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RESEARCH ARTICLE

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Screening of new *Paenibacillus polymyxa* S3 and its disease resistance of grass carp (*Ctenopharyngodon idellus*)

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Abstract

A new strain of *Paenibacillus polymyxa* S3 with antagonistic effects on 11 major fish pathogens (especially *Aeromonas hydrophila*), but had no toxicity to grass carp, was screened from the sediment of fishponds. In vivo colonization studies showed that strain S3 could be colonized and distributed in the gill and abdomen of the grass carp. Bioassay results showed that the weight growth rate of grass carp in the strain S3 oral group (16.01%) and strain S3 immersion group (16.44%) was significantly higher than those of the control group (8.61%). At the same time, the activities of ACP, AKP, CAT and GSH-Px in the serum of grass carp in oral and immersion groups were significantly higher than those of the control group. In addition, the treatment with strain S3 could significantly upregulate the expression of the antioxidant-related genes and immune-related genes *Keap1*, *Nrf2*, *C3*, *LZM*, *IgM*, *TLR-4* and *MyD-88* in grass carp tissues. The challenge test showed that strain S3 treatment significantly increased the survival rate of grass carp infected with *Aeromonas hydrophila*. Whole genome sequencing analysis showed that strain S3 had 16 active metabolite gene clusters, indicating that it had abundant gene resources, which provided important support for its development for fish microecological preparations. In summary, a new strain of *Paenibacillus polymyxa* S3 with antibacterial activity against a variety of fish pathogens was screened in this study and its probiotic function was evaluated, proving its potential value in fisheries.

KEYWORDS
Aeromonas hydrophila, disease resistance, genome, grass carp, *Paenibacillus polymyxa*, probiotic

1 | INTRODUCTION

In recent years, with the development of aquaculture, the mode of high-density and intensive culture has been widely used. At the same time, the risk of fish disease has increased. Studies have shown that viruses, bacteria, fungi and parasites are the main causes of fish diseases, with bacterial diseases accounting for the highest proportion and causing the greatest losses (Garrido-Olvera et al., 2022; Kibenge, 2019; Scholz et al., 2021; Zhang, Han, et al., 2021; Zhang, Xiong, et al., 2021). *Aeromonas hydrophila* is one of the pathogenic bacteria in fish, which can not only cause septicaemia in fish, but also pose a threat to human health. In a study by Saleh et al. (2021), they identified 53.4% of *Aeromonas hydrophila* from 500 Nile tilapia, most of which were pathogenic and multidrug-resistant. Other reported fish pathogens include Vibrio, Streptococcus (Luo et al., 2017), Yersinia (Fernández et al., 2007) and Flavobacterium columnar (LaFrentz et al., 2022). The prevalence of pathogenic bacteria has brought serious challenges to the fish farming industry.

Shijia Yang and Duo Jin contributed equally to this work.

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Antibiotics are often used as antibacterial drugs in aquaculture. Studies have shown that the most frequently used antibiotics are oxolinic acid, flumequine, oxytetracycline and florfenicol in aquaculture (Scarano et al., 2018). These drugs can inhibit a variety of gram-negative pathogens, which is conducive to healthy fish farming. However, the unscientific use of antibiotics brings a series of problems. For example, the emergence of multidrug-resistant bacteria (Algammal et al., 2020) and chronic toxicity to fish (Yang et al., 2020). Therefore, the use of antibiotics to treat fish diseases is no longer the best option. Chinese herbal medicines (Abarike et al., 2019) and vaccines (Wang et al., 2018) are also effective means against pathogenic microbial infections, but they are not suitable for large-scale aquaculture due to their high cost (Adams, 2019).

In recent years, the development of fish probiotics has attracted the interest of many researchers, because these probiotics can not only inhibit the growth of fish pathogenic bacteria, but also without pollution to the environment. Zhang, Han, et al. (2021); Zhang, Xiong, et al. (2021) showed that *Bacillus velezensis* WLYS23 screened in the gut of hybrid snakehead has antagonism to 20 species of fish pathogens such as *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas aquariorum*, *Aeromonas shigelloides* and *Nocardia seriolae*. *Streptomyces amritsarensis* N1-32 screened from soil by Li and Chen (2019) has an antagonistic effect on 10 fish pathogens such as *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas caviae*. Alshammari et al. (2019) screened the *Enterococcus durans* F3 from the intestinal tract of *Catla catla*, which showed good antibacterial activity against *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa*. These bacteria can be further developed to prevent and treat bacterial diseases in freshwater fish farming. Fish probiotics have been studied by many scholars, and some important results have been achieved. However, the natural microbial resources are very rich, and new types of fish probiotics need to be discovered and developed.

