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Single or combined consumption of resveratrol and the probiotic, *Lactobacillus acidophilus* attenuate the effects of crowding stress on growth, immune characteristics, and antioxidant defense in the common carp, (*Cyprinus carpio*)

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ABSTRACT

In the present study, dietary resveratrol (RE) and *Lactobacillus acidophilus* (LAB) were individually or combined added to the diet of common carp (*Cyprinus carpio*) to protect against crowding stress. Fish (30.16 ± 0.7; Mean ± SE) were randomly allocated to seven groups in three replicates, as follows: T_1: basic food as control, T_2: LAB with a concentration of 1.5×10^7 CFU/g, T_3: LAB with a concentration of 3×10^7 CFU/g, T_4: 300 mg resveratrol/kg, T_5: 600 mg resveratrol/kg, and T_6: 1.5×10^7 CFU/g + 300 mg resveratrol/kg and T_7: 3×10^7 CFU/g + 600 mg resveratrol/kg. After 60 days feeding, the supplemented fish had the highest final body weight (FBW), weight gain (WG), and specific growth rate (SGR), and the lowest feed conversion ratio (FCR) as compared with the control group ($P < 0.05$). The activities of amylase, protease and lipase were noticed markedly higher in fish supplemented with 1.5×10^7 CFU/g + 300 mg resveratrol/kg and 1.5×10^7 CFU/g diets compared to the control ($P < 0.05$). Generally, fish in supplemented diets, particularly T_2 and T_6 groups, had the highest lysozyme, alternative complement activity (ACH_{50}), total immunoglobulin (Ig), nitroblue tetrazolium test (NBT), myeloperoxidase (MPO), complement component 3 (C3), complement component 4 (C4), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and lower levels of malondialdehyde (MDA), glucose, cortisol, alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) when were compared with the control before crowding stress ($P < 0.05$). After crowding stress challenge, fish in the supplemented groups, particularly T_2 and T_6, generally showed significantly higher values of lysozyme, ACH_{50}, total Ig, NBT, MPO, C3, C4, SOD, CAT, GPx and lower levels of MDA, glucose, cortisol, ALT, ALP, LDH when compared with the control ($P < 0.05$). Also, recovered fish in the control group demonstrated significantly declined levels of lysozyme, ACH_{50}, total Ig, NBT, MPO, C3, C4, SOD, CAT, GPx and higher levels of MDA, glucose, cortisol, ALT,

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ALP, LDH as compared to other group ($P < 0.05$). In conclusion, a combined administration of RE and LAB effectively improved growth performance and health status as well as protected common carp against crowding stress.

1. Introduction

Today, the use of food additives is increasing in aquaculture due to their role in improving growth, nutrition, reproduction, immune system and resistance against diseases and environmental stressors (Dabrowski and Ciereszko, 2001; Güroy et al., 2012; Mohapatra et al., 2013a, 2013b; Dawood et al., 2018; Pereira da Costa and Campos Miranda-Filho, 2020; Khanjani and Sharifinia, 2021). The movement of aquaculture towards intensive production has increased the risk of crowding stress (Yin et al., 1995; Caipang et al., 2009; Lin et al., 2014; Lin et al., 2018a, 2018b). Many studies have shown that crowding stress seriously reduces the immune system of fish, which may increase the risk of various diseases (Ortuno et al., 2001; Lin et al., 2018a, 2018b; Paray et al., 2020a, 2020b; Adineh et al., 2021a, 2021b, 2021c). In addition, in intensive fish systems, if water quality management is not done efficiently, the increased concentrations of toxic substances such as ammonia may cause fish mortality (Randall and Tsui, 2002; Hargreaves and Tucker, 2004; Datta, 2012). Like other vertebrates, corticosteroids, especially cortisol, play an important role in modulating stress in fish (Pankhurst, 2011; Norris and Hobbs, 2020). During the period of stress, high energetic costs are imposed on the fish, which reduces the growth and immune system of the fish (Tort, 2011; Sadoul and Vijayan, 2016; Schreck and Tort, 2016). The immunosuppressive effects of cortisol have been demonstrated by *in vitro* and *in vivo* studies (Espelid et al., 1996; Esteban et al., 2004; Cortés et al., 2013). During the last decade, a wide range of natural and synthetic immune stimulants (IMS) have been used in aquaculture (Mehana et al., 2015). Herbal supplements and probiotics have been shown to improve effectively the immune system of fish (Banerjee and Ray, 2017; Alagawany et al., 2020; Elumalai et al., 2020a, 2020b). The chemical composition of medicinal herbs includes some compounds with anti-stress and immunogenic properties such as flavonoids, phenolic compounds, carotenoids and terpenes (Shakya, 2016; Tungmunnithum et al., 2018). Probiotics, were defined as "live microbial feed supplements" which beneficially affect the host animal by improving its intestinal microbial balance (Kirihara et al., 2018).

Lactobacillus, *Bacillus*, *Lactococcus*, *Clostridium*, *Leuconostoc*, *Enterococcus*, *Shewanella*, *Carnobacterium*, and *Aeromonas* are among most common probiotics used in aquaculture (Takanashi et al., 2014). Probiotics generally improve the fish immune system mainly through modulating the intestinal microflora, competing with and removing pathogens in the gut, and stimulating the innate immune system (Gatesoupe, 1999; Gómez and Balcázar, 2008; Denev et al., 2009; Nayak, 2010; Aguirre-Guzman et al., 2012). Although the mitigating role of IMS against crowding stress has been studied in fish, (Montero et al., 1999; Reyes-Cerpa et al., 2018a, 2018b; Yousefi et al., 2019a, 2019b; Adineh et al., 2021), studies to find and introduce more effective IMS are always welcome.

RE is a plant-derived polyphenolic compound with antioxidant and anti-inflammatory propertiess (A Santos et al., 2013; Wilson et al., 2015a, 2015b; Colica et al., 2018; Banez et al., 2020). Some studies have also reported the immunogenic effects of resveratrol in fish (Kowalska et al., 2017; Yan et al., 2017; Jia et al., 2019a,2019b,2019c, 2019d; Tan et al., 2019a, 2019b; Giordo et al., 2020; Naderi Farsani et al., 2021). Specific lactic acid bacterial strains, such as LAB, have been considered as probiotics, because of their health benefits (Quinto et al., 2014). LAB is categorized as a probiotic strain because of its beneficial effects in human health and prevention of disease transmission (Hosseini et al., 2016a, 2016b). In the present study, we are trying to investigate the potentials of a plant derived compound, resveratrol (RE) and the probiotic, *Lactobacillus acidophilus* (LAB) and their combination to improve growth and immunity of the common carp, *Cyprinus carpio* under intensive fish culture. The results of the present study may help us to carp aquaculture enhancing.

2. Materials and methods

2.1. Fish and experimental design

900 common carps (30.16 ± 0.7 g; Mean ± SE) were provided from a local farm and kept in 1000 L tanks for 14 days for adaptation and fed basic food. After the adaptation period, fish were distributed in 300 L tanks as seven experimental groups with three replicates with a density of 40 fish per tank and fed experimental diets for 60 days as follows: T_1: basic food as control, T_2: LAB with a concentration of 1.5×10^7 CFU/g, T_3: LAB with a concentration of 3×10^7 CFU/g, T_4: 300 mg resveratrol/kg, T_5: 600 mg resveratrol/kg, and T_6: 1.5×10^7 CFU/g + 300 mg resveratrol/kg and T_7: 3×10^7 CFU/g + 600 mg resveratrol/kg. In addition, biometry of fish was performed every two weeks. The water quality parameters were at optimized range throughout the experiment for temperature: 24 ± 0.5 °C, dissolved oxygen: > 6.5 mg/l, pH: 7.3 ± 0.2 and non-ionized ammonia: < 0.025. The experimental groups were kept under static conditions and the health conditions of each tank were maintained by continuous aeration, siphoning suspended particles and changing 30% of water daily. Feeding was done twice a day at a rate of 2% of biomass (Rajabiesterabadi et al., 2019; Yousefi et al., 2020; Yousefi et al., 2021).

