Received: 25 February 2020 | Revised: 14 August 2020 | Accepted: 21 September 2020

DOI: 10.1111/jwas.12750

APPLIED STUDIES

Journal of the World Aquaculture Society Open Access | WORLD AQUACULTURE Society | WILEY

Lemon, *Citrus aurantifolia*, peel and *Bacillus licheniformis* protected common carp, *Cyprinus carpio*, from *Aeromonas hydrophila* infection by improving the humoral and skin mucosal immunity, and antioxidative responses

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Funding information
University of Zabol, Grant/Award Number: UOZ-GR-9618-104

Abstract

The role of dietary lemon peel (LM) and/or *Bacillus licheniformis* (BL) on the growth, immunity, and resistance against *Aeromonas hydrophila* in common carp, *Cyprinus carpio* was investigated in this study. LM and BL were included in diets at 0% (T0), 10^8 CFU/g BL (T1), 1.5% LM and 10^8 CFU/g BL (T2), and 3% LM and 10^8 CFU/g BL (T3). Fish fed with T1, T2, or T3 had higher weight gain, specific growth rate, white blood cells count, and blood total protein with lower feed conversion ratio than T0 group ($p < .05$). The albumin increased significantly ($p < .05$) in fish fed both BL and LM (T3). The serum superoxide dismutase (SOD), catalase, lysozyme, and bactericidal activities were significantly increased in fish fed both BL and LM (T2 and T3), while serum glutathione peroxidase increased in fish fed BL (T2) ($p < .05$). Fish fed T1, T2, and T3 diets displayed higher SOD and lower malondialdehyde than fish fed T0 ($p < .05$). After the *A. hydrophila* challenge, the mortality rate was significantly lower in T1, T2, and T3 groups than the T0 group ($p < .05$). The obtained results revealed that LM and BL could be used to increase resistance against *A. hydrophila* infection in

carp. However, further field studies should be performed to confirm the obtained results.

KEYWORDS

Bacillus licheniformis, common carp, immunity, lemon peel, oxidative status

1 | INTRODUCTION

Common carp, *Cyprinus carpio* is a freshwater fish that can be cultured under diverse environmental conditions (Dawood, Eweedah, et al., 2020). However, fish cultured in intensive and semi-intensive systems can be easily infected with pathogenic bacteria (Ahmadifar, Moghadam, Dawood, & Hoseinifar, 2019). Antibiotics are usually used to deal with infectious diseases in aquaculture (Liu, Steele, & Meng, 2017). Nonetheless, the excessive use of chemotherapies is associated with drug-resistant properties, environmental pollution, and the deterioration of the microbial biodiversity (Akanmu, 2018). As a result, one of the main challenges in aquaculture is to improve the immunity of fish via eco-friendly immunostimulants to maximize resistance against infectious diseases and at the same time minimize the use of antibiotics and also decrease economic losses (Dawood, Koshio, & Esteban, 2018). Among biomedical additives, the application of dietary supplements such as vitamins (Jakab Sándor, Bor Papp, Ardó, Nagy Biro, & Jeney, 2018), probiotics (Dawood & Koshio, 2016; Van Doan et al., 2019), prebiotics (Dawood, Koshio, Ishikawa, Yokoyama, et al., 2017), and herbal plants (Moustafa et al., 2020).

Lemon, *Citrus aurantifolia* is a polyembryonic plant cultivated in many countries all over the world (Enejoh et al., 2015). This plant has shown antibacterial, anthelmintic, antifungal, antiaflatoxigenic, anticancer, immunomodulatory, antiobesity, antihypertensive, and anti-osteoporosis properties when applied in vivo and in vitro (García Beltrán et al., 2019; Pathirana, Wimalasena, De Silva, Hossain, & Heo, 2018). Studies conducted previously have shown that *C. aurantifolia* contains carbohydrates, sugar, soluble and insoluble fiber, sodium, vitamins (vitamin C), minerals, fatty acids, amino acids, saponins, alkaloids, tannins, phenolics, flavonoids, and terpenoids (Akinsete & Odeniyi, 2016; Al Namani et al., 2018). Recently, the ameliorative effects of lemon peel were reported in aquaculture (Baba, Acar, Öntaş, Kesbiç, & Yılmaz, 2016; Beltrán, Espinosa, Guardiola, & Esteban, 2017; García Beltrán et al., 2019; Laein, Salari, Shahsavani, & Baghshani, 2018; Ngugi, Oyoo-Okoth, & Muchiri, 2017; Toutou, Soliman, Elokaby, & Ahmed, 2018).

