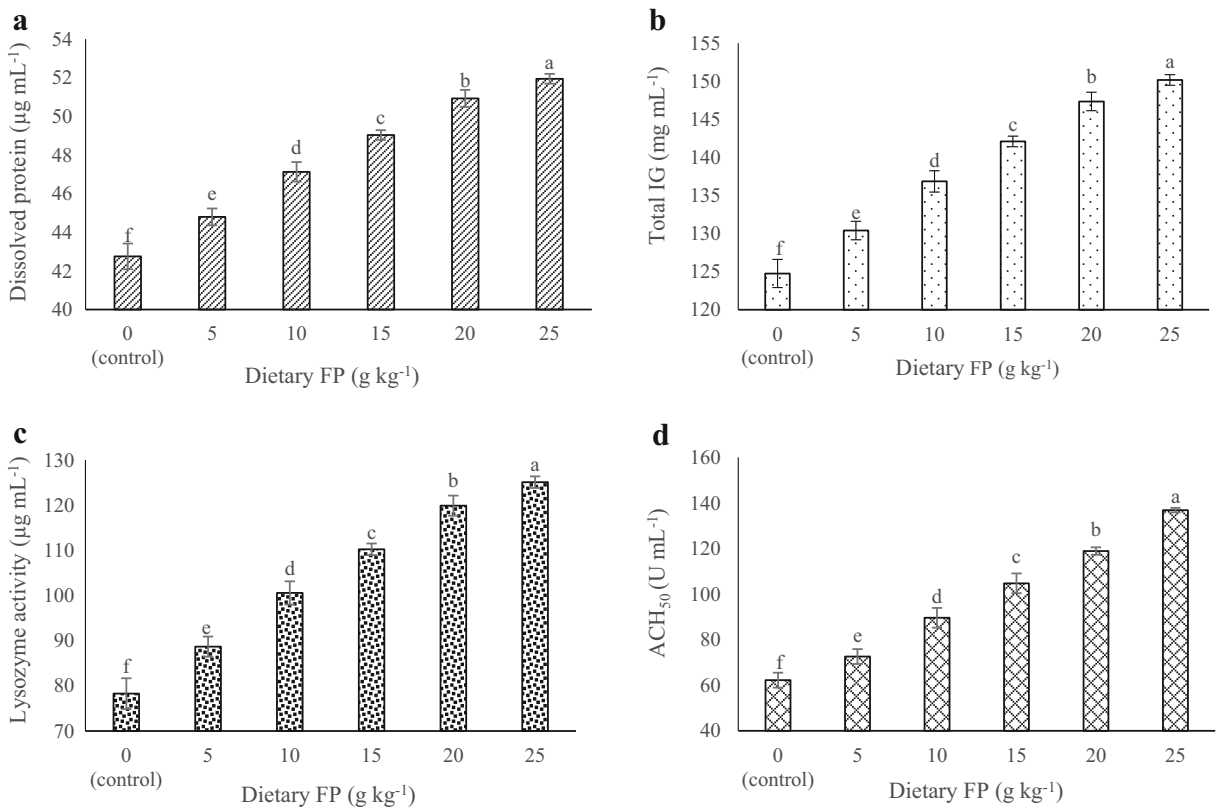

Fig. 1 Means (±SD) of total protein content (μg mL^{-1}) (**a**) and the activities of total immunoglobulin (IG) (μg mL^{-1}) (**b**), lysozyme (μg mL^{-1}) (**c**), and alternative hemolytic complement (ACH_{50}) (U mL^{-1}) (**d**) of koi fish fed with different levels of dietary ferula (*F. asafoetida*) powder (FP) after 63 days of feeding trail. The data with different letters are significantly different (ANOVA, $P < 0.05$)

