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RESEARCH ARTICLE

A new isolate of *Streptomyces lateritius* (Z1-26) with antibacterial activity against fish pathogens and immune enhancement effects on crucian carp (*Carassius auratus*)

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Abstract

The *Streptomyces lateritius* Z1-26 was isolated from soil samples which showed broad-spectrum antibacterial activity against a broad range of fish pathogens. The In Vivo Imaging System (IVIS) monitored that strain Z1-26 could survive and colonize in the gills and abdomen of crucian carp. The effects of dietary supplementation with strain Z1-26 were evaluated with respect to the growth performance, antioxidant capacity, and immune response of crucian carp. The results showed that the Z1-26-fed fish had a significantly higher growth rate than the fish fed the control diet. The immune and antioxidant parameters revealed that the non-specific immune indicators (AKP, SOD, and LZM) of the serum, the expression of immune-related genes (*IgM*, *C3*, and *LZM*), and antioxidant-related genes (*Nrf2* and *Keap1*) of the immune organs were significantly increased, whereas the expression of pro-inflammatory factors (*IL-1β*, *IL-8*, and *TNF-α*) of the immune organs was significantly down-regulated in crucian carp fed strain Z1-26 compared with fish fed a control diet. Moreover, fish fed with Z1-26 supplemented diets showed a significantly improved survival rate after *Aeromonas hydrophila* infection. In addition, the whole genome analysis showed that strain Z1-26 possesses 28 gene clusters, including 6 polyketide synthetase (PKS), 4 non-ribosomal peptide-synthetase (NRPS), 1 bacteriocin, and 1 lantipeptide. In summary, these results indicated that strain Z1-26 could improve the growth performance and disease resistance in crucian carp, and has the potential to be developed as a candidate probiotics for the control of bacterial diseases in aquaculture.

KEYWORDS
antibacterial activity, *Carassius auratus*, genome sequencing, growth, immune response

1 | INTRODUCTION

In the past few decades, the global aquaculture industry has developed rapidly, and the scale and intensity level of aquaculture has been quickly expanding and improving (Anderson et al., 2019; Lu et al., 2020). However, with an increase in the number and density of cultures, outbreaks of aquatic animal diseases have become increasingly serious, which often cause huge economic losses to this industry (Rosado et al., 2019). Notably, bacterial diseases pose a great threat to fish farming, as these diseases are

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highly prevalent with many species that can cause great harm (Assefa & Abunna, 2018). Antibiotic strategies, which can effectively suppress or kill pathogenic microorganisms and play an active role in the control of aquatic diseases, are currently the mainstay for the prevention and management of bacterial fish diseases (Reda et al., 2013). However, the excessive use of antibiotics in aquaculture has resulted in the emergence of a large number of antibiotic-resistant microorganisms and has had a negative impact on human health and the ecological environment (Liu et al., 2017).

As a promising alternative to antibiotics, probiotics can effectively reduce the risk of drug residues and pathogen resistance caused by antibiotic abuse, and have beneficial effects on promoting host growth, enhancing immune function and improving disease resistance. (Serra et al., 2019). At present, the strains most frequently used as probiotics in aquaculture are relatively single, mainly including photosynthetic bacteria, bacillus, lactic acid bacteria, yeasts, and nitrifying bacteria (Fečkaninová et al., 2019; Kuebutornye et al., 2020; Sumardi et al., 2020). In addition to the high stability of *Bacillus*, other probiotics are relatively easy to inactivate during their application process (Cao et al., 2019). *Actinobacteria* can produce a wide range of secondary metabolites and can withstand high temperatures, acids, intestinal enzymes and other extreme environments, thus offering greater advantages in aquaculture than non-spore-forming strains (Johnston & Cross, 2010). In recent years, actinomycetes have been used in aquaculture to control disease outbreaks and have achieved certain results. For example, grass carp fed with *Streptomyces amritsarensis* N1-32 supplemented diets exhibited significant changes in the expression of immune factors in serum and mucus and immune-related genes in immune organs, showing a significant improvement in survival rate compared with the control group after *Aeromonas veronii* infection (Li et al., 2020). Pathological signs that occurred on the body surface, anus and abdominal congestion caused by *A. veronii* infection were alleviated in the group of grass carp injected with the fermentation supernatant of *Streptomyces flavotricini* X101 compared to the control group. Similarly, liver and kidney damage was also relieved in the group of grass carp treated with *S. flavotricini* (Huang et al., 2020). *Actinomycetes* have the functions of biological control and nutritional immunity, and can be used in fish farming, promising great economic benefits and broad application prospects.

In this study, *Streptomyces lateritius* Z1-26 was isolated as a potential probiotic with antibacterial activity against a variety of fish pathogens. The green fluorescent strain Z1-26^{EGFP} was constructed using fluorescent protein labelling technology to analyse the colonization ability of Z1-26^{EGFP} in crucian carp. Subsequently, the effects of dietary supplementation with Z1-26 on growth performance, immunity, and disease resistance were evaluated in crucian carp. Furthermore, the whole genome of Z1-26 was sequenced, and the characteristics of strain Z1-26 were analysed from the perspective of molecular biology.

2 | MATERIALS AND METHODS

2.1 | *Actinomycete* isolation and identification

Soil samples from different regions were collected and serially diluted with sterile water. A 100-μl suspension was spread on GAUZE's agar plates and incubated at 30°C for 5 days. Single actinomycete-like colonies were re-streaked on GAUZE's agar plates fourth and the purified strains were stored in 25% (w/v) glycerol broth at −80°C.

Strain Z1-26 was grown in GAUZE's liquid medium at 30°C for 4 days, and then scanning electron microscopy (SEM, Hitachi Su8010, Japan) was used to observe the morphology of the Z1-26 cell. The biochemical characterization of strain Z1-26 was subsequently analysed. DNA fragments carrying the 16S rRNA gene were amplified with universal primers (27F, 5′ to 3′: AGAGTTTGATCCTGGCTCAG, 1492R, 5′ to 3′: CGGTTACCTTGTTACGACTT) (Behbahani et al., 2019) and sequenced (Sangon, China). The obtained gene sequence was used for an NCBI BLAST search to determine similarities and construct a phylogenetic tree using MEGA 7.0.26 software (Ambrose & Hall, 2019).

2.2 | Antimicrobial assay

Strain Z1-26 was inoculated in AM6 liquid medium at 30°C for 5 days and separated by centrifuging at 12,000 rcf for 10 min to obtain the cell-free supernatant. Then, the cell-free supernatant was sterilized by filtration through a 0.22-μm syringe filter. Fresh overnight cultures of ten pathogenic bacteria (Table S1) at a concentration of approximately 1.0×10^7 cfu/ml were spread on LB agar plates, respectively. Next, sterile oxford cups were placed on the surface of the LB agar plate, and 100 μl of the cell-free supernatant of Z1-26 was added into oxford cups. The diameter of the inhibitory zone around the oxford cup was measured after incubation for 24 h. Oxford cups containing 100 μl of AM6 medium were used as a control, with three replicates per group (Gong et al., 2019).

2.3 | Biosafety test

The hepatic L8824 cell line derived from the liver of grass carp was cultured at 37°C with 5% CO_2 in DMEM (Gibco/Thermo Fisher Scientific, United States) supplemented with 10% foetal calf serum (FCS, BI, Israel) and 1% penicillin–streptomycin solution (BI, Israel). The density of the cells was adjusted to 2×10^5 cells/ml, and then 100 μl of the diluted cells were added to 96-well flat-bottomed plates for pre-cultured for 12 h. The cell-free supernatant (5 or 10 μl) of Z1-26 was added to the L8824 cells and the morphological changes of the cells were observed using an inverted light microscope (Leica Microsystems S.p.A, Italy). The cell-free supernatant of *A. hydrophila* and AM6 medium were used as the positive control and negative control, respectively.

Healthy crucian carp (45 ± 7.8 g) obtained from the Wangcheng fishpond (Changsha, China) were acclimatized in feeding boxes (volume 60 L) equipped with an air pump for 7 days after being transferred to the laboratory. Ninety crucian carp were randomly divided into three groups with three replicates for each group. Experimental fish were intraperitoneally injected with 100 μl of Z1-26 suspension at a concentration of 1×10^8 cfu/ml or 1×10^9 cfu/ml per fish. 100 μl of PBS was used as a control. The living conditions and mortality of the crucian carp were observed and recorded for 14 days.

