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RESEARCH

Effects of dietary *Plantago ovata* seed extract administration on growth performance and immune function of common carp (*Cyprinus carpio*) fingerling exposed to ammonia toxicity

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Abstract

Medicinal plants are powerful antioxidants which can improve well-being and suppress oxidative stress caused by environmental toxins in aquatic animals. In this regard, the present research was designed to show the potential effects of psyllium (*Plantago ovata*) seed extract (PSE) on the growth, and immune responses of common carp *Cyprinus carpio* exposed to acute ammonia toxicity. To perform the study, fish were fed with diets containing 0 (T0), 0.25 (T1), 0.5 (T2), and 1% (T3) PSE for 60 days, and then exposed to ammonia (0.5 mg L^{-1}) for 3 h. The findings showed that fish given the T1 diet outperformed the T3 and control groups in terms of ultimate weight, weight increase, and food conversion ratio. Additionally, the T1 group showed a significantly higher level of total protein and serum lysozyme activity than the other treatment groups. Moreover, the highest serum total immunoglobulin values were recorded in T1 and T2 groups. The results showed that PSE, especially at moderate levels, could successfully upregulate the transcription of immune-related genes (*IFN-γ*, *Hsp70, TNF-a*, *IL-1β*, *IL-10*, and *IgE*) compared to the control group after exposure to ammonia. Furthermore, improving ammonia-induced down regulations of antioxidant-related gene expressions (*CYP1A, SOD*, and *GPX*) was observed in fish fed with PSE-included diets compared to the control one. However, PSE-supplemented diets did not affect the mRNA expression level of *CAT*. Regarding tight junction-associated genes, the higher mRNA expression level of *occludin* was observed in the T1 group, whereas the downregulation of *CLD3* gene occurred in all experimental groups. Conversely, significant upregulation of osmoregulation-associated gene (*NKA*) was recorded in all experimental groups compared to the control one. Therefore, the administration of PSE (0.25% of the diet) for 60 days is recommended to increase growth performance, improve health, and increase the resistance of common carp to oxidative stress caused by ammonia.

Keywords Aquaculture · Herbal medicine · Fish welfare · Toxicant · Common carp

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Introduction

The world's population is rapidly growing which is projected to reach about 10 billion by 2050. In this regard, providing food and especially protein to this huge population has become one of the biggest challenges for countries and nutritionists (FAO 2018). Statistics from the Food and Agriculture Organization (FAO) show that total world fisheries and aquaculture production is 179 million tons per year, about half of which is produced via aquaculture (FAO 2020). Estimates show that the growth rate of increase in fish production via fisheries has stopped in recent decades, but aquaculture is growing at a significant rate so that it has now become the most notable sector with the highest growth rate among other agricultural sectors (Belton and Thilsted 2014). This share of fish output is not yet compelling, and by 2030, it is anticipated that aquaculture would produce more than 109 million tons annually (FAO 2018). Aquaculturists have paid particular attention to the modernisation of fish farming systems and enhanced productivity per unit area via intensive farming methods since inputs/resources (such as land, water, and investment) are not limitless (Yousefi et al. 2021b).Ammonia toxicity, which is a common problem in aquaculture, occurs more severely in intensive farming systems in terms of high biomass density and increased excretion of organic matter produced by fish (Ren et al. 2016). For instance, high level of ammonia may cause a wide range of complications such as impaired internal homeostasis, suppression of the immune system and antioxidant capacity, as well as an increased risk of pathogen release, which can eventually lead to disease and even death of fish (Zhang et al. 2018; Taheri Mirghaed et al. 2019). The prevalence of these disorders in farmed fish was confirmed by previous studies evaluating the expression of genes related to immune, antioxidant, and cytokines systems (Cheng et al. 2015; Qi et al. 2017; Zhang et al. 2018; Kaleo et al. 2019).

Strengthening fish's immune and antioxidant systems and increasing the detoxification of fish bodies via diet manipulation are methods used to combat ammonia toxicity. To this end, using functional feed supplements, including herbs and their derivatives, with antioxidant and anti-inflammatory effects, was extended to prevent ammonia toxicity (Hoseini et al. 2019; Divya et al. 2020; Magouz et al. 2021). For instance, a diet containing *Hibiscus sabdariffa* significantly enhanced immune responses and mitigated inflammation by regulating the expression of cytokine genes in rainbow trout exposed to ammonia (Yousefi et al. 2021a).

The genus of Plantago is one of the most famous medicinal herbs. This herb belongs to the Plantaginaceae family, containing about 270 species (Fierascu et al. 2021). Psyllium (*Plantago ovata*) with the common name of Isapgol/ispaghula belongs to this genus. Psyllium is grown in tropical regions (Egypt, China, Iran, Japan, Korea, etc.), but its primary origin is India and Pakistan, and currently, India is the largest exporter of seeds of this herbal plant (Chevallier 1996; Xing et al. 2017). The seeds of this medicinal herb are a rich source of iridoid glucosides, polysaccharides (cellulose, hemicellulose, and lignin), flavonoids, hydroxycinnamic acids, and minerals, vitamins, alkaloids, terpenoids, terpenes, etc. (Madgulkar et al. 2015; Ziemichód et al. 2019). Psyllium and its derivatives are used to reduce total cholesterol and low-density lipoprotein (LDL) cholesterol, treat Parkinson and cardiovascular diseases and prevent constipation, diabetes, intestinal inflammation, diarrhea, and atherosclerosis (Wei et al. 2009; Madgulkar et al. 2015; Ziemichód et al. 2019). Furthermore, it was shown that they can act as prebiotics and antioxidant (Patel et al. 2016; Xing et al. 2017).

Although this medicinal plant and its derivatives have various and well-known properties, to the best of our knowledge, no study was done to study its effects on common carp exposed to waterborne ammonia. Therefore, present study aimed to evaluate the potential effects of PSE on growth performance, humoral immunity, and cytokine- and antioxidant-associated gene expressions in common carp exposed to ammonia toxicity.

Materials and methods

Diet preparation

P. ovata seed was purchased from a local supplier in Gonbad Kavous, Iran. Plant seeds were powdered in a grinder, and 5 g powdered plant seeds were suspended in 70% ethanol (1:5 v/v) at 45 °C with continuous shaking for 72 h. After the filtration by a Whatman No.1 filter paper, solvent extract was evaporated using a rotary vacuum evaporator (HS-3001, Korea) (Fluke 2001), and a total of 2 g of dry *P. ovata* seed was obtained and kept in a freezer at -80 °C for further examination.

During diet preparation, psyllium seed extract (PSE) was added to basal diet (Table 1) at four incremental levels (0, 0.25, 0.5, and 1% of feed) based on previous studies (Mohammadi et al. 2014a, b). In the experimental diets (Table 1), cellulose was replaced with PSE to attain a similar composition (Mousavi et al. 2020). The prepared diets were stored in plastic bags at 5–8 °C to be used in the feeding trial.