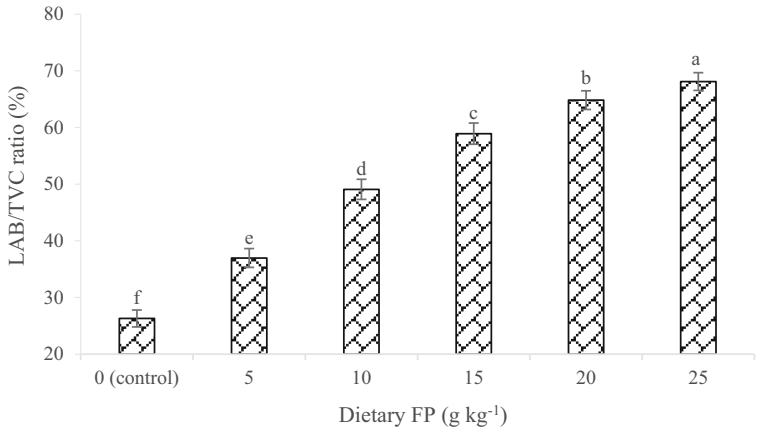

Fig. 2 Lactic acid bacteria (LAB) count (CFU g^{-1}) to total viable heterotrophic aerobic bacteria count (CFU g^{-1}) ratio (%) of intestines extracted from koi fish fed with different levels of dietary ferula (*F. asafoetida*) powder (FP) after 63 days of feeding trial (mean ± SD, $P < 0.05$)

strains than that of other experimental diets ($P < 0.05$). The largest diameter of growth inhibitory zone was formed around *Aeromonas hydrophila* by mucus sampled from koi fish fed with 20 g kg^{-1} FP diet ($P < 0.05$).

Survival rate and growth performance

Initial weight of fish had no significant differences between the treatment groups. Koi fish fed diets supplemented with different levels of FP showed higher final weight and lower FCR value than those of the control diet ($P < 0.05$). The elevation of FP level in experimental diets resulted in an increase in final weight and a reduction in FCR value ($P < 0.05$). All supplemented diets except 5 g kg^{-1} FP diet significantly increased weight gain and SGR value compared with those of the control diet ($P < 0.05$). Feeding koi carp with FP-supplemented diets had no significant effect on the CF value compared with the control diet ($P > 0.05$). The value of survival rate improved in koi carp fed FP-supplemented diets compared with that of the control diet ($P < 0.05$). The highest value of survival rate was recorded in koi carp fed 20 and 25 g kg^{-1} FP diets ($P < 0.05$) (Table 3).

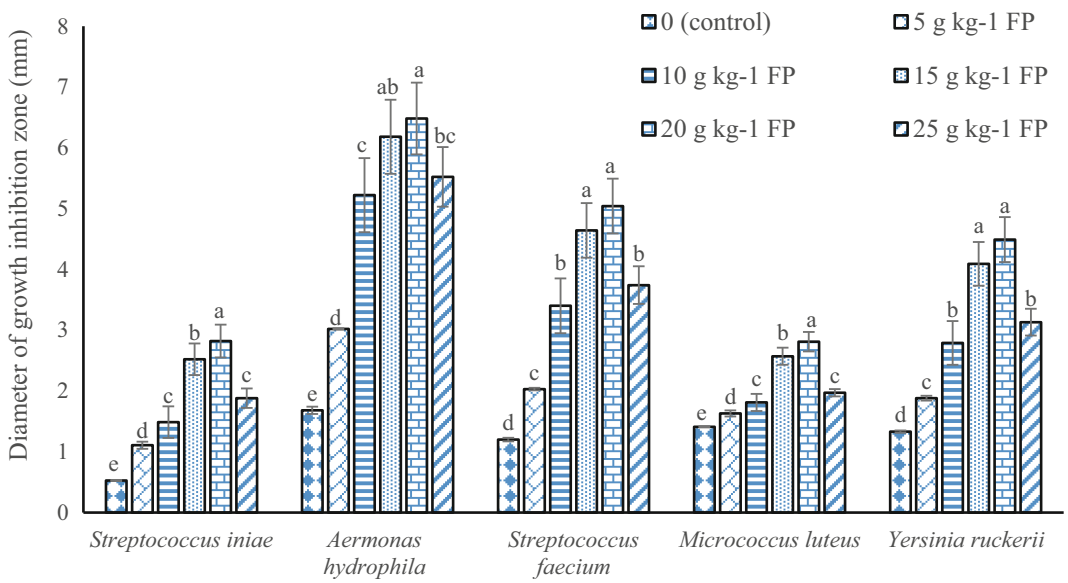

Fig. 3 Diameter of growth inhibition zone (mm) formed by skin mucus of koi fish fed on ferula (*F. asafoetida*) powder (FP)-supplemented diets against five bacterial strains including *Aeromonas hydrophila*, *Streptococcus iniae*, *Streptococcus faecium*, *Micrococcus luteus*, and *Yersinia ruckeri* (mean ± SD, $P < 0.05$)