In this study, a new probiotic strain S3 with good antibacterial activity was successfully screened from fishpond sediment, and it was identified as *Paenibacillus polymyxa*. To determine whether strain S3 has the basic conditions as a probiotic, its safety, in vitro antagonistic effects, and in vivo protective effects were evaluated, all of which achieved ideal results. Finally, the secondary metabolite gene cluster of strain S3 was excavated by whole genome sequencing technology, and it was found that it had the potential to produce a variety of active substances. Strain S3 enriched microbial strain resources and had potential application value as an antagonistic probiotic in fish.

2 | MATERIALS AND METHODS

2.1 | Primers and plasmids

The primers and plasmids used in this study can be found in Table S1 and Figure S1.

2.2 | Isolation and identification of probiotics

Sediment samples from fishponds were diluted with sterile water, and 100 μl of sediment sample diluent was coated on LB Agar (Yeast extract 5 g/L, Tryptone 10 g/L, NaCl 10 g/L, agar 20 g/L) for 24 h. Single colonies were picked and purified twice, then inoculated in CSM Broth (TSB 45 g/L, Glucose 10 g/L, Yeast extract 9 g/L, $MgSO_4·7H_2O$ 2.2 g/L) and incubated at 30°C.

The 11 fish pathogens (Table S2) were cultured in LB liquid medium and diluted to 1×10^7 cfu/ml and then coated on LB plate. Subsequently, 50 μl of the cell-free fermentation broth of strain S3 was added into the hole of plates for 12 h. The strains with inhibition zone were stored in an ultralow temperature refrigerator at −80°C.

The strains with the largest inhibition zone (Three biological replicates) were selected for 16S rRNA sequencing analysis, and their phylogenetic trees were constructed for strain identification.

2.3 | Physiological and biochemical characteristics analysis of strain S3

Glucose, maltose, xylose, sucrose, sorbitol, fructose, xylitol, mannose, lactose, rhamnose, arabinose, hydrolysed starch, *N*-acetylglucosamine and hydrogen sulphide were measured using non-bacterial physiological and biochemical tubes (Hangzhou Microbial Reagent Co., Ltd.). Pick a single clone of strain S3 in the identification tube, and incubate at a constant temperature of 30°C for 48 h. If the culture solution in the identification tube turns yellow, it is positive, and if it does not change colour, it is negative. Pick a single clone of strain S3 in the EP tube, and incubate at a constant temperature of 30°C for 24 h. Then, add 3% H_2O_2 to the EP tube, if bubbles are produced, the contact enzyme is positive, and no air bubbles are negative. Prepare LB broth of 1% NaCl, 2% NaCl, 5% NaCl and 10% NaCl, inoculate a single clone of strain S3 into the above broth, cultivate at 30°C for 24 h, and observe whether the broth becomes turbid.

2.4 | Whole genome sequencing and analysis of strain S3

The cells of strain S3 in logarithmic growth phase were collected, washed with sterile water for three times and frozen in liquid nitrogen. Strain S3 was sent to Wuhan Grandomics for genome sequencing analysis. The genome of strain S3 was annotated for genome function in Clusters of Orthologous Groups of proteins database (COG; http://www.ncbi.nlm.nih.gov/COG/) and Kyoto Encyclopedia of Genes and Genomes database (KEGG; http://www.genome.jp/kegg/). The genome collinearity analysis and homology comparison were predicted using the RAST version 2.0 (https://rast.nmpdr.org/). The secondary metabolite and active substance biosynthetic gene clusters of the genome were analysed with AntiSMASH (https://antismash.secondarymetabolites.org/) platform.

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2.5 | Cytotoxicity experiment of strain S3

The liver cells L8824 of grass carp were spread in a 96-well plate. After cell adherence, 10 μl cell-free fermentation supernatant of strain S3, 10 μl cell-free culture supernatant of *Aeromonas hydrophila* Ah X040 and 10 μl CSM medium were added, respectively. Each operation was repeated for six wells, and the cell was observed every 12 h.

2.6 | Coculture experiment of strain S3 and *Aeromonas hydrophila* AhX040

Preparation of simulated aquaculture water: 0.5 g diet was added per 1 L ddH_2O and sterilized at 121°C for 20 min.

Preparation of bacterial suspension: The bacteria of strain S3 and Ah X040 in the logarithmic growth phase were collected. The bacteria were washed with PBS for three times.

