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# ROSMARINIC ACID ALONE OR IN COMBINATION WITH *LACTOBACILLUS RHAMNOSUS* AMELIORATED RESISTANCE TO AMMONIA STRESS IN THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*: GROWTH, IMMUNITY, ANTIOXIDANT DEFENSE AND LIVER FUNCTIONS

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**Abstract**

**Rosmarinic acid (RS) and *Lactobacillus rhamnosus* (LR) were added singularly or in combination to rainbow trout (*Oncorhynchus mykiss*) diets to test their efficacy in the protection against ammonia stress. Fish (31.4±0.6 g) were randomly allocated to six groups in three replicates, as follows: $T_1$: basic food as control, $T_2$: LR with a concentration of $1.5 \times 10^8$ CFU/g, $T_3$: LR with a concentration of $3 \times 10^8$ CFU/g, $T_4$: 1 g RS/kg, $T_5$: 3 g RS/kg, and $T_6$: $1.5 \times 10^8$ CFU/g LR + 1 g RS/kg and $T_7$: $3 \times 10^8$ CFU/g LR + 3 g RS/kg. After 60 days feeding, fish were exposed to ammonia stress. After the feeding period, the supplemented fish had the highest final body weight (FW), weight gain (WG), and specific growth rate (SGR), and the lowest feed conversion ratio (FCR) as compared with the control group (P<0.05). Amylase, protease and lipase activities were noticed markedly higher in fish supplemented with $1.5 \times 10^8$ CFU/g LR + 1 g RS/kg and $1.5 \times 10^8$ CFU/g LR 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 Ig, nitroblue tetrazolium test (NBT), myeloperoxidase (MPO), complement component 3 (C3), complement component 4 (C4), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx). On the other hand, $T_2$ and $T_6$ groups had the lowest malondialdehyde (MDA), glucose, and cortisol concentrations as well as alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) enzyme levels when compared with the control (P<0.05). After ammonia stress, fish in the supplemented groups, particularly $T_2$ and $T_6$, generally showed significantly higher values of lysozyme, ACH50, 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). In conclusion, a combined administration of RS and *L. rhamnosus* effectively improved growth performance and health status as well as enhanced the resistance of rainbow trout against ammonia toxicity.**

**Key words: rosmarinic acid, probiotic, immunity, *Lactobacillus rhamnosus*, fish, ammonia stress**

The intensive fish farming system was designed in response to increasing needs of human beings to high quality protein resources. However, from the aquaculture point of view, this system has some limitations, including the increased risk of diseases and the continuous elevation of toxic metabolites such as ambient ammonia (Crab et al., 2007; Pulkkinen et al., 2010; Romero et al., 2012; Wang et al., 2021). Water quality management is one of the important ways to reduce toxic substances such as ammonia in fish ponds (Hargreaves and Tucker, 2004; Francis-Floyd et al., 2009). However, it is difficult to continuously maintain water quality in optimized condition, especially in high fish densities. Animal or plant-based supplements have been proven to effectively enhance growth and immunity in fish (Galina et al., 2009; Elumalai et al., 2020). In addition, some studies have shown that nutritional supplements can also be effective in increasing the resistance of fish against environmental stress, especially ammonia stress (Mirghaed et al., 2019 a, b; Rajabiesterabadi et al., 2020 a, b; Hoseini et al., 2021; Magouz et al., 2021).

It is well recognized that ammonia accumulation will cause toxicity signs in fish and in high concentrations may lead to widespread mortalities. Medicinal plants and functional phytochemical compounds extracted from them such as flavonoids, phenolic compounds, carotenoids, and terpenes are well known to improve fish immune system (Shakya, 2016; Tungmunnithum et al., 2018; Ahmadifar et al., 2021; Khanjani et al., 2022 a, b). In addition, they may also support the fish resistance against environmental stressors such as ammonia toxicity.

Probiotics are dietary supplements with immune-stimulatory functions in fish and other vertebrates (Ezema, 2013; Zorriehzahra et al., 2016; Banerjee and Ray, 2017; Abdel-Latif et al., 2023). Probiotics mainly enhance the fish immune system through modulating the intestinal microflora, stimulating the innate immune components, competing with and removing pathogens in the gut (Gómez and Balcázar, 2008; Denev et al., 2009; Nayak, 2010; Aguirre-Guzman et al., 2012; El-Saadony et al., 2021). The probiotic strains such as *Bacillus*,

*Lactococcus* and *Lactobacillus* are among the most important probiotics used in aquaculture (Takanashi et al., 2014; Abdel-Latif et al., 2022; Yilmaz et al., 2022). Generally, few studies have been conducted on the potentials of medicinal plants and probiotics as food supplements in ameliorating toxicity of ammonia in fish. Therefore, finding and introducing effective supplements can help to promote fish farming.

Rosmarinic acid (RS) is a natural polyphenol found in many Lamiaceae herbs such as rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis*), basil (*Ocimum basilicum*), oregano (*Origanum vulgare*), lemon balm (*Melissa officinalis*) and many others (Alagawany et al., 2017; Ahmadifar et al., 2021). Many studies have reported antioxidant, anti-bacterial, anti-inflammatory, and immunomodulatory function for RS (Abedini et al., 2013; Alagawany et al., 2017; Nadeem et al., 2019). However, there are no data on the antioxidant and immuno-stimulant roles of RS in fish. Specific lactic acid bacteria as probiotic (LAB) strains, such as *Lactobacillus rhamnosus*, have been considered as probiotics because of their health benefits (Nikoskelainen et al., 2003). *L. rhamnosus* is categorized as a probiotic strain because of its beneficial effects and prevention of disease transmission (Pirarat et al., 2006; Chen et al., 2016; Westerik et al., 2018; Mindus et al., 2021; Hoseini et al., 2016). In the current research, we have tried to determine the potentials of a plant-derived compound, rosmarinic acid (RS) and the probiotic, *L. rhamnosus* and their combinations in improving growth, immunity, and resistance to ambient ammonia stress in rainbow trout, *Oncorhynchus mykiss*. The results of the present study may help us to enhance the sustainability of rainbow trout aquaculture.

## Material and methods

### Probiotic preparation and rosmarinic acid

*L. rhamnosus* (reference strain: ATCC 7469) was provided from the Center of Industrial Microorganisms of Iran. The bacterium was grown in MRS broth for 24 h at 36°C. One colony of the bacterium was separated by MRS agar, and the colonies were grown again in MRS agar medium for 24 h. After incubation, the grown bacterium on the agar medium was then centrifuged (10,000 × *g* for 10 min at 4°C) and washed by saline solution two times. The experimental bacterial concentrations were prepared using a spectrophotometer (Madreseh et al., 2019). Rosmarinic acid was provided from Sigma-Aldrich Company, USA [purity percentage: >99% (R4033)].