Table 3 Growth performance and survival rate of koi fish fed with different levels of dietary ferula (*F. asafoetida*) powder (FP) after 63 days of feeding trial (mean ± SD, $n = 3$)

	Dietary ferula powder (FP) levels (g kg^{-1})					
	0 (control)	5	10	15	20	25
Initial weight (g)	4.05 ± 0.13^{a}	4.26 ± 0.40^{a}	3.75 ± 0.30^{a}	3.83 ± 0.72^{a}	3.81 ± 0.42^{a}	3.84 ± 0.24^{a}
Final weight (g)	$0.19^{e} \pm 5.23$	$0.17^{d} \pm 5.96$	$0.11^{d} \pm 6.29$	$0.33^{c} \pm 6.97$	$0.08^{b} \pm 7.96$	$0.12^{a} \pm 8.68$
Weight gain (g)	1.17 ± 0.25^{d}	1.70 ± 0.57^{cd}	2.54 ± 0.39^{bc}	3.14 ± 0.90^{b}	4.15 ± 0.42^{a}	4.84 ± 0.36^{a}
Specific growth rate (% BW day^{-1})	$0.08^{c} \pm 0.40$	$0.19^{cd} \pm 0.53$	$0.14^{bc} \pm 0.82$	$0.33^{ab} \pm 0.96$	$0.18^{ab} \pm 1.17$	$0.12^{a} \pm 1.29$
Feed conversion ratio	$0.05^{f} \pm 3.16$	$0.05^{e} \pm 2.73$	$0.05^{d} \pm 2.33$	$0.05^{c} \pm 2.06$	$0.05^{b} \pm 1.86$	$0.05^{a} \pm 1.63$
Condition factor (%)	$0.08^{a} \pm 0.07$	$0.025^{a} \pm 0.08$	$0.028^{a} \pm 0.12$	$0.083^{a} \pm 0.14$	$0.055^{a} \pm 0.15$	$0.032^{a} \pm 0.15$
Survival rate (%)	$2.30^{d} \pm 70.67$	$2.30^{c} \pm 77.33$	$2.30^{b} \pm 82.67$	$2.30^{b} \pm 86.67$	$4.00^{a} \pm 96$	$2.30^{a} \pm 98.67$

Values with different letters within a row are significantly different (ANOVA, $P < 0.05$)

Digestive enzyme activity assay

The protease activity in koi fish fed on FP-supplemented diets was significantly higher than that of the control diet ($P < 0.05$). Koi carp fed 25 g kg^{-1} FP diet showed the highest protease activity ($P < 0.05$). The activity of α-amylase in the koi fish fed with all FP-supplemented diets (except for 5 g kg^{-1} FP diet) increased compared with that of the control group ($P < 0.05$). The supplementation of experimental diets with 5 and 25 g kg^{-1} FP led to an increase in the lipase activity compared with that of the control diet ($P < 0.05$) (Table 4).

Discussion

During the past decade, medical plants in the forms of powder, extract, or derivate (essence) have been considered immunostimulants in aquafeed production industry. The application of immunostimulants to improve fish health and production has been widely increased (Mehana et al. 2015). Ferula is currently being considered because of its bioactive compounds to boost fish immune system and growth performance. Therefore, this study is aimed at investigating the impacts of dietary *F. asafoetida* powder on the immune responses, growth, and survival rate of koi carp.

In teleost fish, phagocytic cells such as neutrophils, macrophages, natural killer (NK) cells, and T and B lymphocytes are known as innate immune mechanisms at a cellular level (Mehana et al. 2015). The potential effects of prebiotics in aquafeed production industry can be investigated using hematological indices as useful indicators (Merrifield et al. 2010; Fric 2007). The results obtained from the present study indicated an increase in RBC, WBC, hematocrit, Hb, MCV, MCH, MCHC, and lymphocyte levels in fish fed dietary FP in a concentration-dependent pattern ($P < 0.05$). A significant reduction in monocyte count was observed in treated groups with an increasing FP level in diet compared with those of the control diet ($P < 0.05$). Inclusion of different levels of FP in diets had no significant effects on neutrophil count ($P > 0.05$). Based on the published data, this was the first study on the effects of dietary FP

