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Research Article

Evaluation of Soil-Derived *Streptomyces chartreusis* KU324443 Effects as Probiotic on Growth Performance, Antioxidant Enzyme Activity, Mucosal and Serum Immune Parameters, and Related Gene Expression in Common Carp (*Cyprinus carpio*) Fingerlings

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The present trial investigates the effects of soil-derived *Streptomyces chartreusis* KU324443 as a probiotic on growth performance, mucosal and serum immune parameters, and immune and antioxidant-associated gene expression in common carp (*Cyprinus carpio*). In a two-month experiment, carps were fed with varying levels of *S. chartreusis* KU324443 (0 (control), 10^5 (S1), 10^6 (S2), and 10^7 (S3) CFU/g). Then, performance, skin mucus, and serum immune parameters besides immune and antioxidant-related gene expression (IL1 and Lyz, CAT and SOD) were measured. Fish fed *S. chartreusis*-supplemented diet showed a significant increase in growth performance parameters ($P < 0.05$) regardless of inclusion levels. Including different doses of *S. chartreusis* significantly increased serum total Ig and lysozyme activity compared to those fed the control diet ($P < 0.05$). While no significant difference was noticed in the case of skin mucus total Ig ($P > 0.05$), lysozyme activity showed a significant increase in *S. chartreusis*-treated carps ($P < 0.05$). No significant differences ($P > 0.05$) were noticed in the case of serum antioxidant enzyme activity (CAT, SOD, and GPx) of *S. chartreusis*-fed carps and control. However, fish fed the control diet recorded the highest SOD and GPx enzyme activity in skin mucus of common carp, while no significant differences were noticed in the case of mucus CAT enzyme activity in common carp ($P > 0.05$). Gene expression studies revealed noticeable alterations between treated fish and control. Fish in S3 treatment had significantly higher CAT, SOD, Lyz, and IL1 expression ($P < 0.05$). These results confirmed the beneficial effects of a soil-derived probiotic (*S. chartreusis*) on the performance and health of common carp.

1. Introduction

The development of aquaculture production increased without attaining the supreme level. The production of fish from the fisheries sector was reported to be around 91 million tons for several years, while production from the aquaculture sector rose to 70 million tons [1]. Presently, fish food supplies an average of 25% of the total protein required for the world population [1]. According to the FAO statistics, common carp is considered a promising and potential freshwater species all

over the world [1]. Intensive and semi-intensive fish production systems are commonly used for culture in different regions of the world that become linked by overcrowding and lack of sanitary management which are associated with microbial pathogen infection and disease outbreaks [2]. Consequently, disease outbreaks could cause countless losses to the aquaculture subdivision [3]. Extreme and severe use of antibiotics causes antibiotic-resistant microbes [4]. Therefore, scientists pay more attention to research to find alternative solutions, such as prebiotics and probiotics which are considered important components of novel strategy and protocol to prevent pathogen infections and control disease outbreaks in aquaculture [5–7]. A widely adopted technique used all over the world is applying immunostimulant substances and bioactive compounds derived from medicinal plants [8, 9] in aquafeeds as a substitute for the administration of inoculations. Boosting the immune system defence mechanism against pathogen infections could be achieved by the protective use of chemicals, prebiotics, and bioactive compounds from medicinal plants that prove valuable to farmer profits, community, and environment [10]. Probiotics are recognized as a beneficial feed additive that can enhance growth, boost the immune system, and control disease outbreaks [11]. The application of probiotics in aquafeeds is slowly becoming common [12–14]. Common probiotic products in aquaculture are from *Lactobacillus* sp. and *Bacillus* sp.; however, there are limited researches regarding the application of Actinobacteria, particularly *Streptomyces* sp., as a probiotic in aquafeeds [15]. Insufficient researches confirming the benefits of using marine Actinobacteria, particularly Streptomyces, as probiotics in aquaculture become a concern needing more effort from scientists [16]. The application of Streptomyces as a probiotic in aquaculture resulting in the promoted growth and survival and controlled disease outbreaks of aquaculture has been stated [15]. However, no information is available about the efficiency of soil-derived *S. chartreusis* as a probiotic on the performance and health of carps. Therefore, the study is aimed at evaluating the administration of *Streptomyces chartreusis* KU324443 in the common carp diet and its possible effects on growth performance, feed utilization, immunity, and antioxidant defence.