Characterization of PSE

The concentration of phenolic compounds of PSE was spectrophotometrically determined using gallic acid, and

Table 1 Formulation and proximate composition of the basal diet (% dry matter)

Compounds	(%)	Chemical composition	(%)
Fish meal	33	Dry matter	89.50
Wheat flour	26	Crude protein	32.40
Soybean meal	12	Crude lipid	8.78
Wheat gluten	6	Ash	5.92
Corn flour	14	Fiber	11.20
Plant oil	3		
Mineral premix[a]	2		
Vitamin premix[a]	2		
Cellulose	1		
Anti fungi[b]	1		

[a]Premix detailed by Hoseinifar et al. (2016)

[b]ToxiBan antifungal (Vet-A-Mix, Shenan-doah, IA)

the final result was shown as gallic acid equivalents in milligrams per gram of dry extract. Inulin was used to calculate the concentration of polysaccharide components in PSE, and the results were shown as inulin equivalents in milligrams per gram of dry extract. The radical scavenging activity of PSE was determined against stable 2,2- diphenyl-2-picryl-hydrazyl hydrate (DPPH) (Brand-Williams et al. 1995) and compared with butylated hydroxytoluene (BHT) as a potent antioxidant.

Fish and experimental design

Common carps with a mean weight of 31.18 ± 1.23 g were purchased from a local fish farm in Gonbad Kavous, Iran, and kept inside a concrete tank (400 L) for 14 days of adaptation. Water temperature (22.27 ± 0.47 °C), dissolved oxygen (6.60 ± 0.42 mg/L), pH (7.57 ± 0.10), and unionized ammonia (0.038 ± 0.020 mg/L) were measured during this experiment. In adaptation phase, fish were carefully examined for diseases and fed with the basal diet (Table 1) at 3% of body weight three times a day (8:30, 13:00, and 17:30 h). Afterward, 180 fish with similar weights were randomly distributed inside 12 concrete tanks (60 L) with a capacity of 15 fish in each tank. During feeding experiment, fish in each experimental group (three tanks per group) were fed diets supplemented with PSE at 0, 0.25, 0.5, and 1% (T0, T1, T2, T3) at 3% of body weight three times a day (8:30, 13:00, and 17:30 h) for 60 consecutive days. Every two weeks, biomass in each tank was determined to calculate the feeding during the study.

At the end of the 60-day trial, all fish in each tank were anesthetized into a clove oil bath (50 µL l^{-1}) to determine the biomass in each tank. Specific growth rate (SGR) and feed conversion ratio (FCR) were calculated as follows:

SGR = final weight (g) − initial weight (g) / feeding days × 100

FCR = total feed intake (g) / weight gain (g)

For the determination of serum immunological parameters, six fish in each tank were randomly selected and bled from the caudal vein with a plastic syringe. Collected blood was centrifuged at 2000 g for 15 min at 4 °C and kept at -20 °C for innate immune parameters, including total protein and immunoglobulin levels, as well as lysozyme activity. Serum total immunoglobulin level was determined by polyethylene glycol (PEG) precipitation and calculating the protein level based on the Bradford method (Bradford 1976). Turbidimetric assay determined Lysozyme activity based on the previous study (Demers and Bayne 1997). In brief, 25 µl of each fish serum was added to 75 µl of *Micrococcus lysodeikticus* suspension and mixed well. Absorbance was recorded for 10 min at 450 nm using a microplate reader (Hiperion, Germany).

Ethics statement

The procedures of this study involving animals and their care were approved by Animal Care and Use Committee of the University of Zabol.

Ammonia challenge test and sampling

After 60-day feeding trial, the remaining fish in each tank ($n = 9$) were exposed to 110 mg L^{-1} ammonium chloride (Merck, Germany, Molecular mass: 53.49 g/mol, Densit: 1.53 g/cm^3) producing 0.5 mg/L ambient unionized ammonia nitrogen based on the water pH and temperature, for 3 h (Hoseini et al. 2019; Rajabiesterabadi et al. 2020). The fish were not fed 24 h before and during the exposure period. Each tank received the necessary quantity of ammonium chloride combined with water throughout the exposure period, and the water remained stationary for 3 h. The water in tanks was daily managed to be the same as in the feeding experiment for this bioassay test. Following the test, each fish's first gill arch on the right side was removed, dissected, and frozen in liquid nitrogen for gene expression research.

Real-time PCR

Total RNA from fish gills was isolated using Gene All Kit (Gene All Biotechnology, Seoul, Korea) according to manufacturer protocol. The concentration and purity of the isolated RNA were measured using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Following the protocol, the cDNA was synthesized by the First Strand cDNA Synthesis Kit (Gene All Biotechnology, Seoul, Korea).The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase

(*GAPDH*) was used to normalize the target gene expressions. Genes used for real-time PCR included the immune-associated genes, namely interferon-γ (*IFN-γ*), interleukin-1β (*IL-1β)*, heat shock protein 70 (*Hsp70*), interleukin-10 (*IL-10*), tumor necrosis factor α (*TNF-α)*, immunoglobulin E (*IgE*); antioxidant-associated genes, including cytochrome P4501A (*CYP1A*), superoxide dismutase (*SOD*), catalase (*CAT*), glutathione peroxidase (*GPX*), tight junction-associated genes, including claudin-3 (*CLD3*) and *occludin*, as well as osmoregulation- associated gene, Na^+/K^+- ATPase (*NKA*) (Table 2). PCR was carried out in a 20 µL reaction mixture containing 10 µl of 2X SYBR green mix (Ampliqon, Denmark), 0.5 µl of forward and reverse primers, 2 µl of Template (cDNA), and 7 µL of nuclease-free water. The thermal profile used for PCR amplification consisted of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The expression of selected genes was initially normalized to that of GAPDH, and then the fold change was calculated by estimating the expression level of differently treated samples divided by that of controls. To minimize the experimental errors, all PCR reactions were repeated three times.

Statistical analysis

The experimental data are expressed as means ± standard error (SE). The normality of data and homogeneity of variances were confirmed by Kolmogorov–Smirnov and Levene tests. All data were then analyzed with a One-way analysis of variance and Tukey in SPSS version 21 software. The p-value less than 0.05 were considered statistically significant.

Results

PSE characterization

PSE contained 3.76 ± 0.82 mg g^{-1} total phenolic compounds expressed as milligram gallic acid per gram of dry extract. The total polysaccharide compounds of PSE were about 447.78 ± 3.06 mg g^{-1} depicted as milligram inulin per gram of dry extract. The antioxidant activity by DPPH method showed that BHT and PSE exhibited 50% free radical scavenging at 11.64 and 44.70 µg ml^{-1}, respectively.