2.4 | Colonization and distribution of Z1-26^{EGFP} strain in crucian carp

The pib139-EGFP plasmid was transformed into strain Z1-26 using the method of conjugal transfer (Zhao et al., 2018). The growth and fluorescence analysis of strain Z1-26^{EGFP} were monitored. The growth performances of Z1-26 and Z1-26^{EGFP} were compared by measuring the dry weight. The fluorescence of strain Z1-26^{EGFP} was detected by the inverted fluorescence microscopy (AXIO OBSERVER A1, Zeiss, Germany). The experiments were performed in triplicate.

The 90 healthy crucian carp (45 ± 7.8 g) were randomly divided into two groups with three replicates for each group. The control group was fed with the basic diet, and the experimental group was fed with Z1-26^{EGFP} supplemented diet at 1×10^9 cfu/g. Take strain Z1-26^{EGFP} suspension, centrifuge at 8000 rcf/min for 5 min to remove the supernatant, collect the bacterial cells, and evenly mix them into the basic feed. The fish were fed at 1% of their body weight twice a day. The experiment lasted for two weeks. The distribution of Z1-26^{EGFP} in crucian carp was monitored using an In Vivo Imaging System (IVIS, Calliper, United States) every two days for 14 days.

2.5 | Diet preparation

The healthy crucian carp (45 ± 7.8 g) with uniform body length and weight were randomly divided into three groups, 30 in each group and three replicates ($n = 10$/replicates). They were fed with different diets: the control group with basic diet, experimental 1 group with Z1-26 supplemented diet at 1×10^8 cfu/g, and experimental 2 group with Z1-26 supplemented diet at 1×10^9 cfu/g. Each treatment was performed in triplicate and all fish were fed twice per day for a total of 2% of their body weight.

2.6 | Growth performance analysis

Fish were weighed on the first (W_0) and the 30th (W_t) day of the experiment. The weight gain rate (WGR), specific growth rate (SGR), feed efficiency (FE) and survival rate (SR) were calculated using the following equations: WGR (%) = $100 \times (W_t - W_0)/W0$; SGR (%) = $100 \times (\ln W_t - \ln W_0)/30$; FE (%) = $100 \times (W_t - W_0)/$(feed intake); SR (%) = 100 × (final number of tested crucian carp)/(initial number of tested crucian carp) (Chen, Li, et al., 2019).

2.7 | Non-specific immune analysis

Blood was collected by the caudal venous blood collection method from 3 animals in each replicate after 30 days of feeding. The blood was allowed to clot at room temperature for 2 h and then for 4 h at 4°C. After centrifuged at 4000 rcf, 4°C for 10 min, the upper serum was obtained. Serum samples were used to determine acid phosphatase (ACP) activity, alkaline phosphatase (AKP) activity (Jia et al., 2019), lysozyme (LZM) activity (Yin et al., 2006) and superoxide dismutase (SOD) activity (Zhou & Progon, 2006). The measurements were performed with commercial kits from Nanjing Jiancheng Institute (Nanjing, China) according to the manufacturer's instructions.

2.8 | RNA isolation and real-time quantitative PCR

The crucian carps were randomly selected from each group to analyse the expression of genes related to growth performance and antioxidant capacity, with three replicates in each group. The muscle and immune organs were collected and frozen under liquid nitrogen, stored at −80°C.

Total RNA was extraction from the muscle, liver, kidney, and spleen of the crucian carp using the method of Gong et al. (2019). Approximately 1 μg of total RNA from each of the samples was reverse transcribed to produce cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions. The expression levels of growth-related genes (*IGF* and *MSTN*) in the muscle, antioxidant-related genes (*Keap1* and *Nrf2*), and immune-related genes (*IgM*, *C3*, *LZM*, *IL-1β*, *IL-8*, and *TNF-α*) in the immune organs were determined using real-time quantitative PCR. The expression of *β-actin* was used as an internal control, and the primers used to detect each gene are listed in Table S2. Real-time quantitative PCR was performed with SYBR Premix Ex Tag™ GC (TaKaRa, Japan) on a 7500 Real-Time PCR system instrument (Applied Biosystems, United States). The relative expression of each gene was calculated with the $2^{-\Delta\Delta CT}$ method.

2.9 | Challenge test

Thirty days post-feeding, 20 fish from each group were intraperitoneally injected with 200 μl of *A. hydrophila* at a concentration of 1×10^8 cfu/ml. The protective effect of strain Z1-26 against *A. hydrophila* was evaluated by the relative per cent survival (RPS) using the formula: RPS = 1 − (mortality in immunized group/mortality in the challenge control group) × 100 (Yi et al., 2018).

2.10 | Whole-genome sequence of Z1-26

Strain Z1-26 was cultivated in AM6 liquid medium to collect the bacteria cells, which were sent to Nextomics Biotechnology Co., Ltd. (Wuhan, China) for whole-genome sequencing. The genomic DNA of Z1-26 was extracted with a Qiagen kit according to the manufacturer's instructions. Single-molecule DNA sequencing was performed by the Oxford Nanopore Technology sequencer PromethION following the manufacturer's recommendations. The sequencing data were assembled by Flye, corrected by Pilon, and optimized with a circulator to obtain the final genomic sequence.

The assembled genome was annotated with the KEGG database (Kanehisa & Sato, 2019). The genome was further analysed with Anti-SMASH for secondary metabolite and prebiotics biosynthesis gene clusters (Blin et al., 2019).

2.11 | Statistical analysis

All data were statistically analysed using SPSS 20 software and presented as the mean ± SE of the mean (SEM). One-way ANOVA was used to compare differences among the treatments (different superscripts represent significant differences, $p < .05$).

3 | RESULTS

3.1 | Isolation and identification of Z1-26

Strain Z1-26, which was isolated from a soil sample from Hebei, had antagonistic effect on ten fish pathogenic bacteria, including *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas sobria*, *Aeromonas caviae*, *Aeromonas allosaccharophila*, *Edwardsiella tarda*, *Erwinia* spp., *Citrobacter freundii*, *Shewanella xiamenensis* and *Plesiomonas shigelloide* (Table 1, Figure S2). Strain Z1-26, which formed concentric annular, dry, opaque, powdery colonies on GAUZE's agar plates, developed mycelium, more and shorter branches (Figure 1a,b). The 16S rRNA gene sequencing analysis showed that Z1-26 had 99% similarity to *S. lateritius* LMG 19372 (GenBank accession no. NR042293.1) (Figures 1c and S2). Therefore, the colonies and cells of strain Z1-26 conformed to the typical characteristics of *actinomycetes* and were named *S. lateritius* Z1-26. The biochemical characteristics of the gram-positive *S. lateritius* Z1-26 are shown in Table 2.

TABLE 1 Antimicrobial spectrum of *S. lateritius* Z1-26

Pathogens	Inhibition zone (mm)
Aeromonas veronii	23.33 ± 1.31
Aeromonas hydrophila	23.13 ± 0.81
Citrobacter freundii	21.35 ± 0.42
Edwardsiella tarda	23.60 ± 0.64
Plesiomonas shigelloide	22.13 ± 0.11
Erwinia spp.	23.93 ± 1.38
Shewanella xiamenensis	25.23 ± 0.88
Aeromonas caviae	22.40 ± 0.64
Aeromonas sobria	26.25 ± 0.35
Aeromonas allosaccharophila	24.93 ± 0.81

3.2 | Biosafety evaluation of Z1-26

In order to evaluate the biosafety of Z1-26 in fish, the cell-free supernatant of Z1-26 was applied to hepatic L8824 cells. After 12 and 24 h of incubation, the cell-free supernatant of Z1-26 did not exhibit toxicity to L8824 cells, which grew well with a full shape and clear outline (Figure 2). Then, 1×10^8 and 1×10^9 cfu/ml Z1-26 were intraperitoneally injected into crucian carp. Within 14 days post-injection, a normal living state was observed without pathological signs or mortality, indicating that Z1-26 is harmless to these fish (Figure S3).