### Diet preparation

The preparation of experimental diets was done according to Rajabiesterabadi et al. (2020 a, b). Briefly, the supplements (LR and RS at adjusted dietary levels) along with little amount of water were added to a basal rainbow trout diet (Faradaneh CO., Iran; pellet size: 4.0±0.3 mg, composition: crude protein: 44%; crude lipid: 16%; crude fiber: 4%; ash content: 11%; moisture content: 11%; phosphorus: 1.5%), and well mixed to make a paste. The paste was processed by a grinder to make pellets and the pellets were finally dried at 36°C. The LR dietary levels (CFU/g diet) were selected based on the results obtained from a previously published study (Nikoskelainen et al., 2003). Also, since the dietary levels of RS have not been determined for fish, we used data related to rosemary, a plant containing high concentrations of rosmarinic acid, to determine its approximate dietary levels, according to Karataş et al. (2020).

### Fish and experimental design

A total number of 630 rainbow trout (initial weight of 31.4±0.6 g) were provided from a local trout farm (Shahrekord, Iran) and kept in 1000 L tanks (210 fish/tank) for 14 days to be adapted to the lab conditions. Fish were fed with the basal diet during adaptation period. After adaptation, fish were distributed in 21 500 L tanks (30 fish/tank) as seven experimental treatments in three replicates and fed experimental diets for 60 days. The experimental groups were as follows: $T_1$: basic food as control, $T_2$: *L. rhamnosus* (LR) with a concentration of $1.5 \times 10^8$ CFU/g, $T_3$: LR with a concentration of $3 \times 10^8$ CFU/g, $T_4$: 1 g rosmarinic acid (RS)/kg, $T_5$: 3 g RS/kg, and $T_6$: $1.5 \times 10^8$ CFU/g + 1 g RS/kg and $T_7$: $3 \times 10^8$ CFU/g + 3 g RS/kg. During feeding experiment, the growth of the fish was investigated by sampling every two weeks. The water quality was checked daily, which was at optimized limitations throughout the experiment for temperature: 15.4±0.3°C, dissolved oxygen: >6.5 mg/L, pH: 7.4±0.1 and non-ionized ammonia: <0.001 mg/L. Feeding of fish was conducted five times a day at a rate of 2.25% of biomass according to the instructions of the feed manufacturer.

### Growth parameters

At the end of feeding period, the fish were starved for about 24 h, anesthetized using tricaine methane sulfonate (at concentration of 60 mg/L) (Wagner et al., 2003) and the growth performance parameters estimated according to following equations:

*Weight gain (WG) = Final weight [FW(g)] – Initial weight [IW (g)]*

*Feed conversion ratio (FCR) = Feed consumed / (FW-IW)*

*Specific growth rate (SGR %) = 100 × [(ln FW – ln IW)/days]*

*Survival rate (SR %) = (Total number of dead fish / Total number of fish) × 100*

### Digestive enzymes

Intestine tissue samples were prepared according to Gisbert et al. (2016) before enzymatic assays. Briefly,

the samples were emptied, homogenized mechanically by a tissue homogenizer in 0.05 M Tris buffer (Heidolph® SilentCrusher-M, Heidolph, Nuremberg, Germany) and centrifuged at 4°C (13000 × *g* for 10 min) and supernatant stored at –80°C. Amylase activity was estimated spectrophotometrically at 600 nm using 2% starch solution as substrate in 0.1 M citrate phosphate buffer (Robyt and Whelan, 1968). Lipase activity was assayed at 405 nm according to 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. Protease activity was determined by using azo-casein in Tris buffer (Tris–HCl 0.1 M; pH=8) as substrate according to García-Carreño (1992).

**Intestinal bacterial load**

Intestinal tissues were prepared before bacterial load examinations. Briefly, fish skin was washed by distilled water and disinfected by 70% ethanol. Intestinal tissue samples were aseptically removed, homogenized in phosphate buffer (PBS, pH = 7.2) by a tissue homogenizer and then diluted in PBS. 100 µL of the bacterial suspension was grown (30°C for 48 h) on MRS agar medium (Merck, Germany) and tryptic soy agar culture medium (TSA; Merck, Germany) to estimate lactic acid bacteria and total intestinal bacteria counts (CFU/g) (Merrifield et al., 2010).

**Blood and mucus sampling**

The blood and mucus samples were collected after feeding experiment and post-ammonia stress. For this purpose, fish (n=3/tank) were anesthetized using tricaine methane sulfonate (at concentration of 60 mg/L) and the blood samples were taken by a 2 ml syringe from the caudal veins and poured into microtubes containing heparin. The samples were kept at room temperature for 2 h, centrifuged at 4°C (13000 × *g* for 8 min) and the supernatants stored at –80°C. For mucus sampling, fish (n=3 fish/tank) were randomly transferred to polyethylene bags containing 10 mL of 50 mM physiological serum. After 4 min, the collected mucus was centrifuged (3000 × *g* for 10 min in 4°C) and the supernatant kept at –80°C (Ross et al., 2000).

**Immune parameters of blood and mucus**

A turbidimetric method (Ellis, 1990) was used to determine lysozyme activity (U/mg) of serum and mucus using *Micrococcus lysodeikticus* bacterial suspension as a target. The serum immunoglobulin (mg/dL) content was measured by calculating the difference in the total protein content before and after addition of polyethylene glycol (Hoseinifar et al., 2016). The components of complement system including C3 and C4 in serum were measured by an ELISA device (Biotek ELx800) using a commercial kit (Pars Azmoon Company, Tehran, Iran). The activity of alternative complement (ACH50) in serum and mucus was determined according to the method of Yano (1992). Briefly, the serum samples were serially diluted by EDTA-GVB (Gelatin Veronal Buffer containing 10 mM EDTA) and then 2% sheep red blood cell was added to the samples as target, incubated for 2 h and absorbance was recorded at 412 nm. Mucus protease activity was assayed according to Hoseinifar et al. (2016). Briefly, 100 µL of mucus was mixed with 100 µL of 100 mM ammonium bicarbonate buffer containing 0.7% azocasein and incubated at 30°C for 20 h. The reaction was stopped using trichloroacetic acid and the supernatant was collected by centrifugation (15,000 × *g* for 5 min). Finally, 0.5 N hydroxide was added to supernatant and the absorbance was read at 450 nm. To measure respiratory burst activity, a nitroblue tetrazolium (NBT) reduction test was used. Briefly, 100 µL of 0.2% NBT solution was mixed with 100 µL of heparinized blood and incubated for 30 min. After incubation, 50 µL of the mixture was mixed with 1 ml of N,N-Dimethylformamide, centrifuged (3000 × g for 5 min) and the adsorption was read at 540 nm (Siwicki et al., 1994).

**Biochemical measurements in blood and mucus**

The cortisol concentrations were determined in serum and mucus by a commercial kit (RK-240CT, Budapest, Hungary) using radioimmunoassay. Glucose concentrations in serum and mucus were assayed by commercial kits (Sigma-Aldrich Co., USA). Serum catalase (CAT) activity (U/mL) was measured by estimating the reduction rate of $H_2O_2$ according to Goth (1991). The activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD) in serum were determined by estimating the oxidation rate of glutathione and reduction rate of cytochrome C, respectively. Malondialdehyde levels (MDA) was determined by thiobarbituric acid method. The activities of hepatic enzymes, ALP, ALT, LDH and AST in serum were assayed an autoanalyzer (Avanti J-26 XPI, Beckman Coulter, CA, USA) by using Sigma-Aldrich commercial kits.