2.2. Probiotic preparation and Resveratrol

LAB (PTCC 1608) was provided from the Center of Industrial Microorganisms of Iran. The bacterium was cultured in MRS broth for 24 h at 35 °C. To obtain a single colony, MRS agar was used, and then one colony from this medium was transferred to MRS broth medium. After 24 h, the medium was centrifuged at 10,000 × *g* for 10 min at 4 °C and washed twice in saline solution. The desired bacterial concentrations were prepared using a spectrophotometer at OD 600 nm (Madreseh et al., 2019). RE was provided from Sigma-Aldrich Company [purity percentage: > 99% (R5010; CAS No: 501–36–0)].

2.3. Diet preparation

To prepare experimental diets, food ingredients (FI) were obtained from different companies and after weighted and mixed well. In the next step, the supplements (LAB and Resveratrol at adjusted concentrations) along with some water were added to FI, and well mixed to form dough. The obtained dough was processed by a meat grinder to form pellets and the pellets were dried at 37 °C (Rajabiesterabadi et al., 2020). The dietary levels of the probiotic and resveratrol were chosen based on the positive results of previous studies (Aly et al., 2008; Al-Dohail et al., 2009; Hoseinifar et al., 2015a, 2015b, 2015c; Torno et al., 2017; 2019a, 2019b,2019c, 2019d; Rohmah et al., 2022).

2.4. Growth parameters

After 60 days feeding period, feeding was stopped for 24 h and then fish were anesthetized using eugenol (100 mg/l) and the growth and nutritional parameters measured according to following formulas (Yousefi et al., 2021):

Weight gain (WG; g) = Final weight [FW(g)] – Initial weigh [IW (g)].

Feed conversion ratio (FCR) = Feed intake/ (FW-IW),

Specific growth rate (SGR; %/d) = 100 × [(ln FW – ln IW)/days],
Survival rate (SR; %) = (Total number of dead fish/ Total number of fish) × 100.
Protein efficiency ratio (PER) = WG⁄ [total protein intake (g)].

2.5. Digestive enzymes

To measure the activity of digestive enzymes, the intestine samples were separated, emptied, and mechanically homogenized using a Tris buffer (Heidolph ® SilentCrusher-M, Heidolph, Nuremberg, Germany) (Gisbert et al., 2016). The homogenized suspension was centrifuged at 4 °C (6000g for 10 min) and supernatant stored at − 80 °C. Amylase activity was measured colorimetrically at 600 nm using a 2% starch solution as substrate in 0.1 M citrate phosphate buffer (Robyt and Whelan, 1968). Lipase enzyme activity was estimated based on the method of Iijima et al. (1998) using polyphenol myristate as substrate dissolved in 0.25 mM Tris HCl (pH=9), 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. In this method, the reaction was stopped by adding 0.7 ml of acetone/n-heptane (5: 2 v/v) and the adsorption was read at 405 nm. Alkaline protease enzyme activity was measured by García-Carreño (1992) method. In this method, azo-casein was used as a substrate in Tris buffer (Tris–HCl 0.1 M; pH=8). The reaction was terminated using trichloroacetic acid (5%) and the mixture was kept at 25 °C for 1 h. After centrifugation, the adsorption in supernatant was read at 440 nm.

2.6. Intestinal load of *Lactobacillus acidophilus*

To determine the intestinal population of bacteria, firstly, fish skin was washed and disinfected by 70% ethanol and then intestine tissues were sampled after dissection. Tissue samples were homogenized in phosphate buffer (PBS, pH=7.2) using a tissue homogenizer. The homogenized solution was diluted in phosphate buffer. Then, 100 µl of the solution was transferred to MRS (Merck, Germany) and tryptic soy agar (TSA) (Merck, Germany) culture medium to estimate lactic acid bacteria and whole intestinal bacteria, respectively. The culture pellets were kept at 30 °C for 48 h and total bacterial count (TBC) and lactic acid bacterial (LABC) colonies (CFU/g) counted (Merrifield et al., 2010).

2.7. Blood and mucus sampling

The blood and mucus samples were collected at three times as follows: a) after feeding period, b) after 6 h exposure to crowding stress, c) after 24 h recovery from crowding stress. Before sampling, fish (three fish from each replicate) were anesthetized using clove powder (100 mg/l; Yousefi et al., 2021). Blood samples were collected using a 2 ml syringe from the caudal vein, poured into microtubes containing heparin and kept at room temperature for 2 h. In the next step, serum samples were separated using centrifugation (12000g for 10 min at 4 °C) and stored at − 70 °C until the measurement of biochemical. To collect mucus samples, fish (3 fish/tank) were randomly caught from each tank and transferred to polyethylene bags containing 10 ml of 50 mM physiological serum. After 3 min, the collected mucus was centrifuged (2500g for 10 min in 4 °C) and the supernatant stored at − 80 °C (Ross et al., 2000). Crowding stress was done after 60 days feeding period by lowering the water level by 80% for 6 h. Also, the recovery process was conducted by increasing the water level by 80% for 24 h (Paray et al., 2020a, 2020b).

2.8. Immune parameters of blood and mucus

Lysozyme activity (u/ml) of serum and mucus was estimated based on the turbidity method described by Ellis (1990) using *Micrococcus lysodeikticus* bacteria as a target in phosphate buffer (0.2 mg ml^{-1} in a 0.05 M sodium phosphate buffer (pH=6.2). The serum total immunoglobulin (Ig) (mg/dl) content was calculated based on the amount of protein before and after the addition of polyethylene glycol (Hoseinifar et al., 2016a, 2016b). The concentration of serum complement components (C3 and C4) (mg/dl) was estimated using an ELISA device (ELX800, BioTek, Vermont, USA) and based on a commercial kit (Pars Azmun Co., Tehran, Iran). The activity of serum and mucus alternative complement (ACH_{50}) was calculated based on the method described by Yano (1992). Briefly, serial dilutions of each serum sample (3.12, 0.625, 1.25, 2.5, 5 and 10) were made in 25 µl of EDTA-GVB (Gelatin Veronal Buffer containing 10 mM EDTA). 2% ship red blood cell in 25 µl of same buffer was added to serum sample, mixed, incubated for 2 h and then absorbance was recorded at 412 nm. Mucus protease activity was measured according to the method of Hoseinifar et al. (2016a), (2016b). In this method, 100 µl of mucus was mixed with 100 µl of 100 mM ammonium bicarbonate buffer containing containing 0.7% azocasein solution and then incubated at 30 °C for 20 h. The reaction was stopped using trichloroacetic acid and the supernatant was collected by centrifugation (15,000g for 5 min). Then, the supernatant was mixed with 0.5 N hydroxide and the absorbance was recorded at 450 nm. Nitroblue tetrazolium (NBT) reduction test was to determine respiratory burst activity in blood samples. Briefly, 100 µl of heparinized blood and 100 µl of 0.2% NBT solution were mixed and incubated for 30 min 50 µl of the mixture was mixed with 1 ml of N, N-Dimethylformamide and centrifuged at 3000g for 5 min and the adsorption was read at 540 nm (Siwicki and Anderson, 1994).

Esterase activity was measured based on Guardiola et al., (2017). In this method, an equal volume of mucus and 0.4 mM nitrophenyl myristate in ammonium bicarbonate buffer containing 0.5% Triton X100 was mixed and incubated at 30 °C and the absorption rate was read at 405 nm.

2.9. Biochemicals in blood and mucus

Serum and mucus cortisol levels (ng/ml) were assayed by ELISA using a commercial kit (IBL Co., Gesellschaft für Immunchemieund Immunbiologie, Germany). The glucose concentrations in serum and mucus (mg/dl) were measured by commercial kits (Pars Azmun Co., Tehran, Iran). The activity of glutathione peroxidase (GPx) (u/ml) and superoxide dismutase (SOD) (u/ml) in serum were assayed by estimating the rate of glutathione oxidation and reduction rate of Cytochrome C respectively (ZellBio GmbH, Veltinerweg). Serum catalase (CAT) activity (u/ml) was calculated by determining the reduction rate of H_2O_2 according to the method described by Goth (1991). The thiobarbituric acid method was used to determine malondialdehyde levels (MDA) by commercial kit ZellBio GmbH, Veltinerweg. The activity of ALP (Alkaline phosphatase), AST (Aspartate amino transferase) and ALT (Alanine amino transferase) enzymes and lactate dehydrogenase in serum (U/L) were measured using Pars Azmun Co., (Tehran, Iran) commercial kits by using an autoanalyzer (Beckman Coulter, Avanti J-26 XPI, CA, USA).

