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INTERACTIVE EFFECTS OF DIETARY PROBIOTIC AND SUCCINIC ACID ON THE GROWTH PERFORMANCE, DIGESTIVE ENZYME ACTIVITIES, IMMUNOMODULATION, ANTIOXIDATIVE CAPACITY, AND DISEASE RESISTANCE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) JUVENILES

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Abstract

The current study was carried out to explore the effects of lactofeed (LA) as a multi-strain probiotic and succinic acid (SA) on growth variables, gut lactic acid bacteria count, digestive enzymes, innate immune responses, antioxidant capacity, and resistance against *Yersinia ruckeri* in rainbow trout juveniles (*Oncorhynchus mykiss)*. Three hundred sixty healthy rainbow trout juveniles (13.21±0.41 g) were randomly divided into twelve tanks (300 L) as four experimental groups. They were fed with basal diet (Control; BD), FT1: BD + 1 g/kg LA, FT2: BD + 5 g/kg SA, and FT3: BD + 1 g/kg LA + 5 g/kg SA for eight weeks. According to the results, final weight (FW), weight gain (WG), protein efficiency rate (PER), and feed conversion rate (FCR) revealed a remarkable discrepancy compared to the control group. In addition, dietary inclusion of LA (FT1 and FT3) significantly increased the specific growth rate (SGR). Dietary supplementation of LA+SA (FT3) remarkably improved total bacteria count (TBC) and amylase activity compared to the unsupplemented group. Gut lactic acid bacteria (LAB) count and digestive protease activity in all supplemented fish were remarkably higher than in unsupplemented group. Blood immunological factors including white blood cell (WBC) count, total immunoglobulin content (Ig), and the activity of lysozyme (LYZ), alternative complement pathway (ACH50), and acid phosphatase (ACP) were significantly enhanced in the supplemented groups. Neutrophil (NEU) count, skin mucus Ig and hepatic glutathione peroxidase (GPX) increased in SA (FT2 and FT3) treatments. In addition, monocyte (MON) count and skin mucus LYZ activity were significantly elevated following feeding with the FT3 diet. Other immunological parameters of skin mucus including protease activity, alkaline phosphatase (ALP) activity, and ACH50 activity, as well as hepatic superoxide dismutase (SOD) and catalase (CAT) activities increased in fish fed with diets containing LA and/or SA. Malondialdehyde (MDA) value was remarkably decreased in all supplemented rainbow trout compared to the fish fed with BD. Disease resistance against *Y. ruckeri* in fish fed with supplemented diets significantly improved with respect to the results obtained in the control specimens. Overall, dietary LA+SA supplementation was beneficial to improve growth performance, gut LAB count, digestive enzyme activities, innate immune responses, antioxidant capacity, and disease resistance in rainbow trout. According to these findings, 1 g/kg LA + 5 g/kg SA is suggested for adding to rainbow trout diet.

Key words: growth rate, health, organic acid, probiotic, rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) has a key role in meeting nutritional requirements and enhancing the economic profit of numerous people worldwide, and its production per unit of space is steadily increasing (Niazi et al., 2023). Compared with traditional rearing models, modern production systems have several special features such as high density, notable feed input levels, significant economic efficiency, and novel management strategies (Lenchenko et al., 2022; Tran et al., 2022). This intensification has led to the induction of stress, disturbance in digestive and metabolic activities, suppression of immune and antioxidant power, and susceptibility of fish to various infections (Van Doan et al., 2020). *Yersinia ruckeri* is a gram-negative opportunistic bacterium causing yersiniosis disease, a common enteric infection in marine and freshwater species mainly salmonids which has notable economic effects on fish farms around the world (Pajdak-Czaus et al., 2019; Sarkar et al., 2021). Fish infected with yersiniosis show diverse clinical symptoms, including reddening of the mouth, pop-eye, lethargy, petechial hemorrhages in different tissues, inflammation in the gut, septicemia in vascularized organs, etc. (Sarkar et al., 2021). Antibiotic therapy is a common way to counteract and treat yersiniosis in fish farms; however, this method in the long run brings unfortunate consequences on the host, the environment, and consumers (Vijayaram

et al., 2023). Hence, the majority of views have shifted to prophylactic attempts (Vijayaram et al., 2022; Dawood et al., 2022). Employing dietary additives can be a strong solution to solve some key issues in the aquaculture industry (Tadese et al., 2022). In this vein, probiotics as live beneficial microorganisms are one of the functional supplements with innumerable beneficial properties in aquaculture nutrition (El-Saadony et al., 2021). Previous studies confirmed that dietary administration of probiotics can support growth characteristics and feed efficiency by ameliorating gut microbiota, stimulating the secretion of digestive enzymes, improving the availability of nutrients, gut morphology, etc (Yousefi et al., 2023 a; Puvanasundram et al., 2021).

In recent years, several probiotic strains also have been tested to boost system defense and resistance against yersiniosis in rainbow trout. For example, *Lactobacillus rhamnosus* (Hooshyar et al., 2020), *L. acidophillus* and *Bifidobacterium bifidum* (Naderi Farsani et al., 2021), *Lactococcus lactis* subsp. *lactis* (Yeganeh et al., 2021), and *L. brevis* (Niazi et al., 2023).

According to the available evidence, multi-species probiotics are more effective than single probiotics because of synergistic effects, reducing the time of the fermentation process, and higher colonization in the intestine (Naderi Farsani et al., 2020; Puvanasundram et al., 2021). Lactofeed® is a multi-strain commercial probiotic with an equal proportion of *L. acidophilus*, *L. casei*, *Enterococcus faecium*, *B. bifidum*. This supplement, due to the presence of different and well-known strains, can exert beneficial effects on the growth and health of the host.

The essential prerequisite for the creation of multiple benefits in the host depends on the successful colonization of probiotics in the gut. In this regard, organic acids (OA) are one of the important promotors for the establishment and development of probiotic bacteria in the gut (Ng and Koh, 2017). These compounds through different mechanisms such as reducing the gastrointestinal tract pH, and inhibiting the growth of harmful bacteria, provide favorable conditions for the growth of lactic acid bacteria (LAB) (Hoseini et al., 2022). On the other hand, these compounds have a significant impact on growth through the breakdown of complex compounds and nutrient bioavailability (Hoseini et al., 2022). In addition, several studies have reported that dietary OA improve immune responses, antioxidant defense, and disease resistance in cultured species (Hoseini et al., 2022; Yousefi et al., 2023 b).

Succinic acid (SA; synonym: butanedioic acid; Hill Formula: $C_4H_6O_4$) is a promising chemical metabolite derived from biological activities (carbohydrate fermentation) that is used as a starter for producing diverse products such as sweeteners, plant-growth promoters, pyrrolidones, etc. (Cukalovic and Stevens, 2008; Kasarci et al., 2021). Besides, it has antimicrobial activity and inhibits the growth of pathogens such as *Escherichia coli*, *Salmonella* sp., and *Candida albicans* (Kumar et al., 2015). Other studies confirmed that succinic acid exerts cytotoxic effects on cancer cells (without side effects on normal cells).

In aquaculture nutrition, few studies reported the effect of SA in the diet on aquatic animals health; based on our literature review, only Duan et al. (2018) investigated the effects of SA on *Litopenaeus vannamei* (Pacific white shrimp), and showed an obvious improvement in growth characteristics and immune responses of *L. vannamei*. However, the effect of SA on immunity responses, antioxidant status, and disease resistance in fish remains unclear, and there is no comprehensive information in this context. In addition, there is no data on the possible synergistic effects of SA and probiotics, particularly in multi-strains form in fish, which requires further studies. Therefore, this study was carried out to clarify the effects of LA, SA, and LA+SA on growth performance, digestive enzyme activities, gut LAB, immune responses, antioxidant capacity, and disease resistance against *Y. ruckeri* in rainbow trout.

Material and methods

Diet preparation and experimental design

In this work effects of dietary supplementation of LA (Tak Gen, Tehran, Iran) and SA (Merck Millipore, Darmstadt, Germany, Catalogue Number 100682) on rainbow trout were evaluated by making four experimental diets including: Control (Basal diet), FT1: BD + 1 g/kg LA, FT2: BD + 5 g/kg SA, and FT3: BD + 1 g/kg LA + 5 g/kg SA. For this, obtained feedstuffs (Table 1) from different companies were weighed, sieved, and appropriately blended for 20 min (SA was added to the diet in exchange for filler). Afterward, soybean oil and fish oil were mixed with the diet components. To create a paste, 300 mL water was added to the aforementioned ingredients. In the next step, pellets were produced by a pelletizing machine. Produced pellets were dried for 24 h, fractured at 3 mm, placed in tagged bags and stored in 4°C (Hoseini et al., 2022).