Growth performance

The growth indices after dietary administration of PSE are shown in Table 3. The fish in all groups were active and no mortality occurred during 60-day feeding trial. Among all experimental diets, fish fed T1 showed growth and improved FCR compared to the control and T3.

Humoral immunity

The lysozyme activity and the serum total protein level significantly increased in the T1 group compared to other groups. However, serum total immunoglobulin level was significantly increased in T1, and T2 treated fish compared to the control fish (Fig. 1).

Immune-relevant gene expressions

A significant increase in *IFN-γ* and *Hsp70* gene expressions was noted in the T1 and T2 groups compared to with the

Table 2 Primers selected for assessing the expression of different genes related to immune and antioxidant systems, tight junction and osmoregulation

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Accession number	product length
INF-γ	GAGAACCTGGACAAGAGCAT	AAAAGATTCTGCTCAGGTTCC	NM_001361222.1	140
Hsp70	TGACCAGGGAAACAGAACAA	GCACAACTGGGTCATCAAAC	XM_019074376.1	158
TNF-α	AGGAGGAAGTCAGGCGTCT	AGCGTGAAGCAGACAGCAG	XM_019080541.1	83
IL-1β	GCTCACTTCCACTGTCACAA	CATCGTTCTGTTGTTTGGTG	XM_019080073.1	104
IL-10	GGCATCAAAGAGAGTCAAGC	TCCATTTGTGCCATATCCTAC	XM_019106061.1	146
IgE	ATTCTTTGGAGGTGTGCATC	AAAGAAGAGAGAAGGGCAAC	XM_019107675.1	102
CYP1A	AGCACATCAGCAAGGAAGGT	TCATCGTCGTGGCTGTAGC	XM_019064218.1	154
CAT	TGGTGGATAATAACAGTTGGG	ACACGATACAACACTGCTGC	XM_019068441.1	139
SOD	TGTGGGGTTCTGCCTCTTG	TGGGAACATAGTGAGGGAGA	XM_019081090.1	155
GPX	CAACAGGAGAATGCCAAGA	AGGAACACGAACAGAGGGT	XM_019093635.1	140
Occulodin	ATCTGGCACTATGGGAAACC	CGCTCTGTCCATTCTGTACG	XM_019113096.1	109
Caludin3	TACACGGGAAAACGAATACC	GTGATGACGGGGATGTGATA	XM_019094928.1	98
NKA	GAGGAACACAGAACCGACAC	GAGAGTCTGCTGAGGGGTTT	XM_019126419.1	123

Table 3 Effects of dietary administration of PSE on carp growth performance

groups	Initial weight (g)	Final weight (g)	Weight gain (g)	SGR		FCR	Survival rate (%)
T0	30.92 ± 0.40	63.32 ± 1.17^{a}	32.41 ± 1.00^{a}	1.28 ± 0.03		3.09 ± 0.09^{b}	100
T1	31.46 ± 0.58	68.27 ± 1.42^{b}	36.82 ± 0.85^{b}		1.38 ± 0.01	2.72 ± 0.06^{a}	100
T2	31.31 ± 0.43	64.84 ± 1.34^{ab}	33.54 ± 1.06^{ab}		1.30 ± 0.02	2.99 ± 0.09^{ab}	100
T3	± 0.6831.05	63.19 ± 1.19^{a}	32.14 ± 1.52^{a}		1.27 ± 0.06	3.12 ± 0.14^{b}	100

Values show means ± SE. Values in each column with different letters were significantly different ($P < 0.05$). For SGR and FCR, $n = 3$ in each treatment group where as for the other parameters $n = 45$

T0, fish fed with the basal diet. T1, fish fed with the treatment diet containing 0.25% PSE. T2, fish fed with the treatment diet containing 0.5% PSE. T3, fish fed with the treatment diet containing 1% PSE

control group. The highest fold change of *TNF-a* and *IL-10* genes were shown in the T1 (0.25 PSE) and T2 groups (0.5 PSE), respectively. Feeding PSE, especially in T1 and T3, could augment the expression of *IL-1ß* when compared to the control group. However, the expression of the *IgE* gene increased in all groups compared to the control group (Fig. 2).

Antioxidant-relevant gene expressions

The expression of *CYP1A* gene was significantly increased in the fish fed with all treated diets when compared to the control group. Conversely, the expression of the *CAT* gene was not altered after the dietary administration of the PSE diet. However, feeding T1 and T3 diets could augment the expression of the *SOD* and *GPX* genes, respectively (Fig. 3).

Tight junction and osmoregulation-relevant genes expression

The upregulation of the *occludin* gene was shown in the T1 group when compared to the other groups. Meanwhile, feeding PSE could downregulate the expression of *CLD3* gene in all treated groups compared to the control group (Fig. 4). Conversely, the upregulation of *NKA* gene was shown in all treated fish compared to the control group (Fig. 5).

Discussion

Choosing proper method to obtain PSE can increase the effectiveness of its polyphenolic compounds to improve fish health and disease resistance. The choice of ethanol was related to the low toxicity and high effectiveness of this solvent to extract the phytonutrients from *Plantago* seed (Shah et al. 2020). Total phenolic content of prepared PSE was 3.76 ± 0.82 mg gallic acid per gram. This result is in agreement with previous study reporting 3.25 and 2.16 mg gallic acid per gram in ethanolic and methanolic extracts of PSE (Shah et al. 2020). Meanwhile, the free radical scavenging ability of PSE was compared with BHT, as a reference antioxidant. The current assay showed the 50% free radical scavenging of BHT and PSE at 11.64 and 44.70 µg ml^{-1}, respectively. This result shows the probable antioxidant ability of PSE compared to a potent antioxidant like BHT.

The beneficial effects of medicinal plants and their derivatives on fish health and growth efficiency, as well as resistance to pathogens and environmental stressors, were proven by numerous studies (Jahazi et al. 2020; Moustafa et al. 2020; Abdel-Tawwab and El-Araby 2021; Yousefi et al. 2021a). Ammonia toxicity is a major abiotic factor that can threaten fish health by causing oxidative stress and tissue damage (Sinha et al. 2014).Furthermore, acute ammonia toxicity under unfavorable aquaculture conditions, in terms of its rapid increase beyond the tolerance threshold of aquatic animals, can lead to the mass mortality of fish on farms (Ip and Chew 2010). Hence, we show the potential of the dietary PSE on growth performance, humoral immunity (lysozyme activity, total protein, and immunoglobulin levels), and immune- (*IFN-γ*, *Hsp70*, *TNF-a*, *IL-1ß, IL-10, and IgE*), antioxidant- (*CYP1A*, *CAT*, *SOD*, and *GPX*), tight junction- (*occludin*, *CLD3*) and osmoregulation- (*NKA*) associated genes in the gills of common carp fingerlings exposed to ammonia.