2.10. Data analysis

After evaluating the normality of the data with the Kolmogorov–Smirnov test, One-way analysis of variance was used to determine the difference between the treatments. Finally, comparison of means was done using Tukey's test at $P < 0.05$. Results were exhibited based on the mean ± standard error. Moreover, the interaction between crowding stress and different concentrations of supplements were done using two-way ANOVA.Table 1.

3. Results

3.1. Growth

After feeding period, the values of FW, WG and SGR were higher in the treatments, T_2 (1.5×10^7 CFU/g LAB), T_4 (300 mg resveratrol/kg),

Table 1
Feedstuffs and compositions of the basal diet (Rajabiesterabadi et al., 2020).

Ingredients	g/kg	Proximate composition	% in dry basis
Fishmeal[a]	160	Crude protein	393
Soybean meal[b]	170	Crude lipid	88.7
Wheat flour (Res)	381	ash	62.1
Poultry meal[c]	150	Dry matter	908
Wheat gluten[d]	100		
Phytase[e]	5		
Fish oil	10		
Lysine[f]	6		
Soybean oil	10		
Methionine[f]	3		
Mineral mix[g]	2.5		
Vitamin mix[h]	2.5		
Total	1000		

[a] Peygir Co (crude protein 55.8%).
[b] Soyabean Co (crude protein 45.5%). [c]Peygir Co (crude protein 50.0%). [d]Shahdineh Aran Co (crude protein 78.3%). [e]CheilJedang Co. [f]Golbid Co (10,000 IU). [g]The premix provided following amounts per kg of diet: Mg: 350 mg; Fe: 13 mg; Co: 2.5 mg; Cu: 3 mg; Zn: 60 mg; NaCl: 3 g; dicalcium phosphate: 10 g. [h]The premix provided following amounts per kg of feed: A: 1000 IU; D3: 5000 IU; E: 20 mg; B5: 100 mg; B2: 20 mg; B6: 20 mg; B1: 20 mg; H: 1 mg; B9: 6 mg; B12: 1 mg; B4: 600 mg; C: 50 mg

T_5 (600 mg resveratrol/kg), T_6 (1.5×10^7 CFU/g LAB+ 300 mg resveratrol/kg) and T_7 (3×10^7 CFU/g LAB + 600 mg resveratrol/kg) compared to control and T3 (3×10^7 CFU/g LAB) (Table 2, $P < 0.05$). There were no significant differences in the growth parameters between the groups, T_3, T_4, T_5 and T_7 (Table 2, $P > 0.05$). The treatment, T6 showed more growth performance compared to other groups ($P < 0.05$), however there were no significant differences between this group with T_2 for FW, with T_4 and T_7 for WG and with T_4, T_5, T_6 and T_7 for FCR (Table 2, $P > 0.05$). Except T_3, the FCR values significantly decreased in the experimental groups compared to control (Table 2, $P < 0.05$). The lowest FCR observed in T_6 (Table 2, $P < 0.05$).

3.2. Digestive enzymes

The activity of digestive enzymes exhibited significant differences between the experimental groups (Table 3, $P < 0.05$). Amylase activity significantly increased in the treatments T_6 and T_7 compared to control and the treatments, T_3-T_5 (Table 3, $P < 0.05$). Also, there were no significant differences in the amylase activity between the groups, T_2, T_6 and T_7 (Table 3, $P > 0.05$). Protease activity significantly increased in the treatments, T_2, T_5, T_6 and T_7 compared to control (Table 3, $P < 0.05$). There were no significant differences in the protease activity between all supplemented fish (Table 3, $P > 0.05$). Lipase activity in the treatments T_2 and T_6 was significantly higher than in control (Table 3, $P < 0.05$). Lipase activity in other groups showed no significant differences with control (Table 3, $P > 0.05$).

3.3. Intestinal load of *Lactobacillus acidophilus*

The intestinal lactic acid bacterial counts (LABC) (CFU/g) significantly increased in the probiotic supplemented groups (Fig. 2A, $P < 0.05$). There were no significant differences in the LABC of control and RE-supplemented fish (Fig. 2A, $P > 0.05$). There were no significant differences in total bacterial concentration (TBC) of intestine between all experimental groups (Fig. 2B, $P < 0.05$).

3.4. Antioxidant enzymes and oxidative stress

3.4.1. Before crowding stress

The activity of CAT and GPx significantly elevated in T_6 compared to control ($P < 0.05$), while SOD activity in other treatments had no differences control (Table 4, $P > 0.05$). MDA showed lower levels in T_6 compared to other experimental groups (Table 4, $P < 0.05$). Except T_6, other groups had no significant differences with control in terms of CAT, GPx and MDA (Table 4, $P > 0.05$).

3.4.2. After crowding stress

CAT and GPx activity increased in T_6 compared to control (Table 4, $P < 0.05$). There were no significant differences between other supplemented groups and control (Table 4, $P > 0.05$). SOD activity showed no differences between all experimental groups (Table 4, $P > 0.05$). MDA levels significantly decreased in T_6 compared to control ($P < 0.05$), while other supplemented groups showed no significant differences with control (Table 4, $P > 0.05$).

3.4.3. After recovery

GPx activity showed higher activity in T_6 compared to control ($P < 0.05$), while other supplemented fish had no differences with control (Table 4, $P < 0.05$). CAT activity significantly elevated in T_2 and

Table 2
Growth parameters of the commpn carp, *Cyprinus carpio* after 60 days feeding with experimental diets. T1: basic food as control, T2: *Lactobacillus acidophilus* (LAB) with a concentration of 1.5×10^7 CFU/ml, T3: LAB with a concentration of 3×10^7 CFU/ml, T4: 300 mg resveratrol/kg, T5: 600 mg resveratrol/kg, and T6: 1.5×10^7 CFU/ml + 300 mg resveratrol/kg and T7: 3×10^7 CFU/ml + 600 mg resveratrol/kg. The means with different letters show significant differences (P<0.05).

Parameters	T1 (control)	T2	T3	T4	T5	T6	T7
IW (g)	30.76±0.31[a]	30.46±0.37[a]	30.16±0.52[a]	30.33±0.21[a]	30.43±0.41[a]	30.60±0.40[a]	30.16±0.44[a]
FW (g)	60.26±1.18[d]	67.00±0.57[ab]	63.00±0.86 [cd]	64.16±0.72[bc]	65.56±0.47[bc]	70.16±0.72[a]	66.50±0.76[abc]
WG (g)	29.50±0.87[c]	36.53±0.49[ab]	32.83±0.56[bc]	33.83±0.54[b]	35.13±0.46[b]	39.56±1.10[a]	36.33±1.16[ab]
FCR	2.02±0.07[a]	1.64±0.02[bc]	1.85±0.03[ab]	1.79±0.03[b]	1.72±0.02[bc]	1.53±0.04[c]	1.66±0.05[bc]
SGR (% d^{-1})	1.12±0.01[c]	1.31±0.01[ab]	1.22±0.01[bc]	1.24±0.01[b]	1.27±0.02[ab]	1.38±0.03[a]	1.31±0.04[ab]
SR (%)	93.00±1.73[a]	96.33±2.02[a]	95.00±1.00[a]	95.33±2.90[a]	95.33±2.90[a]	96.66±3.33[a]	95.33±2.90[a]

Table 3
Activity of digestive enzymes in the common carp, *Cyprinus carpio* after 60 days feeding with experimental diets. T1: basic food as control, T2: *Lactobacillus acidophilus* (LAB) with a concentration of 1.5×10^7 CFU/ml, T3: LAB with a concentration of 3×10^7 CFU/ml, T4: 300 mg resveratrol/kg, T5: 600 mg resveratrol/kg, and T6: 1.5×10^7 CFU/ml + 300 mg resveratrol/kg and T7: 3×10^7 CFU/ml + 600 mg resveratrol/kg. The means with different letters show significant differences ($P < 0.05$).