Application of probiotics as natural immunostimulants and growth promoters is widely accepted in aquaculture (Dawood et al., 2016). *Bacillus* spp. are mostly used in aquaculture due to their highly resistant to environmental stressors, long-lasting shelf life, and functionality (Dawood, Koshio, Abdel-Daim, & Van Doan, 2019; Zaineldin et al., 2018). *Bacillus licheniformis* belongs to the *Bacillus* genus and can improve the performances of aquatic animals (Chen, Lu, Niu, Wang, & Zhang, 2015; Gupta, Gupta, & Dhawan, 2014).

The combination of plant ingredients and probiotics has shown synergistic effects on immunity and infectious diseases resistance in common carp, *C. carpio* (Harikrishnan, Balasundaram, & Heo, 2010), rock bream, *Oplegnathus fasciatus* (Harikrishnan, Kim, Kim, Balasundaram, & Heo, 2011), and Olive flounder, *Paralichthys olivaceus*. In the present study, we have investigated the potential role of *B. licheniformis* and lemon peel on the innate immunity parameters as well as protection against *Aeromonas hydrophila* infection in common carp.

2 | MATERIALS AND METHODS

2.1 | Sources of feed additives and the preparation of the experimental diets

Lemon peel, *C. aurantifolia* dry powdered material was purchased from the local market (Birjand province, Iran). *B. licheniformis* bacteria (IBRC-M 10204, Iranian Biological Center) was cultured for 24 hr at 30°C, then centrifuged

(2,000*g* for 20 min) and suspended in phosphate-buffered saline (PBS). The dose of *B. licheniformis* (10^8 CFU/g) was selected according to Gobi et al. (2018).

Four experimental diets were prepared by incorporating lemon peel and *B. licheniformis* in the basal diet (Table 1) at 0% lemon peel and 0 CFU/g *B. licheniformis* (T0), 10^8 CFU/g *B. licheniformis* (T1), 1.5% lemon peel and 10^8 CFU/g *B. licheniformis* (T2), and 3% lemon peel and 10^8 CFU/g *B. licheniformis* (T3). The ingredients were blended thoroughly in a mixer in the presence of sterilized water and plant oil and then pelleted using a meat grinder. The pellets were then air-dried and kept in sealed bags at 4°C until use. The proximate composition of diets was confirmed according to Association of Official Analytical Chemists (AOAC) standard protocols (AOAC, 1998).

2.2 | Experimental procedure

Common carp (45.67 ± 3.32 g) purchased from a local farm (Zahak, Sistan and Baluchestan, Iran) were transferred to University of Zabol. After arrival, fish were bathed with sodium chloride (2%) for 10 min (García-Magaña, Rodríguez-Santiago, Grano-Maldonado, Jiménez-Vasconcelos, & Guerra-Santos, 2019) and kept in two 1,000 L tanks for 10-day acclimation. Fish were randomly allocated into 12 fiberglass tanks (100 L) with an initial density of 20 common carp per tank (four groups, triplicates). During the experiment, thewater quality parameters, namely temperature, dissolved oxygen and pH were 24.20 ± 2.13°C; 6.6 ± 0.5 mg/L, and 7.3 ± 0.54, respectively. Half of the water in each tank was daily replaced with fresh dechlorinated water. Fish were fed the prepared diets to satiation by hand three times a day (9:00 a.m.; 12:00 p.m.; 3:00 p.m.). After 8 weeks, fish were starved for 24 hr, weighed and counted for growth performance, survival calculation, and blood collection.

2.3 | Blood and mucus sampling

Five fish per tank were randomly selected and anesthetized by clove solution to collect blood (2 ml) from the caudal vein with a nonheparinized syringe. Half of the collected blood was relocated in microtube containing heparin as anti-coagulant for hematological examination, while the second half was kept in microtube without heparin for 2 hr for collection of serum. Sera were separated by centrifugation at 1,500*g* for 20 min and stored at −20°C until use.

TABLE 1 Formulation and proximate composition of the different experimental diets (% 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		
Binder[b]	1		
Anti fungal[c]	1		

[a]Permix detailed by Hoseinifar, Zoheiri, and Caipang (2016).
[b]Amet binder™, Mehr Taban-e-Yazd, Iran.
[c]ToxiBan antifungal (Vet-A-Mix, Shenan-doah, IA).

The mucus samples were collected from five fish per tank following the method described by Balasubramanian et al. (2013). The collected mucus was thoroughly mixed with an equal quantity of sterilized tris-buffered saline (50 mM Tris HCl, pH 8.0, 150 mM NaCl) and centrifuged at 30,000*g* at 4°C for 15 min (Beckman coulter, Avanti J-26 XPI, Brea, CA). The supernatant was then collected and filtered with Whatman no.1 filter paper. The filtrate was collected and kept frozen at −80°C until use.