2. Materials and Methods

2.1. Ethics. For ethics in the animal study, we followed the protocol approved by the committee of ethics of the faculty of sciences of the University of Tehran (357; 8 November 2000).

2.2. Preparation of Experimental Diet

2.2.1. Streptomyces Isolation and Preparation. Streptomyces strain was isolated in a previous study [17]. Blast analysis of the partial 16S rRNA showed that the isolates are nearly identical to *Streptomyces chartreusis*. The isolate was cultured on yeast extract-malt extract (ISP2) broth (10 g L^{-1} malt extract, 4 g L^{-1} yeast extract, 4 g L^{-1} glucose, and 2 g L^{-1} $CaCO_3$, pH 7.4) and incubated at 28°C for 7 days at 150 rpm. Then, Streptomyces was inoculated to basal to make experimental diets.

Table 1: Dietary formulations (%) and proximate composition.

Ingredient	
Fish meal	40.0
Wheat flour	21.0
Soybean meal	13.5
Gluten	5.5
Soybean oil	6.0
Fish oil	6.0
Mineral premix*	3.0
Vitamin premix*	2.0
Binder†	2.0
Antifungi‡	0.5
Antioxidant§	0.5
Proximate analysis	
Dry matter	91.5
Crude protein	36.1
Crude lipid	11.2
Ash	3.5

*Premix detailed by (Hoseinifar et al. 2012). †Amet binder™, Mehr Taban-e-Yazd, Iran. ‡ToxiBan antifungal (Vet-A-Mix, Shenan-doah, IA). §Butylated hydroxytoluene (BHT) (Merck, Germany).

2.2.2. Preparation of Probiotic-Supplemented Diets. A basal diet was prepared for common carp according to previous studies. Different levels of *S. chartreusis* were added to the basal diet (Table 1) at a rate of 10^5 (S1), 10^6 (S2), and 10^7 (S3) CFU/g, thoroughly mixed, and made into pellets using a meat grinder. The pellets were air-dried and stored in a refrigerator until use.

2.3. Experiment Conditions. The present trial was performed in the Aquaculture Lab of GUASNR (Iran). A total of 240 common carp fingerlings were supplied and adapted for 14 days, and then, they were divided into twelve units allocated to four groups with three replicates. The experiment continued for 2 months, and within this period, carps were fed with the basal diet, three times a day and up to apparent satiation. Utmost care was considered to avoid feed loss. The tanks were supplied with an aerator (as the water was static), and to maintain water quality parameters in the optimum range, the tanks were daily cleaned, and every two days, 50% of the tank water was changed with fresh water to keep water quality parameters within recommended levels.

2.4. Assessment of Immune Parameters and Antioxidant Defence

2.4.1. Sampling Blood Serum and Skin Mucus. Blood sampling was obtained at the end of the feeding trial from the caudal vein (nine fish per treatment). The blood serums were separated by centrifuging, and samples were kept at -80°C until analysis. The sampling of carp skin mucus was performed as suggested by Subramanian et al. [18]. The

Table 2: The sequences of the primers used for the real-time PCR.

Primer name	Sequence (5′-3′)	Accession number
IL1-F	CATTGCTTGTACCCAGTCTGG	AJ401031
IL1-R	TCTGAAGAAGAGGAGGCTGTC	
Lyz-F	CAGGTGGAAAGAACAAGTGCA	AB027305
Lyz-R	ACATCTTACGCCCCTTACAGT	
Catalase-F	TCCTGTGGGACGCCTTGTGTTG	GQ376154.1
Catalase-R	CTTGTCTGGGCCGGGCTCAATG	
SOD-F	AGGTCCGCACTTCAACCCT	JF342355.1
SOD-R	GGATCACCATGGTCCTCCC	
Beta actin-F	TCTGCTATGTGGCTCTTGACT	XM_042721308
Beta actin-R	AACCTCTCATTGCCAATGGTG	

method was based on putting anesthetized fish in plastic bags for indirect mucus calculation.