**Ammonia stress**

At the end of feeding period, 15 fish were randomly sampled and exposed to ammonium chloride (Darmstadt, Germany) at concentration of 0.3 g/L for 24 h (Yousefi et al., 2019; Randall and Tsui, 2002). During the challenge test, feeding and water flow of tanks were stopped, and the aeration was continuously conducted by an air pump.

**Data analysis**

The SPSS software (version 16) was used to evaluate data. Data normality was evaluated with the Kolmogorov–Smirnov test before analysis of variance (ANOVA). The proportional and percent data were converted by Arcsin transformation before ANOVA. One-way ANOVA was used to find the significant differences. Finally, the means were compared using Tukey's multiple comparison test at $P<0.05$.

## Results

### Growth

The values of FW, WG and SGR significantly increased in the treatments $T_2$ ($1.5 \times 10^8$ CFU/g LR), $T_4$ (1 g RS/kg), $T_5$ (3 g RS/kg), $T_6$ ($1.5 \times 10^8$ CFU/g LR + 1 g RS/kg) and $T_7$ ($3 \times 10^8$ CFU/g LR + 3 g RS/kg) compared to control and $T_3$ ($3 \times 10^8$ CFU/g LR) (Table 1, $P<0.05$). The growth performance of fish showed no significant differences between the groups, $T_3$, $T_4$, $T_5$ and $T_7$ (Table 1, $P>0.05$). Fish of $T_6$ showed more growth performance compared to other groups ($P<0.05$). Except $T_3$, the supplemented fish showed lower FCR values compared to control (Table 1, $P<0.05$). The $T_6$ group showed the lowest FCR values compared to other groups (Table 1, $P<0.05$).

### Digestive enzymes

Protease activity significantly increased in the treatments $T_2$, $T_5$, $T_6$ and $T_7$ compared to control (Table 2, $P<0.05$). This enzyme showed no differences between supplemented groups (Table 2, $P>0.05$). Amylase activity was significantly elevated in the fish of $T_6$ and $T_7$ compared to control and the treatments, $T_3$, $T_4$ and $T_5$ (Table 2, $P<0.05$). No significant differences were found in the amylase activity among the groups $T_6$, $T_7$ and $T_2$ (Table 2, $P>0.05$). Lipase activity was significantly elevated in the treatments $T_6$ and $T_6$ compared to control (Table 2, $P<0.05$), while other groups exhibited no significant differences with control (Table 2, $P>0.05$).

### Intestinal bacterial load

The intestinal load of lactic acid bacteria (CFU/g) significantly increased in response to probiotic supplementation (Figure 2 A, $P<0.05$). There were no significant differences in the bacterial concentration of control and RS-supplemented fish (Figure 2 A, $P>0.05$). There were no significant differences in total bacterial concentration (TBC) between all experimental groups (Figure 2 B, $P<0.05$).

### Antioxidant enzymes and oxidative stress

*Before ammonia stress*

The antioxidant enzymes, CAT and GPx showed higher activities in the treatment, $T_6$ compared to control ($P<0.05$). Other treatments showed no significant differences in terms of SOD activity (Table 3, $P>0.05$). The levels of MDA were significantly lower in the treatment $T_6$ compared to other groups (Table 3, $P<0.05$). Except $T_6$, other groups exhibited no differences in CAT, GPx and MDA with control (Table 3, $P>0.05$).

*After ammonia stress*

The activity of CAT and GPx was significantly elevated in the treatment $T_6$ compared to control (Table 3, $P<0.05$), while other supplemented fish showed no significant differences with control (Table 3, $P>0.05$). There were no significant differences in SOD activity between all groups (Table 3, $P>0.05$). MDA levels significantly decreased in $T_6$ compared to control ($P<0.05$), while there were no significant differences between other supplemented fish with control (Table 3, $P>0.05$).

Table 1. Growth parameters of rainbow trout, *O. mykiss* after 60 days feeding with experimental diets

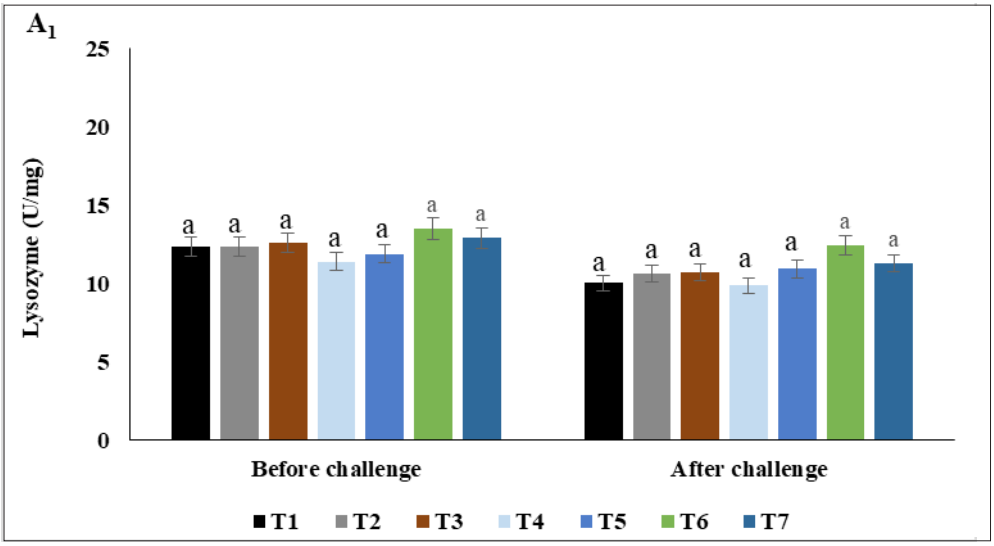
| Parameters | T1 (control) | T2 | T3 | T4 | T5 | T6 | T7 |
|---|---|---|---|---|---|---|---|
| IW (g) | 32.6±0.31 a | 32.5±0.27 a | 33.4±0.42 a | 31.7±0.31 a | 31.63±0.21 a | 3.60±0.30 a | 30.5±0.14 a |
| FW (g) | 59.26±1.16 d | 66.00±0.55 ab | 62.00±0.85 cd | 63.16±0.73 bc | 64.56±0.46 bc | 71.16±0.73 a | 67.50±0.75 abc |
| WG (g) | 28.50±0.77 c | 35.53±0.59 ab | 31.83±0.46 bc | 32.83±0.44 b | 33.13±0.56 b | 40.56±1.20 a | 37.33±1.15 ab |
| FCR | 2.02±0.06 a | 1.53±0.01 bc | 1.74±0.02 ab | 1.68±0.02 b | 1.61±0.03 bc | 1.42±0.03 c | 1.55±0.04 bc |
| SGR (% $d^{-1}$) | 1.12±0.01 c | 1.28±0.01 ab | 1.21±0.02 bc | 1.23±0.01 b | 1.26±0.02 ab | 1.37±0.025 a | 1.32±0.04 ab |
| SR (%) | 94.00±1.63 a | 97.33±2.03 a | 96.00±1.01 a | 96.33±2.80 a | 95.33±2.90 a | 67.66±2.48 a | 96.33±2.80 a |