Before adding LA to the diet, their activity was confirmed. Besides, viability of LA colonies in each experimental diet was investigated every four days by culturing de Man, Rogosa, Sharpe (MRS, Merck, Germany) culture medium, during feeding trial (Yousefi et al., 2023 a). The levels of LA and SA incorporated into three different diets were selected based on previous positive reports (Mirghaed et al., 2018; Duan et al., 2018; Ajdari et al., 2022). The biochemical composition of the experimental diets was measured based on the AOAC method (1995).

Fish culture and growth performance

In this study, a total of four hundred rainbow trout juveniles were purchased from a farm located in Sari city (Mazandaran province, Iran) and transported to a laboratory in Karaj city (Alborz province, Iran). Fish were stored in 1000-L tanks for two weeks; where they acclimated with the water quality variables in the new envi-

ronment including temperature: 15±1°C, dissolved oxygen: 7.7–8.3 mg/L, pH: 7.4–7.5, and unionized ammonia nitrogen: 0.02±0.005 mg/L. Fish were fed with the basal diet *ad libitum*, three times a day (7:00, 13:00, and 19:00). Then, to design an experiment with 4 treatments and triplicate, three hundred sixty fish (13.21±0.41 g) were transferred to 12 tanks (volume: 300 L; 30 specimens per tank (10 g/L) (Hoseini et al., 2022). To maintain water quality, feces, and uneaten food daily were siphoned and all tanks were continuously aerated; moreover, the water flow was adjusted to 0.5 L/min × kg biomass. After each meal, the uneaten pellets were siphoned from the bottom of the tank and dried in the oven. During the experimental period, water temperature, dissolved oxygen, and pH were controlled daily.

After completing the rearing period, all specimens were starved for 24 h and their weight was measured to calculate growth and feed utilization variables (Yousefi et al., 2023 a).

Weight gain (WG, g) = Final weight (FW) − Initial weight (IW)

Specific growth rate (SGR, % / day) = 100 × [(Ln FW – Ln IW) / days]

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

Protein efficiency ratio (PER) = WG (g) / Protein intake (g)

Survival rate (SR, %) = (Final individual numbers / Initial individual numbers) × 100

Sampling

To evaluate the immunological parameters, three fish per tank were randomly harvested and anesthetized using eugenol (100 mg/L) (Hoseini et al., 2022). Blood samples were collected from the caudal peduncle using 2 mm syringes. The obtained blood was divided into two parts: one portion was poured into tubes containing heparin to evaluate leukocyte profiles, and the other portion was centrifuged (3000 g; 10 min; 4°C) to obtain the serum samples for measuring immunity variables. The obtained serum was stored at –70°C (Naderi Farsani et al., 2021).

Table 1. Feedstuffs and compositions of the basal diet (Shekarabi et al., 2022)

Ingredients	Control	FT1	FT2	FT3
Fish meal[a]	495	495	495	495
Soybean meal[b]	127	127	127	127
Wheat four	150	150	150	150
Corn gluten meal[c]	70	70	70	70
Fish oil	45	45	45	45
Soybean oil	35	35	35	35
Minerals mix[d]	10	10	10	10
Vitamins mix[d]	10	10	10	10
Lecithin[e]	0.5	0.5	0.5	0.5
L-threonine	0.4	0.4	0.4	0.4
DL-methionine	0.1	0.1	0.1	0.1
Mono calcium phosphate	5	5	5	5
Cane molasses	20	20	20	20
Antioxidant (BHT)[f]	0.2	0.2	0.2	0.2
Toxin Binder (anti-mycotoxin)	1.8	1.8	1.8	1.8
Filler	30	30	25	25
LA	0	1	0	1
SA	0	0	5	5
Proximate composition% in dry basis				
Crude protein	45.10	45.12	44.90	44.95
Crude lipid	14.14	14.10	14.05	14.0
Crud ash	9.25	9.26	9.30	9.30
Dry matter	91.30	91.40	91.50	91.60

[a]Zafar Fishmeal Factory, Guilan, Iran (crude protein 66.10%).
[b]Soyabean Co., Gorgan, Iran (crude protein 45.2%).
[c]Glucosan Co., Qazvin, Iran.
[d]The premix provided following amounts per kg of feed: A: 1000 IU; D_3: 5000 IU; E: 20 mg; B_5: 100 mg; B_2: 20 mg; B_6: 20 g; B_1: 20 mg; H: 1 mg; B_9: 6 mg; B_{12}: 1 mg; B_4: 600 mg; C: 50 mg; Mg: 350 mg; Fe: 13 mg; Co: 2.5 mg; Cu: 3 mg; Zn: 60 mg Se: 0.3 mg; I: 1.5 mg; Mn: 10 mg). Chinechin Co., Tehran, Iran.
[e]Pouyavision, Tehran, Iran.
[f]Butylated hydroxytoluene (BHT) (Merck, Germany).

Skin mucus was collected using the method described by Ross et al. (2000). In this protocol, three fish per replicate were harvested and after anesthesia, individually placed in polyethylene bags containing 10 mL of 50 mM NaCl. Mucus was obtained by gently rubbing the fish and shaking the bag (for 2 min). Then, the obtained mucus was centrifuged at 2500 ×*g* (for 10 min at 4°C), and the supernatant was stored at –70°C for further analysis.

To calculate the activity of digestive enzymes, three fish from each tank were euthanized using the same anesthetic substance in a high dose, and then the gut was carefully separated and emptied. In the next step, samples were washed using cold distilled water, weighed, and homogenized using Tris buffer (Heidolph® Silent Crusher-M, Heidolph, Nuremberg, Germany) and a homogenizer (UP200H, Hielscher, Germany). The supernatant was obtained by centrifuging suspension at 6000 × *g* for 10 min at 4°C. The obtained product was stored at –80°C (Yousefi et al., 2023 a).

To evaluate the intestinal microbial composition, nine specimens were taken from each treatment. The body surface was disinfected using 70% alcohol, and then the intestines of the fish were used for bacterial culture, immediately after separation.

Liver tissues of euthanized fishes were isolated. Then, tissue samples were cut into small pieces and homogenized using phosphate buffer (0.050 M, pH 7.4). The enzyme source was obtained through centrifugation at 10000 ×*g* for 20 min at 4°C and stored at –80°C until the measurement of antioxidant enzymes (Haghparast et al., 2019).

Gut bacterial assay

The gut samples were washed using sterile phosphate-buffered saline (pH = 7.2; PBS), and then homogenized using a tissue homogenizer (Almagene, Taghiz, Iran). The obtained homogenate was serially diluted in PBS, and then 100 µl of each sample was transferred to MRS (De Man–Rogosa–Sharpe) medium (Merck, Germany) and tryptic soy agar (TSA; Merck, Germany) medium for the calculation of LAB and TBC (total bacterial count), respectively. The plates were kept at 28ºC for 24 h. The colonies of LAB and TBC in rainbow trout gut were counted based on log CFU/g gut.

Digestive enzymes

The dissolved protein content of the samples was determined using the method described by Bradford (1976). To determine the protease activity, 20 µL extract was mixed with 0.5 µL of 2% azocasein solution in 50 mM Tris-HCl and pH = 5.7 and incubated at 25°C for 10 min. Then 0.5 mL of trichloroacetic acid was added to it, the samples were centrifuged for 5 min at 6500g, and OD (optical density) of supernatants was recorded at 440 nm (García-Carreño, 1992). Each lipase test consisted of 7 µL of enzyme extract, 86 µL of sodium cholate, and 2.5 µL 2-Methoxyethanol solution. After adding 5.5 µL of para-nitrophenyl myristate to the above set and incubating the samples at 25°C for 15 minutes, the OD of the samples was read at 405 nm (Iijima et al., 1998). To measure amylase enzyme activity, 250 µL of 1% starch was mixed with 250 µL of enzyme extract. After 3 min, 0.5 mL of dinitrosalicylic acid color reagent was added to the above set. The reaction was placed at 100°C for 5 min. Then 5 mL of distilled water was pipetted to the final cocktail and the OD was read at 540 nm (Bernfeld, 1955).

Immunological assays

White cell count was computed using the Neubauer chamber and method described by Martins et al. (2004). The differential count of leukocytes [neutrophils (NEU), lymphocytes (LYM), and monocytes (MON)] was also carried out by preparing a blood smear and Giemsa staining recommended by Borges et al. (2004). Serum/skin mucus lysozyme (LYZ) activity was assayed based on lysis of the peptidoglycan layer of *Micrococcus luteus* (prepared in phosphate buffer; pH 5.8) (Ellis, 1990). The suspension was blended with serum or skin mucus sample and a reduction in OD was recorded at 450 nm. Alternative complement pathway activity (ACH50) was measured according to the hemolytic activity of serum/skin mucus sample in the hemolysis of sheep erythrocytes and the method described by Yano et al. (1992). The components of veronal buffer as the reaction medium were gelatin, magnesium, and EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid). Sheep erythrocytes were added to the same buffer. Hemolysis of erythrocytes was performed by the complement proteins through cocktail incubation at room temperature. The hemolysis rate of supernatants was recorded at OD 420 nm. The volume of supernatant producing 50% hemolysis is considered as ACH50 activity.