2.4.2. Evaluation of Immune Parameters. The method described by Lowry et al. [19] was followed to measure the total protein levels in serum and skin mucus samples. Then, the total Ig levels were recorded based on the protocol suggested by Siwicki and Anderson [20]. The method was based on precipitation immunoglobulin using polyethylene glycol (Sigma). The lysozyme activity in blood serum and skin mucus of carps was determined after Guardiola et al. [21]. The protocol is based on the turbidimetric method and lysis of *Micrococcus luteus*.

2.4.3. Antioxidant Defence. The levels of antioxidant enzyme activity including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (both in serum and skin mucus) were measured by commercial kits (Zellbio®, Berlin, Germany) and related protocols.

2.5. Molecular Studies. Nine fish were sampled from each treatment and killed with an overdose of clove solution. Thereafter, the intestine samples were obtained and deep-frozen in liquid nitrogen and kept at -80°C until use. Total RNA of samples was isolated and obtained from the tissues by using the BIOZOL RNA extraction kit (Bioflux-Bioer, China). Then, DNase I (Fermentas, Lithuania) was used to remove genomic DNA. The concentration and quality of RNA in each sample were checked by NanoDrop (NanoDrop Technology, Wilmington, DE, USA) and 1.5% agarose gel. To synthesize cDNA, a cDNA synthesis kit (Fermentas, Lithuania) was used, and the protocol suggested by the company was followed [22, 23].

2.5.1. Real-Time PCR. Information on GenBank sequences was used to design primers (Table 2). The Primer3 software was used for primer design. The expression of immune (IL1 and Lyz) and antioxidant (SOD and CAT) was determined by real-time PCR (qPCR) using an SYBR Green qPCR Master Mix (Fermentase, Lithuania) and iCycler (BioRad, USA) [23]. The Pfaffl formula calculated relative gene expression. The REST software analyzed the ratio between targets and housekeeping (Gapdh) genes.

2.6. Growth Performance. Growth parameters were chronicled according to Doan et al. [13]. Growth parameters were intended as follows: weight gain (WG) = (final weight – initial weight), specific growth rate = (Ln of final weight – Ln of initial weight)/period of the trial $*$ 100, feed conversion ratio (FCR) = feed intake/WG, and survival rate (%) = (final number of fish/initial number of fish) × 100.

2.7. Statistical Analysis. The normality of data and homogeneity of variances were checked and confirmed. Then, to check the possible significant difference among treatments at $P < 0.05$, data was subjected to one-way ANOVA followed by the Tukey test. SPSS software was used to perform all statistical analyses.

3. Results

3.1. Growth Performance. The growth performance of carps at the end of two months of feeding with *S. chartreusis* is presented in Table 3. The results showed that carps fed *S. chartreusis* had significantly increased ($P < 0.05$) weight gain (%), weight gain (g), and specific growth rate (% day^{-1}) compared to the control group. This increase was regardless of inclusion levels, and there was no significant difference among different levels ($P < 0.05$). Dietary inclusion of *S. chartreusis* also significantly decreased FCR compared with the control group ($P < 0.05$). No mortality was recorded during the trial, and the survival rate was 100% in all treatments.

3.2. Serum Antioxidant Enzyme Activity. The serum antioxidant enzyme activity of carps following two months of treatment with *S. chartreusis* is summarized in Table 4. The results showed that, although the SOD level in S3 treatment was higher than that in other treatments, the increase was not statistically significant ($P > 0.05$). Similarly, no significant effects were noticed in serum CAT and GPx activities among treatments of serum antioxidant enzyme activity ($P > 0.05$).

3.3. Skin Mucus Antioxidant Enzyme Activity. Table 5 represents the effects of dietary *S. chartreusis* on the skin mucus antioxidant enzyme activity. Fish fed with the control diet had significantly higher SOD activity than those carps fed

Table 3: Growth performance parameters of common carp fed different levels of *Streptomyces chartreusis*. Data in a row assigned with different letters indicate significant differences ($P < 0.05$). Values are reported as the mean ± SD.