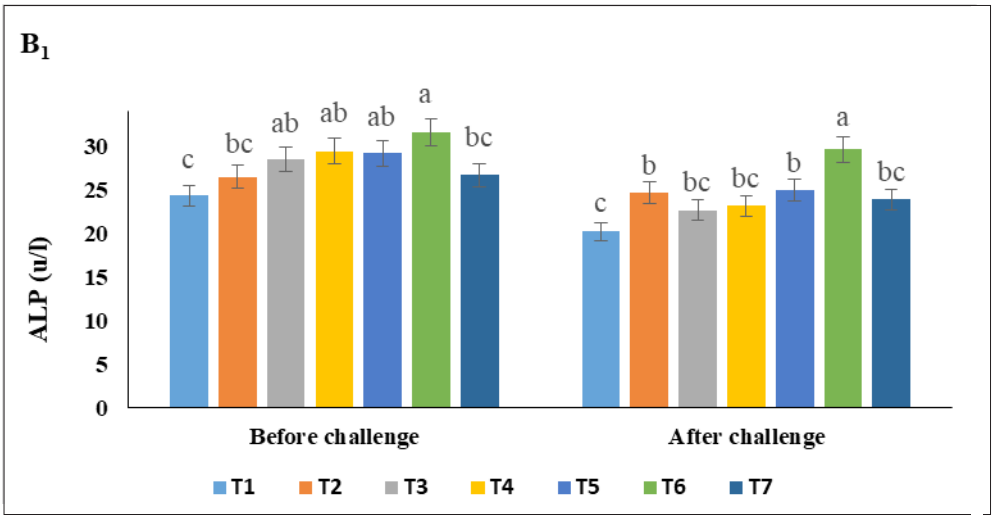
$T_1$: basic food as control, $T_2$: *L. rhamnosus* (LR) with a concentration of $1.5 \times 10^8$ CFU/g, $T_3$: LR with a concentration of $3 \times 10^8$ CFU/g, $T_4$: 1 g RS/kg, $T_5$: 3 g RS/kg, and $T_6$: $1.5 \times 10^8$ CFU/g + 1 g RS/kg and $T_7$: $3 \times 10^8$ CFU/g + 3 g RS/kg. The means with different letters show significant differences ($P<0.05$).

Table 2. Activity of digestive enzymes in rainbow trout, *O. mykiss* after 60 days feeding with experimental diets

| Parameters | T1 (control) | T2 | T3 | T4 | T5 | T6 | T7 |
|---|---|---|---|---|---|---|---|
| Amylase (U/mg protein) | 12.00±0.59 b | 14.25±0.63 ab | 12.25±0.34 b | 12.30±0.60 b | 12.35±0.52 b | 15.95±0.34 a | 15.44±0.46 a |
| Protease (U/mg protein) | 4.80±0.21 c | 7.18±0.33 a | 6.07±0.18 abc | 5.46±0.3 bc | 6.40±0.31 ab | 7.01±0.36 ab | 6.67±0.4 ab |
| Lipase (U/mg protein) | 1.19±0.11 c | 1.73±0.07 ab | 1.58±0.04 abc | 1.41±0.04 bc | 1.43±0.05 bc | 1.85±0.06 a | 1.55±0.07 abc |

$T_1$: basic food as control, $T_2$: *L. rhamnosus* (LR) with a concentration of $1.5 \times 10^8$ CFU/g, $T_3$: LR with a concentration of $3 \times 10^8$ CFU/g, $T_4$: 1 g RS/kg, $T_5$: 3 g RS/kg, and $T_6$: $1.5 \times 10^8$ CFU/g + 1 g RS/kg and $T_7$: $3 \times 10^8$ CFU/g + 3 g RS/kg. The means with different letters show significant differences ($P<0.05$).



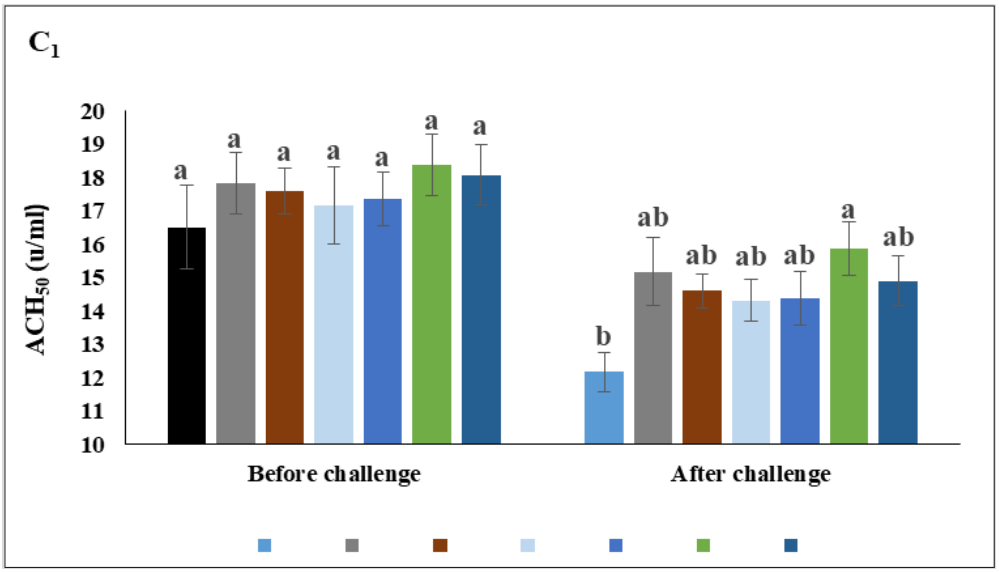
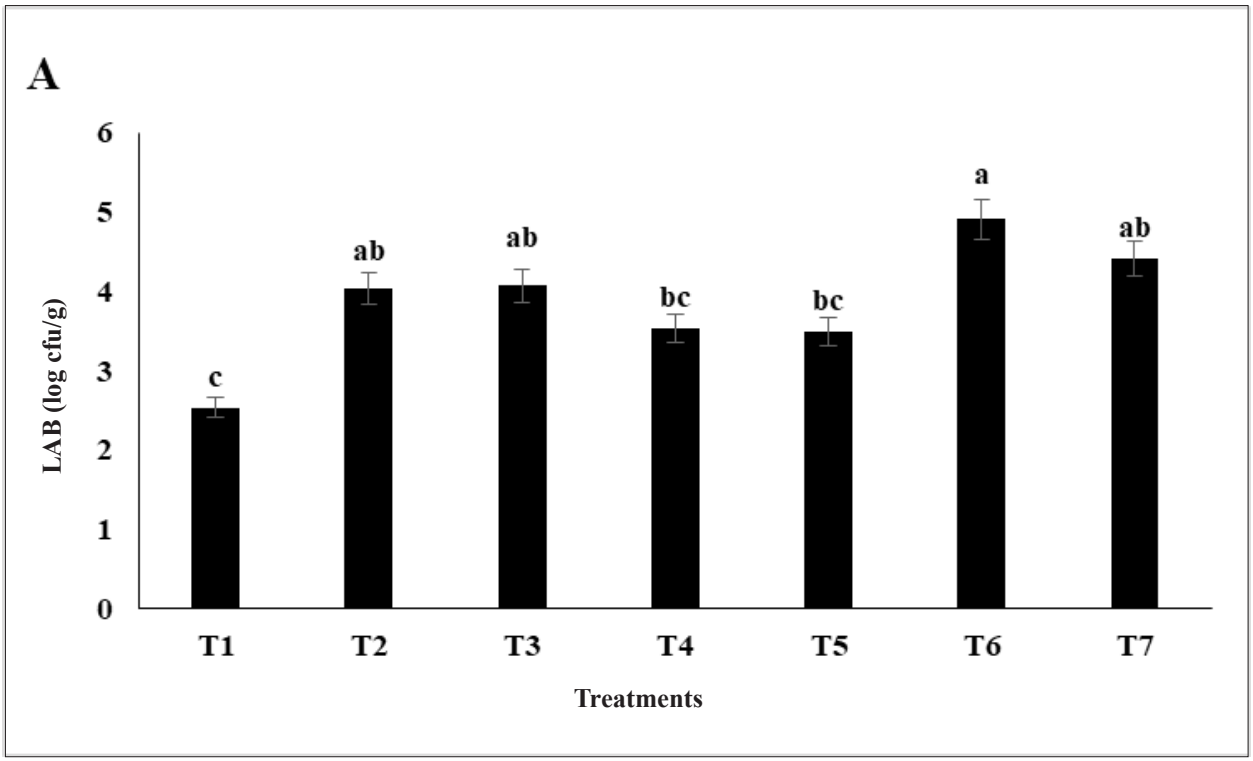