Serum and skin mucus total immunoglobulin (Ig) content was assayed using the protocol suggested by Siwicki (1994). In this method, first, the protein level of the samples was measured using Bradford (1976), then 12% polyethylene glycol (Sigma-Aldrich) was added to the same samples, and the protein content was measured again. The protein difference in these two phases was considered as Ig value. Mucus protease activity was computed in a spectrophotometer based on the azocasein hydrolysis method at 405 nm as explained by Firth et al. (2000). Mucus alkaline phosphatase (ALP) activity was estimated using p-nitrophenyl phosphate as substrate at 405 nm as explained by Kuda et al. (2002). Serum acid phosphatase (ACP) activity was calculated following the protocol of Andersch and Szczypinski (1947), using p-nitrophenyl phosphate disodium salt as substrate and recording the absorbance at 410 nm in a spectrophotometer.

Liver enzymatic assay

Serum superoxide dismutase (SOD) activity was quantified as explained by McCord and Fridovich (1969). Briefly, 100 µl of enzyme source sample was pipetted to 2 mL of reaction cocktail and incubated for 20 min at 37°C. Then, the absorbance was recorded at 560 nm in a spectrophotometer. Catalase (CAT) activity in serum samples was determined using the protocol described by

Abei (1984). Briefly, aliquots of enzyme extract (20 µl) were mixed with 3 mL of reaction mixture (1.98 mL potassium phosphate buffer (50 mM; pH; 7) and 1 mL H_2O_2 (30 mM). The CAT activity was assayed by calculating the disappearance rate of H_2O_2 at 240 nm for 1 min. Glutathione peroxidase (GPx) activity was quantified using the method described by Beutler (1984) and based on the disappearance of NADPH at 340 nm.

The lipid peroxidation index of malondialdehyde (MDA) was quantified by calculating the thiobarbituric acid reaction (TBA) at 532 nm (Ohkawa et al., 1979). An aliquot of the enzyme source was added to 0.67% TBA solution and kept at 95°C for 0.5 h, then OD was recorded.

Bacterial *in-vivo* challenge

The challenge test was carried out using *Y. ruckeri* (KC291153) in a completely separate place and compliance with quarantine and health principles. For this purpose, 15 specimens from each replication were placed in special challenge tanks. Then, they were anesthetized with eugenol (100 mg/L) and infected through intraperitoneal injection (0.1 mL of the bacterium suspension). In this study, bacterial suspension was prepared at a concentration of 1×10^7 cells/mL using the method described by Niazi et al. (2023). The selection of the dose in this study was carried out based on the concentration of *Y. ruckeri* (KC291153) employed in rainbow trout in recent studies (Naderi Farsani et al., 2021; Oroji et al., 2021; Darvishi et al., 2022; Niazi et al., 2023). Fish mortality was recorded daily and the cumulative mortality rate (CMR) was determined on the 14th day.

Statistical analysis

After checking normality data and variance of data by the Kolmogorov-Smirnov test and Levene's test, all data are represented as the mean ± SD and analyzed using SPSS software version no. 20.00 (SPSS Inc., Chicago, IL, USA). Differences between treatments were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test ($P<0.05$).

Results

Growth performance and feed utilization

The effect of experimental diets on growth performance and nutritional parameters is presented in Table 2. Accordingly, the FW, WG, and PER in fish fed with diets enriched with LA or SA were higher than the control group ($P<0.05$). In addition, the highest value of these three parameters was observed in FT3 (LA+SA), which revealed a significant difference compared to the other two treatments ($P<0.05$). Compared to the control group, the specimens fed with FT1 and FT3 diets indicated a significant difference in SGR value ($P<0.05$). Also, the FCR in all groups fed with LA or SA was significantly reduced compared to the control group ($P<0.05$). There was no significant difference in SR and FI between the different experimental groups ($P>0.05$).

Intestinal total and lactic acid bacteria

The gut microbial analysis of fish treated by LA and SA is depicted in Figure 1. The LAB count was significantly increased in all fish fed with dietary supplementation of LA/SA ($P<0.05$); however, the LAB count in the fish gut of the FT2 group was lower than FT1 and FT3 ($P<0.05$). Besides, TBC count only was increased in fish receiving the combined diet (FT3) ($P<0.05$).

Digestive enzyme analysis

The activity level of digestive enzymes in response to experimental diets is illustrated in Figure 2. The specimens fed with dietary supplementation of SA/LA exhibited higher protease activity than the control group ($P<0.05$). Also, dietary supplementation of LA+SA (FT3) in rainbow trout produced a significant increase in amylase activity compared with the fish fed with the basal diet ($P<0.05$). However, no significant differences were found in the lipase activity among the treatments ($P>0.05$).

Table 2. Zootechnical performance of rainbow trout fed with different experimental diets (Control, FT1, FT2, and FT3) after eight weeks

Growth performance	Treatments			
	Control	FT1	FT2	FT3
IW (g)	13.43±0.40 a	13.13±0.41 a	13.30±0.30 a	13.30±0.60 a
FW (g)	68.00±1.50 c	75.50±1.50 b	75.16±1.25 b	79.43±0.6 a
WG (g)	54.56±1.77 c	62.36±1.90 b	61.86±1.55 b	66.43±0.65 a
SGR (%/d)	2.89±0.08 b	3.12±0.09 a	3.09±0.07 ab	3.22±0.08 a
FCR	1.40±0.04 a	1.20±0.06 b	1.21±0.03 b	1.12±0.06 b
PER	1.21±0.04 c	1.38±0.04 b	1.37±0.03 b	1.47±0.01 a
FI (g/fish)	76.83±1.25 a	75.33±1.52 a	75.16±0.76 a	75.00±1.00 a
SR (%)	95.33±4.04 a	95.66±2.30 a	96.66±3.51 a	98.00±1.73 a

Different letters in each column show significant differences among the experimental groups ($P<0.05$). Values are shown as mean ± SD ($n = 3$). Abbreviations: IW: initial weight, FW: final weight, SGR: specific growth rate (%/d), WG: weight gain, FCR: feed conversation rate, PER: protein efficiency rate, FI: feed intake, SR: survival rate, FT1: (basal diet + Lactofeed), FT1: (basal diet + succinic acid) FT3: (basal diet + Lactofeed + succinic acid).

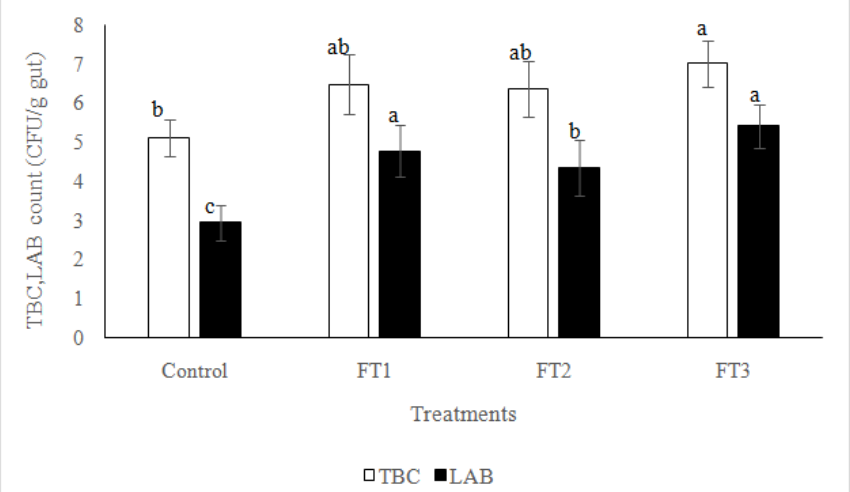


Figure 1. TBC and LAB count in rainbow trout gut fed with the different experimental diets (Control, FT1, FT2, and FT3) after eight weeks. Different letters above the bars show significant differences by Tukey's test (n = 3; P<0.05). All data were analyzed with one-way ANOVA, and values are presented as mean ±SD. Abbreviations: LAB: lactic acid bacteria, TBC: total bacteria count, FT1: (basal diet + Lactofeed), FT2: (basal diet + succinic acid), FT3: (basal diet + Lactofeed + succinic acid)