3.3 | Colonization and distribution of the Z1-26^{EGFP} strain in crucian carp

The broad-spectrum antibacterial activity of Z1-26 against fish pathogens led us to investigate the colonization of Z1-26 in crucian carp. We constructed the green fluorescently labelled strain Z1-26^{EGFP} using the EGFP protein (pIB139-EGFP plasmid). Then, the expression of the EGFP fluorescent protein was observed by inverted fluorescence microscopy. The results showed that strain Z1-26^{EGFP} could emit green fluorescence (Figure 3a). Additionally, the growth performance of the Z1-26 and Z1-26^{EGFP} strains was evaluated using the dry weight method. The results showed that the pIB139-EGFP plasmid did not have a drastic effect on the growth of strain Z1-26, indicating that plasmid pIB139-EGFP may have good stability in strain Z1-26 and can be used instead of Z1-26 to study colonization (Figure S4).

We monitored the distribution of strain Z1-26^{EGFP} by an IVIS, which could accurately observe the real-time localization of Z1-26^{EGFP} in crucian carp without causing damage to the fish. The crucian carp were fed with strain Z1-26^{EGFP} supplemented diet and then observed under the IVIS system every two days for 14 days. On the first day, the green fluorescence signal was detected in the gills of the crucian carp, and over time, the green fluorescence signal could be detected and increased in intensity in the abdomen of the crucian carp. The results indicated that strain Z1-26 could colonize in the crucian carp (Figure 3b).

3.4 | Effects of Z1-26 on the growth of crucian carp

To determine whether Z1-26 can effectively improve the growth performance and feed utilization of crucian carp, the parameters WGR, SGR, and FE were evaluated after 30 days of dietary

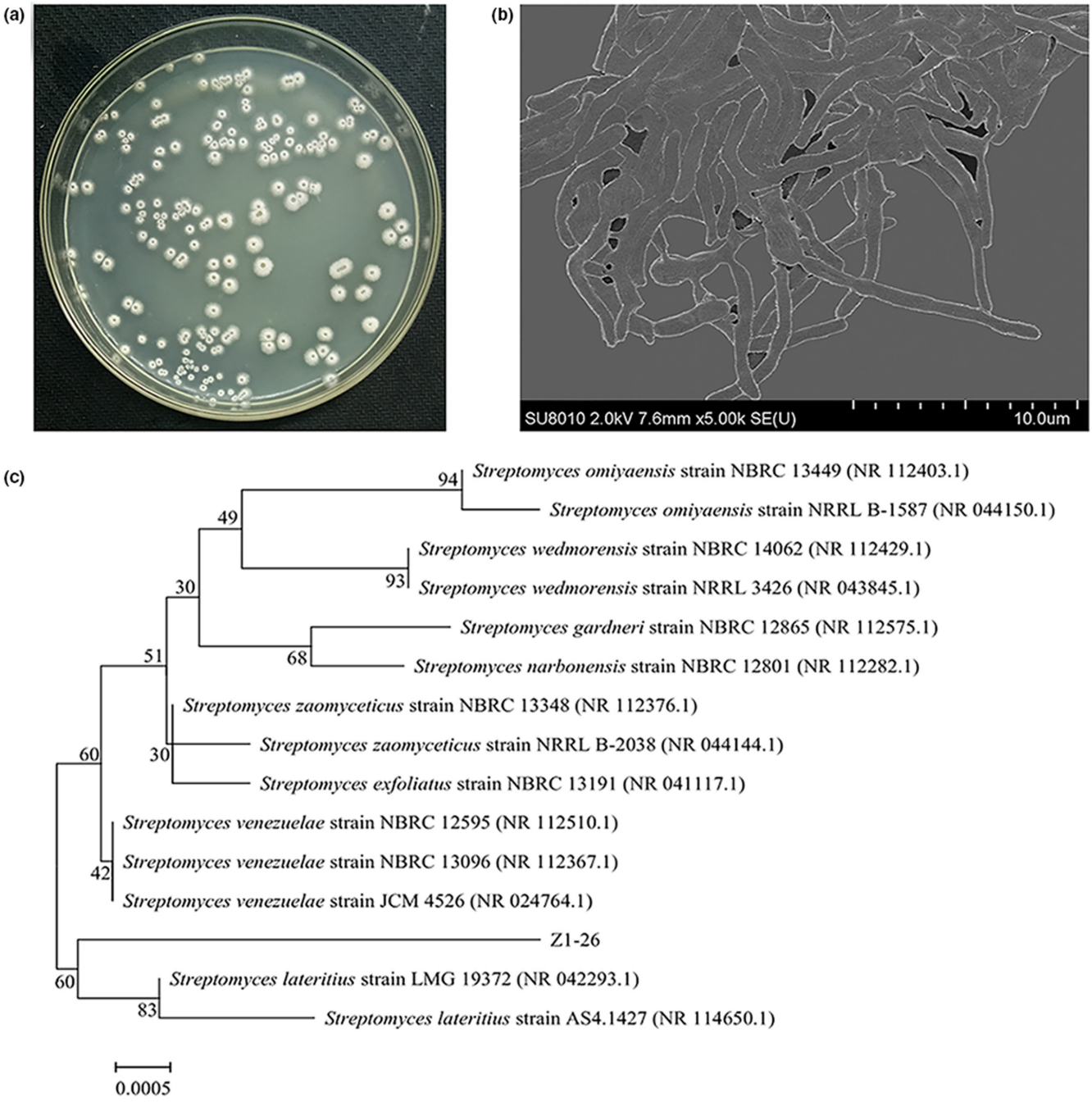

FIGURE 1 The morphological characteristics and identification of *S. lateritius* Z1-26. (a) The colony of strain Z1-26 on Gauze's synthetic agar medium. (b) SEM images of strain Z1-26. (c) The phylogenetic tree based on 16S rRNA sequences analysed evolutionary relationships of strain Z1-26 by the neighbour-joining method.

supplementation with strain Z1-26. The results showed that WGR of 19.00% and SGR of 0.58% for the fish in the 10^9 cfu/g group, which were significantly higher than those of the control group (15.42% and 0.48%, respectively). The WGR and SGR of the 10^8 cfu/g group were 17.90% and 0.55%, respectively, which were also higher than those of the control group albeit not statistically significant. The FE value of crucian carp fed with strain Z1-26 supplemented diets was significantly higher than that of the control group. There were no significant differences in WGR, SGR, and FE between crucian carp fed with 10^8 cfu/g or 10^9 cfu/g of Z1-26 (Table 3). These results suggested that dietary supplementation with Z1-26 could enhance the growth performance and feed utilization of crucian carp.

Subsequently, we analysed the expression of muscle growth-related genes using real-time quantitative PCR. Compared with the control, the expression levels of myostatin (*MSTN-1* and *MSTN-2*) were significantly down-regulated in the Z1-26-treated groups, while the expression levels of insulin-like growth factor (*IGF-1* and *IGF-2*) were significantly up-regulated. The expression of the detected genes showed significant differences between the 10^8 and 10^9 cfu/g groups (Figure 4).

TABLE 2 Biochemical characterization of *S. lateritius* Z1-26

Test item	Result
Gram staining	+
Lactose	−
Galactose	+
Mannitol	−
Fructose	−
Glucose	+
Xylose	+
Arabinose	+
Rhamnose	−
Trehalose	+
Sucrose	−
Gelatin liquefaction	−
Milk peptonization	−
H_2S production	+
Cellulose hydrolysis	−
Starch hydrolysis	+

Note: "+"Positive; "−"Negative.

3.5 | Effects of Z1-26 on non-specific immune responses

The non-specific immune parameters in the serum of crucian carp were detected 30 days post-feeding with Z1-26. The acid phosphatase (ACP) activity in the Z1-26 groups was higher than that of the control group, but there was no significant difference. The activities of alkaline phosphatase (AKP) and lysozyme (LZM) in the 10^9 cfu/g group were significantly higher than those in the control group. The fish also showed significantly higher superoxide dismutase (SOD) activity in the Z1-26 groups than in the control group (Figure 5). There was no significant difference in ACP, AKP, SOD, and LZM activity between the two Z1-26 groups.