Figure 1. Immune components of mucus in rainbow trout, *O. mykiss* after 60 days feeding with experimental diets and after ammonia stress. $T_1$: basic food as control, $T_2$: *L. rhamnosus* (LR) with a concentration of $1.5 \times 10^8$ CFU/g, $T_3$: LR with a concentration of $3 \times 10^8$ CFU/g, $T_4$: 1 g RS/kg, $T_5$: 3 g RS/kg, and $T_6$: $1.5 \times 10^8$ CFU/g + 1 g RS/kg and $T_7$: $3 \times 10^8$ CFU/g + 3 g RS/kg. The means with different letters show significant differences ($P<0.05$)



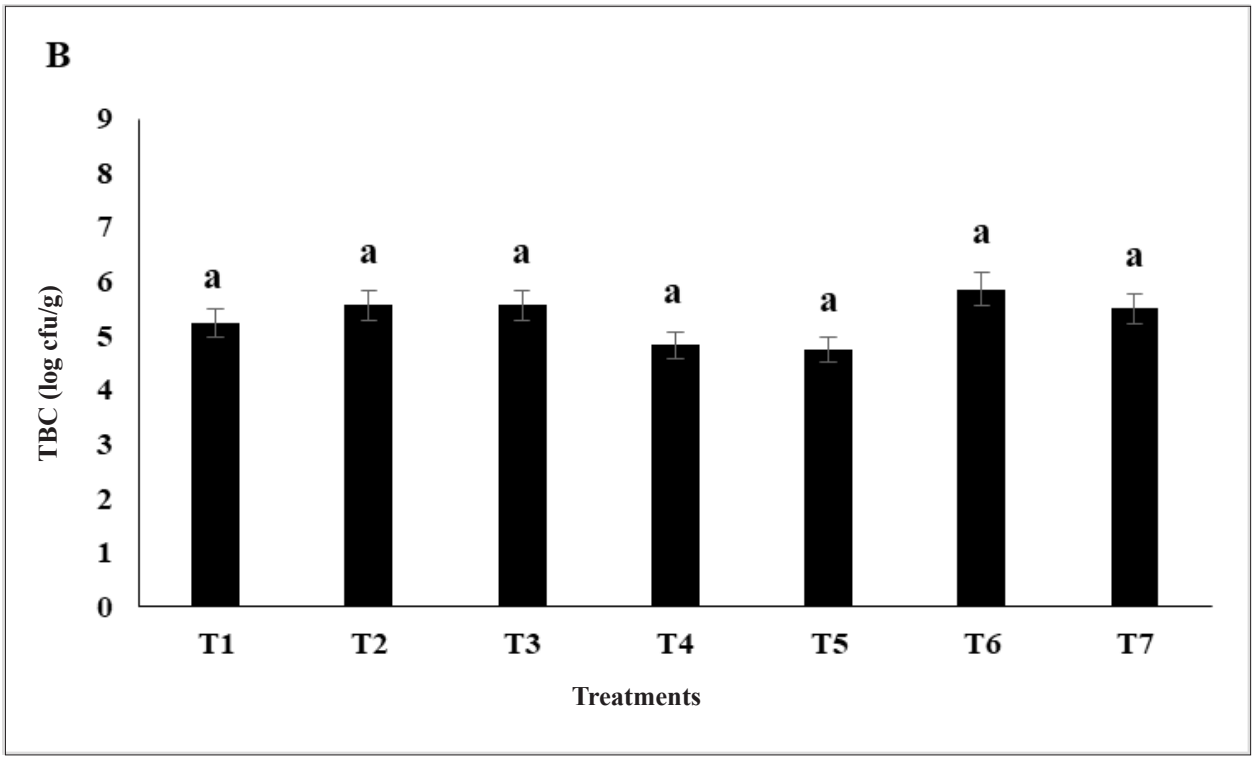




Figure 2. The intestinal lactic acid bacteria load in rainbow trout, *O. mykiss* after 60 days feeding with experimental diets. $T_1$: basic food as control, $T_2$: *L. rhamnosus* (LR) with a concentration of $1.5 \times 10^8$ CFU/g, $T_3$: LR with a concentration of $3 \times 10^8$ CFU/g, $T_4$: 1 g RS/kg, $T_5$: 3 g RS/kg, and $T_6$: $1.5 \times 10^8$ CFU/g + 1 g RS/kg and $T_7$: $3 \times 10^8$ CFU/g + 3 g RS/kg. The means with different letters show significant differences ($P<0.05$)

Table 3. Antioxidant enzyme activity and oxidative stress indices (MDA) in rainbow trout, *O. mykiss* after 60 days feeding with experimental diets and after ammonia stress

| Status | Parameters | T1 (control) | T2 | T3 | T4 | T5 | T6 | T7 |
|---|---|---|---|---|---|---|---|---|
| Before challenge | MDA (nm/L) | 38.45±1.10 a | 35.36±1.85 ab | 36.20±0.86 ab | 36.46±0.80 ab | 33.81±1.14 ab | 32.63±1.2 b | 34.86±1.11 ab |
| | SOD (U/mL) | 24.00±1.15 a | 25.43±1.15 a | 26.00±1.41 a | 25.50±1.33 a | 26.50±1.21 a | 27.43±1.02 a | 24.80±0.80 a |
| | CAT (U/mL) | 103.33±2.65 b | 117.36±2.13 ab | 111.83±1.9 ab | 112.20±2.27 b | 112.00±2.11 ab | 126.66±1.23 a | 111.00±1.20 b |
| | Gpx (U/mL) | 134.00±1.42 b | 136.66±1.66 ab | 136.83±2.58 ab | 136.80±1.11 ab | 138.26±1.83 ab | 143.50±1.22 a | 139.66±2.23 ab |
| After challenge | MDA (nm/L) | 41.23±1.26 a | 36.13±1.93 b | 36.13±1.01 ab | 37.10±1.05 ab | 34.91±1.24 b | 33.73±1.32 b | 35.63±1.25 b |
| | SOD (U/mL) | 21.66±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) | 96.00±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) | 128.00±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 |

$T_1$: basic food as control, $T_2$: *L. rhamnosus* (LR) with a concentration of $1.5 \times 10^8$ CFU/g, $T_3$: LR with a concentration of $3 \times 10^8$ CFU/g, $T_4$: 1 g RS/kg, $T_5$: 3 g RS/kg, and $T_6$: $1.5 \times 10^8$ CFU/g + 1 g RS/kg and $T_7$: $3 \times 10^8$ CFU/g + 3 g RS/kg. The means with different letters show significant differences (P<0.05).