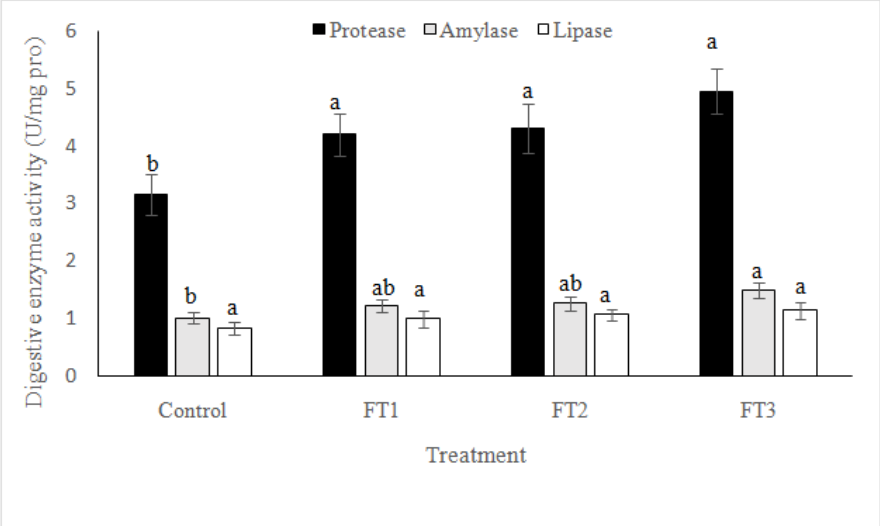


Figure 2. Digestive enzymes activity in rainbow trout fed with the different experimental diets (Control, FT1, FT2, and FT3) after eight weeks. Different letters above the bars show significant differences by Tukey's test (n = 3; P<0.05). All data were analyzed with one-way ANOVA and values are presented as mean ±SD. Abbreviations: FT1: (basal diet + Lactofeed), FT2: (basal diet + succinic acid), FT3: (basal diet + Lactofeed + succinic acid)

Table 3. Blood immunological parameters of rainbow trout fed with the different experimental diets (Control, FT1, FT2, and FT3) after eight weeks

Parameters	Control	FT1	FT2	FT3
WBC (× 10^3/ mm^3)	14.91±0.62 b	17.46±0.55 a	18.53±1.23 a	17.90±1.05 a
LYM (%)	77.56±1.90 a	76.10±1.24 a	74.36±1.70 a	73.60±1.70 a
NEU (%)	15.93±1.15 b	17.83±0.90 ab	18.30±0.60 a	18.93±0.81 a
Mon (%)	3.60±0.43 b	3.80±0.26 b	4.56±0.45 ab	5.16 ± 0.65 a
Total Ig (mg/mL)	8.66±0.76 c	12.83±1.04 b	14.33±1.25 ab	16.23±1.25 a
Lysozyme (U/mL)	12.00±0.80 c	20.23±1.25 b	19.83±1.04 ab	22.90±1.01 a
ACH50 (U/mL)	99.16±3.40 c	112.16±4.25 b	122.33±3.05 a	122.83±3.01 a
ACP (U/mL)	20.00±1.74 b	27.08±2.55 a	30.46±2.36 a	31.00±1.98 a

Different letters in each column show significant differences among the experimental groups (P<0.05). Values are shown as mean ± SD (n = 3). Abbreviations: WBC: white blood cells, LYM: lymphocytes, NEU: neutrophils, Mon: monocyte, Ig: immunoglobulin, ACH50: alternative complement pathway activity, ACP: acid phosphatase, FT1: (basal diet + Lactofeed), FT1: (basal diet+ succinic acid), FT3: (basal diet + Lactofeed + succinic acid).

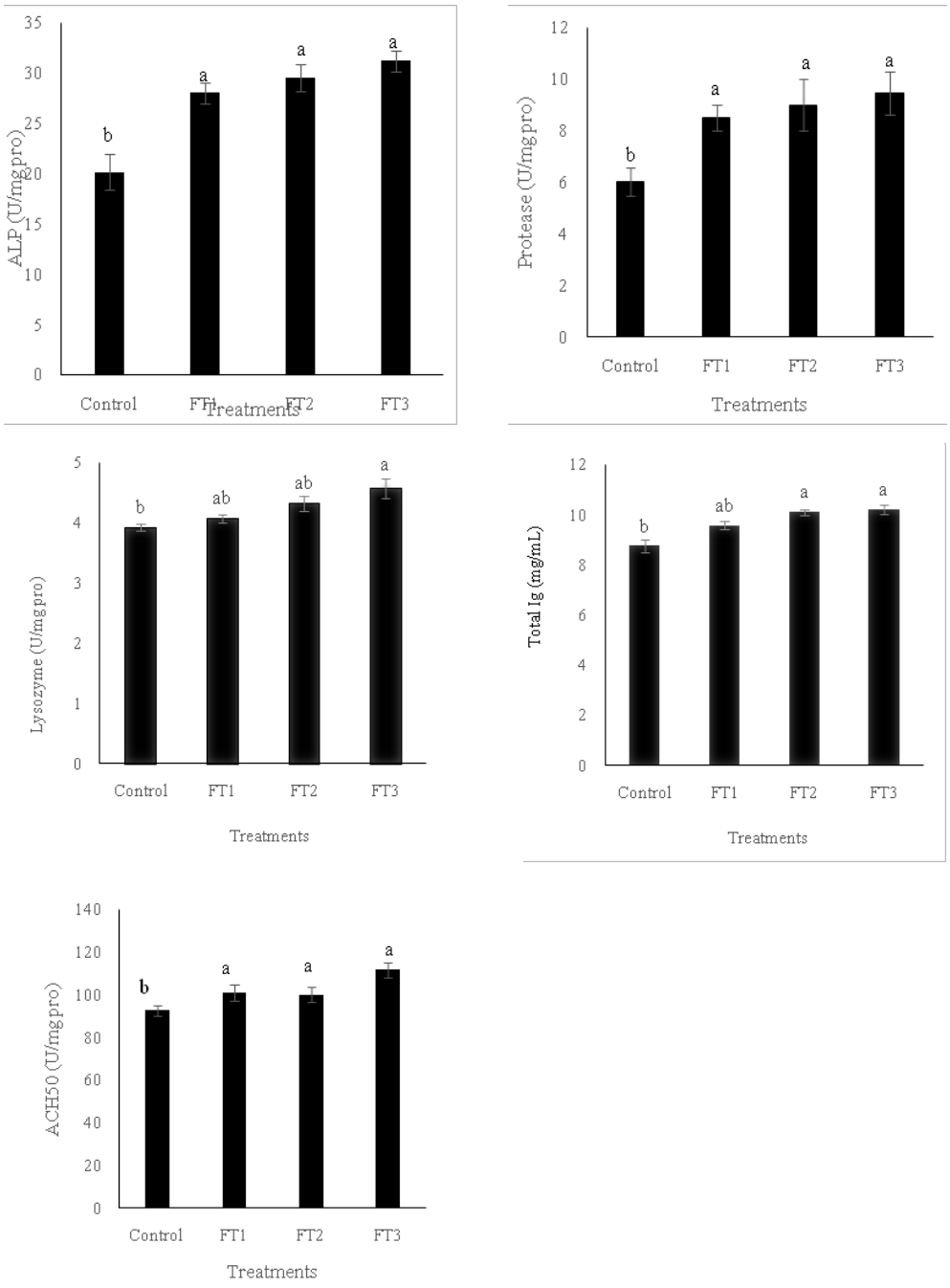


Figure 3. Skin immunity responses in rainbow trout fed with the different experimental diets (Control, FT1, FT2, and FT3) after eight weeks. Different letters above the bars show significant differences by Tukey's test (n = 3; P<0.05). All data were analyzed with one-way ANOVA and values are presented as mean± SD. Abbreviations: ACH50: alternative complement pathway activity, ALP: alkaline phosphatase, Ig: immunoglobulin, FT1: (basal diet + Lactofeed), FT2: (basal diet + succinic acid), FT3: (basal diet + Lactofeed + succinic acid)

Blood immunological responses

Dietary supplementation of LA/SA significantly affected WBC count and ACP compared to the control group (Table 3), with a similar statistical pattern (P<0.05). FT2 and FT3 diets significantly increased NEU count compared to the basal diet (P<0.05). The MON count only was affected in fish receiving the combined diet (FT3). Dietary supplementation of LA/SA remarkably improved Ig value and LYZ activity compared to the control group (P<0.05); moreover, the highest values were recorded in those fed with the FT3 diet. Dietary supplementation of LA/LS indicated a remarkable improvement in ACH50 activity compared to the control group (P<0.05); however, the maximum ACH50 activity was found in FT2 and FT3 groups (P<0.05). No significant discrepancy in LYM count was detected among different experimental groups (P>0.05).