3.6 | Effects of Z1-26 on the expression of antioxidant-related genes

The expression levels of the Kelch-like ECH-related protein 1 gene (*Keap1*) and nuclear factor E2-related factor 2 gene (*Nrf2*) in fish were measured. Compared with the control group, the expression of *Keap1* in the liver, kidney, and spleen was significantly up-regulated in the Z1-26 groups, and the expression of *Keap1* in the kidney and spleen showed significant differences between the 10^8 and10^9 cfu/g group. The expression of *Nrf2* in the liver was significantly higher in the 10^9 cfu/g group than that in the control group, but there was no significant difference in Nrf2 expression between the Z1-26 groups and the control group in the kidney and spleen (Figure 6). These results suggested that the Z1-26 strain could increase the expression level of antioxidant-related genes and enhance the antioxidant capacity of crucian carp.

3.7 | Effects of Z1-26 on the expression of immune-related genes

We further analysed the expression of immune-related genes in crucian carp fed with Z1-26 for 30 days. The results showed that the expression of immunoglobulin M (*IgM*) and complement 3 (*C3*) in the liver and spleen was significantly increased in the Z1-26 groups, and the expression of lysozyme (*LZM*) in the kidney was significantly increased in the Z1-26 group compared to the control group. Compared with the control group, the expression of interleukin-1β (*IL-1β*) in the liver, kidney and spleen showed a significantly lower value in the Z1-26 groups. The expression of interleukin-8 (*IL-8*) in the liver of the fish in the 10^9 cfu/g group and the kidney of the fish in both Z1-26 groups was significantly down-regulated. Similarly, the expression of tumour necrosis factor-α (*TNF-α*) in the liver of the fish in the Z1-26 groups and the spleen of the fish in the 10^9 cfu/g group were significantly decreased compared with the control group (Figure 7). These results revealed that the Z1-26 strain could regulate the expression of immune-related genes in the organs and tissues of crucian carp.

3.8 | Protection of Z1-26 against *A. hydrophila* infection

The crucian carp were challenged intraperitoneally with *A. hydrophila* after 30 days of feeding with Z1-26. The survival rates of the 10^8 and 10^9 cfu/g group at 7 days post-infection were 50.0% and 57.4%, respectively, which were significantly higher than that of the control group (7.5%), with relative per cent survival (RPS) values of 44.4% and 55.6%, respectively (Figure 8). Strain Z1-26 could therefore protect crucian carp against *A. hydrophila* infection.

3.9 | Whole-genome analysis of Z1-26

The whole genome of strain Z1-26 was sequenced using the PromethION sequencing platform, and the original data gave a total of 1,987,190,725 bp. After assembly and optimization, the resulting genome sequence had a size of 7,932,528 bp and an overall GC content of 72.14%. The genome of Z1-26 consists of a nuclear genome sequence with a size of 7,761,152 bp and a plasmid genome sequence with a size of 171,376 bp. The genome of Z1-26 was annotated with the NCBI prokaryotic genome annotation pipeline, resulting in 7096 coding DNA sequences (CDSs), 73 tRNAs, 21 rRNAs, and 1 CRISPR (Figure 9a).

The KEGG database was used to annotate and group the encoded proteins according to their function. The results showed that 2516 coding proteins were classified into six categories. Among these annotated proteins, 1067 encoded proteins were related to metabolism, accounting for 42.4% of the total annotated proteins. In addition, 290 encoded proteins were related to environmental information processing, accounting for 11.5% of the total annotated proteins. Moreover, carbohydrate metabolism and amino acid

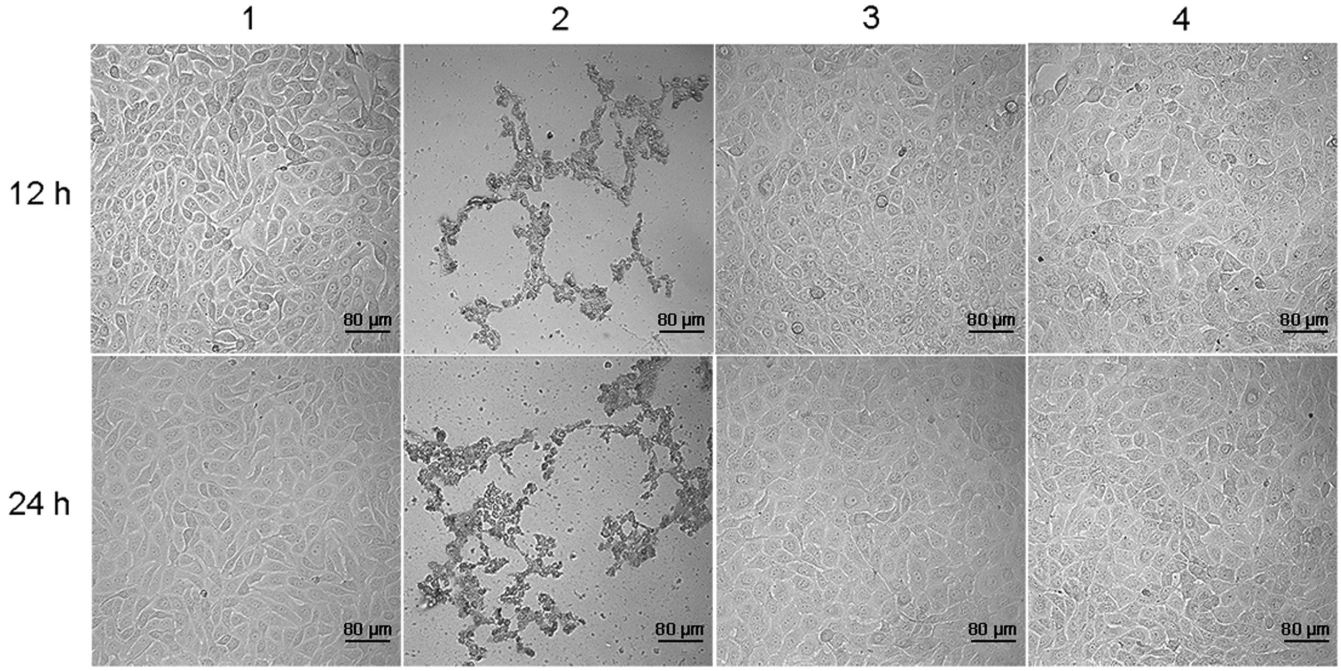

FIGURE 2 The morphology of grass carp hepatic L8824 cell line treated with fermentation supernatant of *S. lateritius* Z1-26 was observed under the inverted light microscope. 1, Medium (negative control); 2, cell-free culture supernatant of *A. hydrophila* (positive control); 3, 5 μl of fermentation supernatant of strain Z1-26; 4, 10 μl of fermentation supernatant of strain Z1-26.

metabolism accounted for large proportions, containing 291 and 303 encoded proteins respectively (Figure 9b).

Subsequently, a total of 28 secondary metabolite synthesis gene clusters were found in the Z1-26 genome using Anti-SMASH software, including 6 polyketide synthetase (PKS), 4 non-ribosomal peptide-synthetase (NRPS), 1 bacteriocin, and 1 lantipeptide. In addition, a gene cluster related to the synthesis of siderophores was predicted. Some of the gene clusters could be matched to specific homologous gene clusters, including bacilysin, carotenoid, salinomycin, thiolutin, and so on (Figure 10). These results indicated that strain Z1-26 has the potential to synthesize multiple secondary metabolites.

4 | DISCUSSION

S. lateritius, which can produce diverse secondary metabolites that are important sources of substances with bactericidal activity, insecticidal activity, and antitumor activity, has been used in the biological control of plant diseases and insect pests (Awla et al., 2017). *S. lateritius* has antibacterial activity against a variety of bacteria, including *Salmonella typhi*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger* (Gurovic & Olivera, 2017). However, the application of *S. lateritius* in aquaculture is relatively rare, and its use as a probiotic for disease prevention and control in cultured fish species has not been previously reported. The present study isolated *S. lateritius* strain Z1-26 from a soil sample taken from Hebei Province, China. Strain Z1-26 displayed broad-spectrum antibacterial activity against bacterial pathogens that commonly occur in fish. Therefore, strain Z1-26 has great value in the development of new and improved methods for the prevention and control of fish diseases.

The colonization ability of microorganisms in their host is a key factor in the interaction between the microorganism and the host. Probiotics with colonization capacity could adhere to the host intestinal mucosa, occupy a special ecological niche, form a symbiotic micro-ecosystem with the host, and finally survive and play an important role in the intestinal tract (Liang et al., 2020). It has been reported that *Bacillus velezensis* BvL03 could colonize grass carp after intraperitoneal injection (Cao et al., 2019). In this study, the colonization and distribution of strain Z1-26 in crucian carp were studied using the Z1-26 strain labelled with a green fluorescent protein, and a stable green fluorescent signal could be detected in the gills and abdomen of the crucian carp. These results indicated that strain Z1-26 could survive and colonize in crucian carp, which laid an important foundation for its probiotic function in this species.