2.4 | Hematological and biochemical assays

The red blood cell (RBC) and white blood cell (WBC) counts were determined using a Neubauer hemocytometer. Hematocrit (Hct) was measured using the standard microhematocrit method and reported in percentage. Hemoglobin levels (Hb) were obtained by the cyanomethemoglobin spectrophotometry method (Blaxhall & Daisley, 1973). The blood indices, including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Wintrobe (2008). To estimate the differential leukocyte counts (lymphocytes and neutrophils), the blood smears were prepared, air-dried, fixed in methanol, and stained using May-Grunwald-Giemsa solution (Sepperumal & Saminathan, 2013).

Blood total protein, albumin, triglyceride, cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were assessed using commercial kits (Pars Azmoon, Tehran, Iran) and a biochemical autoanalyzer instrument (Eurolyser, Belgium) (Binaii et al., 2014). The globulin content was calculated by deducting the albumin from the total blood protein.

2.5 | Immunological assays

Serum lysozyme activity was measured using a modified turbidimetric method described by Demers and Bayne (1997). The lysozyme activity for each sample was determined compared to the standard and expressed as μg/ml serum samples.

In vitro bactericidal activity of mucus, samples as examined against *A. hydrophila* (JF313402). *A. hydrophila* was briefly cultured in tryptic soy broth (TSB) and then incubated at 25°C for 24 hr and finally adjusted to 1×10^6 CFU/ml. Optical density of the suspension was adjusted to 0.5 at 546 nm (Rao, Das, Jyotyrmayee, & Chakrabarti, 2006). Bacterial suspension was serially diluted (1:10) five times with PBS. Mucus bactericidal activity was determined by incubating 2 μl of the diluted *A. hydrophila* suspension with 20 μl of mucus in a micro-vial for 1 hr at 37°C. For the control group, mucus was replaced with the PBS. The number of viable bacteria was determined by counting the colonies grown on a nutrient agar plate for 24 hr at 37°C (Rao et al., 2006).

2.6 | Antioxidant assays

Glutathione peroxidase activity (GPX) was analyzed using NADPH oxidation protocol (Greenwald, 2018). Superoxide dismutase (SOD) was measured using SOD Assay Kit (Sigma-Aldrich, 19160) according to the manufacturer's instructions (Shahkar et al., 2015). Catalase (CAT) assay was performed using the spectrophotometric determination of hydrogen peroxide (H_2O_2), which forms a stable complex with ammonium molybdate that absorbs at 405 nm (Goth, 1991). Malondialdehyde (MDA) activity was determined using Buege and Aust (1988) method.

2.7 | Challenge test

A. hydrophila (ATCC 7966) was used for experimental infection as described earlier (Soltanian & Fereidouni, 2016). Stock cultures were maintained at −70°C in a suspension of TSB containing 15% glycerol. For the preparation of bacteria for challenge test, *A. hydrophila* from stock was cultured for 24 hr at 25°C in TSB. The cells were centrifuged (3,000*g* for 15 min) and washed three times with sterile PBS (pH 7.2). The bacterial suspension was adjusted to an optical density of 0.5 at 540 nm, which corresponded to approximately 10^8 CFU/ml.

After 8 weeks of feeding with the experimental diets, 10 fish were randomly sampled from each experimental unit and injected with *A. hydrophila* (10^8 CFU) via intraperitoneal route as described by Hoseinifar et al. (2016). The dead fish from each tank were counted and removed daily. The mortality (%) of fish in each treatment was calculated after 14 days post-challenge as follows: the mortality (%) = (number of dead fish/total number of fish) × 100.

2.8 | Statistical analysis

The data were analyzed using the SPSS software version no. 21 (SPSS Inc., Chicago, IL). The statistical analysis was done using a one-way analysis of variance followed by Duncan's multiple range tests. *p*-value of <.05 was considered significant.

3 | RESULTS

3.1 | Growth performance

The final body weight, weight gain, and specific growth rate of carps fed *B. licheniformis* (T1) or both lemon peel, and *B. licheniformis* (T2 and T3) showed higher values than T0 ($p < .05$) (Table 2). Final body length was significantly higher in fish fed both *B. licheniformis* and lemon peel at 3% (T3) ($p < .05$). Fish fed *B. licheniformis* (T1) or both lemon peel and *B. licheniformis* (T2 and T3) showed lower feed conversion ratio than T0 group ($p < .05$) (Table 2).

3.2 | Blood hematology and biochemistry

The obtained blood hematological results (RBCs, WBCs, Hb, Hct, MCV, MCH, MCHC, lymphocyte, and neutrophil count) tended to be within the normal values for common carp without significant differences among the groups ($p > .05$) (Table 3). The WBCs increased significantly in fish fed both lemon peel and *B. licheniformis* (T2 and T3) when compared to the control. Fish fed T2 diet displayed non-significant differences with fish fed *B. licheniformis* only (T1) ($p > .05$).