Table 4. Liver antioxidant enzymes biomarkers of rainbow trout fed with the different experimental diets (Control, FT1, FT2, and FT3) after eight weeks

Parameters	Control	FT1	FT2	FT3
SOD (U/mg tissue protein)	32.75±2.00 c	37.58±1.25 b	40.25±1.80 ab	43.58±1.75 a
GPx (U/mg tissue protein)	88.83±1.52 b	92.76±1.91 ab	96.66±2.08 a	97.50±2.29 a
CAT (U/mg tissue protein)	19.01±2.05 c	23.50±1.50 b	25.28±0.77 ab	29.01±1.12 a
MDA (nmol/mg protein)	17.95±1.13 a	13.83±1.25 b	11.33±0.71 bc	9.91±0.76 c

Different letters (a–c) in the same row indicate significant differences (P<0.05). Values are presented as the mean ± SD, n=3. Abbreviations: SOD: superoxide dismutase, GPx: glutathione peroxidase, CAT: catalase, MDA: malondialdehyde, FT1: (basal diet + Lactofeed), FT1: (basal diet + succinic acid), FT3: (basal diet + Lactofeed + succinic acid).

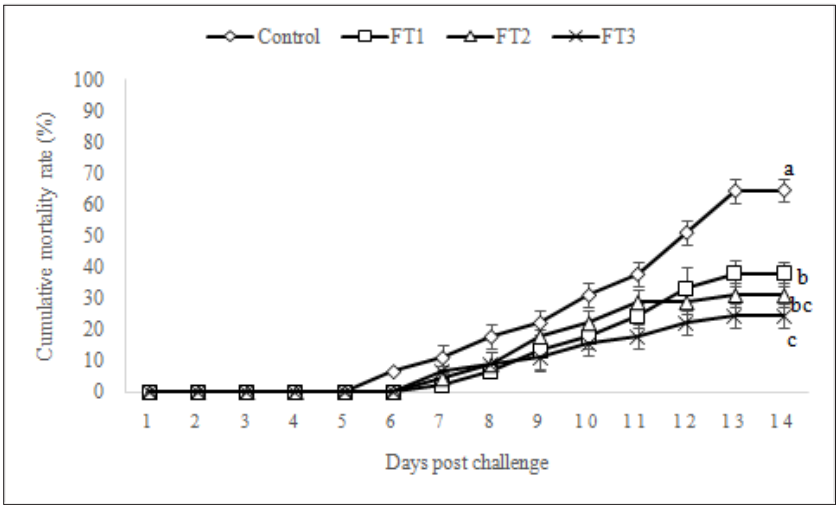


Figure 4. Cumulative mortality rate after challenge with *Y. ruckeri* for two weeks in rainbow trout fed with the different experimental diets (Control, FT1, FT2, and FT3) after eight weeks. Abbreviations: FT1: (basal diet + Lactofeed), FT2: (basal diet + succinic acid), FT3: (basal diet + Lactofeed + succinic acid)

Skin mucus immune responses

Dietary inclusion of LA/SA resulted in a significant increase in ALP, protease, and ACH50 activity when compared to the control group at the end of the eighth week (Figure 3) (P<0.05). Total Ig content in the FT2 and FT3 groups was significantly higher than the control group (P<0.05). A peak LYZ activity was found in fish fed with the diet containing LA+SA (FT3) (P<0.05).

Liver antioxidant parameters

Dietary inclusion of LA/SA produced a significant increase in SOD and CAT activities compared to the control group (P<0.05); the activity of these two parameters in fish fed with the FT3 diet was higher than the FT1 group. GPx activity was notably elevated in SA-added treatments compared to the control treatment (P<0.05). MDA value significantly decreased in fish fed with dietary supplementation of LA+SA compared to the control group (P<0.05); besides, the MDA value in the FT3 group was lower than the FT1 group (P<0.05) (Table 4).

***In vivo* fish infection model**

The mean survival rate of rainbow infected with *Y. ruckeri* is shown in Figure 4. Dietary supplementation of LA/SA significantly boosted the disease resistance of rainbow trout against *Y. ruckeri* compared to the control group (P<0.05). Death during the challenge period in the control group had a steeper slope and started earlier (the sixth day) than the treated groups (the seventh day). Meanwhile, death in groups receiving dietary supplementation of LA/SA had a slower slope. On the fourteenth day, the cumulative mortality rate (CMR) of specimens fed dietary of FT3 (24.4%) decreased to about 2.63-fold compared to the control group (64.66%) (P<0.05).

Discussion

Improving the feed efficiency of fish diets through feed additives is one of the main ways to improve the growth, FCR, and biosecurity of farming species (Dawood et al., 2018; Vijayaram et al., 2023). The results of the current study indicated that LA and SA, especially

their mixture, could act as a growth promotor in rainbow trout. In line with our results, growth performance and FCR significantly increased in rainbow trout fed with a diet supplemented with butyric acid and *Bacillus licheniformis* (Taherpour et al., 2023). Moreover, a significant enhancement was observed in growth performance and feed utilization in *Acipenser baerii* (Siberian sturgeon) fed with a diet containing acetic acid and protexin (probiotic) (Zare et al., 2021). Scrutiny of the results showed that the growth performance significantly increased in the supplemented groups, without a significant effect on the FI. On the other hand, the improvement of FCR and PER in this study directly affected the growth markers. The improvement of these two nutritional parameters reflects a significant improvement in the activity of digestive enzymes and the increase in accessibility to nutrients, which leads to the retention of more protein and subsequently elevated growth performance (Yousefi et al., 2023 a).

Recent findings showed that OA through inhibiting the proteolysis process and increasing the protein deposition rate increase growth variables (Li et al., 2022). In addition, OA can help the colonization of the lactic acid bacteria population by reducing the intestinal pH and inhibiting the growth of pathogenic microbes (Hoseini et al., 2022). Our findings showed that dietary supplementation of LA/SA led to an improvement in the proliferation of lactic acid bacteria. Similarly, the LAB count increased in tilapia fed with dietary supplementation of potassium diformate (Elala and Ragaa, 2015). Besides, *Pontastacus leptodactylus* (narrow-clawed crayfish) exhibited a LAB count higher than the control group, when fed by dietary supplementation of OAB (organic acid blend; Na-acetate, Na-butyrate, Na-lactate, and Na-propionate) (Safari et al., 2021). Based on documented evidence, modulating gut microbiota towards LAB strongly affects the activity of digestive enzymes (Niazi et al., 2023; Yousefi et al., 2023 a; Hoseini et al., 2022). In this study, dietary administration of LA+SA significantly increased the activity of protease and amylase enzymes. These findings may be related to the SA capability for producing energy for epithelial cells and reduced pH, which is needed to activate the digestive enzymes (El-Sharkawy et al., 2023). Besides, dietary SA can modulate the abundance of gut microbiota with the capability of secreting exogenous or endogenous enzymes related to the digestion process (Ng and Koh, 2017).

The defense reactions of aquatic animals are mainly dependent on non-specific immunity (Mokhtar et al., 2023). Therefore, boosting the innate immune system can provide many benefits for the host such as resistance to stressful and infectious agents, manipulations as well as better growth (Hoseinifar et al., 2020). Leukocytes are one of the vital components of the immune system that implement several immune reactions such as cytotoxic activities, respiratory bursts, phagocytosis, and the production of antibacterial enzymes against microbial invasion in aquatic animals (Fischer et al., 2006). In the current study, the count of WBC, neutrophils and monocytes as phagocytic cells was remarkably increased in fish fed with enriched pellets LA+SA. Also, earlier findings have well confirmed the improvement of WBC count and phagocytic cell counts via consuming diets containing probiotics and OA. For instance, Taherpour et al. (2023) reported that a diet containing butyric acid + *Bacillus licheniformis* elevated the count of WBC, Mon, and NEU. Also, Santos et al. (2023) reported that the simultaneous use of *Bacillus spp*., *B. licheniformis* and benzoic acid in *Oreochromis niloticus* (Nile tilapia) diet increased total leukocyte count. Previous findings stated that probiotics and OA boosted the proliferation of circulating leukocyte counts by improving the hematopoietic system (Yousefi et al., 2023 a; Taherpour et al., 2023).

The components of humoral immunity are a set of soluble molecules that have preventive functions by inhibiting the growth of pathogens and neutralizing enzymes that are associated with them (Magnadóttir, 2006). In this study, immune parameters measured in serum including LYZ, ACH50, Ig, and ACP were enhanced in all diets enriched with LA or SA; however, the highest values were obtained in the groups supplemented with the combined form (LA+SA). There is no information about the effect of LA and SA combined on the humoral immunity of aquatic organisms. However, in some studies the improvement of serum immune indices was observed following the inclusion of probiotics and OA in the diet of different species such as butyric acid + *B. licheniformis* in rainbow trout (Taherpour et al., 2023), formic acid + *Pediococcus acidilactici* in common carp (Heshmatfar et al., 2023). Lysozyme is one of the most well-known serum lytic enzymes that work in the lysis of the peptidoglycan layer of bacteria (Mokhtar et al., 2023). The increment in the level of lysozyme in this work can be related to the improvement of its producing sources (*i.e.,* neutrophils and monocytes), a hypothesis that was documented in several studies (Niazi et al., 2023; Yousefi et al., 2023 a). The complement system is responsible for the connection between innate and adaptive immune responses. This system performs a variety of immune functions, including the lysis of infected cells and pathogenic agents, phagocytosis of stranger germs, the clearing of apoptotic cells, and bactericidal activity (Loh et al., 2020). Immunoglobulins (Igs) are other key components of the defense system with diverse functions such as phagocytosis of pathogens, neutralization of viruses and toxins, and triggering of the complement cascade (Mokhtar et al., 2023). The increment in ACP and ACH50 activity, total Ig concentration in the serum indicating hepatoprotective and immunomodulatory effects of LA and SA on liver and lymphoid tissues (spleen and kidney) of rainbow trout, which may be the outcome of manipulation of the intestinal microbiota, the production of compounds with antioxidant properties such as folate and exopolysaccharides, and other valuable metabolites such as SCFA (short-chain fatty acids) (Naderi Farsani et al., 2021; Niazi et al., 2023).