Parameters	T1 (control)	T2	T3	T4	T5	T6	T7
Amylase (u/mg protein)	10.00 ± 0.69[b]	12.16 ± 0.72[ab]	10.16 ± 0.44[b]	10.20 ± 0.70[b]	10.23 ± 0.62[b]	13.83 ± 0.44[a]	13.33 ± 0.56[a]
Alkaline protease (u/mg protein)	3.90 ± 0.22[c]	6.29 ± 0.34[a]	5.18 ± 0.19[abc]	4.57 ± 0.31[bc]	5.50 ± 0.32[ab]	6.11 ± 0.37[ab]	5.78 ± 0.41[ab]
Lipase (u/mg protein)	1.23 ± 0.12[c]	1.76 ± 0.08[ab]	1.61 ± 0.05[abc]	1.44 ± 0.05[bc]	1.46 ± 0.06[bc]	1.88 ± 0.07[a]	1.57 ± 0.08[abc]

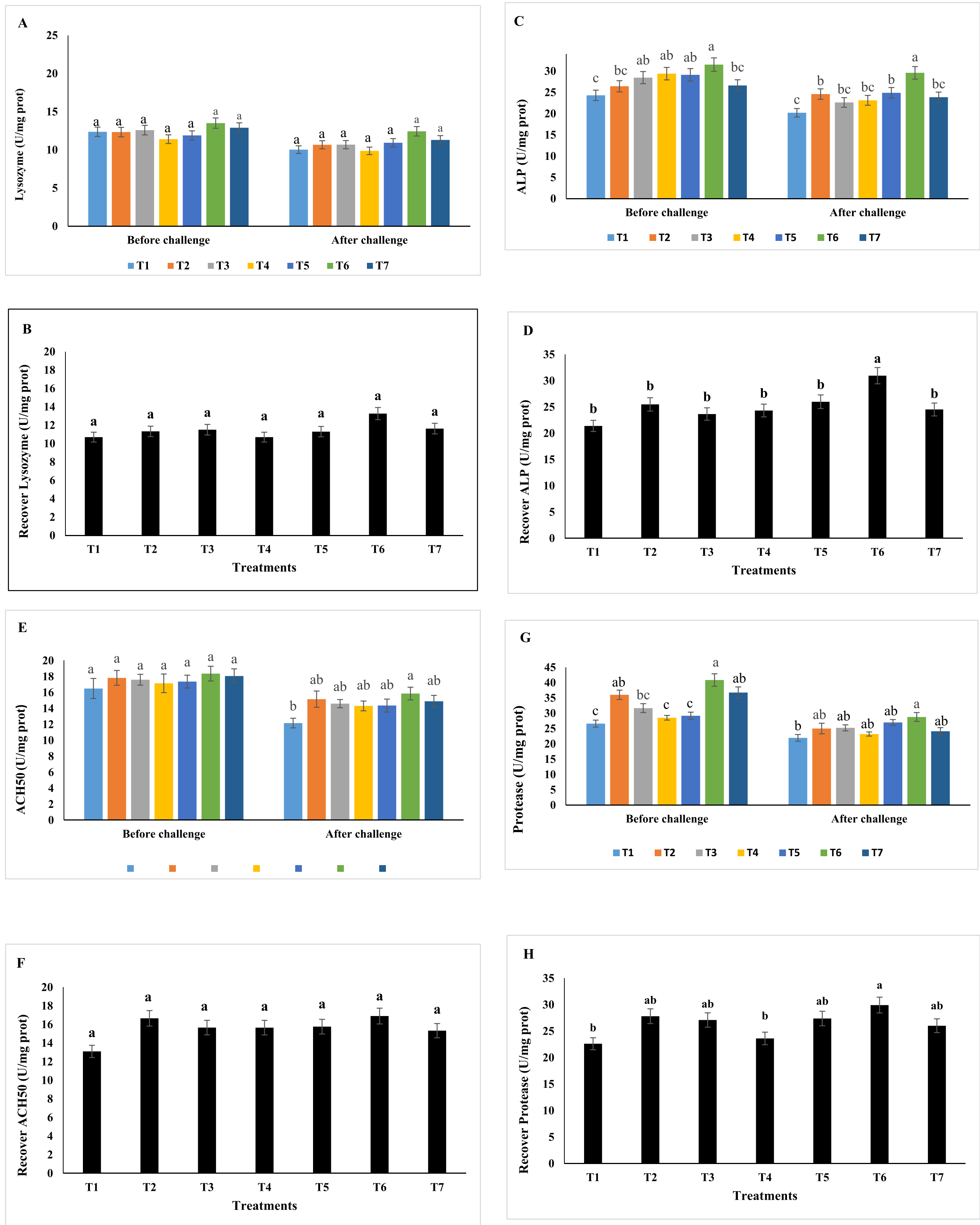

Fig. 1. Immune components of mucus in the common carp, *Cyprinus carpio* after 60 days feeding with experimental diets, crowding stress and recovery. T1: basic food as control, T2: *Lactobacillus acidophilus* (LAB) with a concentration of 1.5×10^7 CFU/ml, T3: LAB with a concentration of 3×10^7 CFU/ml, T4: 300 mg resveratrol/kg, T5: 600 mg resveratrol/kg, and T6: 1.5×10^7 CFU/ml + 300 mg resveratrol/kg and T7: 3×10^7 CFU/ml + 600 mg resveratrol/kg. The means with different letters show significant differences ($P < 0.05$).

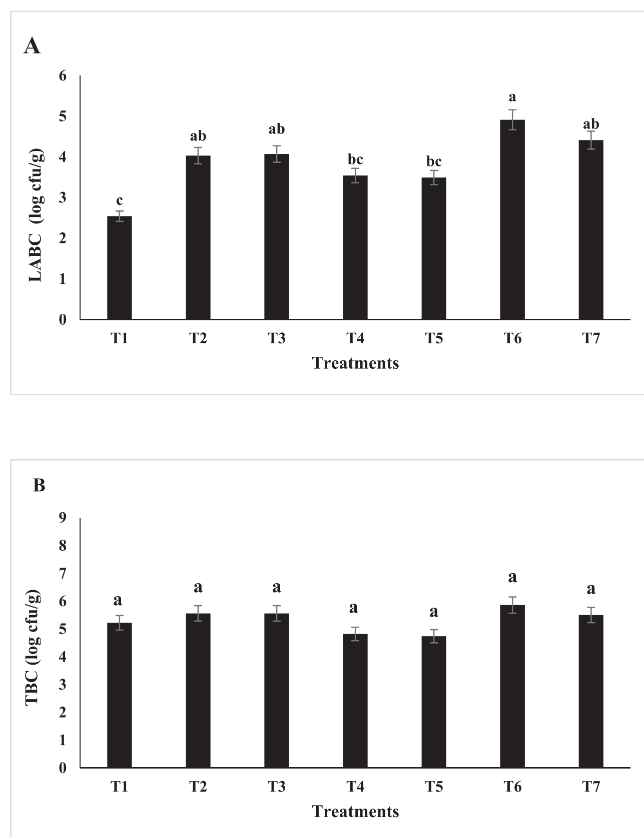

Fig. 2. The intestinal lactic acid bacteria load in the common carp, *Cyprinus carpio* after 60 days feeding with experimental diets. T1: basic food as control, T2: *Lactobacillus acidophilus* (LAB) with a concentration of 1.5×10^7 CFU/ml, T3: LAB with a concentration of 3×10^7 CFU/ml, T4: 300 mg resveratrol/kg, T5: 600 mg resveratrol/kg, and T6: 1.5×10^7 CFU/ml + 300 mg resveratrol/kg and T7: 3×10^7 CFU/ml + 600 mg resveratrol/kg. The means with different letters show significant differences ($P < 0.05$).

T_6 compared to control (Table 4, $P < 0.05$). Other groups showed no significant differences with control (Table 4, $P > 0.05$). SOD activity exhibited no differences between all experimental groups (Table 4, $P > 0.05$). MDA levels significantly decreased in T_2, T5, T_6 and T_7 compared to control (Table 4, $P < 0.05$).

3.5. Immune components of serum

3.5.1. Before crowding stress

Lysozyme activity and Ig content significantly increased after feeding period in the supplemented fish compared to control (Table 5, $P < 0.05$). The treatment T_6 showed higher values of lysozyme activity and Ig compared to other experimental groups (Table 5, $P < 0.05$). Furthermore, other treatments had no differences in terms of lysozyme activity and Ig content (Table 5, $P > 0.05$). NBT and MPO values were significantly higher in the treatments, T_2 and T_6 than in control (Table 5, $P < 0.05$). There were no significant differences in these components between the other groups with control (Table 5, $P > 0.05$). ACH_{50} activity significantly elevated in the groups, T_2, T_6 and T_7 compared to control (Table 5, $P < 0.05$). The values of C3 showed significant increases in T_3 and T_6 compared to control (Table 5, $P < 0.05$). The treatments T_2 and T_7 had no differences in C3 values with control (Table 5, $P > 0.05$). C4 values were significantly higher in the treatments, T_3-T_5 than in control ($P < 0.05$), while other supplemented fish had no differences with control (Table 5, $P < 0.05$).