Table 4. Immune components of serum in rainbow trout, *O. mykiss* after 60 days feeding with experimental diets and after ammonia stress

| Status | Parameters | T1 (control) | T2 | T3 | T4 | T5 | T6 | T7 |
|---|---|---|---|---|---|---|---|---|
| Before challenge | Lysozyme (U/mg) | 19.60±0.60 c | 26.60±0.47 b | 24.36±0.62 b | 23.43±0.63 b | 23.63±0.67 b | 29.33±0.85 a | 24.45±1.02 b |
| | $ACH_{50}$ (U/L) | 110.73±0.62 c | 121.06±1.37 b | 113.60±1.15 bc | 114.73±1.54 bc | 115.40±1.67 bc | 131.30±2.02 a | 120.70±1.50 b |
| | Total Ig (mg/dL) | 14.44±0.66 b | 18.44±0.70 a | 20.24±0.57 a | 18.61±0.31 a | 18.77±0.46 a | 20.67±0.46 a | 19.04±0.33 a |
| | NBT (540 nm) | 0.31±0.05 b | 0.65±0.04 a | 0.4±0.06 ab | 0.38±0.05 ab | 0.45±0.06 ab | 0.65±0.05 a | 0.51±0.06 ab |
| | MPO (450 nm) | 1.15±0.12 b | 2.32±0.29 a | 1.72±0.16 ab | 1.65±0.17 ab | 1.85±0.18 ab | 2.35±0.24 a | 1.69±0.15 ab |
| | C3 (g/dL) | 24.43±1.05 b | 27.83±1.05 ab | 32.83±1.30 a | 34.02±1.36 a | 33.23±1.45 a | 32.63±1.30 a | 28.30±1.02 ab |
| | C4 (g/dL) | 9.40±0.65 b | 11.42±0.54 ab | 14.00±0.36 a | 14.10±0.51 a | 13.13±0.16 a | 12.41±0.28 ab | 11.54±0.48 ab |
| After challenge | Lysozyme (U/mg) | 14.12±0.76 c | 20.29±0.16 ab | 19.12±0.6 b | 18.42±0.49 b | 18.22±0.41 b | 23.32±0.63 a | 19.01±0.40 b |
| | $ACH_{50}$ (U/L) | 102.00±1.63 b | 111.33±2.16 ab | 105.70±1.41 b | 106.83±1.20 b | 108.50±1.09 b | 123.06±1.56 a | 108.80±1.06 b |
| | Total Ig (mg/dL) | 10.32±0.76 b | 13.32±0.80 a | 15.00±0.40 a | 13.60±0.23 a | 13.86±0.22 a | 14.70±0.45 a | 14.30±0.35 a |
| | NBT (540 nm) | 0.3±0.04 c | 0.62±0.03 ab | 0.48±0.05 bc | 0.36±0.04 bc | 0.38±0.03 bc | 0.58±0.04 a | 0.46±0.05 abc |
| | MPO (450 nm) | 0.731±0.11 b | 2.11±0.33 a | 1.34±0.22 ab | 1.41±0.16 ab | 1.54±0.15 ab | 2.20±0.22 a | 1.45±0.16 ab |
| | C3 (g/dL) | 16.85±0.75 d | 19.55±0.87 cd | 27.62±2.02 ab | 27.75±1.43 ab | 30.69±1.26 a | 29.72±0.71 a | 24.39±1.03 bc |
| | C4 (g/dL) | 6.63±0.61 b | 9.33±0.70 ab | 9.00±0.46 ab | 10.10±0.60 a | 11.33±0.23 a | 11.36±0.41 a | 10.2±0.26 a |

$T_1$: basic food as control, $T_2$: *L. rhamnosus* (LR) with a concentration of $1.5 \times 10^8$ CFU/g, $T_3$: LR with a concentration of $3 \times 10^8$ CFU/g, $T_4$: 1 g RS/kg, $T_5$: 3 g RS/kg, and $T_6$: $1.5 \times 10^8$ CFU/g + 1 g RS/kg and $T_7$: $3 \times 10^8$ CFU/g + 3 g RS/kg. The means with different letters show significant differences (P<0.05).

**Immune components of serum**

*Before ammonia stress*

Lysozyme activity and Ig content increased in the supplemented groups compared to control (Table 4, P<0.05). The highest lysozyme activity and Ig content was found in the treatment $T_6$ (Table 4, P<0.05). NBT and MPO values were significantly elevated in the treatments $T_2$ and $T_6$ compared to control (Table 4, P<0.05), while no significant differences were observed between other groups with control (Table 4, P>0.05). $ACH_{50}$ activity significantly increased in the treatments $T_2$, $T_6$ and $T_7$ compared to control (Table 4, P<0.05). The values of C3 were significantly elevated in $T_3$ and $T_6$ compared to control (Table 4, P<0.05). The fish of $T_2$ and $T_7$ showed no differences in C3 values with control (Table 4, P>0.05). C4 values were significantly elevated in the treatments, $T_3$, $T_4$ and $T_5$ compared to control (P<0.05), while other supplemented fish showed no differences with control (Table 4, P<0.05).

*After ammonia stress*

The supplemented fish showed higher lysozyme activity and Ig content compared to control, with highest levels in $T_6$ (Table 4, P<0.05). $ACH_{50}$ activity was significantly elevated in $T_4$ compared to control and other supplemented fish (P<0.05), while other supplemented fish exhibited no differences with control (Table 4, P>0.05). Significant increases were observed for NBT and MPO in the treatments $T_2$ and $T_6$ compared to control (P<0.05), while other supplemented fish had no differences with

control (Table 4, P>0.05). C3 and C4 values were significantly elevated in the fish of $T_3$-$T_7$ compared to control (P<0.05).

**Immune components of mucus**

*Before ammonia stress*

There were no significant differences in lysozyme (Figure 1 A1) and $ACH_{50}$ (Figure 1 C1) activities between all groups (P<0.05). ALP (Figure 1 B1) activity was considerably elevated in the $T_3$-$T_6$ compared to control (P<0.05). Protease activity (Figure 1 D1) significantly increased in $T_2$, $T_6$ and $T_7$, while other treatments showed no differences with control (Figure 1 D1, P>0.05).