Skin mucus is a fully organized structure that through the secretion of pathogenic antagonists such as bacteriocin, lysozyme, Ig, complement proteins, antimicrobial peptides, and growth inhibitors, provides proper immunity against external perturbations (Salinas and Magadán, 2017). Several studies have confirmed the properties of probiotics or OA in regulating mucosal immune responses by influencing mucosal-associated lymphoid tissues (MALT) (Ahmadifar et al., 2022; Sotoudeh and Esmaeili, 2022). In this study, the peak activity of protease, ALP, and ACH50 was observed in the groups supplemented with LA+SA. Also, diets supplemented with SA increased mucus Ig content and lysozyme activity (i.e. FT2 and FT3). Previous studies have reported that using organic acid or probiotics in aquafeed can stimulate immune responses of skin mucus. For instance, Hoseinifar et al. (2017) reported that dietary supplementation of sodium propionate significantly improved skin mucus immune parameters particularly Ig value, lysozyme level, and protease activity of *Danio rerio* (zebrafish). Similarly, Yousefi et al. (2023 b) reported that LYZ, ALP, Ig, and ACH50 of *Cyprinus carpio* (common carp) increased when basal diet was combined with dietary malic acid. On the other hand, Mirghaed et al. (2018) showed that dietary administration of PrimaLac® as a multi-strain probiotic had stimulating effects on the mucus immune responses (ALP, protease, and LYZ) of *Rutilus frisii kutum* (Caspian white fish) fingerlings. The mechanisms of action of multi-strain probiotics and OA on the immune system of fish have been explained in several studies (Saravanan et al., 2021; Naderi Farsani et al., 2021). Overall, the modulation of the intestinal microbial flora and the effect of its beneficial products on the lines involved in the secretion of immune responses leads to an increase in fish health (Niazi et al., 2023).

Oxidative stress in aquatic organisms is the outcome of excessive production of reactive oxygen species (ROS) such as superoxide anion ($O2^-$), hydroxyl radical (OH), hydroxyl ion (OH-) and singlet oxygen which cause serious damage to the cell by stealing electrons from proteins, lipids, and nucleotides (Bal et al., 2021). In this status, the fish defense system protects the integrity of the cellular constituents by increasing the activity of antioxidant enzymes such as SOD, CAT, and GPx (Aliko et al., 2018). The evaluation of the activity of antioxidant enzymes in this study showed that the inclusion of LA or SA in the basal diet led to the boosting of the antioxidant enzyme activity, however, the level of these enzymes in the group fed with dietary supplementation of LA+SA was better than the other two groups. Also, the lowest MDA content as an index of lipid peroxidation was observed in the FT3 group. Antioxidant properties of probiotics and OA were reported in several studies. A marked increase was detected in the activity of antioxidant enzymes in rainbow trout fed with dietary sodium butyrate (Mirghaed et al., 2019), malic acid (Yousefi et al., 2023 b), *L. acidophilus* + *B. bifidum* (Naderi Farsani et al., 2021) and *L. rhamnosus* (Hajirezaee and Khanjani, 2023). In the last few years, there has been tremendous attention on the beneficial effects of probiotics in response to oxidative stress. These reactions often include inhibiting the growth of abnormal bacteria and preventing the production of endotoxins in the blood, the production of antioxidant metabolites such as exopolysaccharides, folate, butyrate, and glutathione (GSH), the ability to chelate metal ions (Fe^{2+} or Cu^{2+}) and enhancing the activity of antioxidant enzymes (Wang et al., 2017; Hoseinifar et al., 2020). The antioxidant properties of dietary OA on the oxidative stress of aquatic animals have yet not been sufficiently elucidated. However, a few studies stated that dietary inclusion of OA increased redox power and their intermediates (Zhang et al., 2020). In addition, these compounds can increase up-regulated expression of genes related to antioxidant enzymes (Safari et al. 2020).

One of the main objectives of this study was to investigate the disease resistance of rainbow trout against a common infection, yersiniosis, which often causes high mortality and huge economic losses to rainbow trout farms. In this work, dietary administration of LA + SA (FT3) increased rainbow trout resistance against *Y. ruckeri* up to 65%. The effect of LA and SA in single or blend form has not yet been tested to evaluate the survival rate in rainbow trout; however, previous findings have confirmed the protective effects of dietary probiotics or OA such as *L. acidophilus* and *B. bifidum* (Naderi Farsani et al., 2021) against *Y. ruckeri*, *Lactococcus lactis* subsp. *lactis* against *Y. ruckeri* (Yeganeh et al., 2021), *Enterococcus casseliflavus* against *S. iniae* (Safari et al., 2016), sodium butyrate against *S. iniae* (Mirghaed et al., 2019), *B. subtilis* and trans-cinnamic against *Y. ruckeri* (Yılmaz et al., 2020). It seems that the modulation of the gut microbiota, the enhancing serum and mucus immune parameters, leukocyte count, as well as the boosting of the antioxidant capacity had a prominent role in increasing disease resistance in the treatments.

Conclusion

Overall, this is the first study to examine a combination of succinic and probiotics on rainbow trout. The dietary supplementation of LA or SA had promoting effects on the growth and health of rainbow trout. However, the diet containing LA plus SA could more effectively improve the gut microbiota and digestive enzyme activity, which in turn led to higher growth performance. Furthermore, dietary supplementation of LA+SA boosted resistance to disease against *Y. ruckeri* through beneficial effects on WBC count, serum and mucus immune responses, and antioxidant capacity. The results of the current research clearly showed the synergistic effects of LA+SA on zootechnical characteristics, immunological parameters, and disease resistance. Therefore, the combination of LA+SA can be a functional compound for the farming of rainbow trout. However, more evaluations in the next studies are suggested to clarify the effects of LA+SA supplementation at the molecular level as well as its potential against common stresses in rearing conditions such as crowding,

hypoxia, and toxic compounds (e.g., ammonium, nitrite, heavy metals, etc.).

Data availability

The data that support the findings of this study are available upon reasonable request to the corresponding authors.

Compliance with ethical standards

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

Conflict of interest

Authors have no conflict of interest to declare for the publication of the present work.

References

Abei H. (1984). Catalase *in vitro*. Method Enzymol., 105: 121–126.

Ahmadifar E., Eslami M., Kalhor N., Zaretabar A., Mohammadzadeh S., Moghadam M.S., Yousefi M., Ahmadifar M., Hoseinifar S.H., Pusadee T., van Doan H. (2022). Effect of a diet enriched with sodium propionate on growth performance, antioxidant property, innate-adaptive immune response, and growth-related genes expression in critically endangered beluga sturgeon (*Huso huso*). Fish Shellfish Immunol., 125: 101–108.

Ajdari A., Ghafarifarsani H., Hoseinifar S.H., Javahery S., Narimanizad F., Gatphayak K., Van Doan H. (2022). Effects of dietary supplementation of primaLac, inulin, and biomin imbo on growth performance, antioxidant, and innate immune responses of common carp (*Cyprinus carpio*). Aquac. Nutr., 2022: 1–13.

Aliko V., Qirjo M., Sula E., Morina V., Faggio C. (2018). Antioxidant defense system, immune response and erythron profile modulation in gold fish, *Carassius auratus*, after acute manganese treatment. Fish Shellfish Immunol., 76: 101–109.

Andersch M.A., Szczypinski A.J. (1947). Use of p-nitrophenylphosphate as the substrate in determination of serum acid phosphatase. Am. J. Clin. Pathol., 17: 571–574.

AOAC (1995). Official Methods of Analysis of the Association of Official Analytical Chemists. Association of Official Analytical Chemists, Washington, DC, USA.

Bal A., Panda F., Pati S.G., Das K., Agrawal P.K., Paital B. (2021). Modulation of physiological oxidative stress and antioxidant status by abiotic factors especially salinity in aquatic organisms. Comp. Biochem. Physiol., 241: 108971.

Bernfeld P. (1955). Amylase. In: Methods in enzymology, Colowick S.P., Kaplan N.O. (eds). Academic Press, New York, pp. 149–158.