	Control	S1	S2	S3
Initial weight (g)	14.16 ± 1.25^a	13.66 ± 1.00^a	14.60 ± 1.15^a	13.83 ± 1.25^a
Final weight (g)	25.96 ± 3.66^b	32.00 ± 3.56^a	29.83 ± 2.02^a	31.63 ± 2.65^a
Weight gain (g)	11.80 ± 3.21^b	18.30 ± 3.09^a	15.58 ± 1.40^a	27.44 ± 0.62^a
Weight gain (%)	83.53 ± 13.66^b	135.25 ± 15.98^a	103.57 ± 3.63^a	129.60 ± 14.13^a
SGR (% day^{-1})	1.07 ± 0.23^b	1.51 ± 0.20^a	1.26 ± 0.05^a	1.47 ± 0.17^a
FCR	2.39 ± 0.33^b	1.78 ± 0.32^a	2.12 ± 0.39^a	1.83 ± 0.34^a
Survival (%)	100%	100%	100%	100%

Table 4: Antioxidant enzyme activity in serum of common carp fed different levels of *Streptomyces chartreusis*. Data in a row assigned with different letters indicate significant differences ($P < 0.05$). Values are reported as the mean ± SD.

	Control	S1	S2	S3
SOD (U/ml)	469.40 ± 48.49^a	487.94 ± 15.79^a	483.21 ± 30.43^a	523.40 ± 64.64^a
CAT (ml/min/n)	0.32 ± 0.02^a	0.31 ± 0.01^a	0.34 ± 0.02^a	0.33 ± 0.2^a
GPx (nmol/min/ml)	166.28 ± 1.10^a	166.33 ± 9.29^a	164.06 ± 3.28^a	162.60 ± 2.41^a

Data are presented as the mean ± S.D. Data assigned with different superscripts in a column present a significant difference at $P < 0.05$.

Table 5: Antioxidant enzyme activity in the skin mucus of common carp fed different levels of *Streptomyces chartreusis*. Data in a row assigned with different letters indicate significant differences ($P < 0.05$). Values are reported as the mean ± SD.

	Control	S1	S2	S3
SOD (U/ml)	403.78 ± 13.40^a	332.86 ± 25.38^b	353.19 ± 48.53^b	305.91 ± 30.89^b
CAT (ml/min/n)	0.32 ± 0.21^a	0.31 ± 0.09^a	0.31 ± 0.01^a	0.31 ± 0.03^a
GPx (nmol/min/ml)	156.42 ± 1.64^a	151.82 ± 2.21^b	142.03 ± 1.05^c	140.89 ± 1.80^c

Data are presented as the mean ± S.D. Data assigned with different superscripts in a column present a significant difference at $P < 0.05$.

S. chartreusis-supplemented diets ($P < 0.05$). In the case of skin mucus CAT activity, no significant difference was recorded between the treated groups and control ($P > 0.05$). However, feeding with *S. chartreusis* caused a significant decrease in GPx activity compared with that in the control group ($P < 0.05$). Indeed, the highest GPx activity was noticed in carps fed with the control diet.

3.4. Serum and Skin Mucus Immunity. Figures 1 and 2 represent the serum and skin mucus immune parameters of carps fed with *S. chartreusis* for two months. The total Ig levels in the blood serum of fish fed *S. chartreusis*-supplemented diets were significantly higher than those in the control group ($P < 0.05$), regardless of the inclusion level (Figure 1). The same results were obtained in the case of serum lysozyme activity. Significant increases in lysozyme activity were noticed in all carps fed supplemented diet ($P < 0.05$). In the case of skin mucus lysozyme activity, the same trend was noticed. Feeding with *S. chartreusis* remarkably increased lysozyme activity compared with the control ($P < 0.05$). This increase was not dose-dependent, and there was no significant differences among treatments ($P > 0.05$). However, in the case of skin mucus total Ig, the results were different, and no significant alteration was noticed when compared to the control group ($P < 0.05$).