The following concentrations of S3 and Ah X040 were added at 1% inoculation volume ratio. Group A: 1×10^6 cfu/ml Ah X040; Group B: 1×10^6 cfu/ml Ah X040 and 1×10^5 cfu/ml strain S3; Group C: 1×10^6 cfu/ml Ah X040 and 1×10^6 cfu/ml strain S3; Group D: 1×10^6 cfu/ml Ah X040 and 1×10^7 cfu/ml strain S3. Determination of *Aeromonas hydrophila* concentrations in each group every 2 h.

2.7 | Culture and domestication of experimental fish

The body weight of healthy juvenile grass carp used in the experiment was 30 ± 5 g. Each 20 fish was cultured in 50 L water, and 1/3 of water was replaced every 3 days. Room temperature was controlled at 25 ± 2°C. Before the experiment was officially started, the experimental fish were starved for 1 week to adapt to the change in environment. Experimental fish fed a 1% body weight diet twice a day. All fish were killed after the experiment.

2.8 | Colonization analysis of strain S3 on grass carp

The plasmid pMUTIN4-GFP was electrotransformed into strain S3 using the method of Cao et al. (2019) as follows. The pMUTIN4-GFP plasmid from *E. coli* DH5α was isolated using the SanPrep Column Plasmid Mini-Preps Kit (Sangon Biotech). Strain S3 was grown in CSM to an optical density at 600 nm (OD_{600} nm) of 1.0 and sedimented by centrifugation at 6010 rcf/min for 10 min at 4°C and the cells subjected to two cycles of resuspension in electroporation buffer (0.5 M sucrose plus 10% glycerol). Then, 5 μl of pMUTIN4-GFP plasmid was added to 100 μl of the bacterial suspension and was electroporated at 2.5 kV in a 0.2-cm cuvette using an Eporator (Eppendorf), obtaining a time constant of 2.5–5 ms. Transformants were selected in CSM-agar supplemented with Amp at 100 μg/ml. The obtained recombinant strain was designated $S3^{GFP}$.

A total of 80 domesticated experimental fish were divided into two groups with 40 fish in each group. The control group was fed with normal diet. The experimental group: fed 1×10^8 cfu/g $S3^{GFP}$ mixed diet. During the experiment, the distribution of $S3^{GFP}$ in grass carp at 0, 4, 7, 10 and 13 days was detected in Calliper IVIS Spectrum system.

2.9 | Bioassay of strain S3 on grass carp

A total of 180 domesticated experimental fish were divided into three groups with 60 fish in each group, three replicates per group. The control group was fed with normal diet. The experimental fish in the oral group were fed with 1×10^8 cfu/g strain S3 mixed diet, and the experimental fish in the immersion group were immersed in strain S3 with a concentration of 1×10^5 cfu/ml for 30 days. The detailed processing procedures are provided in the Supplementary Materials and Methods.

After 30 days of feeding, the fish were starved for 24 h. Ten fish in each group were randomly selected and weighed, and the weight gain rate (WGR), specified growth rate (SGR) and survival rate (SR) of grass carp were calculated. WGR (%) = $100 \times [(W_t - W_0)/W_0]$; SGR (%) = $100 \times (\ln W_t - \ln W_0)/30$ days; SR (%) = number of fish at the end of the test/number of initial fish at the test × 100.

The serum, liver, kidney, spleen and intestine of four experimental fish were taken from each group, that is, four biological replicates. The enzyme activities of ACP (acid phosphatase), AKP (alkaline phosphatase), GSH-Px (glutathione peroxidase) and CAT (catalase) in the serum of experimental fish were determined, and the mRNA expression levels of antioxidant-related genes *Keap1* and *Nrf2* and immune-related genes *C3* (Alexin), *LZM* (Fish lysozyme), *IgM* (Immunoglobulin M), *TLR-4* and *MyD-88* in liver, kidney, spleen and intestine were determined by quantitative real-time PCR.

Forty grass carps in each group were injected with 200 μl 1×10^6 cfu/ml *Aeromonas hydrophila* Ah X040, and the survival rate of the experimental fish was recorded within 15 days.

2.10 | Analysis of the data

All statistical analyses were performed using IBM SPSS Statistics 21. One-way ANOVA was used to compare differences among the treatments. *, ** and *** indicate $p < .05$, $p < .01$ and $p < .001$, respectively. All data are expressed as the mean ± SD.