According to the results, diet supplemented with 0.25% PSE could significantly improve final weight, weight gain, and FCR in fish (T1) after 60 days. Previous studies showed that this improvement in growth efficiency can occur via the beneficial effects of herbal medicines on intestinal morphology, intestinal bacteria, fish appetite, as well as the activity of intestinal digestive enzymes (Chakraborty et al. 2014; Ferreira et al. 2016; Ahmadifar et al. 2020; Dawood et al. 2021). Improved intestinal characteristics can be explained by the presence of various compounds in PSE, including polysaccharides, flavonoids, vitamins, alkaloids, etc. (Kawashty et al. 1994; Rezaeipoor et al. 2000; Ziemichód et al. 2019). It was shown that psyllium can act as a probiotic, thereby increasing the efficiency of digestion and absorption in the intestine (Xing et al. 2017). Similarly, Yilmaz (2019) reported an increase in the growth performance of Nile tilapia fed at a moderate dose (15 g kg^{-1}) of blackberry syrup. Moreover, common carp fed a diet incorporated with medlar (*Mespilus*

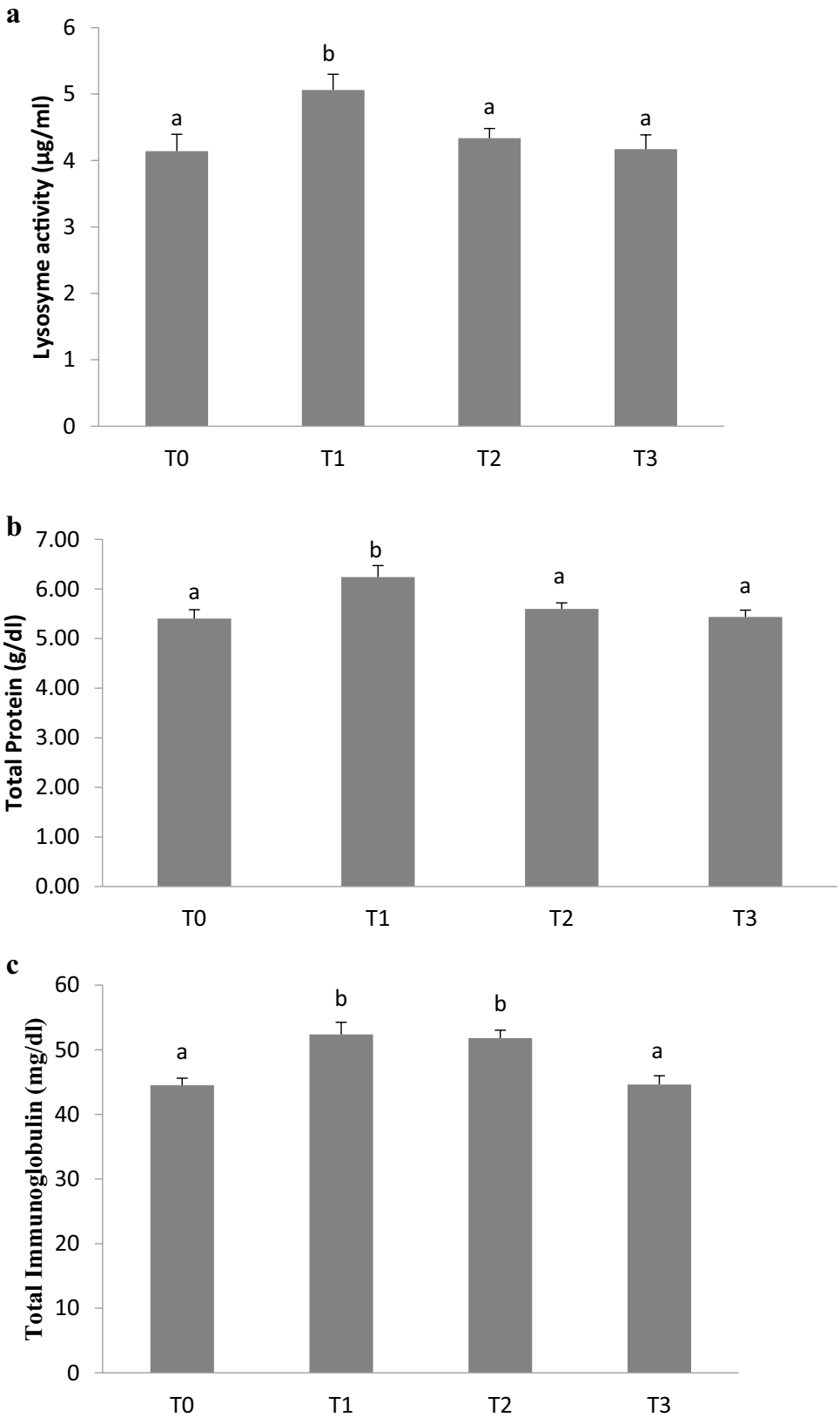


Fig. 1 Effects of dietary administration of PSE and exposure with ammonia on carp humoral immunity, including serum lysozyme activity (1.a), total protein level (1.b) and total immunoglobulin (1.c). Values show means ± SE. Bars with different letters were significantly different ($P < 0.05$). Fish grouping are as mentioned in Table 3. For each treatment group $n = 18$