3.5. Gene Expression Results. Figures 3 and 4 summarize the expression levels of immunity and antioxidant defence-related genes and the results of gene expression studies. Fish fed diet supplemented with S3 recorded the highest expression of antioxidant-related genes (CAT and SOD) ($P < 0.05$). No significant difference was noticed between S1 and S2 and control group ($P > 0.05$). Parallel outcomes were noticed in cases of immune-related genes (IL1 and Lyz) where the highest values were noticed in S3 treatment.

4. Discussion

Aquaculture is considered a main food-producing industry that has been confronted with several challenges within the past years; the two most important challenges are microbe infection and disease outbreaks. Scientists pay more attention to discovering probiotics since they are widely employed to control the multidrug resistance of infectious pathogens in the aquaculture industry [24]. Probiotics are progressively used in aquafeeds to favourably alter gastrointestinal microbiota, thereby improving growth, feed utilization, immune response, health status, and survival [13]. The results of this study clearly showed that feeding with *S. chartreusis* remarkably improved growth response and feed utilization. These results are in parallel with [25] who

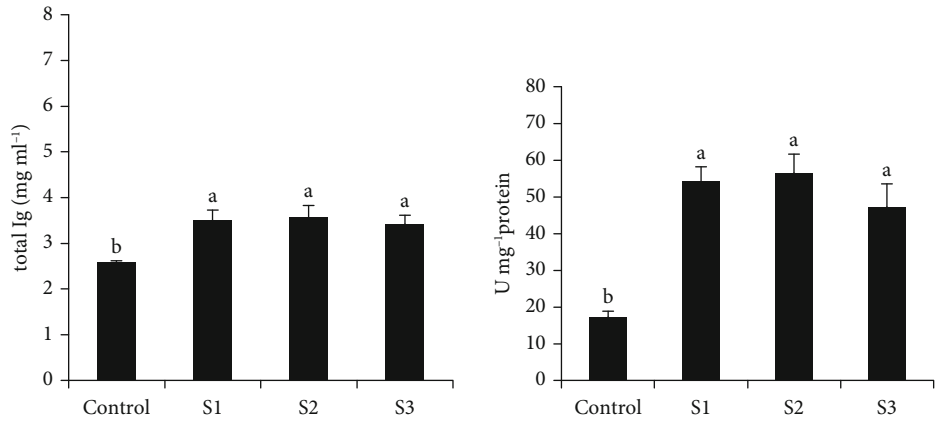

Figure 1: The effects of different levels of dietary *Streptomyces chartreusis* on serum immune parameters of common carp (*Cyprinus carpio*). Bars assigned with different letters indicate significant differences ($P < 0.05$). Values are reported as the mean ± SD.

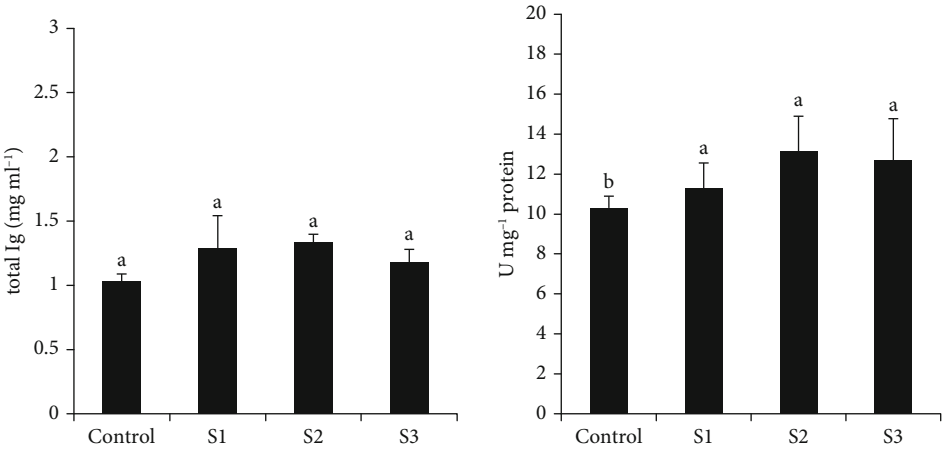

Figure 2: The effects of different levels of dietary *Streptomyces chartreusis* on skin mucus immune parameters of common carp (*Cyprinus carpio*). Bars assigned with different letters indicate significant differences ($P < 0.05$). Values are reported as the mean ± SD.