3 | RESULTS

3.1 | Isolation and identification of strain S3

Strain S3 with broad-spectrum antimicrobial activity was successfully screened from the sediment of fishponds. Strain S3 has inhibitory effects on 11 major fish pathogens such as *Aeromonas*

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hydrophila and *Aeromonas salmonicida* (Figure 1, Table S2). The colony of strain S3 is large, moist, white and opaque, and its microstructure is long and rod-shaped (Figure 2a). The results of 16s rRNA gene blast comparison and phylogenetic tree show that strain S3 has the highest homology with *Paenibacillus polymyxa* (Figure 2b). The physiological and biochemical characteristics of strain S3 are also consistent with those of *Paenibacillus polymyxa* (Table S3). However, compared with the 16s rRNA genes of other *Paenibacillus polymyxa* that have been reported, the strain S3 has at least six base differences, indicating that this strain is a new strain of *Paenibacillus polymyxa*.

3.2 | Whole genome sequencing and comparative genome analysis of strain S3

The de novo genome assembly revealed that strain S3 genome size was 5,740,272 bp with GC content (45.6%), coding sequences (5086) and total RNAs (153) (CP102865) (Figure 3a). The sequence was searched against the COGs and KEGG databases to annotate the genome, and 3952 CDSs were annotated as functional proteins (approximately 77.70% of the total CDSs). For example, a total of 3865 CDSs were counted in the COG database. The top three categories are carbohydrate transport and metabolism, transcription and signal transduction mechanisms in order (Figure 3b). A total of 2471 CDSs were annotated in the KEGG database. The top three pathways are metabolism, environmental information processing and genetic information processing in order (Figure 3c). Meanwhile, the secondary metabolite biosynthetic gene cluster of strain S3 was predicted by antiSMASH platform. It was found that strain S3 contained gene clusters for the synthesis of active substances such as aurantinin B/C/D, nostamide A, polymyxin, fusaricidin B, paenibacillin, paeninodin, marthiapeptide A, paenilan and tridecaptin (Table 2). The results showed that strain S3 had an active metabolic system and strong biosynthetic potential, among which carbon metabolism was the most vigorous, and they were regulated by complex and precise molecular signal networks.

Strain S3 is phylogenetically coherent bacteria to Strain CF05 based on 16s rRNA sequence analysis. The availability of their whole genome sequences has enabled the knowledge about molecular bases of the genetic and biochemistry of these bacteria. The collinearity analysis showed that the two strains had some regions with poor collinearity (Figure 3d). Sequence homology analysis showed that there were 3215 genes in strain S3, and their sequences were different from those of strain CF05(Figure 3e). In addition, 486 genes were unique to strain S3. These results further confirmed that strain S3 is a new strain of *Paenibacillus polymyxa*.

3.3 | Cytotoxicity test of strain S3

The cell-free fermentation broth of strain S3, cell-free supernatant of *Aeromonas hydrophila* Ah X040 and CSM medium were incubated with L8824 cells (grass carp liver cells). The L8824 cells in the cell-free supernatant of strain Ah X040 were all lysed at 12 h, while the cells in the cell-free fermentation broth of strain S3 were in normal state. The cell density of L8824 cells treated with cell-free fermentation broth of strain S3 and CSM medium increased at 24 h compared with 12 h, indicating that the two groups of L8824 cells proliferated normally (Figure 4). It is concluded that the fermentation supernatant of strain S3 has no toxicity to liver cells of grass carp, indicating that strain S3 is a safe probiotic for fish.

3.4 | In vitro antagonistic effect of strain S3 on *Aeromonas hydrophila* Ah X040

Different concentrations of strain S3 and 1×10^6 cfu/ml Ah X040 were cocultured in simulated aquaculture water. In Group A without strain S3, the biomass of Ah X040 continued to increase throughout the experiment. In Group B with 1×10^5 cfu/ml strain S3, the biomass of Ah X040 decreased continuously after 12 h. In Group C with 1×10^6 cfu/ml strain S3, the biomass of Ah X040 began to decrease from 10 h, and the decline rate was greater than that of