The total serum protein of carps fed *B. licheniformis* (T1) or both lemon peel, and *B. licheniformis* (T2 and T3) showed higher values than the T0 group ($p < .05$) (Table 4). While the measured blood albumin increased significantly ($p < .05$) in fish fed both *B. licheniformis* and 3% lemon peel (T3) when compared to the control and T1 groups without differences with fish fed T2 diet. The levels of globulin, cholesterol, triglycerides, ALT, AST, and ALP were not significantly influenced by dietary *B. licheniformis* (T1) or both lemon peel and *B. licheniformis* (T2 and T3) ($p > .05$).

TABLE 2 Growth performance and feed utilization of fish fed the test diets for 8 weeks

Item	T0	T1	T2	T3
Initial weight (g)	46.33 ± 0.50	46.73 ± 0.70	47.27 ± 1.03	46.80 ± 0.60
Final weight (g)	66.80 ± 0.53a	70.93 ± 1.53b	71.87 ± 0.50b	74.27 ± 0.50c
Initial length (cm)	13.77 ± 0.60	13.85 ± 0.43	13.87 ± 0.55	13.69 ± 0.62
Final length (cm)	15.55 ± 0.21a	15.80 ± 0.39a	15.94 ± 0.24a	16.66 ± 0.22b
Weight gain (g)[a]	20.47 ± 0.61a	24.20 ± 1.83b	24.60 ± 1.39b	27.47 ± 0.92c
Specific growth rate[b]	0.65 ± 0.03a	0.75 ± 0.06b	0.76 ± 0.03bc	0.83 ± 0.03c
Feed conversion ratio[c]	2.88 ± 0.09c	2.45 ± 0.19b	2.40 ± 0.14b	2.15 ± 0.07a
Survival rate (%)[d]	100	100	100	100

Note: Values expressed as means ± *SE*. Different letters indicate significant differences for each pairwise comparison between treatments.

[a]Weight gain = W2 (g) − W1 (g).

[b]Specific growth rate = 100 (Ln W2 − Ln W1)/T.

[c]Feed conversion ratio = feed intake (g)/weight gain (g).

[d]Survival rate (%) = (final amount of fish/initial amount of fish) × 100. Where W1 is the initial weight, W2 is the final weight and T is the number of days in the feeding period.

TABLE 3 Blood hematology of fish fed the test diets for 8 weeks

	T0	T1	T2	T3
RBC ($\times 10^5$/μl)	1.18 ± 0.10	1.16 ± 0.04	1.25 ± 0.09	1.20 ± 0.07
WBC ($\times 10^3$/μl)	7.83 ± 0.2a	8.83 ± 2.02ab	9.4 ± 0.2b	10.6 ± 5.86c
Hb (g/dl)	4.60 ± 0.36	4.43 ± 0.31	4.97 ± 0.61	4.67 ± 0.58
Hct (%)	24.00 ± 1	25.00 ± 0.58	24.67 ± 0.58	25.33 ± 1.53
MCV (fl)	214.60 ± 1.15	215.40 ± 4.31	214.93 ± 3.54	220.26 ± 2.01
MCH (pg)	39.40 ± 1.31	39.50 ± 1.73	39.53 ± 2.97	41.43 ± 1.47
MCHC (g/dl)	17.10 ± 1.15	18.36 ± 1.02	18.33 ± 1.09	18.83 ± 1.16
Lymphocyte (%)	98.66 ± 0.23	94.33 ± 0.41	94.00 ± 0.57	91.00 ± 0.58
Neutrophil (%)	1.33 ± 0.33	5.66 ± 0.28	6.00 ± 0.57	9.00 ± 0.58

Note: Values show means ± *SE* of three replicates. Values in each column with different letters were significantly different ($p < .05$).

Abbreviations: Hb, hemoglobin; Hct, hematocrit; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RBC, red blood cell; WBC, white blood cell.

3.3 | Immunity

The lysozyme activity of fish fed *B. licheniformis* (T1) or both lemon peel and *B. licheniformis* (T2 and T3) showed higher values than T0 ($p < .05$) (Figure 1).

The number of bacterial colonies incubated with the mucus samples of fish fed T1, T2, and T3 diets were lower than the control group, with the lowest values in T2 and T3 groups ($p < .05$) (Figure 2). The mucus bactericidal activity of fish fed *B. licheniformis* (T1) or both lemon peel and *B. licheniformis* (T2 and T3) showed higher values than the T0 group ($p < .05$) (Figure 2). Interestingly, the highest activity was observed in fish fed *B. licheniformis* and lemon peel at 1.5 or 3% (T2 and T3).