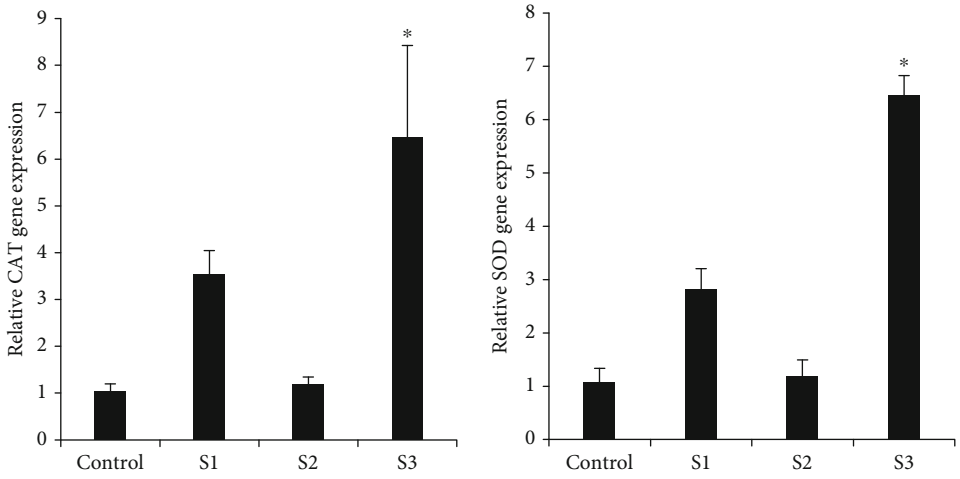

Figure 3: The effects of different levels of dietary *Streptomyces chartreusis* on the expression of antioxidant enzyme (CAT and SOD) genes in the intestine of common carp (*Cyprinus carpio*). Bars assigned with asterisks indicate significant differences ($P < 0.05$). Values are reported as the mean ± SD.