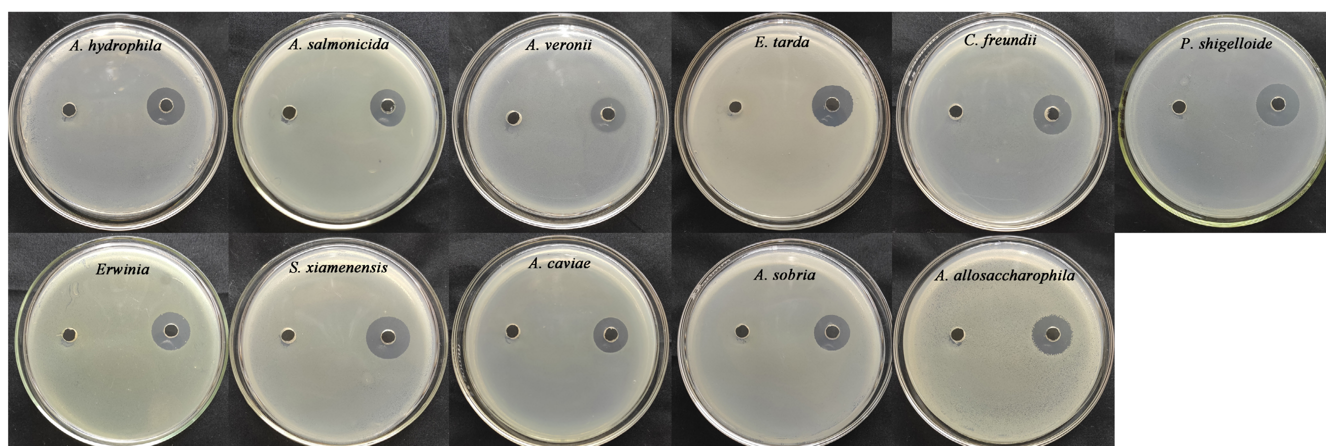

FIGURE 1 Antibacterial activity of fermentation supernatant of strain S3 on fish pathogens.

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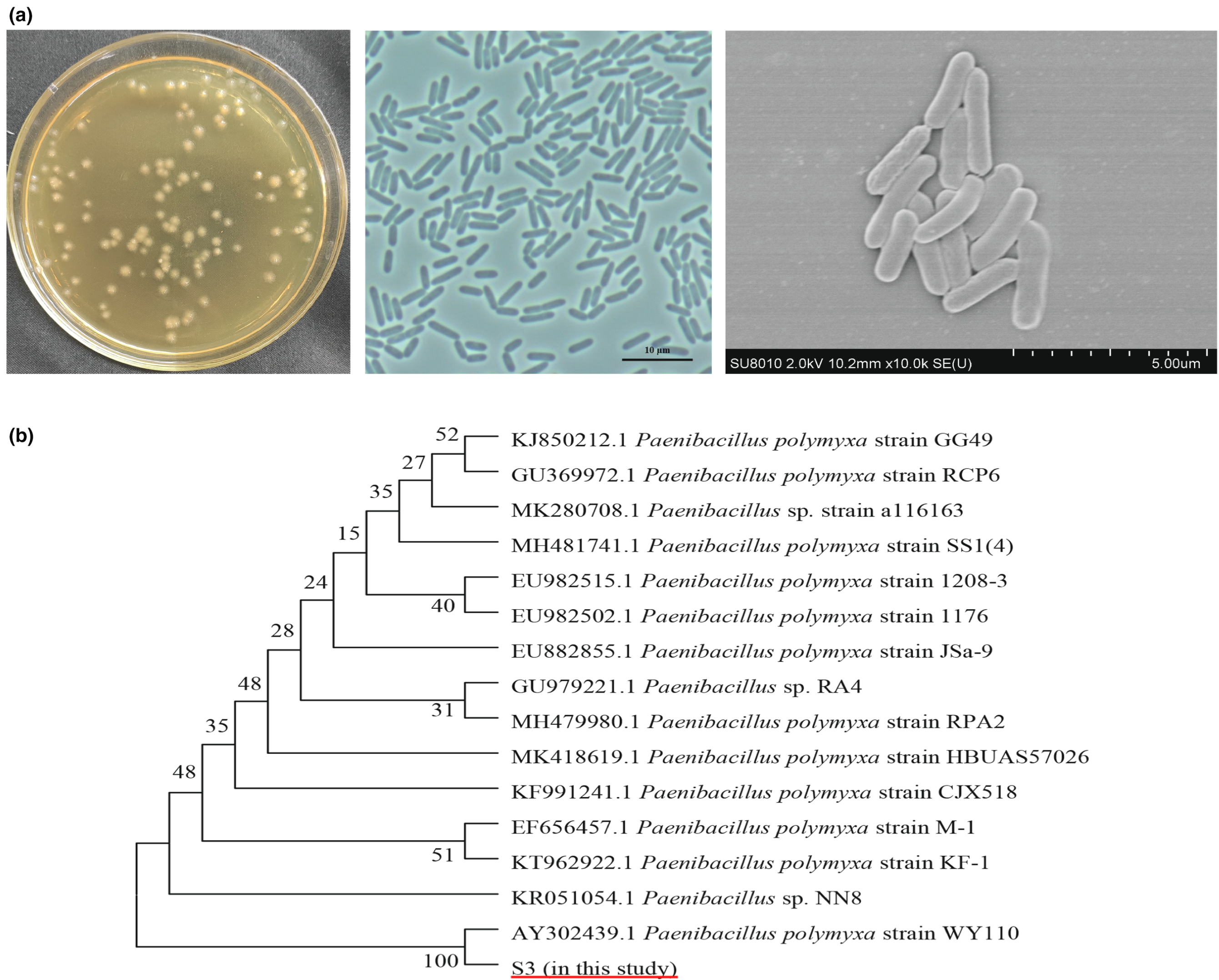