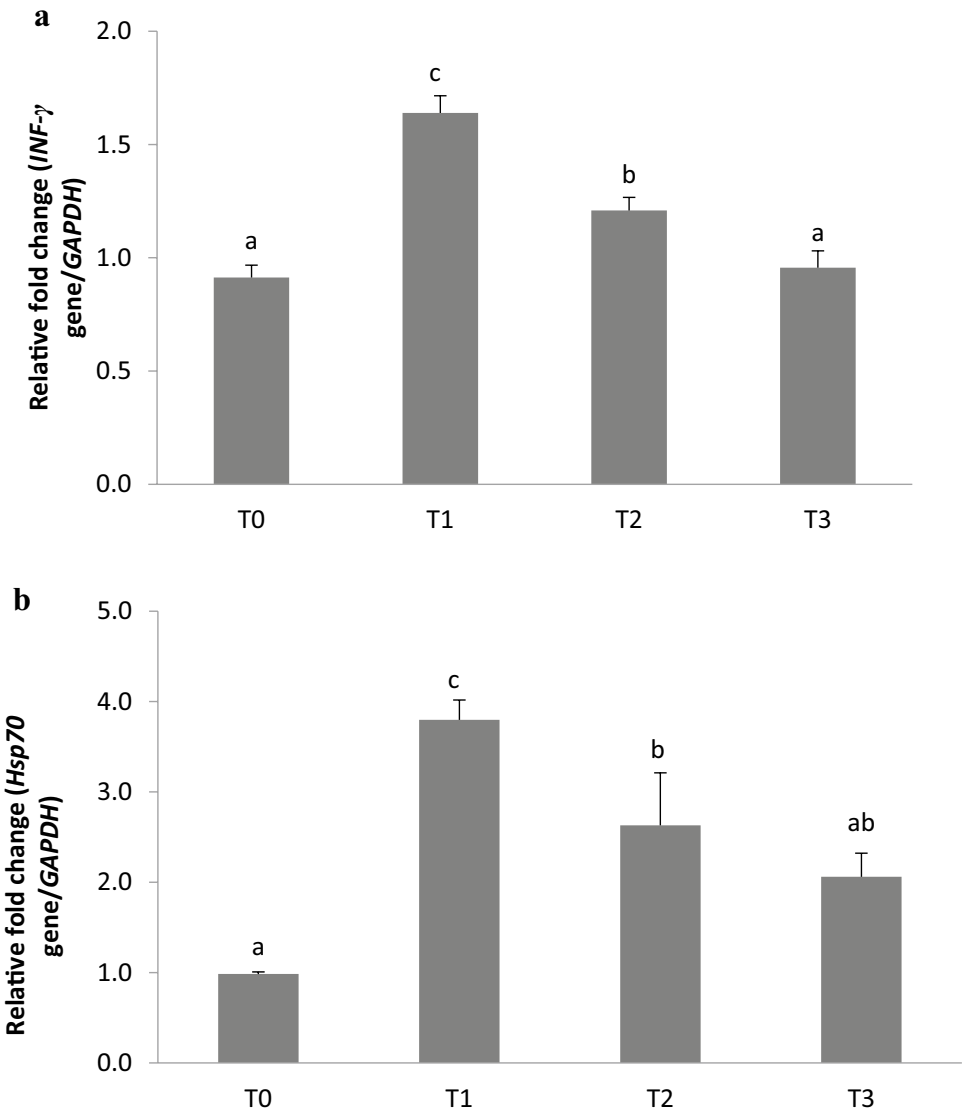


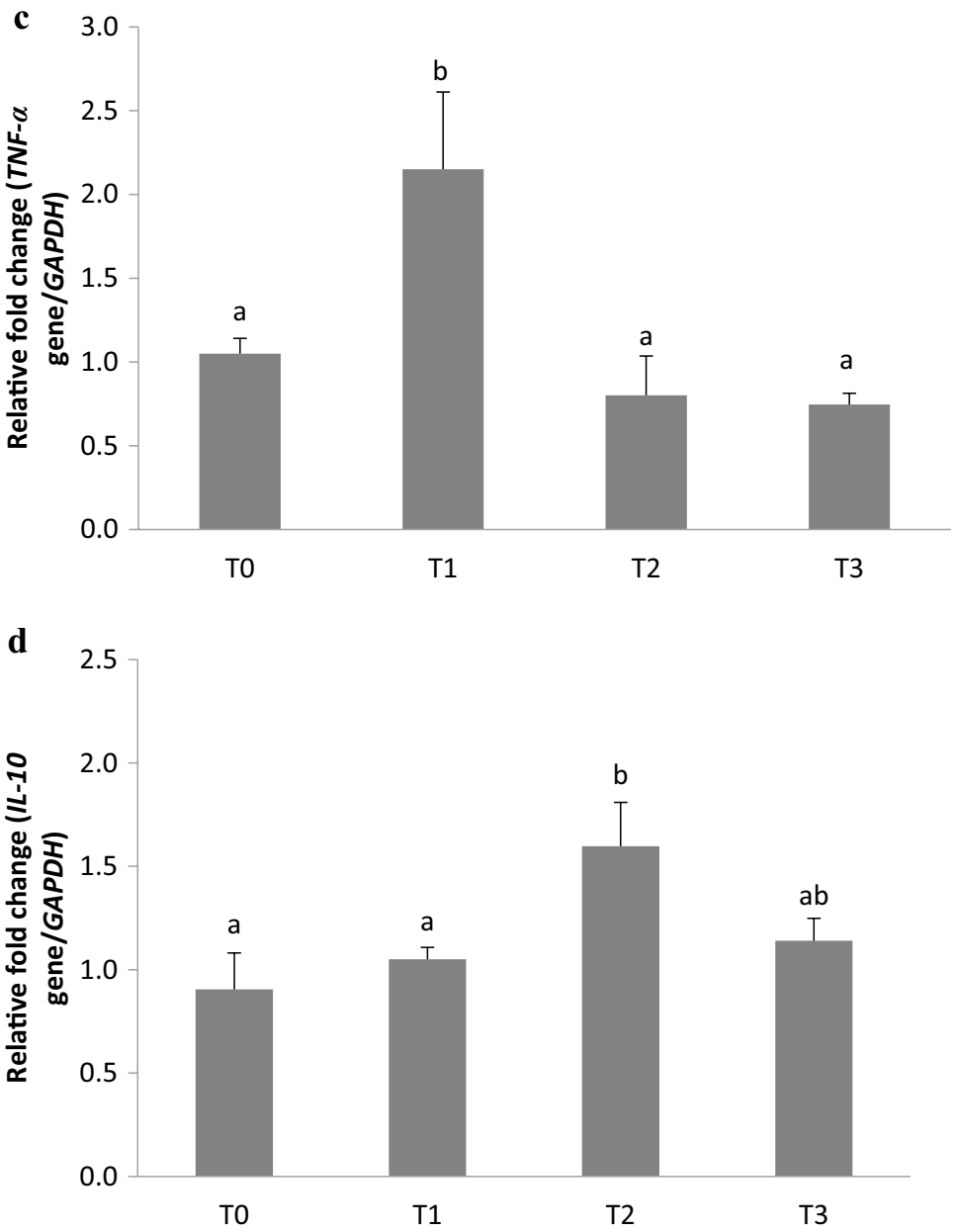
Fig. 2 Effects of dietary administration of PSE and exposure with ammonia on carp immune-related genes including interferon-γ (*IFN-γ*) (2.a), interleukin-1β (*IL-1β)* (2.b), interleukin-10 (*IL-10*) (2.c), heat shock protein 70 (*Hsp70)* (2.d), tumor necrosis factor α (*TNF-a)* (2.e) and immunoglobulin E (*IgE*) (2.f). Values show means ± SE. Bars with different letters were significantly different ($P < 0.05$). Fish grouping are as mentioned in Table 3. For each treatment group $n = 27$

germanica) leaf extract showed increased growth performance and feed conversion ratio (Hoseinifar et al. 2017).