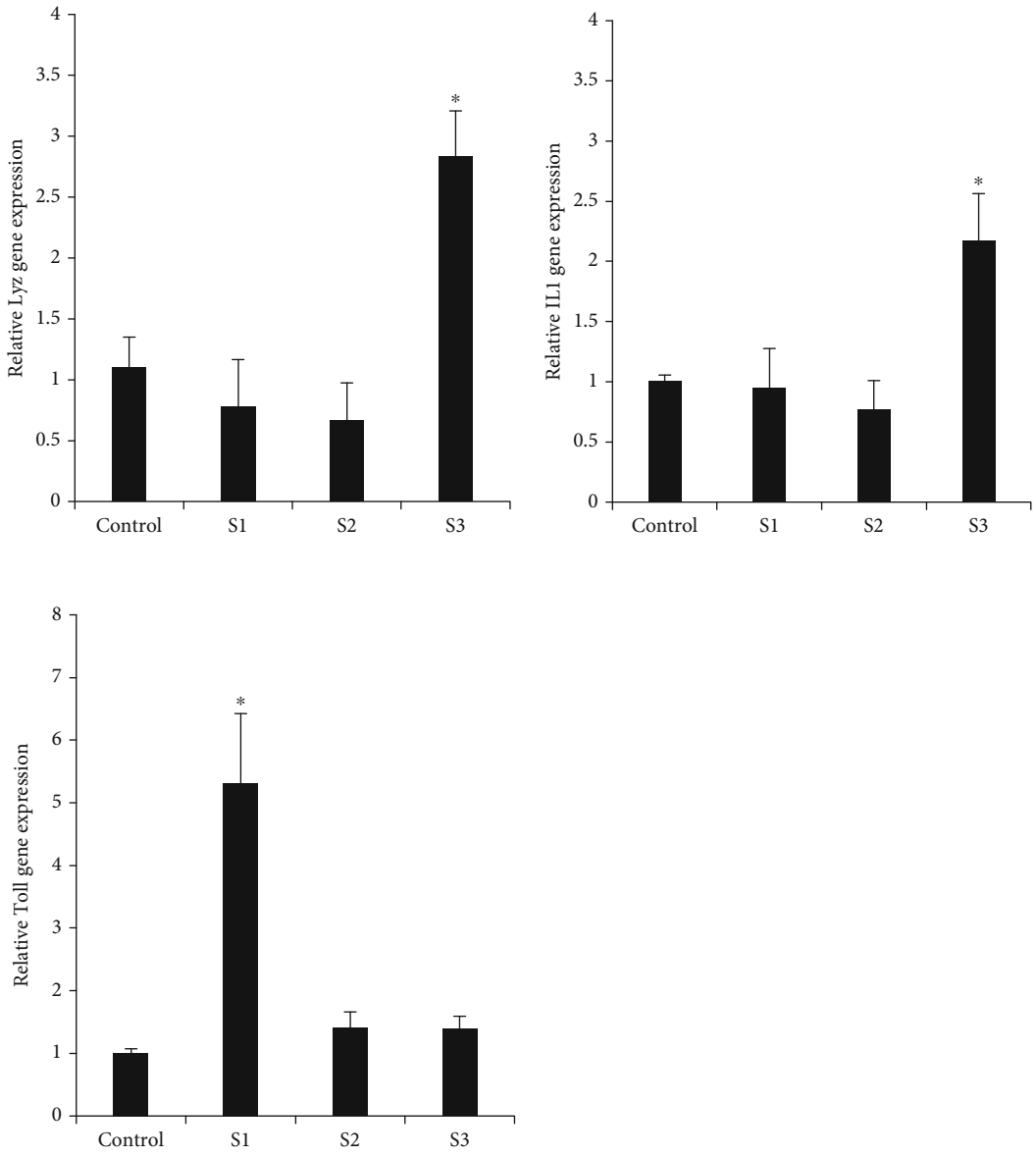

Figure 4: The effects of different levels of dietary *Streptomyces chartreusis* on the expression of immune-related genes (IL1 and Lyz) in the intestine of common carp (*Cyprinus carpio*). Bars assigned with asterisks indicate significant differences ($P < 0.05$). Values are reported as the mean ± SD.

found that tiger shrimp (*Penaeus monodon*) fed diets supplemented with *Streptomyces fradiae* and *Bacillus megaterium* as probiotics has improved growth performance and feed efficiency. Furthermore, García-Bernal et al. [26] found that the inclusion of *Streptomyces* sp. positively affected shrimp postlarva growth performance. In addition, Das et al. [27] found positive impacts on growth performance of black tiger shrimp fed diets supplemented with *Streptomyces* spp. Also, Dharmaraj and Dhevendaran [28] reported that *Xiphophorus helleri* fed diet supplemented with *Streptomyces* sp. has improved performance and feed efficiency. Recently, García-Bernal et al. [26] reported that the inclusion of *Streptomyces* spp. alone or combined with *Bacillus* sp. and *Lactobacillus* sp. improved the growth performance of *L. vannamei*. The present findings may be attributed to the role of *Streptomyces* in several mechanisms to improve performance: (i) production of antibiotics, vitamins, growth promoters, and important secondary metabolites that can improve digestive enzyme activities in the digestive tract [29], (ii) the breakdown of starch and protein that enhance the digestion and nutrient absorption, (iii) improved gut microbial balance, (iv) beneficial properties associated with metabolites produced by Actinobacteria which have transformed these microorganisms into possible candidates for use as probiotics improving nutrient digestion and assimilation, (v) the production of growth-endorsing molecules by probiotics as Streptomyces that boost the performance of different fish species [28], (vi) Streptomyces being an indirect type of protein that has better food conversion efficiency and acts as growth promoters for fish and shrimp [30], and

(vii) *Streptomyces* sp. which is reported to increase the intestine absorption surface, which per se improved nutrient assimilation [31].