TABLE 4 Blood biochemistry of fish fed the test diets for 8 weeks

	T0	T1	T2	T3
Total protein (g/dl)	2.53 ± 0.12a	3.06 ± 0.38b	3.47 ± 0.22bc	4.10 ± 0.23c
Albumin (g/dl)	1.10 ± 1.06a	1.13 ± 0.03a	1.47 ± 0.17ab	1.93 ± 0.28b
Globulin (g/dl)	1.43 ± 0.52	1.93 ± 0.24	2 ± 0.71	2.17 ± 0.39
Cholesterol (mg/dl)	228.50 ± 20.61	238.87 ± 11.13	217.00 ± 11.53	244.17 ± 8.34
Triglyceride (mg/dl)	101.40 ± 23.25	95.10 ± 29.70	73.40 ± 16.93	83.30 ± 11.22
AST (IU/L)	140.50 ± 15.94	121.90 ± 11.20	176.63 ± 39.81	177.53 ± 19.33
ALP (IU/L)	209.10 ± 15.46	236.87 ± 13.28	216.67 ± 12.88	242.83 ± 16.95
ALT (IU/L)	15.00 ± 5.61	15.90 ± 5.02	10.90 ± 3.43	16.00 ± 2.42

Note: Values show means ± *SE* of three replicates. Values in each column with different letters were significantly different ($p < .05$).
Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

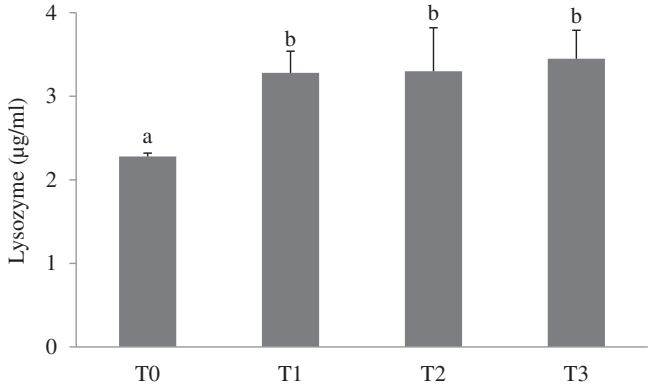

FIGURE 1 Serum lysozyme activity of fish fed test diets for 8 weeks. Values are expressed as mean ± *SE*from triplicate groups. Bars with different letters are significantly different from those of control group ($p < .05$)

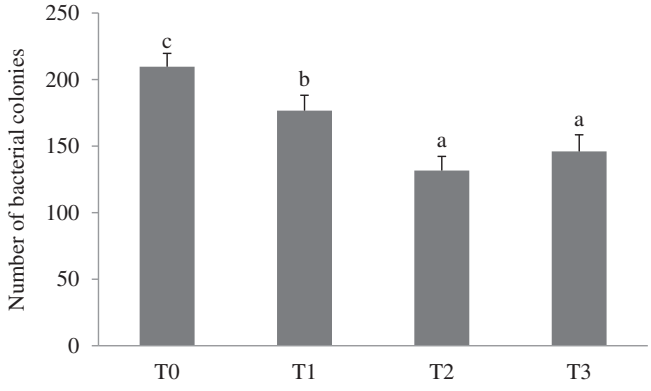

FIGURE 2 Mucus bactericidal activity of fish fed test diets for 8 weeks. Values are expressed as mean ± *SE*from triplicate groups. Bars with different letters are significantly different from those of control group ($p < .05$)

3.4 | Antioxidative related enzymes

Catalase activity was significantly increased in fish fed both *B. licheniformis* and lemon peel at 1.5 or 3% (T2 and T3) ($p < .05$) (Figure 3). The GPX increased significantly in fish fed both *B. licheniformis* and lemon peel at 1.5% (T2) ($p < .05$) (Figure 4). The SOD of fish fed *B. licheniformis* (T1) or both lemon peel and *B. licheniformis* (T2 and T3)

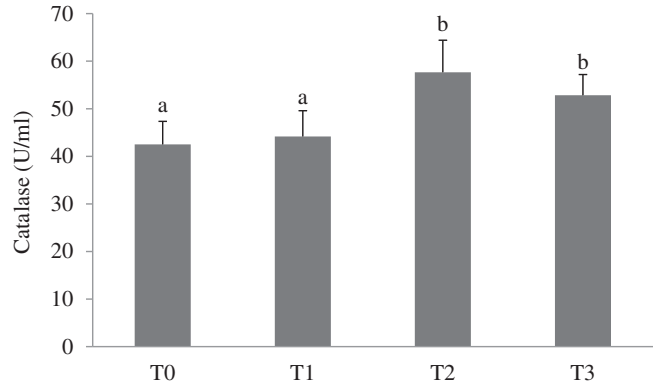

FIGURE 3 Serum catalase activity (CAT) of fish fed test diets for 8 weeks. Values are expressed as mean ± *SE*from triplicate groups. Bars with different letters are significantly different from those of control group ($p < .05$)