3.5.2. After crowding stress

Lysozyme activity and Ig content significantly increased in all supplemented fish compared to control, with highest values in T_6 (Table 5, $P < 0.05$). ACH_{50} activity significantly increased in T_4 compared to control and other supplemented fish ($P < 0.05$), while other supplemented fish showed no differences with control (Table 5, $P > 0.05$). NBT and MPO significantly increased in the treatments, T_2 and T_6 compared to control ($P < 0.05$), while other supplemented fish had no differences with control (Table 5, $P > 0.05$). C3 and C4 values significantly increased in the treatments T_3-T_7 compared to control ($P < 0.05$), while there was no significant difference between them (Table 5, $P > 0.05$).

3.5.3. After recovery

Lysozyme activity and Ig content was significantly higher in all supplemented fish compared to control (Table 5, $P < 0.05$). The treatment T_6 showed higher Ig content compared to other groups (Table 5, $P < 0.05$). ACH_{50} activity significantly increased in T_2, T_6 and T_7 compared to control (Table 5, $P < 0.05$). The highest ACH_{50} activity was observed in T_6 (Table 5, $P < 0.05$). NBT and MPO significantly increased in the treatments, T_2, T_6 and T_7 compared to control ($P < 0.05$), while other supplemented fish showed no differences with control (Table 5, $P < 0.05$). C3 and C4 values significantly increased in all supplemented fish compared to control ($P < 0.05$), while there was no significant

Table 4
Antioxidant enzyme activity and oxidative stress indices (MDA) in the common carp, *Cyprinus carpio* after 60 days feeding with experimental diets, crowding stress and recovery. T1: basic food as control, T2: *Lactobacillus acidophilus* (LAB) with a concentration of 1.5×10^7 CFU/ml, T3: LAB with a concentration of 3×10^7 CFU/ml, T4: 300 mg resveratrol/kg, T5: 600 mg resveratrol/kg, and T6: 1.5×10^7 CFU/ml + 300 mg resveratrol/kg and T7: 3×10^7 CFU/ml + 600 mg resveratrol/kg. The means with different letters show significant differences ($P < 0.05$).

Status	Parameters	T1 (control)	T2	T3	T4	T5	T6	T7
Before challenge	MDA (nmol/ml)	40.56 ± 1.10^a	37.46 ± 1.86^{ab}	38.30 ± 0.87^{ab}	38.56 ± 0.80^{ab}	35.91 ± 1.15^{ab}	34.73 ± 1.21^b	36.96 ± 1.12^{ab}
	SOD (U/ml)	27.00 ± 1.25^a	29.43 ± 1.26^a	29.00 ± 1.52^a	29.50 ± 1.44^a	30.50 ± 1.32^a	31.43 ± 1.03^a	27.80 ± 0.90^a
	CAT (U/ml)	110.33 ± 3.75^b	120.36 ± 3.24^{ab}	114.83 ± 3.03^{ab}	113.20 ± 3.37^b	115.00 ± 3.21^{ab}	129.66 ± 2.33^a	114.00 ± 2.30^b
	GPx (U/ml)	137.00 ± 1.52^b	140.66 ± 1.76^{ab}	139.83 ± 2.68^{ab}	139.80 ± 1.21^{ab}	141.26 ± 1.93^{ab}	146.50 ± 1.32^a	142.66 ± 2.33^{ab}
After challenge	MDA (nmol/ml)	45.23 ± 1.17^a	39.13 ± 1.84^b	40.13 ± 0.90^{ab}	41.10 ± 0.97^{ab}	37.91 ± 1.15^b	36.73 ± 1.21^b	38.63 ± 1.16^b
	SOD (U/ml)	24.66 ± 1.92^a	26.43 ± 0.69^a	26.33 ± 1.85^a	26.16 ± 1.74^a	27.43 ± 1.26^a	28.76 ± 0.76^a	26.80 ± 2.57^a
	CAT (U/ml)	100.00 ± 4.04^b	114.70 ± 3.46^{ab}	111.50 ± 2.56^{ab}	103.53 ± 2.59^b	111.33 ± 3.84^{ab}	124.83 ± 2.74^a	108.00 ± 3.21^b
	GPx (U/ml)	132.00 ± 1.52^b	139.00 ± 2.30^{ab}	136.50 ± 2.78^{ab}	136.13 ± 0.99^{ab}	137.63 ± 1.59^{ab}	142.16 ± 1.58^a	136.33 ± 2.02^{ab}
After recovery	MDA (nmol/ml)	44.06 ± 0.78^a	38.33 ± 1.76^b	39.36 ± 0.90^{ab}	39.43 ± 0.80^{ab}	36.81 ± 1.13^b	35.73 ± 1.21^b	37.83 ± 1.01^b
	SOD (U/ml)	24.83 ± 1.36^a	26.93 ± 1.09^a	26.50 ± 1.25^a	26.33 ± 1.45^a	28.76 ± 1.57^a	29.46 ± 0.72^a	26.93 ± 2.13^a
	CAT (U/ml)	101.33 ± 3.75^c	117.70 ± 3.46^{ab}	113.16 ± 2.89^{abc}	105.20 ± 2.27^{bc}	113.16 ± 3.81^{abc}	127.16 ± 2.45^a	110.33 ± 2.33^{bc}
	GPx (U/ml)	132.50 ± 1.80^b	139.86 ± 1.95^{ab}	137.33 ± 2.61^{ab}	136.36 ± 1.59^{ab}	139.56 ± 1.79^{ab}	144.26 ± 1.50^a	139.00 ± 2.64^{ab}

Two-way ANOVA (P-value)	MDA (nmol/ml)	SOD (U/ml)	CAT (U/ml)	GPx (U/ml)
supplements	*0.000*	*0.299*	*0.000*	*0.001*
stress	*0.178*	*0.707*	*0.516*	*0.264*
supplements × stress	*0.999*	*0.998*	*0.990*	*0.996*

Table 5
Immune components of serum in the common carp, *Cyprinus carpio* after 60 days feeding with experimental diets, crowding stress and recovery. T1: basic food as control, T2: *Lactobacillus acidophilus* (LAB) with a concentration of 1.5×10^7 CFU/ml, T3: LAB with a concentration of 3×10^7 CFU/ml, T4: 300 mg resveratrol/kg, T5: 600 mg resveratrol/kg, and T6: 1.5×10^7 CFU/ml + 300 mg resveratrol/kg and T7: 3×10^7 CFU/ml + 600 mg resveratrol/kg. The means with different letters show significant differences (P < 0.05).