This study's results showed that growth parameters were dose-dependent on using PSE in fish diets. Similarly, previous studies showed that herbal extracts affect fish growth in a dose-dependent manner. In general, growth improvement happens by administering the optimum level of dietary extract and thereafter it decreases by increasing extract inclusion levels (Gabriel et al. 2019). For instance, based on the second-order polynomial regression model, a dietary caraway inclusion level of 12.5 g kg^{-1} diet was found most suitable to support maximum growth in Nile tilapia (Ahmad and Tawwab 2011). Samad et al. 2014 showed that supplemented diet with 1% of ethanolic katuk extract (*Sauropus androgynous*) stimulated appetite, growth, and improved food utilization (lower feed conversion ratio) in grouper *Ephinephelus coioides*. However, *E. coioides* fed with enriched diets with higher percentages of katuk extract (2.5 and 5%) presented lower growth (Samad et al. 2014). Weak or neutral effects of herbal extracts on fish growth, which are mostly observed at higher inclusion levels, seem to be in terms of high concentrations of anti-nutrients, toxic compounds, and allergic reactions (Irkin et al. 2015; Yilmaz and Ergün 2018). These results indicate the importance of the appropriate dose to obtain the desired effects, and therefore the need for further studies to chemically identify the extracts to quantify the active molecules and create sufficient doses in diets.

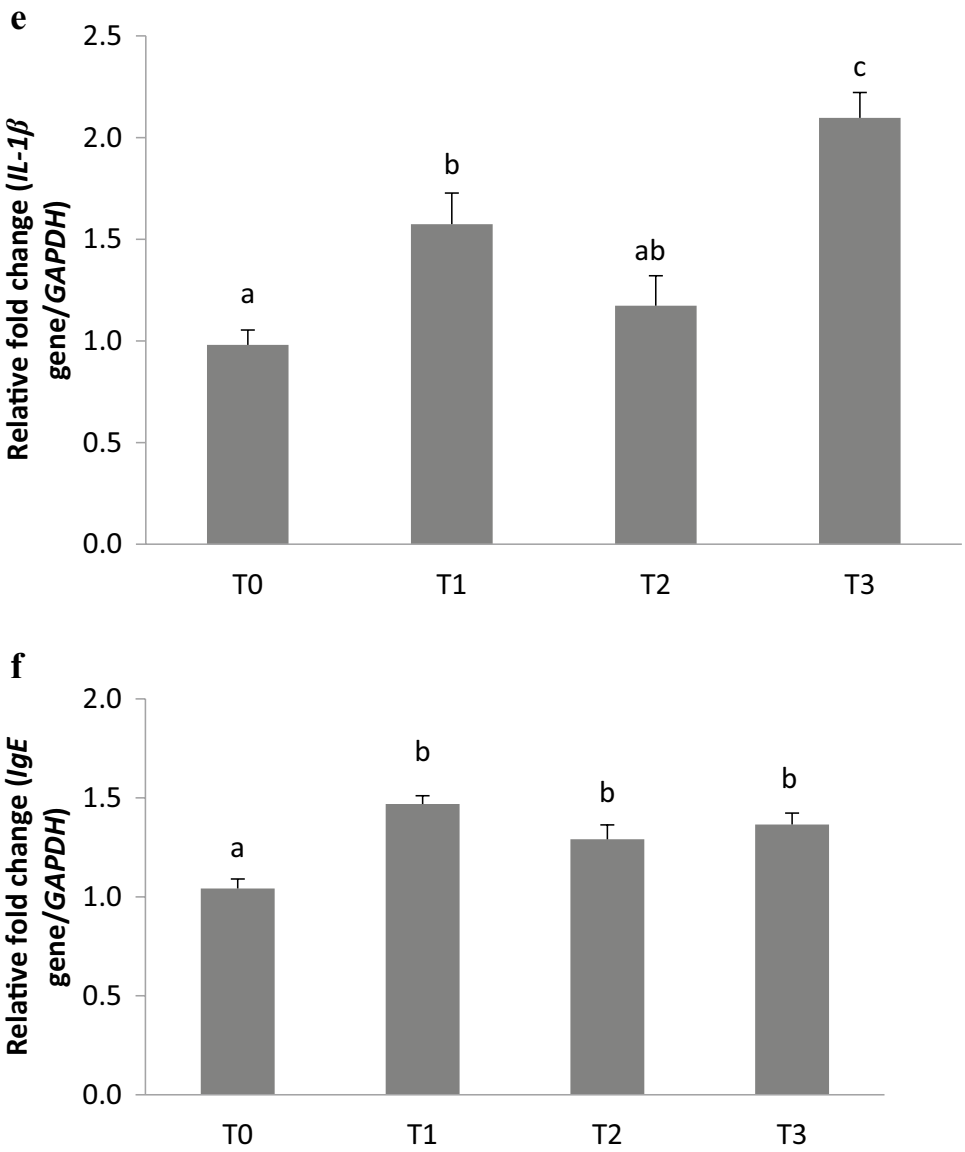
Lysozyme, total immunoglobulin, and total protein are a few of the immunological measures that are often used as markers to evaluate the impact of feed additives in the diets of aquatic animal models (Awad and Awaad 2017). The most important function of lysozyme, a non-specific immune parameter, is to counteract the harmful effects of

Fig. 2 (continued)



pathogenic microorganisms (Oliver and Wells 2015). Total Immunoglobulin, as an indicator of innate and acquired immunity of fish with complement activation, is involved in the lysis and opsonization of pathogens (Mashoof and Criscitiello 2016). Moreover, it was shown that total protein concentration indirectly reflects the innate immune status of fish, and its variations compared to baseline are used as an indicator in assessing health status or stress in different fish species (Peyghan et al. 2014). The modulation of the specific immune system by medicinal plants via augmenting the protease inhibitors and lytic enzymes of immune cells and molecules was proved in fish species (Awad and Awaad 2017). All above-mentioned immune parameters were improved in fish-fed diets containing PSE compared to the control group. Similar results were observed in common carp-fed diets supplemented with *Phoenix dactylifera* (Hoseinifar et al. 2015), *Achillea wilhelmsii* (Adel et al. 2016), and *Lawsonia inermis* (Soltanian and Fereidouni 2016) extracts. The seed of psyllium is a rich source of iridoid glucosides, polysaccharides, flavonoids, hydroxycinnamic acids, alkaloids, terpenoids, terpenes, etc. (Madgulkar et al. 2015; Ziemichód et al. 2019). These compounds might act as modulators of active sites and receptors in the fish's innate immune system and activate immune-related signaling pathways to enhance

Fig. 2 (continued)



humoral and cellular immune parameters (Stratev et al. 2018; Ahmadifar et al. 2021).