Probiotics, which contain a large number of nutrients or can produce nutrients through metabolism and the enzymes needed for aquaculture animal metabolism to improve their growth performance and feed utilization, are widely used as nutritional additives in aquaculture (Olmos et al., 2020). For example, the growth rate and protein content significantly improved in a group of Nile tilapia fed with 1% *Rhodotorula mucilaginosa* diets compared to the control group (Chen, Liu, et al., 2019). The growth performance and feed utilization of juvenile Atlantic salmon supplemented with *Bacillus velezensis* V4 and *Rhodotorula mucilaginosa* compound were markedly higher than those of the control fish (Wang et al., 2019). Similarly, after 30 days of feeding with Z1-26, the specific growth rate and feed efficiency

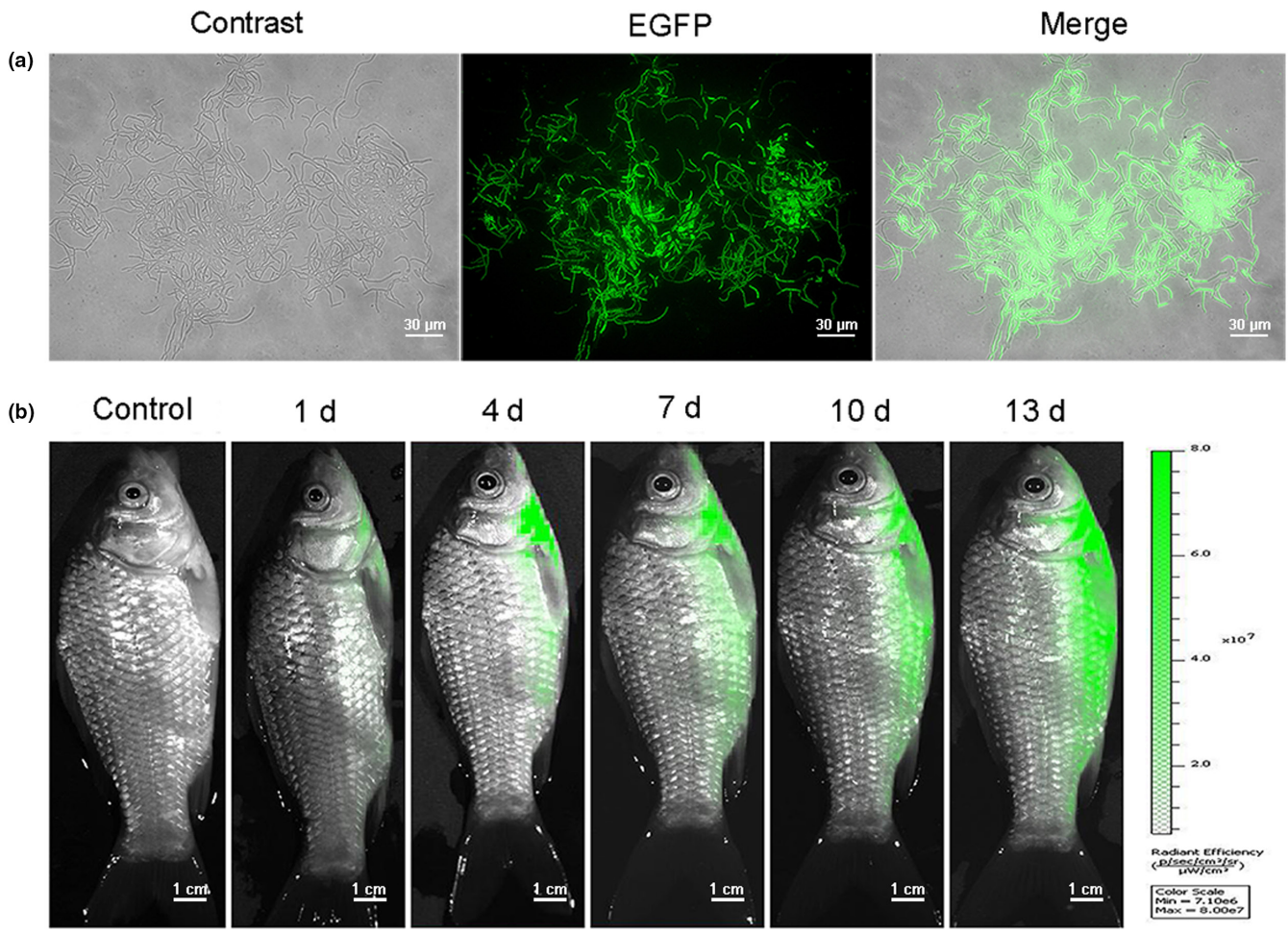

FIGURE 3 Fluorescent labelling and distribution of *S. lateritius* Z1-26 in crucian carp. (a) The bacterial cells of strain Z1-26^{EGFP} were observed under the inverted fluorescence microscope. (b) Dynamic distribution of labelled strain Z1-26^{EGFP} in crucian carp observed by IVIS.

TABLE 3 Effects of the *S. lateritius* Z1-26-supplemented diet on the growth of crucian carp

	Treatments		
Parameters	Control	10^8 cfu/g	10^9 cfu/g
W_0 (g)	45.06 ± 7.20^a	45.45 ± 8.29^a	45.05 ± 8.22^a
W_t (g)	49.67 ± 8.78^a	53.59 ± 10.64^a	53.59 ± 11.17^a
WGR (%)	15.42 ± 0.03^a	17.90 ± 0.01^{ab}	19.00 ± 0.01^b
SGR (%)	0.48 ± 0.0008^a	0.55 ± 0.0001^{ab}	0.58 ± 0.0003^b
FE (%)	62.15 ± 0.08^a	73.20 ± 0.03^b	76.88 ± 0.01^b
SR (%)	100^a	100^a	100^a

Note: Data (mean ± SD) in the same row with different letters show significant differences ($p < .05$).

of the crucian carp in this study were improved. Furthermore, the expression levels of the myostatin genes (*MSTN-1* and *MSTN-2*) in crucian carp in the Z1-26 supplement groups were down-regulated, while the expression levels of insulin-like growth factor genes (*IGF-1* and *IGF-2*) were up-regulated. Previous studies have shown that myostatin (MSTN) has a negative regulatory effect on the skeletal muscle growth of fish such as zebrafish, channel catfish, and flounder, whereas insulin-like growth factor (IGF) has a positive regulatory effect on skeletal muscle growth by stimulating cell growth. This study confirmed that strain Z1-26 could promote the growth of crucian carp at both the individual and molecular levels.

Oxidative stress refers to the overproduction of reactive oxygen species (ROS) that can cause cell and tissue damage in the body when an organism encounters a harmful stimulus, which eventually leads to a variety of diseases (Wang et al., 2020). The Nrf2/Keap1 pathway, an important antioxidant pathway, protects the body from oxidative damage by up-regulating antioxidant enzymes and removing excess reactive oxygen species. Among them, nuclear factor

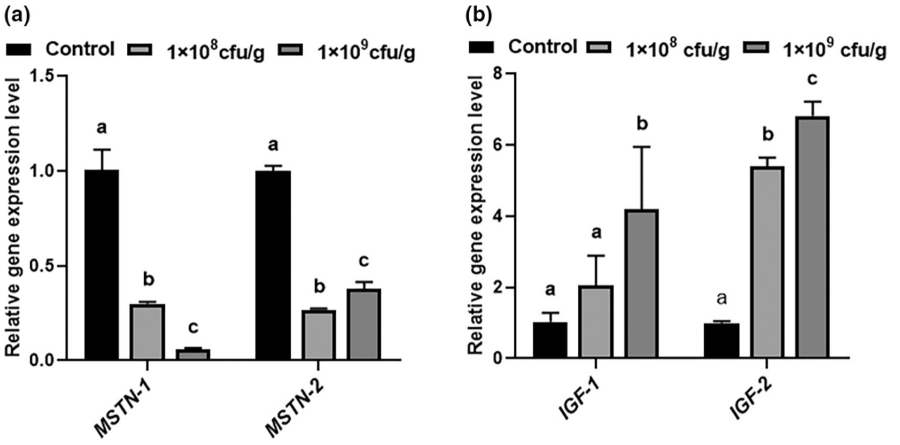

FIGURE 4 The effect of *S. lateritius* Z1-26 on expression levels of muscle growth-related genes of crucian carp. (a) Myostatin (*MSTN-1* and *MSTN-2*). (b) Insulin-like growth factor (*IGF-1* and *IGF-2*). Data represented mean ± SEM. Bars with different superscripts are significantly different ($p < .05$).