*After ammonia stress*

The lysozyme activity (Figure 1 A1) was similar between all treatments (P<0.05). ALP (Figure 1 B1) activity showed significant elevations in $T_2$, $T_5$ and $T_6$ compared to control (P<0.05). $ACH_{50}$ (Figure 1 C1) and protease (Figure 1 D1) activities considerably increased in $T_6$ compared to control, while other groups showed no significant differences with control (P>0.05).

**Stress related components**

*Before ammonia stress*

The glucose concentrations showed similar levels in all groups (Table 5, P>0.05). Cortisol concentrations exhibited lower levels in $T_5$ than in control (P<0.05), while other groups had no differences with control (Table 5, P>0.05).

*After ammonia stress*

Glucose concentrations showed similar levels in all groups (Table 5, P>0.05). The cortisol levels significantly declined in the supplemented fish compared to control (P<0.05).

**Liver metabolic enzymes of blood**

*Before ammonia stress*

The concentration of liver metabolic enzymes in serum significantly decreased in almost all supplemented fish compared to the control (Table 6, P<0.05). ALT activity in the treatments $T_2$, $T_3$, $T_6$ and $T_7$ significantly decreased compared to control (Table 6, P<0.05). A decreasing trend was also observed for ALP in $T_2$, $T_3$ and $T_7$ and for LDH in $T_2$, $T_3$, $T_6$ and $T_7$ (Table 6, P<0.05). AST activity showed no differences between all groups (Table 6, P>0.05).

*After ammonia stress*

ALT activity in $T_2$ and $T_7$ significantly declined compared to control (Table 6, P<0.05). Except $T_4$, LDH significantly decreased in the supplemented fish compared to control (Table 6, P<0.05). The lowest LDH activity was found in $T_6$ (Table 6, P<0.05). AST and ALP activities showed no differences between all experimental groups (Table 6, P>0.05).

Table 5. Stress related components of serum in rainbow trout, *O. mykiss* after 60 days feeding with experimental diets and after ammonia stress

| Status | Parameters | T1 (control) | T2 | T3 | T4 | T5 | T6 | T7 |
|---|---|---|---|---|---|---|---|---|
| Before challenge | Glucose (mg/dL) | 62.33±2.16 a | 59.40±1.43 a | 61.23±1.44 a | 62.00±1.03 a | 58.60±1.20 a | 58.10±0.84 a | 60.86±0.83 a |
| | Cortisol (ng/mL) | 99.21±2.11 a | 92.50±1.43 ab | 90.50±1.81 ab | 89.61±2.67 ab | 88.34±1.74 b | 91.61±1.71 ab | 90.51±2.28 ab |
| After challenge | Glucose (mg/dL) | 67.10±3.05 a | 63.82±1.20 a | 64.15±2.12 a | 64.00±1.22 a | 63.42±0.53 a | 61.41±1.45 a | 63.84±2.52 a |
| | Cortisol (ng/mL) | 107.56±2.52 a | 97.16±1.88 b | 96.50±1.81 b | 95.73±2.67 b | 92.03±1.46 b | 94.16±1.29 b | 97.73±1.60 b |

$T_1$: basic food as control, $T_2$: *L. rhamnosus* (LR) with a concentration of $1.5 \times 10^8$ CFU/g, $T_3$: LR with a concentration of $3 \times 10^8$ CFU/g, $T_4$: 1 g RS/kg, $T_5$: 3 g RS/kg, and $T_6$: $1.5 \times 10^8$ CFU/g + 1 g RS/kg and T7: $3 \times 10^8$ CFU/g + 3 g RS/kg. The means with different letters show significant differences (P<0.05).

Table 6. Hepatic metabolic enzymes in serum of rainbow trout, *O. mykiss* after 60 days feeding with experimental diets and after ammonia stress

| Status | Parameters | T1 (control) | T2 | T3 | T4 | T5 | T6 | T7 |
|---|---|---|---|---|---|---|---|---|
| Before challenge | ALT (U/L) | 23.41±0.64 a | 17.21±0.77 c | 18.81±1.02 bc | 23.11±0.56 ab | 19.20±1.34 abc | 18.12±0.62 c | 17.82±0.47 c |
| | AST (U/L) | 78.62±2.29 a | 72.15±2.78 a | 76.55±1.65 a | 75.13±2.80 a | 77.10±1.20 a | 72.42±1.71 a | 69.62±2.21 a |
| | ALP (U/L) | 119.72±2.52 a | 105.15±2.02 b | 111.22±3.06 ab | 114.55±2.80 ab | 111.10±1.78 ab | 102.55±2.50 b | 103.22±2.85 b |
| | LDH (U/L) | 207.00±2.88 a | 188.22±2.33 bc | 191.12±2.02 bc | 198.00±2.07 ab | 196.00±2.06 abc | 175.40±3.01 c | 191.50±2.35 bc |
| After challenge | ALT (U/L) | 28.44±1.22 a | 20.24±0.79 c | 23.51±1.03 abc | 27.47±0.52 ab | 24.44±1.09 abc | 23.07±1.49 abc | 21.51±1.74 bc |
| | AST (U/L) | 85.62±1.73 a | 77.72±2.23 a | 82.00±1.62 a | 78.55±2.22 a | 80.10±1.24 a | 76.75±2.21 a | 77.40±2.50 a |
| | ALP (U/L) | 131.72±4.78 a | 126.81±3.22 a | 129.22±3.03 a | 130.55±4.64 a | 121.00±2.66 a | 118.44±3.26 a | 128.54±3.08 a |
| | LDH (U/L) | 225.53±4.41 a | 203.63±3.49 bc | 220.00±3.16 ab | 219.33±4.47 a | 202.33±3.60 c | 189.16±3.48 c | 197.66±5.40 c |

$T_1$: basic food as control, $T_2$: *L. rhamnosus* (LR) with a concentration of $1.5 \times 10^8$ CFU/g, $T_3$: LR with a concentration of $3 \times 10^8$ CFU/g, $T_4$: 1 g RS/kg, $T_5$: 3 g RS/kg, and $T_6$: $1.5 \times 10^8$ CFU/g + 1 g RS/kg and $T_7$: $3 \times 10^8$ CFU/g + 3 g RS/kg. The means with different letters show significant differences (P<0.05).

## Discussion

Probiotics and medicinal herbs have been well recognized to improve fish growth performance and immune system (Nayak, 2010; Shakya et al., 2017; Elumalai et al., 2020). In this study, potentials of the probiotic, *L. rhamnosus* (LR) and herb-derived compound, rosmarinic acid (RS) only and in combination were investigated on growth, immunity, blood biochemicals and resistance to ambient ammonia stress in rainbow trout. Based on the results, LR and RS only or in combination efficiently improved the fish growth indices compared to the non-supplemented individuals. A combination of $1.5 \times 10^8$ CFU/g LR and 1 g RS/kg resulted in better performance compared to other treatments. However, use of probiotic only and at high dosage ($T_3$: $3 \times 10^8$ CFU/g LR) showed lower performance, which suggest the optimizing of probiotic levels in the diet. In addition, dietary probiotics efficiently modulated the intestinal bacterial flora since the load of lactic acid bacteria in the intestine increased in response to dietary supplementation. The growth enhancing effects of *L. rhamnosus* in this study may be attributed to the improving effects of probiotics on digestion and absorption of the nutrients, intestinal microbial balance, gut morphology, activity of digestive enzymes and their role on production of growth-promoting metabolites, as previously reported in other studies (Balcázar et al., 2006; Pirarat et al., 2011; Dawood et al., 2016; Xia et al., 2018; Sadeghi et al., 2020; Assan et al., 2022). The potential of RS on fish growth has not been reported yet. However, various studies have reported the promoting effect of rosemary, a plant containing rosmarinic acid, on fish growth (Turan and Yiğitarslan, 2016; Yousefi et al., 2019; Ebrahimi et al., 2020; Karataş et al., 2020). The improving effects of RS on fish growth are mainly attributed to the role of RS in ameliorating intestinal damages (Cai et al., 2019), enhancing antioxidant and immune defense (Lamaison et al., 1991; Adomako-Bonsu et al., 2017; Nadeem et al., 2019).