FIGURE 2 Morphological characteristics and identification of strain S3. (a) Colony morphology and microstructure of strain S3. (b) The phylogenetic tree of strain S3.

group B. At 18 h, the biomass of Ah X040 was 0. In Group D with 1×10^7 cfu/ml strain S3, the biomass of AhX040 began to decrease at 6 h and reached 0 at 12 h. With the increase in the concentration ratio of strain S3 to AhX040, the decline time of Ah X040 biomass gradually increased and the biomass gradually decreased (Figure S2). The experiment showed that the growth of Ah X040 was inhibited in the presence of strain S3 and that Ah X040 could be completely killed when the concentration of S3 was comparable to that of Ah X040. Therefore, strain S3 has the potential to inhibit Ah X040 in fishponds.

3.5 | Colonization distribution of strain S3 in grass carp

The strain $S3^{GFP}$ carrying pMUTIN4-GFP was successfully constructed (Figure 5a). The $S3^{GFP}$ mixed diet was fed to grass carp to observe the dynamic distribution of strain $S3^{GFP}$ in grass carp. On the 4th day, the gill of grass carp showed obvious fluorescence, and the abdomen showed weak fluorescence. Then, with the extension of time, the fluorescence signal gradually increased in the gill and abdomen of grass carp (Figure 5b). This experiment showed that strain S3 could colonized the gills and abdomens of grass carp, which provided a basis for the probiotic function of strain S3.

3.6 | Effect of strain S3 treatment on the growth of grass carp

The results of the 30-day bioassay showed that the WGR and SGR of grass carp in the control group were 8.61% and 0.28%, respectively. The WGR and SGR of grass carp in the oral group were significantly higher than those of the control group, which were 16.01% and 0.50%, respectively. The WGR and SGR of grass carp in the immersion group were significantly higher than those in the control group, which were 16.44% and 0.51%, respectively (Table 1). This