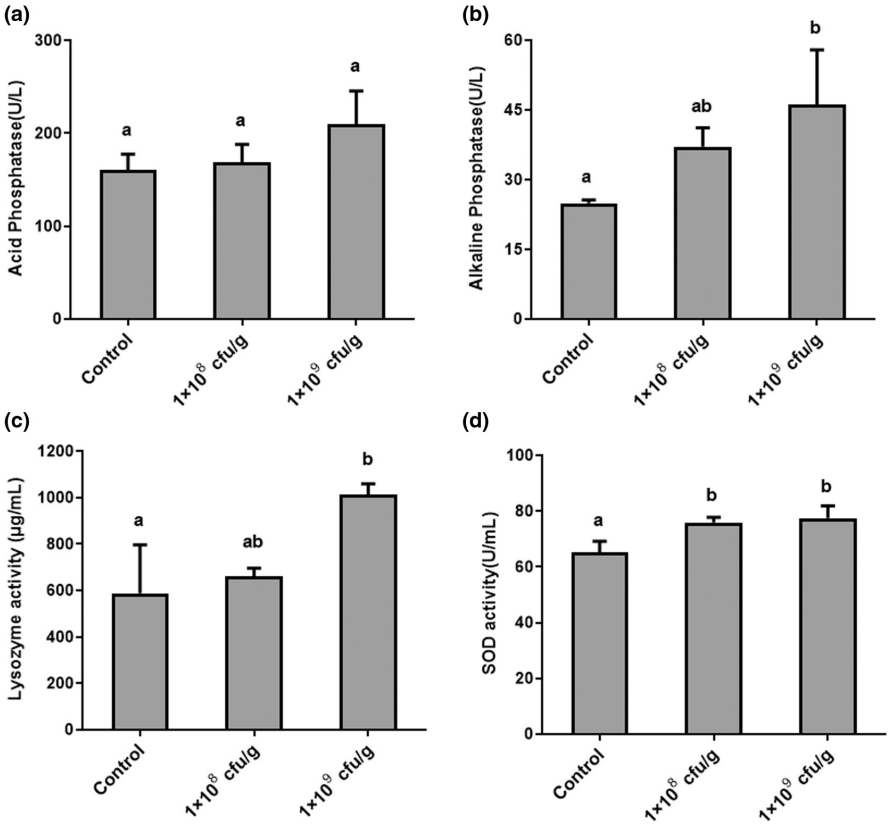

FIGURE 5 The effect of *S. lateritius* Z1-26 on non-specific immune indicators in serum of crucian carp. (a) Acid phosphatase activity. (b) Alkaline phosphatase activity. (c) Lysozyme enzyme activity. (d) Superoxide dismutase enzyme activity. Data represented mean ± SEM. Bars with different superscripts are significantly different ($p < .05$).

E2-related factor (*Nrf2*), whose activity is regulated by Kelchlike ECH-related protein 1 (*Keap1*), is a key transcription factor that regulates the expression of antioxidant enzyme genes (Hu et al., 2020). Here, we found that dietary strain Z1-26 in feed significantly up-regulated the expression of *Keap1* in the liver, kidney, and spleen of crucian carp ($p < .05$) and significantly up-regulated the expression of *Nrf2* in the liver of crucian carp ($p < .05$). Our results are consistent with a previous report by Yang et al., in which the authors observed a significant increase in the expression levels of *Keap1* and *Nrf2* in Pengze crucian carp supplemented with *Bacillus cereus* compared with those of fish fed a basal diet (Yang et al., 2020). We speculate that strain Z1-26 enhances the antioxidant capacity of crucian carp by regulating the Keap1/Nrf2 signalling pathway, thereby reducing the oxidative stress caused by unfavourable environmental conditions and pathogenic bacteria.

Acid phosphatase (ACP) and alkaline phosphatase (AKP) are lysosomal marker enzymes that are important humoral immune factors in fish. These markers are usually used as the main indicators to evaluate the strength of the non-specific immunity of fish, and a decrease in their activity will cause a decrease in non-specific immunity (Liu et al., 2020). In our study, strain Z1-26 increased the ACP and AKP activity in crucian carp in a dose-dependent manner. Lysozyme (LZM) catalyses the hydrolysis of the bacterial cell wall, leading to bacterial lysis and death (Zhang et al., 2020). Superoxide dismutase (SOD) protects cells from potential oxidative damage caused by pathogen infection by reducing destructive oxidative

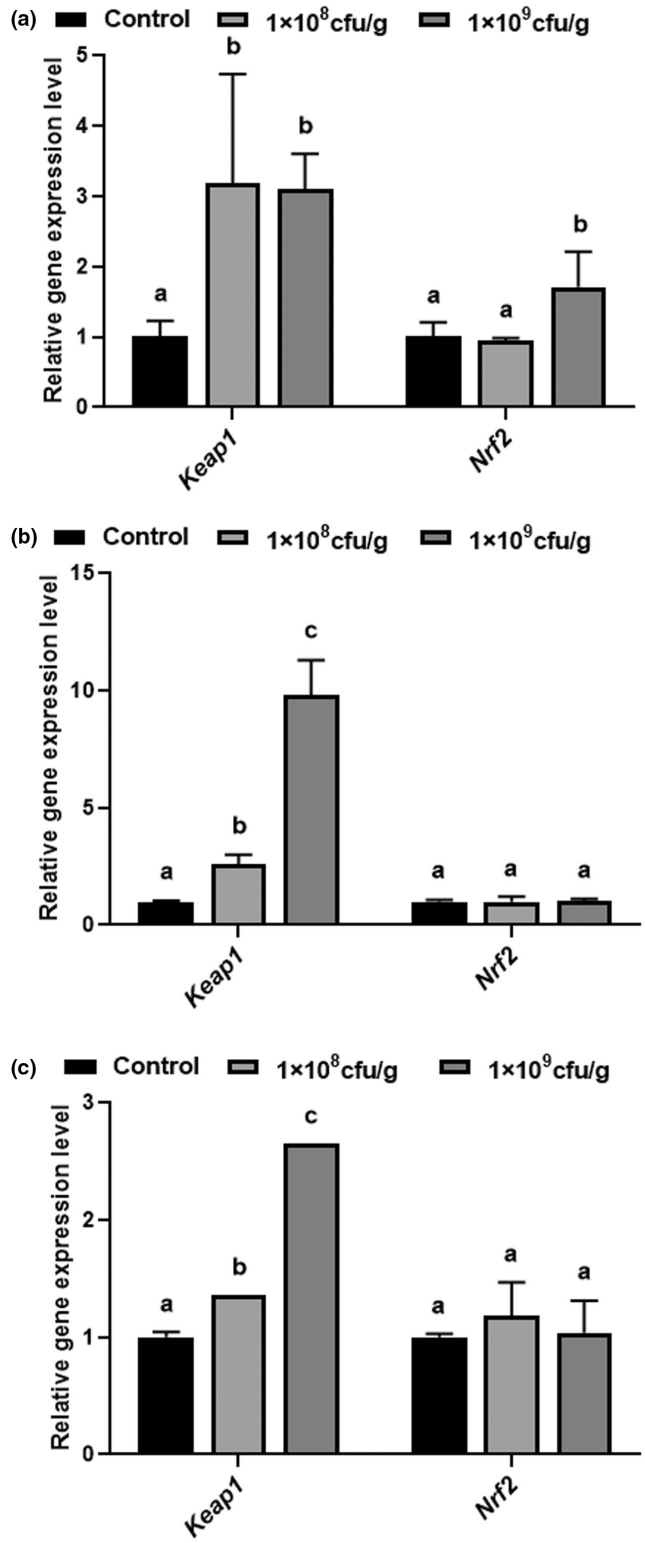

FIGURE 6 The effect of *S. lateritius* Z1-26 on expression levels of antioxidant-related genes in tissues of crucian carp. (a) Liver. (b) Kidney. (c) Spleen. Data represented mean ± SEM. Bars with different superscripts are significantly different ($p < .05$).