Fish gills, the most important organ of gas exchange, have several other roles, such as ion and osmotic regulation, acid–base balance, and hormone production. This organ plays a pivotal role in the excretion of ammonia and the immune system of fish (Secombes and Wang 2012). For this purpose, fish gill tissue was used to examine the expression of different genes after the ammonia stress test. The immune genes indicated above are among the most significant indicators used to assess the health state of fish species. The results demonstrated that after exposure to ammonia, PSE significantly enhanced the transcription of these immune-related genes as compared to the control group. It was shown that herbal medicines and their bioactive compounds can mitigate/inhibit the toxic effects of water pollutants by activating the immune/inflammatory signaling pathway (Ahmadifar et al. 2021). For example, Pre-treatment with 0.5% myrcene could significantly inhibit the down-regulation of immune-related genes (*Hsp70, TNF-a, IL-1β*) induced by water-born copper sulfate in the gill of common carp. In another study, negative effects of ammonia exposure on transcription of *TNFa*, *IL-1β*, *IL-8*, and *Hsp70* genes were attenuated in fish fed with Roselle, *Hibiscus sabdariffa* supplemented diets (Yousefi et al. 2021a). In our study, *IL-1β and TNF-α* were upregulated in T1 treatment compared to the control group, while *IL-10* gene, an anti-inflammatory cytokine were down-regulated in this group. Similarly, this reverse trend was observed between proinflammatory and anti-inflammatory cytokines in other studies (Wang et al. 2015a, b). The optimal dose of herbal medicines and their derivatives was shown to successfully regulate the immune/inflammatory

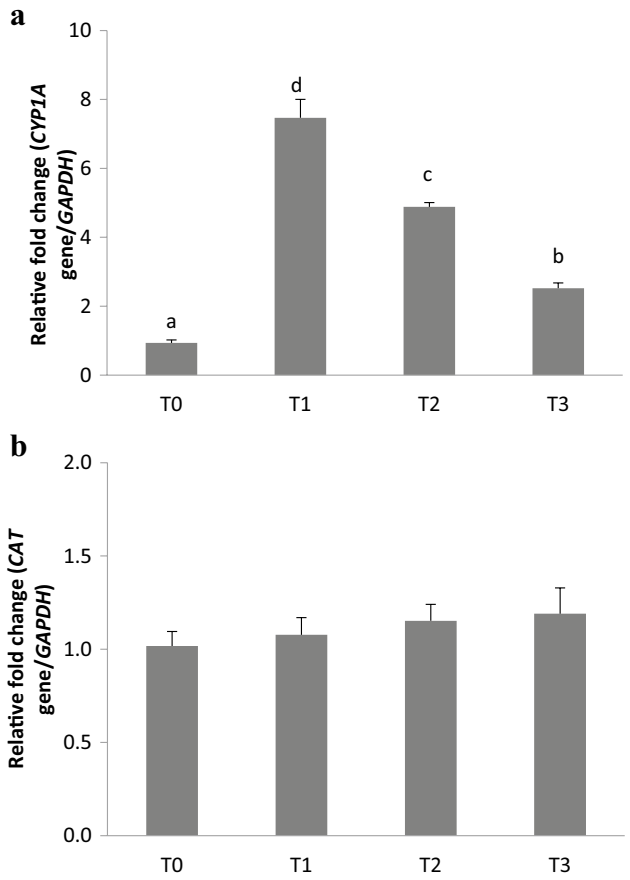


Fig. 3 Effects of dietary administration of PSE and exposure with ammonia on carp antioxidant-related genes, including cytochrome P4501A (*CYP1A*) (3.a), superoxide dismutase (*SOD*) (3.b), catalase (*CAT*) (3.c), glutathione peroxidase (*GPX*) (3.d). Values show means ± SE. Bars with different letters were significantly different ($P < 0.05$). Fish grouping are as mentioned in Table 3. For each treatment group $n = 27$

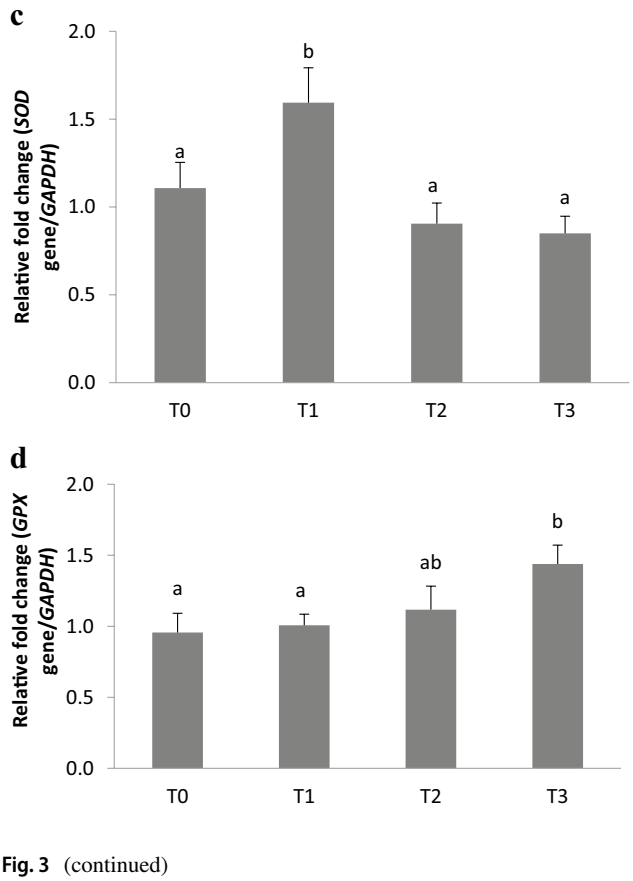


Fig. 3 (continued)

responses of fish by activating the corresponding signaling pathway, but their overdose may have a negative effect (Ming et al. 2020; Ahmadifar et al. 2021). Therefore, it seems that the trend of regulatory changes in gene expression in T1 is more logical than other experimental groups, and can provide a more accurate interpretation of the effect of PSE to reduce the negative effects of ammonia exposure.

It was shown that antioxidant agents, such as medicinal herbs can engage the antioxidant-associated signaling pathway such as *Nrf2* (nuclear factor erythroid 2 [NF-E2]-related factor 2) (Tsai et al. 2011; Ka et al. 2015). *Nrf2,* as a pivotal signaling pathway in regulating oxidative stress and antioxidant genes expression, can inhibit the activation of *NLRP3* (NOD-like receptor pyrin domain-containing-3) and *NF-κB* (nuclear factor kappa light chain enhancer of activated B cells) pathways by suppressing reactive oxygen species (ROS) production (Saha et al. 2020). *NLRP3* and *NF-κB* are pro-inflammatory pathways which cause damage to cells (Liu et al. 2017). In one study, isoliquiritigenin (a flavonoid compound) successfully suppressed *NLRP3* and *NF-κB* pathways by inhibiting ROS and engaging the *Nrf2*/antioxidant reaction (ARE) signaling pathway, leading to reduced lipopolysaccharide-induced lung damage in mice (Liu et al. 2017). Our results showed that PSE could successfully upregulate the transcription of most antioxidant-related genes (except *CAT*) compared to the control group after exposure to ammonia. Exposure to ammonia suppresses the expression of antioxidant-related genes which are dangerous to fish health and can lead to disease and death (Hoseini et al. 2020). However, the improvement of these genes by PSE can help the fish overcome this condition. Similarly, Hoseini et al. (2020) reported that dietary myrcene could markedly improve the down-regulation of antioxidant-related genes (*SOD, CAT, GPX, GST, GR*) induced by exposure to copper sulfate in common carp (Hoseini et al. 2020). Future research is proposed to evaluate the mRNA expression of *NRF2*, *NLRP3,* and other related genes to show the above mechanism in the antioxidant system of fish fed with PSE.