In the present study, increases in the activity of digestive enzymes were observed mainly in the fish fed a combination of RS and LR (i.e. $T_6$ and $T_7$). However, the supplementation with LR ($T_2$) and RS ($T_5$) increased only the protease activity compared to control as well. These results strengthen the possibility that the improved growth in the supplemented fish can be due to the stimulating effect of the supplements on the activity of digestive enzymes. Although the positive potential of LR on fish growth has been reported in various studies (Gonçalves et al., 2011; Dawood et al., 2016; Xia et al., 2018; Sewaka et al., 2019; Hooshyar et al., 2020), there are few studies related to the effect of this probiotic in combination with herbs (Harikrishnan et al., 2010). The antioxidant system of fish is the first line against oxidative stress (Hoseinifar et al., 2020; Hajirezaee and Khanjani, 2021). Under oxidative stress conditions, antioxidant enzymes and related compounds protect cells against damages caused by free radicals. The activity of antioxidant enzymes increased only in response to $1.5 \times 10^8$ CFU/g LR + 1 g RS/kg both before and after ammonia stress. Therefore, this result clearly shows that a dietary combination of LR and RS can effectively improve the fish antioxidant defense. Probiotics have been recognized to protect fish and other vertebrates against oxidative stress-related free radicals (Kullisaar et al., 2012; Heshmati et al., 2018; Hoseinifar et al., 2020). Although the protective mechanism of probiotics on oxidative stress has not yet been fully found in fish, this function may be attributed to the production of some metabolites with antioxidant properties such as folate, glutathione, butyrate and exopolysaccharides by probiotics, inhibition of free radical production through inhibiting the activity of cyclo-oxygenase enzyme (an enzyme responsible for free radical production), and stimulation of antioxidant enzymes (Lin and Yen, 1999; Hussain et al., 2003; Brzozowski et al., 2006). The stimulating effect of probiotics on fish antioxidant enzymes has been reported in some studies. A diet containing *B. licheniformis* prompted SOD and GPx activities in Mozambique tilapia, *O. mossambicus* (Giri et al., 2013). In gilthead seabream *Sparus aurata*, a diet containing *Shewanella putrefaciens* and *Bacillus* increased the expression of SOD and GPx (Esteban et al., 2014). The antioxidant properties of RS have been reported by some studies (Truong et al., 2018). In the current study, we observed a significant decrease in MDA (as the most important indicator of oxidative stress) levels in most groups fed $1.5 \times 10^8$ CFU/g LR + 1 g RS/kg, which could be due to the antioxidant properties of the RS. MDA is known as most important indicator of oxidative stress in fish and other vertebrates. In this study, dietary probiotic, and RS alone or in combination promoted the immune components of serum and mucus both before and after ammonia stress, mostly in the fish of $T_2$, $T_6$ and $T_7$. The immune-stimulating action of probiotics and medicinal herbs has been widely studied in fish (Galina et al., 2009; De et al., 2014; Kuebutornye et al., 2020). The immune-boosting properties of *L. rhamnosus* was also reported in fish (Pirarat et al., 2011; Dawood et al., 2016; Xia et al., 2018; Sewaka et al., 2019).

In general, probiotics strengthen the fish immune system in several major ways, including changing the balance of intestinal microbes, stimulating immune compounds, and competing with pathogens in the gut and replacing them (Gómez and Balcázar, 2008; Denev et al., 2009; Nayak, 2010; Aguirre-Guzman et al., 2012). The immune-enhancing mechanism of RS on the fish is not yet recognized, study of vertebrates has demonstrated that RS may exert immunogenic action through producing cytokines and modulating inflammatory reactions (Luo et al., 2020; Fasolo et al., 2021). Also, some studies have reported the anti-microbial properties of RS (Alagawany et al., 2017; Noor et al., 2022). The stress-inducing effects of ammonia is reported by some studies in fish (Cheng et al., 2015; Zhang et al., 2018; Dawood et al., 2021; Liang et al., 2022). An increase in cortisol levels followed by an increase in glucose concentration of

blood are the most important physiological changes in fish upon stressful conditions. Cortisol acts on liver to break down the glycogen stores and release glucose to blood to meet the energetic costs of stress (Vijayan et al., 2010). In the present study, the blood cortisol levels declined in the supplemented groups compared to control after ammonia stress, suggesting a stress ameliorating function for RS and the probiotic. Similarly, the ameliorating effects of probiotics (Salama et al., 2016; Yang et al., 2021; Gopi et al., 2022) and medicinal herbs (Chen et al., 2016; Esmaeili et al., 2017; Magouz et al., 2021; Zuo et al., 2022) on ammonia stress has been also reported in many studies. The probiotic and herb related stress mitigating mechanisms are not yet recognized in fish. However, this effect may be related to the reducing effect of these supplements on oxidative stress (Mohapatra et al., 2013; Gioacchini et al., 2014; Hamed and El-Sayed, 2019; Jia et al., 2019; Shukry et al., 2021). In addition, there are some studies reporting the protective effects of RS against oxidative stress in fish and other vertebrates (Mushtaq et al., 2015; Rong et al., 2018; Zhao et al., 2020; Javed et al., 2021).

The increased concentration of liver metabolic enzymes in blood is usually considered as an indicator of liver lesions or dysfunctions (Ghelichpour et al., 2020). Toxicity of ammonia on fish liver and following increases in liver enzymes have been reported in some studies (Abbas, 2006; Rama and Manjabhat, 2014; Hoseini et al., 2019; Mirghaed et al., 2019 a, b). In this study, the levels of the liver enzymes in blood decreased in almost all supplemented fish both before and after ammonia stress, which was more significant in the fish fed a combination of the probiotic and RS. Therefore, it seems that the supplements have protective effects, as previously observed in other fishes with herbs and probiotics (Banaee et al., 2011; Adorian et al., 2019; Rafieepour et al., 2019). The protective mechanism of rosmarinic acid on liver is not yet understood in fish. However, a few studies on other vertebrates have indicated that RS may protect liver through enhancing hepatic antioxidant capacity (Mushtaq et al., 2015; Elufioye and Habtemariam, 2019).

In conclusion, the findings of this study showed that both probiotics and RS and their combinations in the diet of fish can improve growth, immune system and ameliorate the ammonia stress. However, dietary combinations of $1.5 \times 10^8$ CFU/g LR + 1 g RS/kg showed more performance in the fish.

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