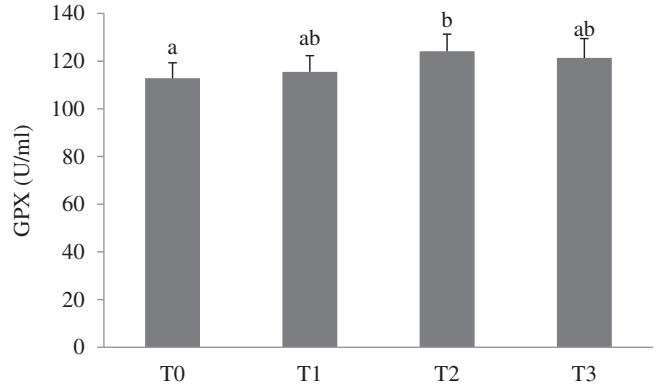

FIGURE 4 Serum glutathione peroxidase activity (GPX) of fish fed test diets for 8 weeks. Values are expressed as mean ± *SE*from triplicate groups. Bars with different letters are significantly different from those of control group ($p < .05$)

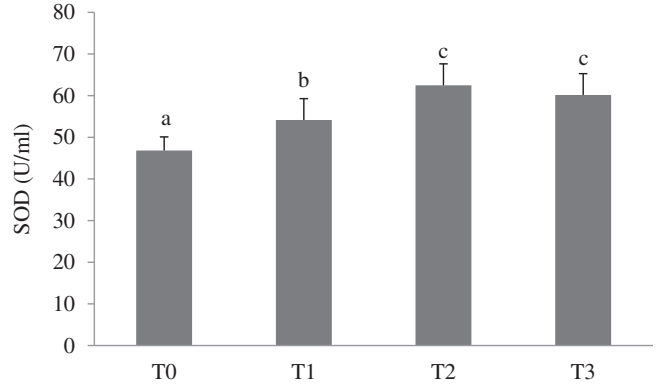

FIGURE 5 Serum superoxide dismutase activity (SOD) of fish fed test diets for 8 weeks. Values are expressed as mean ± *SE*from triplicate groups. Bars with different letters are significantly different from those of control group ($p < .05$)

showed significantly higher values than T0 group ($p < .05$) (Figure 5). On the other hand, the blood MDA of fish fed *B. licheniformis* (T1) or both lemon peel and *B. licheniformis* (T2 and T3) showed lower values than T0 group ($p < .05$) (Figure 6). Interestingly, the highest SOD and the lowest MDA were noticed in fish fed both *B. licheniformis* and lemon peel at 1.5 or 3% (T2 and T3).

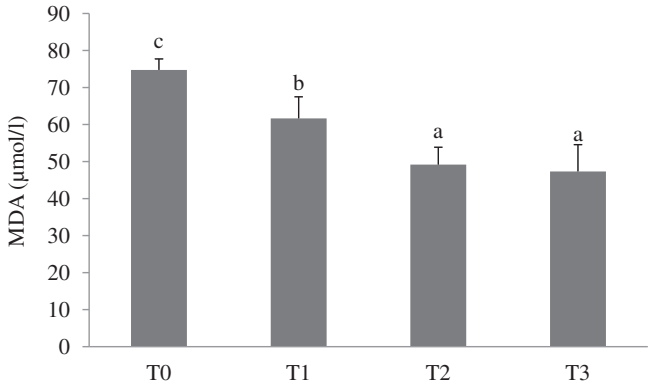

FIGURE 6 Serum malondialdehyde activity (MDA) of fish fed test diets for 8 weeks. Values are expressed as mean ± *SE*from triplicate groups. Bars with different letters are significantly different from those of control group ($p < .05$)

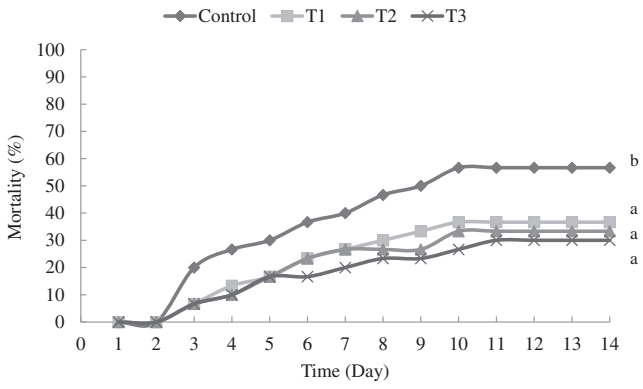

FIGURE 7 Mortality (%) of common carp challenged with*Aeromonas hydrophila*after feedingontest diets for 8 weeks. The mortality rate in the supplemented groups was significantly lower ($p < .05$) than that in the control group. Different letters are significantly different from those of control group ($p < .05$)