Table 4 Digestive enzyme activity (U mg protein^{-1} min^{-1}) of koi fish fed with different levels of dietary ferula (*F. asafoetida*) powder (FP) after 63 days of feeding trial (mean ± SD, $n = 3$)

	Dietary ferula powder (FP) levels (g kg^{-1})					
	0 (control)	5	10	15	20	25
Protease (U mg protein^{-1} min^{-1})	$0.04^{f} \pm 0.38$	$0.05^{e} \pm 058$	$0.07^{d} \pm 0.89$	$0.08^{c} \pm 1.15$	$0.06^{b} \pm 1.53$	$0.08^{a} \pm 1.80$
α-Amylase (U mg protein^{-1} min^{-1})	$0.01^{e} \pm 0.74$	$0.02^{e} \pm 0.85$	$0.01^{d} \pm 1.08$	$0.16^{c} \pm 1.28$	$0.01^{b} \pm 1.76$	$0.07^{a} \pm 2.26$
Lipase (U mg protein^{-1} min^{-1})	$0.01^{b} \pm 0.52$	$0.02^{ab} \pm 0.55$	$0.01^{ab} \pm 0.54$	$0.01^{ab} \pm 0.53$	$0.01^{b} \pm 0.52$	$0.01^{a} \pm 0.56$

Values with different letters within a row are significantly different (ANOVA, $P < 0.05$)

on hematological parameters in fish. The effects of other medical plants on hematological indices of different fish species have been documented in literatures. For example, feeding fingerling of Asian sea bass, *Lates calcarifer*, with diets supplemented with various levels of lipopolysaccharide and *Cissus quadrangularis* plant (stem) showed a significant increase in hematological parameters including leucocytes and Hb levels (Devakumar and Chinnasamy 2017). Safari and Sarkheil (2018) also reported a significant concentration-dependent elevation of Hb, Ht, MCH, and MCV levels in koi carp fingerlings fed diets supplemented with eryngii mushroom, *Pleurotus eryngii.*

Innate immune parameters at a humoral level in teleost fish includes hemolysin, lysozyme, alternative hemolytic complement, transferrin, and C-reactive protein (Magnadóttir 2006; Mehana et al. 2015). The results of this study displayed a significant improvement in total protein content, lysozyme, total immunoglobulin, and ACH_{50} in the serum of koi fish fed with FP-supplemented diets compared with those of the control diet in a concentration-dependent pattern ($P < 0.05$). Safari et al. (2016) showed that the serum lysozyme activity of common carp (*C. carpio*) only enhanced in fish fed with 5 g kg^{-1} FP diet, whereas total immunoglobulin and ACH_{50} levels had no significant changes compared with the control diet.

Ferula spp. and essential oils derived from these species are important source of unique organosulfur compounds (Kasaian et al. 2016; Kavoosi and Rowshan 2013). It has been reported that plant-derived organosulfur compounds exhibit immunomodulatory activity (Schafer and Kaschula 2014). Considering type of species, feeding period, initial weight, nutritional history, and rearing conditions, formulating a standard diet and the kind of effective ingredients in plant materials is critical to interpret results. However, further studies are needed to identify the mechanisms of dietary FP on metabolic pathways and gene expression in koi fish in the future.

It is well known that the intestinal microbiota of fish is modulated through genetic, nutritional, and environmental factors) Perez et al. 2010). The improvement of health status and growth of aquatic animals through changes in gastrointestinal (GI) morphology and microbial balance toward potentially effective populations such as lactic acid bacteria (LAB) by using prebiotics, probiotics, and synbiotics have been reported (Abid et al. 2013Hoseinifar et al. 2015). According to findings of the present study, the supplementation of koi carp diet with FP resulted in 1.4, 1.86, 2.24, 2.46, and 2.59 times increase in the LAB/TVC ratio (%) in 5, 10, 15, 20, and 25 g kg^{-1} of dietary FP, respectively. Safari and Sarkheil (2018) reported the enhancement of the LAB/TVC ratio (%) in koi carp fingerling feeding on eryngii mushroom (*P. eryngii*) powder for 63 days. Hoseinifar et al. (2015) also showed the elevation of the LAB level in an intestinal microbiota population of common carp (*C. carpio*) larvae fed on short-chain fructooligosaccharide (sc-FOS) for 7 weeks. This result may be related to the carbohydrate content of FP, especially crude fiber that acts as a prebiotic. Therefore, the measurement of short-chain fatty acids (SCFAs) and the recognition of microbial population profile in the digestive tract may help better interpret future results. However, further in vitro and in vivo studies are needed to evaluate the interaction between effective ingredients (and/or hydrolyzed by-products) existing in plant products and digestive microbial population.