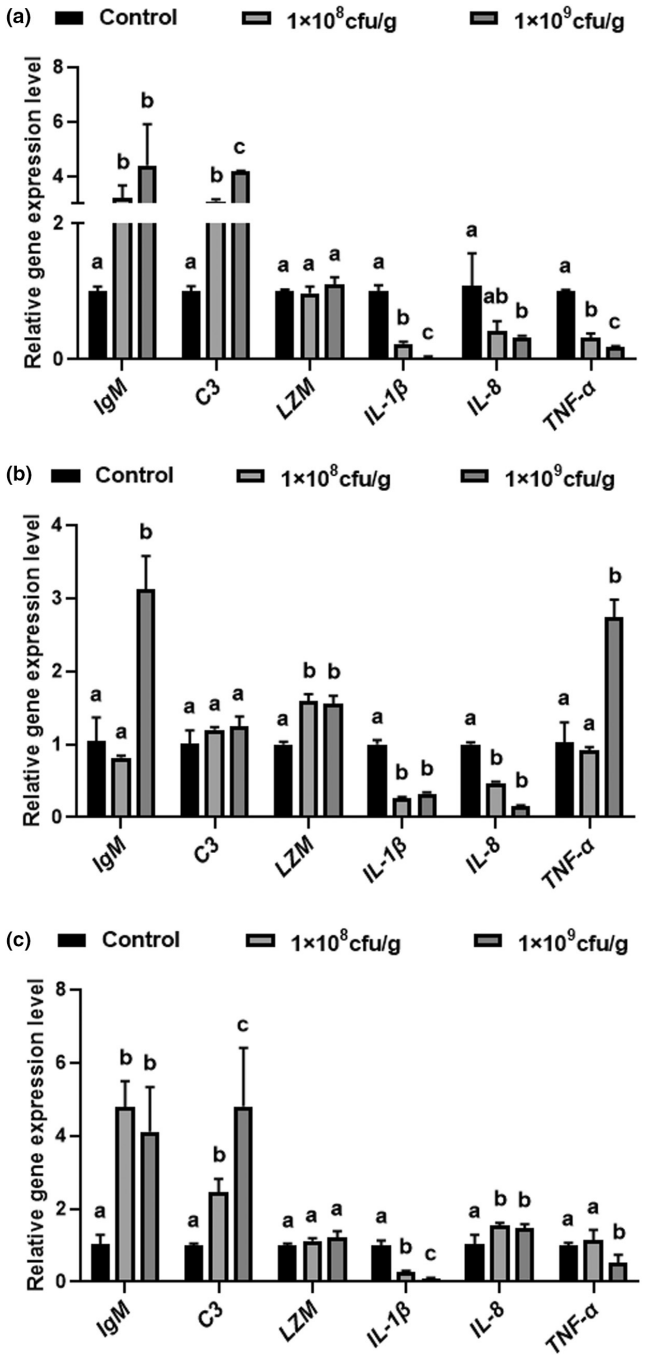

FIGURE 7 The effect of *S. lateritius* Z1-26 on expression levels of immune-related genes in tissues of crucian carp. (a) Liver. (b) Kidney. (c) Spleen. Data represented mean ± SEM. Bars with different superscripts are significantly different ($p < .05$).

stress (Ooi et al., 2020). In this study, LZM activity was significantly increased in the serum of crucian carp fed Z1-26 at a concentration of 10^9 cfu/g compared with that of crucian carp fed the control diet, and the SOD activity in the serum of crucian carp in the Z1-26 feeding groups was also significantly higher than that of the control group. Immunoglobulin M (IgM), the major immunoglobulin of bony fishes, is widely distributed in various tissues and plays an important role in the humoral immune response (Leya et al., 2021). In this study, it was found that the expression of immunoglobulin M (*IgM*) in the liver, kidney, and spleen of crucian carp increased significantly after feeding with strain Z1-26 ($p < .05$). Complement is a

component of the innate immune system, which protects the host from foreign pathogens. Complement component 3 (C3), which plays a key role in the activation of the complement pathway, is the core of the complement system (Chen, Zhao, et al., 2019). In our study, the expression level of complement component 3 (*C3*) in the liver and spleen and the expression level of *LZM* in the kidney of crucian carp were significantly up-regulated after feeding with strain Z1-26 ($p < .05$), which was consistent with the serum immunoassay results. Previous research had reported that *Lactobacillus delbrueckii* could significantly down-regulate the expression of *IL-1β*, *IL-8*, and *TNF-α* in *Cyprinus carpio* (Zhang et al., 2017). This study showed similar results in crucian carp. After feeding with diets containing strain Z1-26, the expression of *IL-1β* in the liver, kidney, and spleen significantly decreased, the expression of *IL-8* in the liver and kidney significantly decreased, and the expression of *TNF-α* in the liver and spleen also significantly decreased, indicating that strain Z1-26 could down-regulate the expression of pro-inflammatory cytokine in crucian carp. We speculated that strain Z1-26 might have anti-inflammatory properties. In summary, strain Z1-26 could enhance the immunity of crucian carp by regulating the expression of factors related to the innate and specific immune responses.

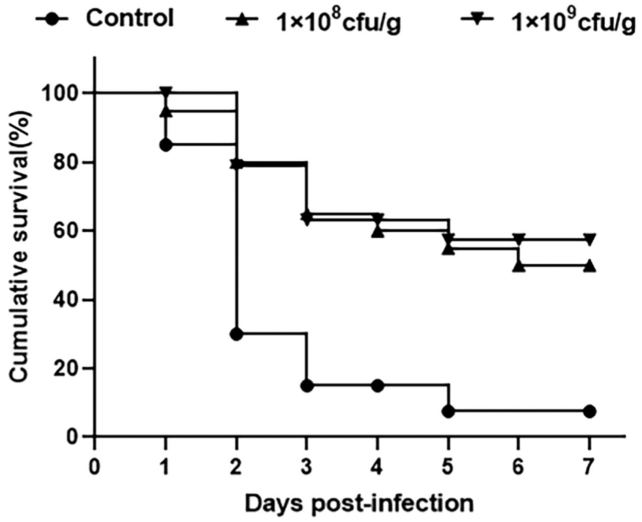

FIGURE 8 The cumulative survival of crucian carp fed with *S. lateritius* Z1-26-supplemented diet after challenged with *A. hydrophila* for 7 days.

Genome sequencing revealed that most of the genes of strain Z1-26 were associated with carbon and amino acid metabolism, indicating that metabolism in strain Z1-26 was relatively active, which might be related to its potential to synthesize diverse secondary metabolites. With this background, the secondary metabolite gene clusters in the genome of strain Z1-26 were predicted using Anti-SMASH. A total of 28 gene clusters involved in the synthesis of secondary metabolites were identified in the genome of strain Z1-26, including PKS, NRPS, terpene, bacteriocin, lanthipeptide, and siderophore. Among them, gene cluster 1.17, gene cluster 1.19, gene cluster 1.22, and gene cluster 1.24 showed high similarity to the biosynthetic gene clusters of desferrioxamin B, carotenoid, bacilysin, and ectoine (100%, 63%, 57%, and 100%, respectively) (Wu et al., 2015). Desferrioxamin B, an iron chelator, can inhibit the growth of pathogenic bacteria by reducing the bioavailability of iron required by pathogenic bacteria in the environment (Tiwari et al., 2019). Carotenoids and their derivatives not only have antioxidant effects but can also be used as a source of vitamin A to enhance immunity in humans and animals