3.5 | The challenge against *A. hydrophila* infection

Recording mortalities in the control group started on the second-day post-challenge. After 14 days, the cumulative mortality rate was the lower in fish fed *B. licheniformis* (T1) or both lemon peel and *B. licheniformis* (T2 and T3), while the highest rate was recorded in T0 group fed the basal diet ($p < .05$) (Figure 7).

4 | DISCUSSION

The combined effects of dietary *B. licheniformis* and lemon peel on the growth performance, blood and skin mucus immune responses, antioxidant capacity, and resistance of common carp to *A. hydrophila* were investigated in the current study. The results showed that a fish-fed mixture of *B. licheniformis* and 3% lemon peel resulted in the highest growth performance. This finding could be explained in part by the antimicrobial effect of lemon peel, which suppresses the harmful microorganisms in the intestinal flora and promotes the development of *B. licheniformis*. It is well known that *Bacillus* spp. increases the synthesis of vitamins, cofactors, and enzymatic activity (Han et al., 2015). Similarly, dietary supplementation of lemon peel resulted in significantly improved growth performance of *Labeo victorianus* fingerlings (Ngugi et al., 2017). Beneficial bacteria can secrete a wide range of exoenzymes which help in with digestion through production of extracellular enzymes (e.g., protease, lipase, and carbohydrase), decomposition of indigestible compounds and production of antibacterial compounds and vitamins and have a significant effect on growth and survival rate (Dawood, 2020; Ringø et al., 2020). Thus, the combined addition of *B. licheniformis* with

lemon peel powder might have enhanced the growth of common carp by improving the digestibility and absorption of feed.

Innate-immune responses, serum biochemical, and hematological indices are commonly measured to evaluate the effects of dietary additives on fish health (El Basuini et al., 2020; Sarhadi, Alizadeh, Ahmadifar, Adineh, & Dawood Mahmoud, 2020; Yılmaz, Ergün, & Çelik, 2016). The obtained results also revealed that the count of WBC increased in common carp fed with *B. licheniformis* or a mix of *B. licheniformis* and lemon peel supplemented diets. Comparable with our study, dietary *Bacillus* induced the counts of WBC in Nile tilapia (Hassaan, Soltan, Jarmołowicz, & Abdo, 2018). Basophils, neutrophils, eosinophils, monocytes, and lymphocytes are immune cells present in the blood. Lymphocytes are capable of producing antibodies and also boosting macrophages activity in fish during disease attack (Jalali, Ahmadifar, Sudagar, & Takami, 2009). Additionally, fish have a specific innate-complex defense system, including WBCs, which may become more significant against pathogenic bacteria (Low, Mariana, Maha, Chee, & Fatimah, 2016). Interestingly, the number of lymphocytes was not significantly altered by *B. licheniformis* and/or lemon peel, which can be attributed to the stable health condition and the absence of infection under the current trial conditions. Similar results were obtained in different fish and shellfish species fed diets containing single-use of *B. licheniformis* (Gobi et al., 2018; Zhang et al., 2015).

From the results of the present study, the serum albumin and total protein levels in fish fed *B. licheniformis* and/or lemon peel were significantly higher compared to the control group. It was already confirmed that the total serum protein represents a large part of the serum immunity (Binaii et al., 2014). Similarly, dietary *Bacillus* has been reported to increased the levels of blood total protein, albumin, and globulin values of Nile tilapia (Hassaan et al., 2018). Similar to our finding, total serum protein, and WBC counts have also been increased in Mozambique tilapia fed with diet containing lemon peel (Baba et al., 2016). Ngugi et al. (2017) added 1.0 to 8.0% lemon peel extract to the diets of *L. victorianus* and demonstrated an improvement and increase of hematological values, serum biochemical status, and non-specific immunity Hct ratios, Hb levels, WBC counts, neutrophil percentages, and serum total protein levels. Moreover, the application of dehydrated lemon peel powder to *Sparus aurata* feed at two different rates (1.5 and 3%), resulted in increased leukocyte count (Beltrán et al., 2017).