The establishment and growth of the epithelium, the preservation of tissue integrity, and the control of TJ protein permeability are the most significant functions of tight junction (TJ) proteins in vertebrates (Kolosov et al. 2014).Based on the results, PSE was somehow useful in counteracting

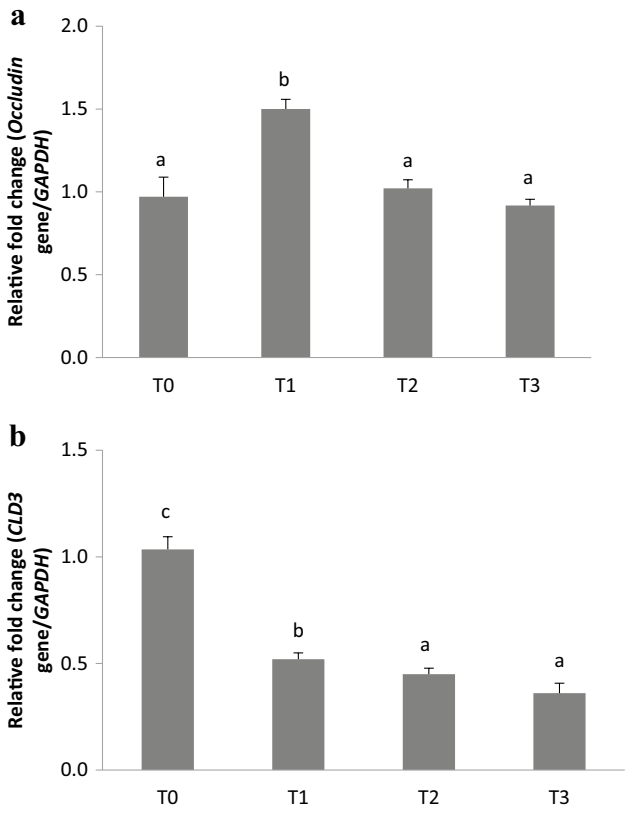


Fig. 4 Effects of dietary administration of PSE and exposure with ammonia on carp tight junction-associated genes, including *occludin* (4.a) and claudin 3 (*CLD3*) (4.b). Values show means ± SE. Bars with different letters were significantly different ($P < 0.05$). Fish grouping are as mentioned in Table 3. For each treatment group $n = 27$

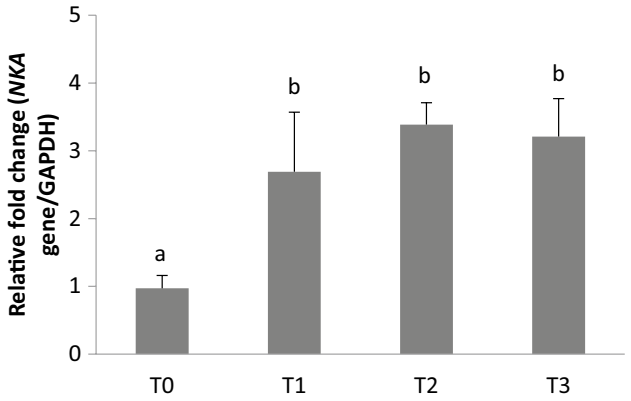


Fig. 5 Effects of dietary administration of PSE and exposure with ammonia on carp osmoregulation- associated gene, $Na^+/K.^+$- ATPase (*NKA*). Values show means ± SE. Bars with different letters were significantly different ($P < 0.05$). Fish grouping are as mentioned in Table 3. For each treatment group $n = 27$

the negative effects of ammonia exposure on TJ proteins, characterized by alleviated down-regulation in *occludin* mRNA expression levels. In a previous study, a myrcene supplemented diet showed to be beneficial for reducing the suppressive effects of copper sulfate on mRNA expression levels of *cld7* in common carp gill. However, it failed to restore gene expression of other TJ proteins (*occludin*, *cld3*) to pre-stress levels (Hoseini et al. 2020). Moreover, *Mangifera indica* Seed Kernel Extract has been shown to prevent intestinal barrier dysfunction in high-fat diet-Induced obese mice, characterized by restoring the expression of TJ protein *zonula occludens-1* (ZO-1) and *claudin-1* (Mujawdiya et al. 2020).

Previous studies showed that environmental toxins (Wang et al. 2015a, b) and hypersalinity stress (Zhu et al. 2018) can impair Na + /K + -ATPase activity in fish. While, antioxidant agents such as medicinal herbs can protect membrane enzymes and enhanced their related gene expression levels against oxidative stress caused by environmental factors (Dawood 2021). Therefore, because fish fed with PSE showed higher levels of *NKA* gene expression than the control group, it can be concluded that this medicinal plant acts as a useful antioxidant in inappropriate rearing conditions. The potential mechanism is the attenuating effects of PSE on the *Nrf2* (discussed above) and other related signaling pathways. A future study is proposed to prove this theory.

Conclusion

In conclusion, our results showed that PSE is not only a growth promotor but also an immunostimulant in common carp exposed to ammonia via improving serum immunity parameters and the expression of the immune/antioxidant-related gene. Thus, based on obtained results, it seems that the diet containing PSE (especially in 0.25% of the diet) is suitable for use in aquaculture to increase growth efficiency and strengthen the immunity of fish during ammonia exposure.

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Authors' contributions Ehsan Ahmadifar: Data curation, writing; Naser Kalhor: Data curation, gene expression analysis; Morteza Yusefi: Writing and Reviewing and editing; Hossein Adineh: Data curation, Writing, Review and Editing; Mohsen Shahriari Moghadam: formal analysis, methodology; Najmeh Sheikhzadeh: Reviewing and editing; Tossapol Moonmanee: editing; Seyed Hossein Hoseinifar: Reviewing and editing; Hien Van Doan: Reviewing and editing.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable

Declarations

Ethics approval All experiments were performed following the protocol approved by the committee of ethics of the faculty of sciences of the University of Tehran (357; 8 November 2000).

Consent to participate Authors, have permission to participate.

Consent for publication Authors have permission for publication.

Conflicts of interest/Competing interests The authors have no relevant financial or non-financial interests to disclose and no conflicts of interest to declare that are relevant to the content of this article.

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