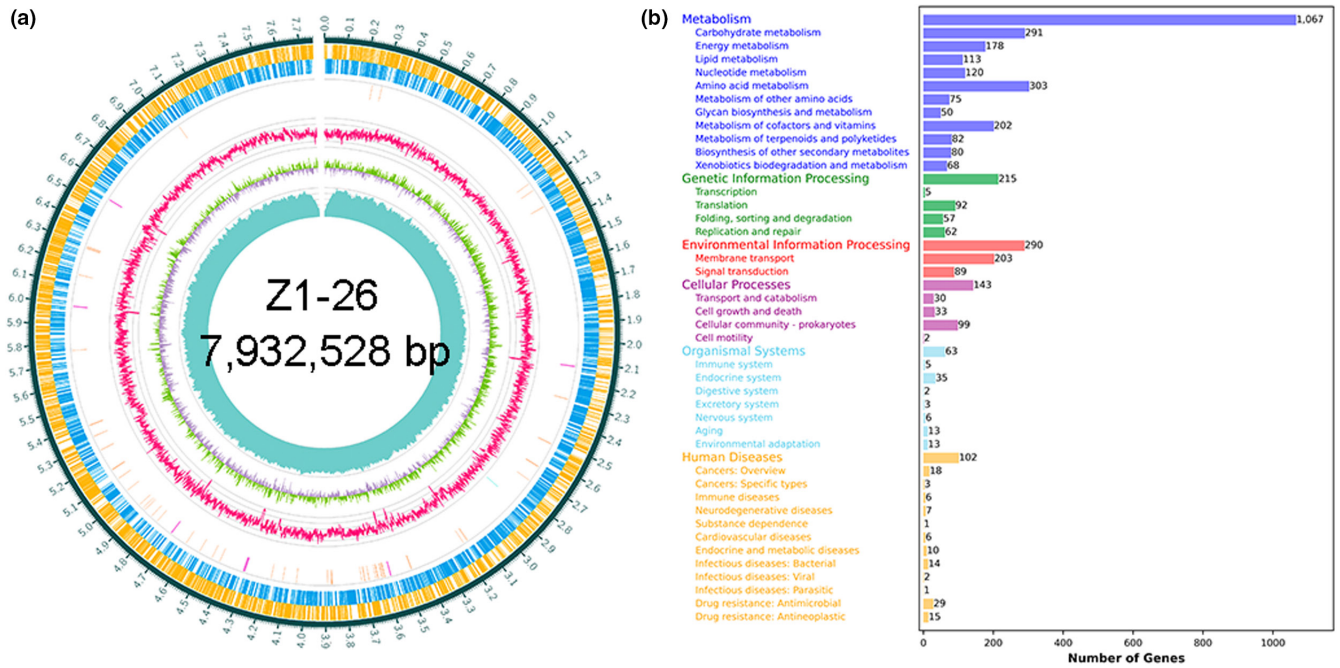

FIGURE 9 The analysis of the whole genome of *S. lateritius* Z1-26. (a) Circular representation of the genome. (b) Statistics of functional classification of Kyoto Encyclopedia of Genes and Genomes (KEGG) protein.

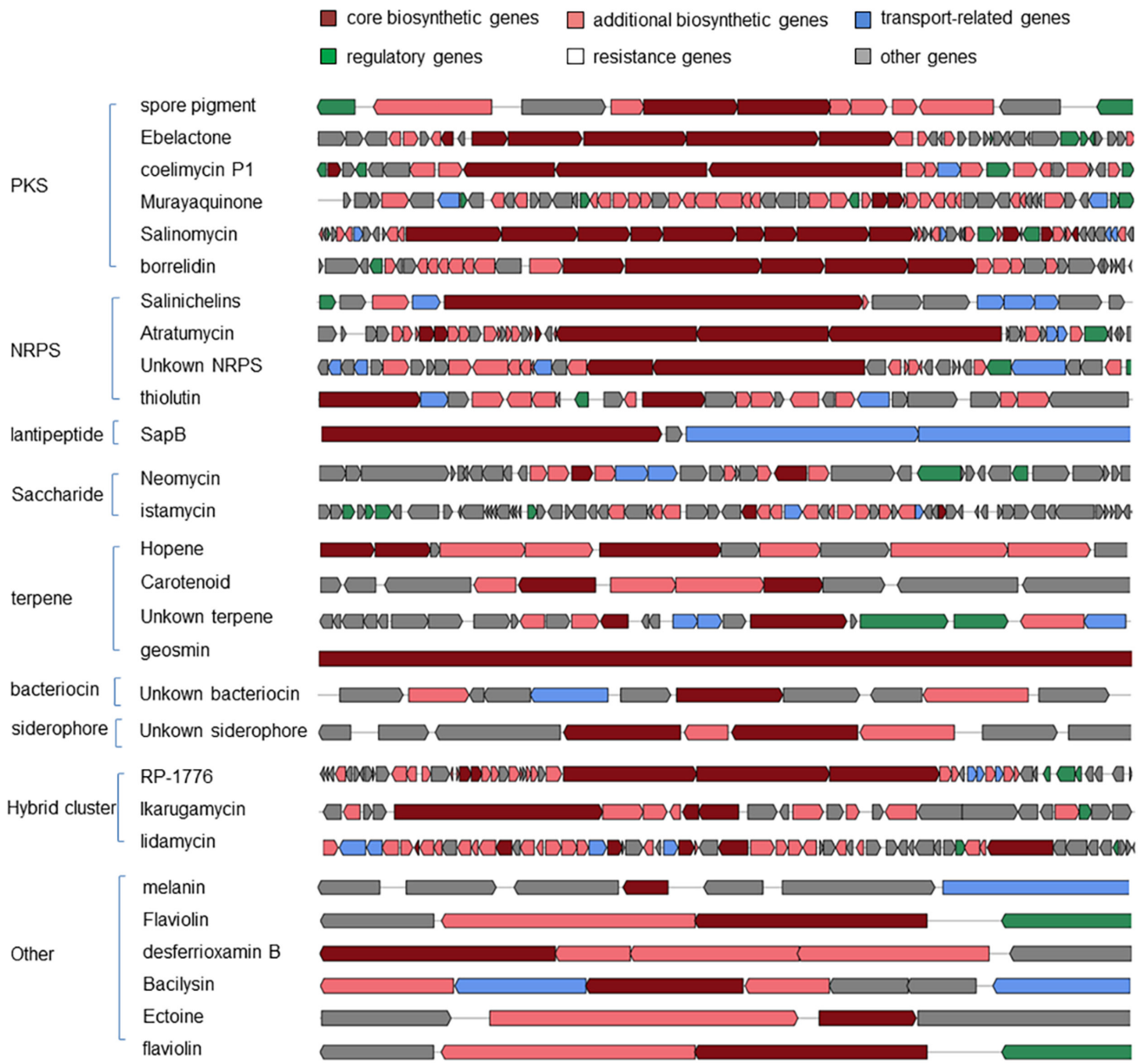

FIGURE 10 Gene clusters of secondary metabolites in the genome of *S. lateritius* Z1-26.

(Nabi et al., 2020). Bacilysin has good antibacterial activity against pathogenic bacteria (Nannan et al., 2021). Ectoine is an electrolyte that has protective effects on its producer under extreme conditions such as high osmotic pressure, low temperature, high temperature and dryness (Szabelak et al., 2019). These potential secondary metabolites might be responsible for the colonization of strain Z1-26 in crucian carp and the improvement in growth ability and disease resistance of crucian carp.

In summary, the application of *S. lateritius* as a probiotic for disease control in fish farming was reported for the first time. The in vitro antibacterial test showed that strain Z1-26 has broad-spectrum antibacterial activity against a variety of fish pathogens bacteria. Strain Z1-26 could survive and colonize in crucian carp fed with strain Z1-26 diets. Strain Z1-26 could enhance the growth performance and immune response of crucian carp, and also could improve the survival rate of crucian carp after *A. hydrophila* challenge. In addition, multiple antibacterial and probiotic gene clusters were identified in the genome of strain Z1-26. This research provides an important theoretical basis for the further development of strain Z1-26 as a probiotic strain with good application prospects in aquaculture.

AUTHOR CONTRIBUTIONS

Yahui Yang and Duo Jin designed the experiments. Yahui Yang, Duo Jin, Wensu Long and Ximiao Lai contributed to perform the experiments. Yahui Yang, Duo Jin, Yibo Hu, Pan Wang, Yunjun Sun and Xixun Zhou analysed the data. Yahui Yang and Duo Jin wrote a draft of the manuscript. Liqiu Xia, Yibo Hu and Ganfeng Yi supervised the research.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The work described has not been published previously.

DATA AVAILABILITY STATEMENT

This manuscript contains previously unpublished data. The name of the repository and accession number are not available.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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