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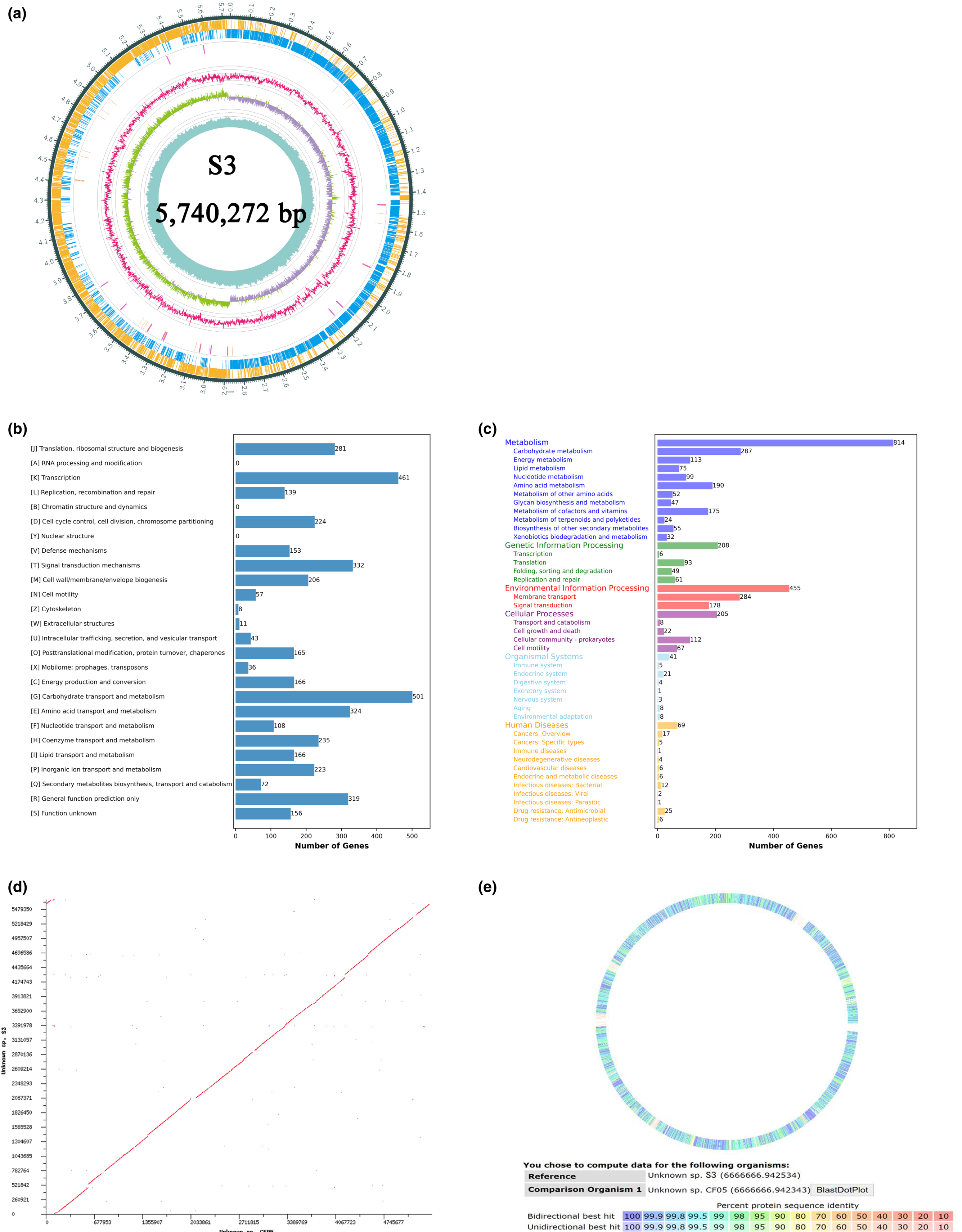

FIGURE 3 Whole genome sequencing analysis of strain S3. (a) Nuclear genome map of strain S3. From outside to inside are coding genes (sense chain), coding genes (negative sense chain), tRNA (orange) and rRNA (purple), CRISPR (blue) and gene islands (green), GC ratio, GC-skew, sequencing depth; (b) Statistics of COG functional classification of the encoded protein of strain S3; (c) Statistics of KEGG functional classification of the encoded protein of strain S3; (d) The collinearity analysis of strain S3 and strain CF05; (e) Sequence homology analysis of strain S3 and strain CF05.

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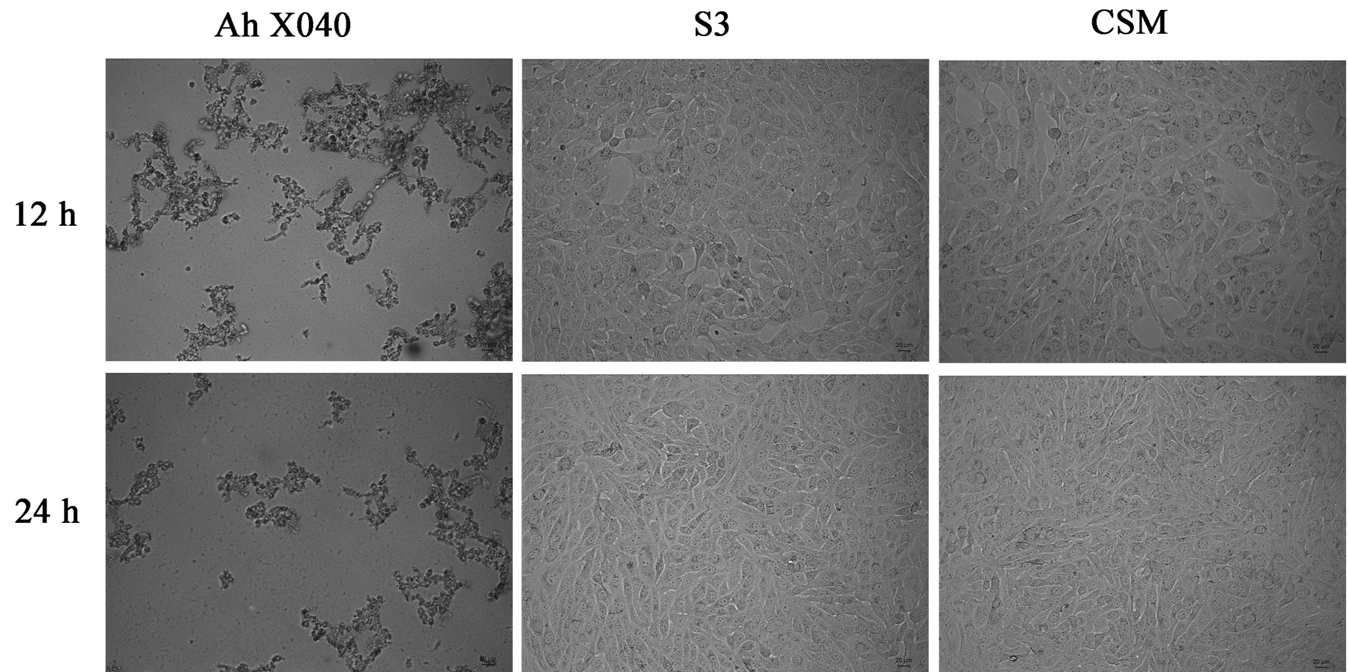