Beutler E. (1984). Red cell metabolism: A manual of biochemical methods, 2nd Ed., Biochemistry, 72: 248–254.

Borges A., Scotti L.V., Siqueira D.R., Jurinitz D.F., Wassermann G.F. (2004) Hematologic and serum biochemical values for jundiá (*Rhamdia quelen*). Fish Physiol. Biochem., 30: 21–25.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248–254.

Cukalovic A., Stevens C.V. (2008). Feasibility of production methods for succinic acid derivatives: a marriage of renewable resources and chemical technology. Biofuel Bioprod. Biorefin., 2: 505–529.

Darvishi M., Shamsaie Mehrgan M., Khajehrahimi A.E. (2022). Effect of licorice (*Glycyrrhiza glabra*) extract as an immunostimulant on serum and skin mucus immune parameters, transcriptomic responses of immune-related gene, and disease resistance against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*). Front. Vet. Sci., 9: 811684.

Dawood M.A., Koshio S, Esteban M.Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. Rev. Aquac., 10: 950–974.

Dawood M.A., El Basuini M.F., Yilmaz S., Abdel-Latif H.M., Alagawany M., Kari Z.A., Abdul Razab M.K.A., Hamid N.K.A., Moonmanee T., Van Doan H. (2022). Exploring the roles of dietary herbal essential oils in aquaculture: A review. Animals, 12: 823.

Duan Y., Wang Y., Zhang J., Sun Y., Wang J. (2018). Dietary effects of succinic acid on the growth, digestive enzymes, immune response and resistance to ammonia stress of *Litopenaeus vannamei*. Fish Shellfish Immunol., 78: 10–17.

Elala N.M.A., Ragaa N.M. (2015). Eubiotic effect of a dietary acidifier (potassium diformate) on the health status of cultured *Oreochromis niloticus*. J. Adv. Res., 6: 621–629.

Ellis A.E. (1990). Lysozyme assays: In: Techniques in fish immunology, Stolen J.S., Fletcher T.C., Anderson D.P., Roberson B.S., Van Muiswinkel W.B. (eds). SOS Publications, Fair Haven, NJ, pp. 101–103.

El-Saadony M.T., Alagawany M., Patra A.K., Kar I., Tiwari R., Dawood M.A. Abdel-Latif H.M. (2021). The functionality of probiotics in aquaculture: An overview. Fish Shellfish Immunol., 117: 36–52.

El-Sharkawy E.A., Abd El-Razek I.M., Amer A.A., Soliman A.A., Shukry M., Gewaily M.S., Téllez-Isaías G., Kari Z.A., Dawood M.A. (2023). Effects of sodium butyrate on the growth performance, digestive enzyme activity, intestinal health, and immune responses of Thinlip Grey Mullet (*Liza ramada*) juveniles. Aquac. Rep., 30: 101530.

Firth K.J., Johnson S.C., Ross N.W. (2000). Characterization of proteases in the skin mucus of Atlantic salmon (*Salmo salar*) infected with the salmon louse (*Lepeophtheirus salmonis*) and in whole-body louse homogenate. J. Parasitol., 86: 1199–1205.

Fischer U., Utke K., Somamoto T., Köllner B., Ototake M., Nakanishi T. (2006). Cytotoxic activities of fish leucocytes. Fish Shellfish Immunol., 20: 209–226.

García-Carreño F.L. (1992). Protease inhibition in theory and practice. Biotechnol. J., 3: 145–150.

Haghparast R.J., Moghanlou K.S., Mohseni M., Imani A. (2019). Effect of dietary soybean lecithin on fish performance, hemato-immunological parameters, lipid biochemistry, antioxidant status, digestive enzymes activity and intestinal histomorphometry of pre-spawning Caspian brown trout (*Salmo trutta caspius*). Fish Shellfish Immunol., 91: 50–57.

Hajirezaee S., Khanjani M.H. (2023). 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. J. Anim. Sci., 23: 819–831.

Heshmatfar F., Safari R., Shabani A., Hoseinifar S.H., Ghaffari H., Shokohian B., Ullah M.R., Siddik M.A. (2023). The effects of combined or singular administration of formic acid and *Pediococcus acidilactici* on stress resistance, growth performance, immune responses and related genes expression in common carp, *Cyprinus carpio*. Aquac. Rep., 29: 101474.

Hooshyar Y., Abedian Kenari A., Paknejad H., Gandomi H. (2020). Effects of *Lactobacillus rhamnosus* ATCC 7469 on different parameters related to health status of rainbow trout (*Oncorhynchus mykiss*) and the protection against *Yersinia ruckeri*. Probiotics Antimicrob. Proteins, 12: 1370–1384.

Hoseini S.M., Rajabiesterabadi H., Abbasi M., Khosraviani K., Hoseinifar S.H., Van Doan H. (2022). Modulation of humoral immunological and antioxidant responses and gut bacterial community and gene expression in rainbow trout, *Oncorhynchus mykiss*, by dietary lactic acid supplementation. Fish Shellfish Immunol., 125: 26–34.

Hoseinifar S.H., Safari R., Dadar M. (2017). Dietary sodium propionate affects mucosal immune parameters, growth and appetite related genes expression: Insights from zebrafish model. Gen. Comp. Endocrinol., 243: 78–83.

Hoseinifar S.H., Sun Y.Z., Zhou Z., Van Doan H., Davies S.J. Harikrishnan R. (2020). Boosting immune function and disease biocontrol through environment-friendly and sustainable approaches