Status	Parameters	T1 (control)	T2	T3	T4	T5	T6	T7
Before challenge	Lysozyme (u/ml)	22.60 ± 0.70^{c}	28.60 ± 0.58^{b}	27.36 ± 0.73^{b}	26.43 ± 0.74^{b}	26.63 ± 0.68^{b}	32.43 ± 0.86^{a}	27.56 ± 1.02^{b}
	ACH_{50} (u/ml)	119.83 ± 0.72^{c}	130.16 ± 1.48^{b}	122.70 ± 1.27^{bc}	123.83 ± 1.64^{bc}	124.50 ± 1.77^{bc}	140.40 ± 2.12^{a}	129.80 ± 1.60^{b}
	Total Ig (mg/ml)	16.43 ± 0.76^{b}	20.43 ± 0.80^{a}	22.23 ± 0.67^{a}	20.60 ± 0.41^{a}	20.76 ± 0.56^{a}	22.66 ± 0.56^{a}	21.03 ± 0.44^{a}
	NBT(540)	0.52 ± 0.06^{b}	0.86 ± 0.05^{a}	0.61 ± 0.07^{ab}	0.59 ± 0.06^{ab}	0.66 ± 0.07^{ab}	0.86 ± 0.06^{a}	0.71 ± 0.07^{ab}
	MPO (450)	1.26 ± 0.14^{b}	2.41 ± 0.30^{a}	1.83 ± 0.17^{ab}	1.76 ± 0.20^{ab}	1.96 ± 0.20^{ab}	2.46 ± 0.26^{a}	1.80 ± 0.17^{ab}
	C3 (g/dl)	26.53 ± 1.25^{b}	29.93 ± 1.09^{ab}	34.93 ± 1.50^{a}	36.03 ± 1.46^{a}	35.43 ± 1.55^{a}	34.83 ± 1.30^{a}	30.50 ± 1.04^{ab}
	C4 (g/dl)	11.50 ± 0.86^{b}	13.53 ± 0.75^{ab}	16.00 ± 0.57^{a}	16.20 ± 0.72^{a}	15.26 ± 0.37^{a}	14.53 ± 0.49^{ab}	13.66 ± 0.69^{ab}
After challenge	Lysozyme (u/ml)	16.33 ± 0.88^{c}	22.50 ± 0.28^{ab}	21.33 ± 0.72^{b}	20.63 ± 0.61^{b}	20.43 ± 0.53^{b}	25.53 ± 0.75^{a}	21.16 ± 0.60^{b}
	ACH_{50} (u/ml)	104.00 ± 2.64^{b}	113.33 ± 3.17^{ab}	107.70 ± 2.42^{b}	108.83 ± 2.20^{b}	110.50 ± 1.89^{b}	125.06 ± 2.66^{a}	110.80 ± 2.07^{b}
	Total Ig (mg/ml)	12.43 ± 0.76^{b}	15.43 ± 0.80^{a}	17.00 ± 0.50^{a}	15.60 ± 0.34^{a}	15.86 ± 0.34^{a}	16.70 ± 0.56^{a}	16.30 ± 0.47^{a}
	NBT(540)	0.41 ± 0.05^{c}	0.73 ± 0.04^{ab}	0.50 ± 0.06^{bc}	0.48 ± 0.05^{bc}	0.50 ± 0.05^{bc}	0.80 ± 0.05^{a}	0.58 ± 0.04^{abc}
	MPO (450)	0.93 ± 0.12^{b}	2.31 ± 0.34^{a}	1.56 ± 0.23^{ab}	1.63 ± 0.17^{ab}	1.75 ± 0.16^{ab}	2.40 ± 0.23^{a}	1.66 ± 0.17^{ab}
	C3 (g/dl)	18.96 ± 0.76^{d}	21.66 ± 0.88 ^{cd}	29.73 ± 2.03^{ab}	29.86 ± 1.44^{ab}	32.80 ± 1.27^{a}	32.83 ± 0.72^{a}	26.50 ± 1.04^{bc}
	C4 (g/dl)	8.73 ± 0.72^{b}	11.43 ± 0.80^{ab}	11.00 ± 0.57^{ab}	12.20 ± 0.70^{a}	13.43 ± 0.34^{a}	13.46 ± 0.51^{a}	12.10 ± 0.37^{a}
After recovery	Lysozyme (u/ml)	18.70 ± 0.70^{c}	25.50 ± 0.50^{ab}	23.66 ± 0.88^{ab}	22.63 ± 0.52^{b}	23.13 ± 0.63^{b}	26.63 ± 0.69^{a}	24.20 ± 0.41^{ab}
	ACH_{50} (u/ml)	105.16 ± 2.20^{d}	120.66 ± 2.33^{ab}	109.16 ± 2.61 ^{cd}	110.83 ± 1.87^{bcd}	113.40 ± 2.83^{bcd}	131.40 ± 2.56^{a}	117.63 ± 1.61^{bc}
	Total Ig (mg/ml)	14.43 ± 0.76^{c}	19.66 ± 0.66^{ab}	19.23 ± 0.61^{ab}	18.26 ± 0.75^{b}	18.53 ± 0.43^{ab}	21.20 ± 0.41^{a}	18.73 ± 0.28^{ab}
	NBT(540)	0.46 ± 0.05^{c}	0.80 ± 0.04^{ab}	0.53 ± 0.05^{bc}	0.53 ± 0.06^{bc}	0.59 ± 0.07^{abc}	0.84 ± 0.07^{a}	0.64 ± 0.05^{abc}
	MPO (450)	1.10 ± 0.11^{b}	2.40 ± 0.32^{a}	1.70 ± 0.28^{ab}	1.70 ± 0.23^{ab}	1.83 ± 0.19^{ab}	2.40 ± 0.26^{a}	1.70 ± 0.17^{ab}
	C3 (g/dl)	19.63 ± 0.64^{b}	28.83 ± 1.01^{a}	30.23 ± 2.21^{a}	32.20 ± 1.49^{a}	34.46 ± 1.58^{a}	34.00 ± 1.04^{a}	28.16 ± 0.72^{a}
	C4 (g/dl)	9.60 ± 0.51^{b}	12.80 ± 0.56^{a}	13.16 ± 0.60^{a}	14.43 ± 0.61^{a}	14.56 ± 0.63^{a}	14.10 ± 0.49^{a}	13.13 ± 0.73^{a}

Two-way ANOVA (P-value)	LYZ (u/ml)	ACH50 (u/ml)	Total Ig (mg/ml)	NBT(540)	MPO (450)	C3 (g/dl)	C4 (g/dl)
supplements	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*
stress	*0.000*	*0.000*	*0.014*	*0.144*	*0.796*	*0.020*	*0.000*
supplements × stress	*0.653*	*0.110*	*0.577*	*0.993*	*0.990*	*0.202*	*0.801*

difference between them (Table 5, P > 0.05).

3.6. Immune components of mucus

3.6.1. Before crowding stress

Lysozyme (Fig. 1A) and ACH_{50} (Fig. 1 E) activities showed no significant differences between all experimental groups (P < 0.05). ALP (Fig. 1C) activity were significantly higher in the T_3-T_6 compared to control (P < 0.05). Protease activity (Fig. 1G) significantly increased in T_2, T_6 and T_7, while other groups had no significant differences with control (P > 0.05).

3.6.2. After crowding stress

Lysozyme (Fig. 1A) showed no significant differences between all treatments (P < 0.05). ALP (Fig. 1C) activity significantly increased in T_2, T_5 and T_6 compared to control (P < 0.05). ACH_{50} (Fig. 1 E) and protease (Fig. 1G) activities significantly increased in T_6 compared to control, while other groups had no significant differences with control (P > 0.05).

3.6.3. After recovery

Lysozyme (Fig. 1 B) and ACH_{50} (Fig. 1F) activities showed no significant differences between all experimental groups (P < 0.05). ALP (Fig. 1 D) and protease (Fig. 1 H) activities were significantly higher in the T_6 compared to control (P < 0.05), while other treatments had no differences with control (P > 0.05).

Table 6
Stress related components of serum in the common carp, *Cyprinus carpio* after 60 days feeding with experimental diets, crowding stress and recovery. T1: basic food as control, T2: *Lactobacillus acidophilus* (LAB) with a concentration of 1.5×10^7 CFU/ml, T3: LAB with a concentration of 3×10^7 CFU/ml, T4: 300 mg resveratrol/kg, T5: 600 mg resveratrol/kg, and T6: 1.5×10^7 CFU/ml + 300 mg resveratrol/kg and T7: 3×10^7 CFU/ml + 600 mg resveratrol/kg. The means with different letters show significant differences (P < 0.05).

Status	Parameters	T1 (control)	T2	T3	T4	T5	T6	T7
Before challenge	Glucose (mg/dl)	64.43 ± 2.17^{a}	62.50 ± 1.44^{a}	63.33 ± 1.45^{a}	64.00 ± 1.04^{a}	61.60 ± 1.30^{a}	60.10 ± 0.95^{a}	62.86 ± 0.94^{a}
	Cortisol (ng/ml)	103.33 ± 2.12^{a}	96.60 ± 1.44^{ab}	94.60 ± 1.81^{ab}	93.83 ± 2.68^{ab}	92.56 ± 1.74^{b}	93.83 ± 1.71^{ab}	94.73 ± 2.28^{ab}
After challenge	Glucose (mg/dl)	69.10 ± 3.05^{a}	65.83 ± 1.30^{a}	66.16 ± 2.12^{a}	66.00 ± 1.32^{a}	65.43 ± 0.63^{a}	62.43 ± 1.55^{a}	65.86 ± 2.62^{a}
	Cortisol (ng/ml)	110.66 ± 2.52^{a}	100.26 ± 1.89^{b}	99.60 ± 1.81^{b}	99.83 ± 2.68^{b}	96.03 ± 1.56^{b}	97.16 ± 1.39^{b}	100.73 ± 1.70^{b}
After recovery	Glucose (mg/dl)	67.10 ± 2.74^{a}	64.00 ± 1.00^{a}	64.66 ± 1.45^{a}	65.50 ± 1.44^{a}	63.80 ± 0.92^{a}	61.26 ± 1.15^{a}	64.53 ± 1.27^{a}
	Cortisol (ng/ml)	107.66 ± 1.92^{a}	97.66 ± 1.76^{b}	96.93 ± 1.57^{b}	96.83 ± 1.74^{b}	94.86 ± 1.69^{b}	95.16 ± 1.95^{b}	98.73 ± 2.53^{ab}

Two-way ANOVA (P-value)	Glucose (mg/dl)	Cortisol (ng/ml)
supplements	*0.178*	*0.000*
stress	*0.052*	*0.024*
supplements × stress	*0.996*	*0.996*

3.7. Stress related components

3.7.1. Before crowding stress

Glucose concentrations had no significant differences between all experimental groups (Table 6, $P > 0.05$). Cortisol levels showed lower levels in T_5 than in control ($P < 0.05$), while other treatments had no differences with control (Table 6, $P > 0.05$).