The results displayed that the inclusion of *S. chartreusis* improved the immune parameters (total Ig level and lysozyme activity) in serum and mucus. The present findings are consistent with previous research conducted by [32–34] and who found that fish fed diet supplemented with probiotics have enhanced immune parameters and oxidative status. Also, Line et al. [35] stated that zebrafish fed diet supplemented with probiotics *B. amyloliquefaciens* and *B. amyloliquefaciens* R8 have enhanced innate immunity.

The present findings showed that *S. chartreusis* could significantly upregulate immune and oxidative-related genes. Indeed, the effects were pronounced when the highest level of *S. chartreusis* was used. The present results were *consistent with* Khan et al. [36] who found that the inclusion of *Bacillus amyloliquefaciens* significantly upregulated the immune-related genes *viz. IL-1β* and *TNF-α* in head-kidney and liver tissues. In addition, the present findings are in parallel with Selim and Reda [37] who found that Nile tilapia fed diet enriched with *B. amyloliquefaciens* has significantly upregulated expression of IL-1β and TNF-α. Also, He et al. [38] also noticed that tilapia fed diet supplemented with *Bacillus* probiotic has significantly improved gene expression of IL-1β and TNF-α. Furthermore, Lin et al. [35] found that zebrafish fed diet supplemented with probiotics have a significantly increased expression of innate immune-related genes. However, the present results are inconsistent with those results stated by Picchietti et al. [39] where they observed downregulation of IL-1β and TNF-α after the administration of probiotics. Recently, more importance is being given to studying immunostimulants as probiotics in fish and shrimp farms to boost immune system response and control microbe pathogen infection of probiotics which are often attributed to the stimulation of the immune system [12, 13, 36]. The improvement of immune response and expression of related genes could be attributed to (i) the immune protective effect of *Streptomyces* sp. following modulation of gut microbiota, which produced the antimicrobial peptides and regulated the inflammatory rejoinder; (ii) *Streptomyces* sp. producing various secondary metabolites which could modulate host immune responses and increase the chances of long-term survival; (iii) the anti-inflammatory and antirheumatic arthritis activity of polysaccharides derived from *Streptomyces* sp.; and (iv) *Streptomyces* sp. having been recognized as a source of antibiotics such as cephalosporins, chloramphenicol, and tetracycline.

The antioxidant defence system plays a crucial role in the host as a provider of protection against antioxidant stress and free radicals [40]. The antioxidant defence and related gene expression are highly influenced by diet composition and feed additives [41]. In the present trial, the inclusion of different doses of *S. chartreusis* displayed significant differences in mucus oxidative enzyme activity as GPx and SOD and significantly upregulates oxidative gene expression versus the basal diet. The highest expression was noticed in fish fed S3. The present results are confirmed by previous research conducted by Aravindan et al. [42] who stated the role of Streptomyces in the production of important metal chelation and enhancing antioxidant enzyme activities and consequently boosting oxidative gene expression and enzyme activity. Similarly, Tibaldi et al. [43] found that *Streptomyces carpaticus* produced EPS with high DPPH radical-scavenging capacity that supports the role of Streptomyces as an antioxidant that boosts enzymes and genes associated with oxidative stress. Consistently, Lin et al. [35] found that zebrafish fed diet supplemented with Streptomyces-induced 3-HAD and CS suggested higher mitochondrial integrity and lipid β-oxidation. Therefore, the results suggest that supplementation with *B. amyloliquefaciens* R8 possibly increases the utilization of XOSs and enhances liver glucose and lipid metabolism.

The positive impact of *S. chartreusis* inclusion to enhance the antioxidant enzyme activity and related gene expression could be attributed to several mechanisms: (i) Streptomyces boost and enhance the antioxidant protection enzymes against oxidative stress, (ii) Streptomyces produced a wide range of biological properties that include antioxidant and antitumor Lin et al. [35]; and (iii) Streptomyces produced Exopolysaccharide (EPS) that proved robust DPPH radical scavenging, prevent the deleterious function of free radicals in various tissues, superoxide hunting, and metal-trivalent activities, and moderate suppression of lipid peroxidation [35, 44].

Data Availability

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

Conflicts of Interest

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

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