in finfish aquaculture: herbal therapy scenarios. Rev. Fish Sci. Aquac., 28: 303–321.
Iijima N., Tanaka S., Ota Y. (1998). Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiol. Biochem., 18: 59–69.
Kasarci G., Ertugrul B., Iplik E. S., Cakmakoglu B. (2021). The apoptotic efficacy of succinic acid on renal cancer cell lines. Med. Oncol., 38: 144.
Kuda T., Matsumoto C., Yano T. (2002). Changes in acid and alkaline phosphatase activities during the spoilage of raw muscle from horse mackerel *Trachurus japonicus* and gurnard *Lepidotriga microptera*. Food Chem., 76: 443–447.
Kumar M.S., Karthikeyan S., Ramprasad C., Aruna P.R., Mathivanan N., Velmurugan D., Ganesan S. (2015). Investigation of phloroglucinol succinic acid dendrimer as antimicrobial agent against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Nano. Biomed. Eng., 7: 62–74.
Lenchenko E., Lenchenko S., Sachivkina N., Kuznetsova O., Ibragimova A. (2022). Interaction of *Cyprinus carpio* Linnaeus with the biofilm-forming *Aeromonas hydrophila*. Vet. World, 15: 2458.
Li R. X., Zhou W.H., Ren J., Wang J., Qiao F., Zhang M.L., Du Z.Y. (2022). Dietary sodium lactate promotes protein and lipid deposition through increasing energy supply from glycolysis in Nile tilapia (*Oreochromis niloticus*). Aquaculture, 550: 737858.
Loh J.Y., Chan H.K., Yam H.C., In L.L.A., Lim C.S.Y. (2020). An overview of the immunomodulatory effects exerted by probiotics and prebiotics in grouper fish. Aquac. Int., 28: 729–750.
Magnadóttir B. (2006). Innate immunity of fish (overview). Fish Shellfish Immunol., 20: 137–151.
Martins M., Tavares-Dias M., Fujimoto R., Onaka E., Nomura D. (2004). Haematological alterations of *Leporinus macrocephalus* (Osteichtyes: Anostomidae) naturally infected by *Goezia leporini* (Nematoda: Anisakidae) in fish pond. Arq. Bras. Med. Vet. Zootec., 56: 640–646.
McCord J.M., Fridovich I. (1969). An enzymic function for erythrocuprein. J. Biol. Chem., 244: 6049–6055.
Mirghaed A.T., Yarahmadi P., Hosseinifar S.H., Tahmasebi D., Gheisvandi N., Ghaedi A. (2018). The effects singular or combined administration of fermentable fiber and probiotic on mucosal immune parameters, digestive enzyme activity, gut microbiota and growth performance of Caspian white fish (*Rutilus frisii kutum*) fingerlings. Fish Shellfish Immunol., 77: 194–199.
Mokhtar D.M., Zaccone G., Alesci A., Kuciel M., Hussein M.T., Sayed R.K. (2023). Main components of fish immunity: An overview of the fish immune system. Fishes, 8: 1–24.
Naderi Farsani M., Bahrami Gorji S., Hoseinifar S.H., Rashidian G., Van Doan H. (2020). Combined and singular effects of dietary PrimaLac® and potassium diformate (KDF) on growth performance and some physiological parameters of rainbow trout (*Oncorhynchus mykiss*). Probiotics Antimicrob. Proteins, 12: 236–245.
Naderi Farsani M., Meshkini S., Manaffar R. (2021). Growth performance, immune response, antioxidant capacity and disease resistance against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*) as influenced through singular or combined consumption of resveratrol and two-strain probiotics. Aquac. Nutr., 27: 2587–2599.
Ng W.K., Koh C.B. (2017). The utilization and mode of action of organic acids in the feeds of cultured aquatic animals. Rev. Aquac., 9: 342–368.
Niazi A., Shamsaie Mehrgan M., Rajabi Isalmi H. (2023). Optimizing turmeric and green tea fermentation with *Lactobacillus brevis* to enhance growth performance, digestive enzymes, and immunity in rainbow trout. Aquaculture, 577: 739962
Ohkawa H. (1979). Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. Anal. Biochem., 44: 276–278.
Oroji E., Mehrgan M.S., Islami H.R., Sharifpour I. (2021). Dietary effect of *Ziziphora clinopodioides* extract on zootechnical performance, immune response, and disease resistance against *Yersinia ruckeri* in *Oncorhynchus mykiss*. Aquac. Rep., 21: 100827.
Pajdak-Czaus J., PlattSamoraj A., Szweda W., Siwicki A.K., Terech-Majewska E. (2019). *Yersinia ruckeri* – a threat not only to rainbow trout. Aquac. Res., 50: 3083–3096.
Puvanasundram P., Chong C.M., Sabri S., Yusoff M.S., Karim M. (2021). Multi-strain probiotics: Functions, effectiveness and formulations for aquaculture applications. Aquac. Rep., 21: 100905.
Ross N.W., Firth K.J., Wang A., Burka J.F., Johnson S.C. (2000). Changes in hydrolytic enzyme activities of naive Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. Dis. Aquat. Organ., 41: 43–51.
Safari R., Adel M., Lazado C.C., Caipang C.M.A., Dadar M. (2016). Host-derived probiotics *Enterococcus casseliflavus* improves resistance against Streptococcus iniae infection in rainbow trout (*Oncorhynchus mykiss*) via immunomodulation. Fish Shellfish Immunol., 52: 198–205.
Safari O., Paolucci M., Ahmadniaye Motlagh H. (2021). Effect of dietary encapsulated organic salts (Na-acetate, Na-butyrate, Na-lactate and Na-propionate) on growth performance, haemolymph, antioxidant and digestive enzyme activities and gut microbiota of juvenile narrow clawed crayfish, *Astacus leptodactylus leptodactylus* Eschscholtz, 1823. Aquac. Nutr., 27: 91–104.
Safari R., Hoseinifar S.H., Dadar M., Nejadmoghaddam S., Van Doan H. (2020). Effect of dietary sodium acetate on skin mucus immune parameters and expression of gene related to growth, immunity and antioxidant system in common carp (*Cyprinus carpio*) intestine. Ann. Anim. Sci., 20: 1441–1452.
Salinas I., Magadán S. (2017). Omics in fish mucosal immunity. Dev. Comp. Immunol., 75: 99–108.
Santos G.G., Libanori M.C.M., Pereira S.A., Ferrarezi J.V.S., Ferreira M.B., Soligo T.A., Yamashita E., Martins M.L., Mouriño J.L.P. (2023). Probiotic mix of *Bacillus* spp. and benzoic organic acid as growth promoter against *Streptococcus agalactiae* in Nile tilapia. Aquaculture, 566: 739212.
Saravanan K., Sivaramakrishnan T., Praveenraj J., Kiruba-Sankar R., Haridas H., Kumar S., Varghese B. (2021). Effects of single and multi-strain probiotics on the growth, hemato-immunological, enzymatic activity, gut morphology and disease resistance in Rohu, *Labeo rohita*. Aquaculture, 540: 736749.
Sarkar P., Issac P.K., Raju S.V., Elumalai P., Arshad A., Arockiaraj J. (2021). Pathogenic bacterial toxins and virulence influences in cultivable fish. Aquac. Res., 52: 2361–2376.
Shekarabi S.P.H., Javarsiani L., Mehrgan M.S., Dawood M.A., Adel M. (2022). Growth performance, blood biochemistry profile, and immune response of rainbow trout (*Oncorhynchus mykiss*) fed dietary Persian shallot (*Allium stipitatum*) powder. Aquaculture, 548: 737627.
Siwicki A. (1993). Nonspecific defense mechanisms assay in fish: II. Potential killing activity of neutrophils and macrophages, lysozyme activity in serum and organs and total immunoglobulin level in serum. Fish Dis. Diag. Prevent. Met., 105–112.
Sotoudeh E., Esmaeili N. (2022). Effects of Biotronic® Top3, a feed additive containing organic acids, cinnamaldehyde and a permeabilizing complex on growth, digestive enzyme activities, immunity, antioxidant system and gene expression of barramundi (*Lates calcarifer*). Aquac. Rep., 24: 101152.
Tadese D.A., Song C., Sun C., Liu B., Liu B., Zhou Q., Xu P., Ge X., Liu M., Xu X., Tamiru M. (2022). The role of currently used medicinal plants in aquaculture and their action mechanisms: A review. Rev Aquac., 14: 816–847.
Taherpour M., Roomiani L., Islami H.R., Mehrgan M.S. (2023). Effect of dietary butyric acid, *Bacillus licheniformis*' (probiotic), and their combination on hemato-biochemical indices, antioxidant enzymes, immunological parameters, and growth performance of Rainbow trout (*Oncorhynchus mykiss*). Aquac. Rep., 30: 101534.
Tran N.T., Yang W., Nguyen X.T., Zhang M., Ma H., Zheng H., Zhang Y., Chan K.G., Li S. (2022). Application of heat-killed probiotics in aquaculture. Aquaculture, 548: 737700.
Van Doan H., Hoseinifar S.H., Ringø E., Angeles Esteban M., Dadar M., Dawood M.A., Faggio C. (2020). Host-associated probiotics: a key factor in sustainable aquaculture. Rev. Fish. Sci. Aquac., 28: 16–42.
Vijayaram S., Sun Y.Z., Zuorro A., Ghafarifarsani H., Van Doan H., Hoseinifar S.H. (2022). Bioactive immunostimulants as health-

promoting feed additives in aquaculture: A review. Fish Shellfish Immunol., 130: 294–308.

Vijayaram S., Ringø E., Zuorro A., van Doan H., Sun Y. (2023). Beneficial roles of nutrients as immunostimulants in aquaculture: A review. Aquac. Fish., https://doi.org/10.1016/j.aaf.2023.02.001

Wang Y., Wu Y., Wang Y., Xu H., Mei X., Yu D., Wang Y., Li W. (2017). Antioxidant properties of probiotic bacteria. Nutrients, 9: 521.

Yano T. (1992). Assays of hemolytic complement activity, Stolen J.S., Fletcher T.C., Anderson D.P., Kaattari S.L., Rowley A.F. (eds). Fish Shellfish Immunol., 2: 131–141.

Yeganeh S., Adel M., Nosratimovafagh A., Dawood M.A. (2021). The effect of *Lactococcus lactis* subsp. *lactis* PTCC 1403 on the growth performance, digestive enzymes activity, antioxidative status, immune response, and disease resistance of rainbow trout (*Oncorhynchus mykiss*). Probiotics Antimicrob. Proteins., 13: 1723–1733.

Yılmaz S., Ergun S., Yigit M., Çelik E.Ş. (2020). Effect of combination of dietary *Bacillus subtilis* and trans-cinnamic acid on innate immune responses and resistance of rainbow trout, *Oncorhynchus mykiss* to *Yersinia ruckeri*. Aquac. Res., 51: 441–454.

Yousefi M., Farsani M.N., Ghafarifarsani H., Raeeszadeh M. (2023 a). Dietary *Lactobacillus helveticus* and Gum Arabic improves growth indices, digestive enzyme activities, intestinal microbiota, innate immunological parameters, antioxidant capacity, and disease resistance in common carp. Fish Shellfish Immunol., 135: 108652.

Yousefi M., Ghafarifarsani H., Raissy M., Yilmaz S., Vatnikov Y.A., Kulikov E.V. (2023 b). Effects of dietary malic acid supplementation on growth performance, antioxidant and immunological parameters, and intestinal gene expressions in rainbow trout, *Oncorhynchus mykiss*. Aquaculture, 563: 738864.

Zare R., Abedian Kenari A., Yazdani Sadati M. (2021). Influence of dietary acetic acid, protexin (probiotic), and their combination on growth performance, intestinal microbiota, digestive enzymes, immunological parameters, and fatty acids composition in Siberian sturgeon (*Acipenser baerii*, Brandt, 1869). Aquac. Int., 29: 891–910.

Zhang L., Zhang P., Xia C., Cheng Y., Guo X., Li Y. (2020). Effects of malic acid and citric acid on growth performance, antioxidant capacity, haematology and immune response of *Carassius auratus gibelio*. Aquac. Res., 51: 2766–2776.

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