The liver-related function enzymes (ALT, AST, and ALP) are associated with liver condition, and their high levels indicates liver damage (Ueno & Komatsu, 2017). Interestingly the present study displayed non-significant differences among the groups received *B. licheniformis* and mix of *B. licheniformis* and lemon peel powder supplemented diets, which shows that the tested additives have no negative impact on the liver function. Along with the stable blood total cholesterol and triglycerides values, it can be inferred that dietary *B. licheniformis* and lemon peel powder are recommended to maintain common carp's health condition.

The results of our study using common carp as well showed a rise in the lysozyme activity in fish fed with *B. licheniformis* and mix of *B. licheniformis* and lemon peel powder supplemented diets. Lysozyme activity attacks the peptidoglycans in the cell wall of bacteria and inhibits their severity on the host (Ángeles Esteban, 2012). In the present study, fish fed with both *B. licheniformis* and lemon peel displayed increased lysozyme activity. Similarly, the lysozyme activity showed increased activities when the red sea bream fed *B. subtilis* (Zaineldin et al., 2018).

The secretion of mucus from the fish skin is the first defensive response during environmental stressors and outbreaks (Adel, Dawood, Shafiei, Sakhaie, & Shekarabi, 2020; Dawood, Koshio, Ishikawa, El-Sabagh, et al., 2017). The fish skin mucus contains many immune-related components that protect against pathogens (Ángeles Esteban, 2012). Feeding with probiotics, prebiotics, and immunostimulants in several fish species are known to enhance immunity (Adel, Yeganeh, Dadar, Sakai, & Dawood, 2016; Dawood, Koshio, El-Sabagh, et al., 2017; Dawood, Koshio, et al., 2020; Srichaiyo et al., 2020). The present study revealed that the bactericidal activity of skin mucus of common carp fed both of *B. licheniformis* and lemon peel powder was higher than those of the control fish. The present findings are in line with previous results reported in gilthead seabream fed with lemon peel (Beltrán et al., 2017) and Mozambique tilapia fed with *B. licheniformis* (Gobi et al., 2018). Moreover, findings of the study in common carp showed that feeding fish with *B. licheniformis* or a mix of *B. licheniformis* and lemon peel powder for eight weeks resulted in decreased mortality following *A. hydrophila* infection. Similarly. Gobi et al. (2018) reported that dietary *B.*

licheniformis reduced the mortality rate of Mozambique tilapia challenged with *A. hydrophila*. The efficacy of probiotics in fish or shellfish culture is often attributed to elevated immune responses against pathogens.

In the present study, serum SOD, CAT, and GPX levels were elevated in fish after feeding with *B. licheniformis* or a mix of *B. licheniformis* and lemon peel powder. Previous studies showed that fish fed diets containing single-use of *B. licheniformis* increased the antioxidant enzyme responses (Gobi et al., 2018). Dehydrated lemon peel in the diet increased the activity of the antioxidant enzymes in gilthead seabream (García Beltrán et al., 2019). However, Han et al. (2015) reported that SOD activity was not significantly affected by *B. licheniformis* supplementation. Gilthead seabream fed with dehydrated lemon peel powder incorporated diet at 1.5–3% diet for 30 days revealed no effect on the liver glutathione reductase, SOD, and CAT levels (Beltrán et al., 2017). These different results might be associated with various factors such as experimental conditions, fish species, and feeding duration. In this study, probiotic and lemon peel addition to the diet resulted in the decreased MDA level and the increased antioxidant enzyme activities in serum.

The disease challenges usually applied to test the possibility of using medicinal plant-derived ingredients as an alternative strategy to alleviate the risk of diseases in aquatic animals (Ahmadifar et al., 2019). *A. hydrophila* infection threatens the aquaculture industry by increasing the mortality rate of aquatic animals (Zhou et al., 2019). In the current study, the enhancement of humoral and skin mucus immunity and the antioxidative response confirmed the beneficial role of *B. licheniformis* or/and lemon peel against *A hydrophila* infection in common carp.

5 | CONCLUSION

In the light of the findings obtained in this study, it can be concluded that application of dried lemon peel powder with *B. licheniformis* can effectively improve growth performance, immune responses, and resistance of common carp against *A. hydrophila* infection.

ACKNOWLEDGMENTS

There is no conflict of interest about this article. This publication was supported by the University of Zabol under Grant code: UOZ-GR-9618-104.

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How to cite this article: Sadeghi F, Ahmadifar E, Shahriari Moghadam M, Ghiyasi M, Dawood MAO, Yilmaz S. Lemon, *Citrus aurantifolia*, peel and *Bacillus licheniformis* protected common carp, *Cyprinus carpio*, from *Aeromonas hydrophila* infection by improving the humoral and skin mucosal immunity, and antioxidative responses. *J World Aquac Soc*. 2021;52:124–137. https://doi.org/10.1111/jwas.12750