3.7.2. After crowding stress

Glucose concentrations showed no significant differences between all experimental groups (Table 6, $P > 0.05$). The levels of cortisol significantly decreased in all supplemented fish compared to control ($P < 0.05$), while there were no significant differences between them (Table 6, $P > 0.05$).

3.7.3. After recovery

There were no significant differences in glucose concentrations between control and the supplemented groups (Table 6, $P > 0.05$). Except T_7, cortisol levels significantly decreased in the supplemented fish compared to control (Table 6, $P < 0.05$).

3.8. Liver metabolic enzymes of blood

3.8.1. Before crowding stress

The levels of hepatic metabolic enzymes in serum almost showed a decreasing pattern in the supplemented groups compared to the control (Table 7, $P < 0.05$). ALT levels in T_2, T_3, T_6 and T_7 treatments were significantly lower than in control (Table 7, $P < 0.05$). A similar pattern was observed for ALP in T_2, T_3 and T_7 treatments and LDH in T_2, T_3, T_6 and T_7 treatments (Table 7, $P < 0.05$). AST levels showed no differences between all experimental groups (Table 7, $P > 0.05$).

3.8.2. After crowding stress

ALT levels in T_2 and T_7 treatments significantly decreased compared to control (Table 7, $P < 0.05$). Except T_4, LDH decreased in the supplemented groups compared to control (Table 7, $P < 0.05$). The lowest LDH was observed in T_6 (Table 7, $P < 0.05$). AST and ALP levels showed no differences between all experimental groups (Table 7, $P > 0.05$).

3.8.3. After recovery

ALT levels in T_2, T_6 and T_7 treatments significantly decreased compared to control (Table 7, $P < 0.05$). Except T_4, LDH levels decreased in the supplemented groups compared to control (Table 7, $P < 0.05$). ALP activity showed significant decreases in T_2 and T_6 compared to control (Table 7, $P < 0.05$). AST levels showed no differences between all experimental groups (Table 7, $P < 0.05$).

4. Discussion

Dietary supplements including probiotics and herbs are used in aquaculture to improve growth performance and immunity (Nayak, 2010; Shakya et al., 2017; Elumalai et al., 2020a, 2020b). In the present study, we evaluated the potentials of the probiotic, LAB and a herb-derived compound, RE only and in combination on growth, immunity, biochemicals and resistance against crowding stress in the common carp. According to results, LAB and RE only or in combination efficiently improved the growth performance (FW, WG; SGR and FCR) compared to non-supplemented fish. A combination of 1.5×10^7 CFU/g LAB and 300 mg RE/kg showed higher performance compared to other groups. However, use of probiotic only and at high dosage (T_3: 3×10^7 CFU/g LAB) had no positive effect on growth, which shows the necessity of optimizing the concentration of probiotics in the diet. The intestinal lactic acid bacterial counts increased in the probiotic supplemented fish, indicating the efficient modulation of intestinal bacterial flora by the dietary probiotic. The prompting effects of LAB on growth in the present study may be related to the functional role of probiotics in improving digestion and absorption of the nutrients, stimulating the activity of digestive enzymes, producing the growth-inducing metabolites, competing with and excluding the pathogenic bacteria in gut, as previously demonstrated by other researchers (Balcázar et al., 2006; Assan et al., 2022).

In this study, the activity of digestive enzymes increased almost all in response to a combination of RE and LAB (*i.e.* T_6 and T_7). However, the protease activity in treatments, T_2 and T_5 was higher than in control. Therefore, the improved growth in the treatments may also be due to the prompting effect of the supplements on the activity of digestive enzymes. Although the positive potentials of LAB on fish growth has been reported in various studies (Al-Dohail et al., 2009; Faramarzi et al., 2011; Wang, 2011; Hoseinifar et al., 2015a, 2015b, 2015c; Hosseini et al., 2016a, 2016b), there are few studies related to the effect of this probiotic in combination with herbs (Abidin et al., 2022). The prompting effect of RE on fish growth has been widely studied (Wilson et al., 2015a, 2015b; Zhang et al., 2018; Salomão et al., 2019; Naderi Farsani et al., 2021; Tian et al., 2021). However, there is very little information about the combined use of RE and probiotics in the diet (Tan et al., 2019a, 2019b). In the study of Tian et al. (2019), a dietary

Table 7
Hepatic metabolic enzymes in serum of the common carp, *Cyprinus carpio* after 60 days feeding with experimental diets, crowding stress and recovery. T1: basic food as control, T2: *Lactobacillus acidophilus* (LAB) with a concentration of 1.5×10^7 CFU/ml, T3: LAB with a concentration of 3×10^7 CFU/ml, T4: 300 mg resveratrol/kg, T5: 600 mg resveratrol/kg, and T6: 1.5×10^7 CFU/ml + 300 mg resveratrol/kg and T7: 3×10^7 CFU/ml + 600 mg resveratrol/kg. The means with different letters show significant differences ($P < 0.05$).

Status	Parameters	T1 (control)	T2	T3	T4	T5	T6	T7
Before challenge	ALT (U/L)	26.53 ± 0.75^a	20.33 ± 0.88^c	21.93 ± 1.02^{bc}	26.23 ± 0.66^{ab}	22.30 ± 1.45^{abc}	21.16 ± 0.72^c	20.93 ± 0.58^c
	AST (U/L)	89.73 ± 2.39^a	83.16 ± 2.89^a	88.66 ± 1.76^a	87.33 ± 2.90^a	87.10 ± 1.30^a	84.53 ± 1.81^a	80.73 ± 2.32^a
	ALP (U/L)	121.83 ± 2.61^a	107.16 ± 2.12^b	114.33 ± 3.17^{ab}	117.66 ± 2.90^{ab}	113.20 ± 1.68^{ab}	105.66 ± 2.60^b	106.33 ± 2.96^b
	LDH (U/L)	308.00 ± 2.88^a	289.33 ± 2.33^{bc}	292.33 ± 2.02^{bc}	299.00 ± 2.08^{ab}	297.00 ± 2.08^{abc}	286.50 ± 3.01^c	292.50 ± 2.46^{bc}
After challenge	ALT (U/L)	30.53 ± 1.31^a	22.33 ± 0.88^c	25.60 ± 1.02^{abc}	29.56 ± 0.61^{ab}	26.53 ± 1.18^{abc}	25.16 ± 1.58^{abc}	23.60 ± 1.83^{bc}
	AST (U/L)	96.73 ± 1.82^a	88.83 ± 2.31^a	93.00 ± 1.73^a	89.66 ± 2.33^a	91.10 ± 1.35^a	87.86 ± 2.31^a	88.40 ± 2.60^a
	ALP (U/L)	133.83 ± 3.89^a	128.83 ± 2.31^a	131.33 ± 2.02^a	132.66 ± 3.75^a	123.00 ± 1.77^a	120.33 ± 3.17^a	131.66 ± 4.09^a
	LDH (U/L)	326.33 ± 4.40^a	304.33 ± 3.48^{bc}	321.00 ± 3.05^{ab}	321.33 ± 3.17^a	303.33 ± 2.60^c	288.16 ± 2.48^c	296.66 ± 4.40^c
After recovery	ALT (U/L)	28.86 ± 0.73^a	21.33 ± 0.88^c	24.26 ± 0.72^{abc}	27.90 ± 0.95^{ab}	24.86 ± 1.50^{abc}	22.16 ± 1.30^c	23.26 ± 0.63^{bc}
	AST (U/L)	93.73 ± 2.19^a	84.50 ± 2.92^a	92.33 ± 1.45^a	88.66 ± 2.02^a	89.10 ± 1.11^a	85.53 ± 1.81^a	87.73 ± 2.32^a
	ALP (U/L)	129.00 ± 1.73^a	114.83 ± 1.83^{bc}	123.66 ± 2.02^{ab}	124.00 ± 2.08^{ab}	118.33 ± 1.60^{abc}	112.66 ± 2.84^c	121.66 ± 2.96^{abc}
	LDH (U/L)	320.33 ± 3.71^a	296.33 ± 1.85^c	310.00 ± 2.88^{ab}	312.33 ± 3.52^{ab}	300.16 ± 1.58^{bc}	286.83 ± 3.21^c	293.66 ± 2.02^c