Antibacterial properties of *Ferula* species and their constituents well documented in literatures (Abedi et al. 2009; Akaberi et al. 2015; Sahebkar 2010). Al-Yahya et al. (1998) found that the sesquiterpene 14-(O-hydroxycinnamoyloxy)-dauc-4,8-diene extracted from *Ferula communis* possessed strong activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus durans*, and *Enterococcus faecalis.* Antibacterial activity of essential oil extracted from *F. asafoetida* also has been reported against *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* as Gram-positive bacteria and some of Gram-negative bacteria like *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* (Samadi et al. 2016). In the present study, the skin mucus obtained from koi carp fingerlings fed on dietary FP showed significant antibacterial activity against *Streptococcus iniae*, *Aeromonas hydrophila*, *Micrococcus luteus*, *Streptococcus faecium*, and *Yersinia ruckeri*. The highest antibacterial activity of skin mucus was observed in 2 g kg^{-1} of dietary FP. However, antibacterial activity of skin mucus samples decreased significantly with the elevation of the dietary FP level to 25 g kg^{-1}. Some researchers expressed that bactericidal properties of skin mucus is related to its nitrogenous compounds such as proteases, lysozyme, immunoglobulins, lectins, and mucins (Angeles Esteban 2012). However, the reason for this is not clear. It needs more researches in the near future. Evaluating effective materials (e.g., organosulfur compounds) of

FP on physiological pathways via nutrigenomic can help better interpret results.

The results of the present study showed that the survival rate and growth of koi fish fingerlings were significantly affected by diets supplemented with different levels of FP. Final weight and survival rate values of fish increased coincident with the increasing level of FP, but FCR values inversely decreased ($P < 0.05$). Fish fed with 10, 15, 20, and 25 g kg^{-1} of dietary FP significantly showed higher WG and SGR values compared with the control group ($P < 0.05$). Safari et al. (2016) reported a higher expression of appetite and growth-related genes (GH, IGF1, and Ghrl) of common carp (*C. carpio*) fed on diets supplemented with 10 and 20 g kg^{-1} FP. On the contrary, dietary inclusion of *Ferula coskunii* at 15, 30, and 40 g kg^{-1} levels negatively affected the growth and FCR value in common carp (*C. carpio*) (Yilmaz et al. 2006). In the present study, condition factor (CF) value of koi carp fingerlings fed FP-supplemented diets also had no significant difference compared with the control ($P > 0.05$). Shademani et al. (2015) revealed that feeding broiler chicken with dietary FP resulted in increased digestive enzyme activity and thus enhanced growth performance. Oral administration of *F. asafoetida* powder to albino rats at a level of 250 mg kg^{-1} for 8 weeks stimulated activity of pancreatic amylase, lipase, and chymotrypsin enzymes (Platel and Srinivasan 2000). In the present study, dietary FP significantly affected digestive enzyme activity in koi fish fingerlings. The protease and α-amylase activities in FP-supplemented diets increased significantly compared with those of the control diet in a concentration-dependent manner. A significant increase in the lipase activity was also observed in 5 and 25 g kg^{-1} of dietary FP. Further researches need to model the effects of phytogenics on the up-/down-regulation of genes involved in the secretion of materials with endocrine and exocrine origins.

Conclusion

Disease outbreak plays an important role in decreasing aquaculture product. Instead of using antibiotics and synthetic drugs to control diseases, a proper herbal feed supplementation can be effective in the enhancement of overall health and growth performance of fish. The outcomes of this study revealed remarkable impacts of dietary FP powder on growth and humoral immune status of koi fish. The growth performance, survival rate, protease and α-amylase enzyme activity, humoral innate immune responses, intestinal microbiota, and antibacterial properties of skin mucus improved in koi carp fingerlings fed FP-supplemented diets especially at levels of 20 and 25 g kg^{-1}.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement FUM animal ethic rights were applied to all experiments on koi fish. The authors followed all suitable international, national, and/or institutional guidelines for the care and use of aquatic animals.

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