Two-way ANOVA (P-value)	ALT (U/L)	AST (U/L)	ALP (U/L)	LDH(U/L)
supplements	*0.000*	*0.008*	*0.000*	*0.000*
stress	*0.024*	*0.119*	*0.000*	*0.003*
supplements × stress	*0.922*	*0.975*	*0.732*	*0.619*

combination of resveratrol (400–800 mg/kg) and the probiotics, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* improved the growth performance in rainbow trout. The improving effects of resveratrol on fish growth are mainly attributed to the role of RE in ameliorating of intestinal damages (Tan et al., 2019a, 2019b), decreasing protein degradation (Wilson et al., 2015a, 2015b), enhancing antioxidant defense (Salomão et al., 2019) and lipid and glucose metabolism (Zhang et al., 2018).

The antioxidant system in aquatic animals is the first line against oxidative stress (Hoseinifar et al., 2020a, 2020b,2020c, 2020d). Antioxidant enzymes and compounds protect cells from damage caused by free radicals during oxidative stress. In this study, the activity of antioxidant enzymes increased only in response to 1.5×10^7 CFU/g LAB + 300 mg RE/kg both before and after crowding stress. Therefore, it seems that a combination of RE and the probiotic at optimized dietary level, improves more efficiently the antioxidant defense system in the fish. After the recovery period, increases in antioxidant enzyme activities also contained the treatments, T_2, T_5 and T_7 in addition to T_6. This result may demonstrated the positive effect of the supplements alone and in combination on the antioxidant system during the recovery from the crowding stress. The protecting function of probiotics against oxidative stress and free radicals is reported by some studies in fish (Hoseinifar et al., 2020a, 2020b,2020c, 2020d) and other vertebrates (Kullisaar et al., 2012; Heshmati et al., 2018).

The mechanism and mode of action of probiotics has not yet been fully understood in fish. However, Probiotics probably suppress oxidative stress by producing metabolites with antioxidant properties such as glutathione, butyrate, folate, and exopolysaccharides, inhibiting the activity of Cyclo-oxygenase (an enzyme involved in the production of free radicals), and by stimulating the antioxidant enzymes (Lin and Yen, 1999; Hussain et al., 2003; Brzozowski et al., 2006). In mozambique tilapia, *O. Mossambicus*, diets containing *B. licheniformis* stimulated the activity of SOD and GPx (Giri et al., 2013). The supplementation of the gilthead seabream *Sparus aurata*, with diet enriched by *Shewanella putrefaciens and Bacillus* up-regulated the expression of SOD and GPx (Esteban et al., 2014). The protective function of RE against oxidative stress may be related to its antioxidant properties (Truong et al., 2018; Tan et al., 2019a, 2019b). Our findings are also supported by this fact that MDA levels significantly decreased almost all in fish supplemented with 1.5×10^7 CFU/g LAB + 300 mg RE/kg. MDA is known as most important indicator of oxidative stress in fish and other vertebrates (Valenzuela, 1991; Gaweł et al., 2004).

In the present study, crowding stress depressed the immunity mostly in the non-supplemented fish. The results obtained from previous studies have shown that crowding stress may reduce immunity in fish in various ways, including changing the amount of proteins in the serum, destroying the activity of phagocytic cells, changing the metabolic rate, and as a result, energy waste (Yin et al., 1995) and cell apoptosis in spleen, a main tissue involved in production of immune cells (Lin et al., 2018a, 2018b). The use of the probiotic and RE alone or in combination improved the immune components of serum and mucus both before and after crowding stress and during recovery period, which mostly contained the treatments, T_2, T_6 and T_7. The immune-prompting role of probiotics and herbal supplements has been widely studied in fish (Galina et al., 2009; C De et al., 2014; Kuebutornye et al., 2020). The immune-boosting properties of LAB also reported in fish (Aly et al., 2008; Hosseini et al., 2016a, 2016b; Foysal et al., 2020). Generally, probiotics mainly improve the fish immune through modulating the intestinal bacterial flora, competing with and eliminating pathogenic bacteria in the gut, and stimulating the immune components (Gómez and Balcázar, 2008; Denev et al., 2009; Nayak, 2010; Aguirre-Guzman et al., 2012). The mode of action of RE on the fish immune system is not yet known, but studies on other vertebrates have shown that RE may play a role in the immune system by producing cytokines and modulating inflammatory responses (Falchetti et al., 2001; Malaguarnera, 2019).

The increased levels of cortisol and glucose in blood usually occur in response to acute or chronic stress in fish. As the main stress hormone, cortisol breaks down the glycogen stores of liver to produce glucose to meet the energetic costs of stress (Vijayan et al., 2010). In this study, cortisol levels decreased in the supplemented fish compared to control after crowding stress and also during recovery period. This result suggest a stress mitigating effect for RE and the probiotic, as previously observed in some studies (Hoseinifar et al., 2015a, 2015b, 2015c; Rohmah et al., 2022). In agreement with our results, the mitigating effects of probiotics and herbs on crowding stress has been also reported in many studies (Xie et al., 2008; Gonçalves et al., 2011; Tapia-Paniagua et al., 2014; Reyes-Cerpa et al., 2018a, 2018b; Yousefi et al., 2019a, 2019b; Paray et al., 2020a, 2020b; Adineh et al., 2021). Although the mechanism of the stress mitigating effects by probiotics and medicinal plants is not yet fully known in fish, it may be related to the protective properties of these supplements against oxidative stress (Mueller et al., 2010; Hamed and El-Sayed, 2019; Hoseinifar et al., 2020a, 2020b,2020c, 2020d).

Release of hepatic metabolic enzymes into the blood usually occurs following liver damage or dysfunction (Obomanu et al., 2009; Ghelichpour et al., 2020). Although this issue is not specific, it is usually considered as an indicator of liver dysfunction in biological studies. In the present study, the concentration of liver enzymes in the blood decreased in almost all supplemented fish compared to the control, especially in the fish supplemented with a combination of the probiotic and RE. This result may suggest a protective role for the supplements on the liver, as previously reported for other herbs and probiotics (Banaee et al., 2011; Adorian et al., 2019; Rafieepour et al., 2019).

5. Conclusion

In conclusion, the results of this study showed that both probiotics and RE and their combinations in the diet can improve growth, antioxidant and immunity system and mitigate the crowding stress in fish. However, it seems that the combinations of the probiotic and RE gives a more favorable result. In this regard, a combination of 1.5×10^7 CFU/g LAB + 300 mg RE/kg gives the most favorable results in terms of the above parameters.

Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

CRediT authorship contribution statement

Gamal A. Gabr: Writing – original draft, Supervision. **Yousif Saleh Ibrahim:** Conceptualization. Sarmad Ghazi Al-Shawi: Supervision. Munther Abosaooda: Conceptualization. Jitendra Gupta: Writing – review & editing. **Khulood H. Oudaha:** Writing – review & editing. **Khudargan Mavlonov:** Formal analysis. **Abduladheem Turki Jalil:** Writing – original draft, Formal analysis. **Karkaz M. Thalij:** Writing – review & editing, Resources. **Yasser Fakri Mustafa:** Writing – review & editing, Resources. **Mohammad Khodadadi:** Methodology. **Mahnaz Dadras:** Writing – original draft, Methodology.

Author statement

All people who meet authorship criteria are mentioned as authors, and all authors certify that they have taken part sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. In addition, every author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Aquaculture Reports.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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