FIGURE 4 Effect of fermentation supernatant of strain S3 on L8824 cells of grass carp. CSM: Medium; Ah X040: Cell-free supernatant of Ah X040; S3: Cell-free supernatant of strain S3.

experiment showed that strain S3 promoted the growth of juvenile grass carp.

3.7 | Effect of strain S3 treatment on the activity of immune-related enzymes in grass carp serum

After 30 days of treatment, the serum of grass carp was collected to determine the activities of ACP, AKP, CAT and GSH-Px. Compared with the grass carp in the control group, the activities of the four enzymes in the grass carp of the oral group and the immersion group were significantly increased, especially for the CAT activity and the GSH-Px activity (Figure 6). This experiment showed that strain S3 could improve the activity of immune-related enzymes in grass carp serum, thereby improving the immunity of grass carp.

3.8 | Effect of strain S3 treatment on antioxidant- and immune-related genes in grass carp

After 30 days of treatment, the liver, kidney, spleen and intestine of grass carp from the three groups were obtained, and the expression levels of the antioxidant-related genes *Keap1* and *Nrf2* were detected by qRT-PCR (Figures 6a–d). Compared with the control group, there was a statistically significant difference in the expression of *Keap1* only in the liver and kidney ($p > .01$) (Figure 7a,b). Especially in the immersion group, the relative expression of *Keap1* in the liver of grass carp was 16.56, which was at least 15 times higher than that in the control group (Figure 7a). The expression of *Nrf2* was significantly different only in the intestine ($p > .01$), and it was increased by 7.35 times and 7.05 times in the oral group and the immersion group, respectively (Figure 7d). The above detection results indicated that the strain S3 could promote the expression of genes related to antioxidant factors in grass carp, promote the opening of antioxidant pathways and improve the antioxidant capacity of grass carp.

In addition, the liver, kidney, spleen and intestine of the three groups of experimental grass fish were also detected for the effects of immune-related genes in grass carp by qRT-PCR. The expression levels of *C3*, *LZM*, *IgM*, *TLR-4* and *MyD-88* in grass carp in the oral group and immersion group were compared with those in the control group, and the following results were obtained (Figures 7e–h). Compared with the control group, the expression of *C3* was only significantly different in the liver and spleen ($p > .001$). Especially in the liver of the immersion group and the spleen of the oral group, the relative expression levels were 15.95 and 35.14, respectively, which were 15.08 times and 37.90 times that of the control group (Figure 7e,g). The expression of *LZM* was only significantly different in the kidney ($p > .001$), and its expression in the kidney of the oral group and the immersion group was 4.45 and 3.71 times that of the control group, respectively (Figure 7f). There was a statistically significant difference in the expression of *IgM* mainly in the liver and kidney ($p > 0001$), especially in the kidney, the expression levels of the oral group and the immersion group were 8.92 and 7.37 times that of the control group, respectively (Figure 7e,f). In addition, in the spleen, there were also significant differences in the expression of *IgM* in the immersion group ($p > .001$) (Figure 7g). The expression of *TLR-4* only in the kidney of grass carp was significantly different ($p > .001$), and its expression in the oral group and the immersion group was 8.48 and 6.44 times that of the control group, respectively (Figure 7f). The expression level of *MyD-88* was only

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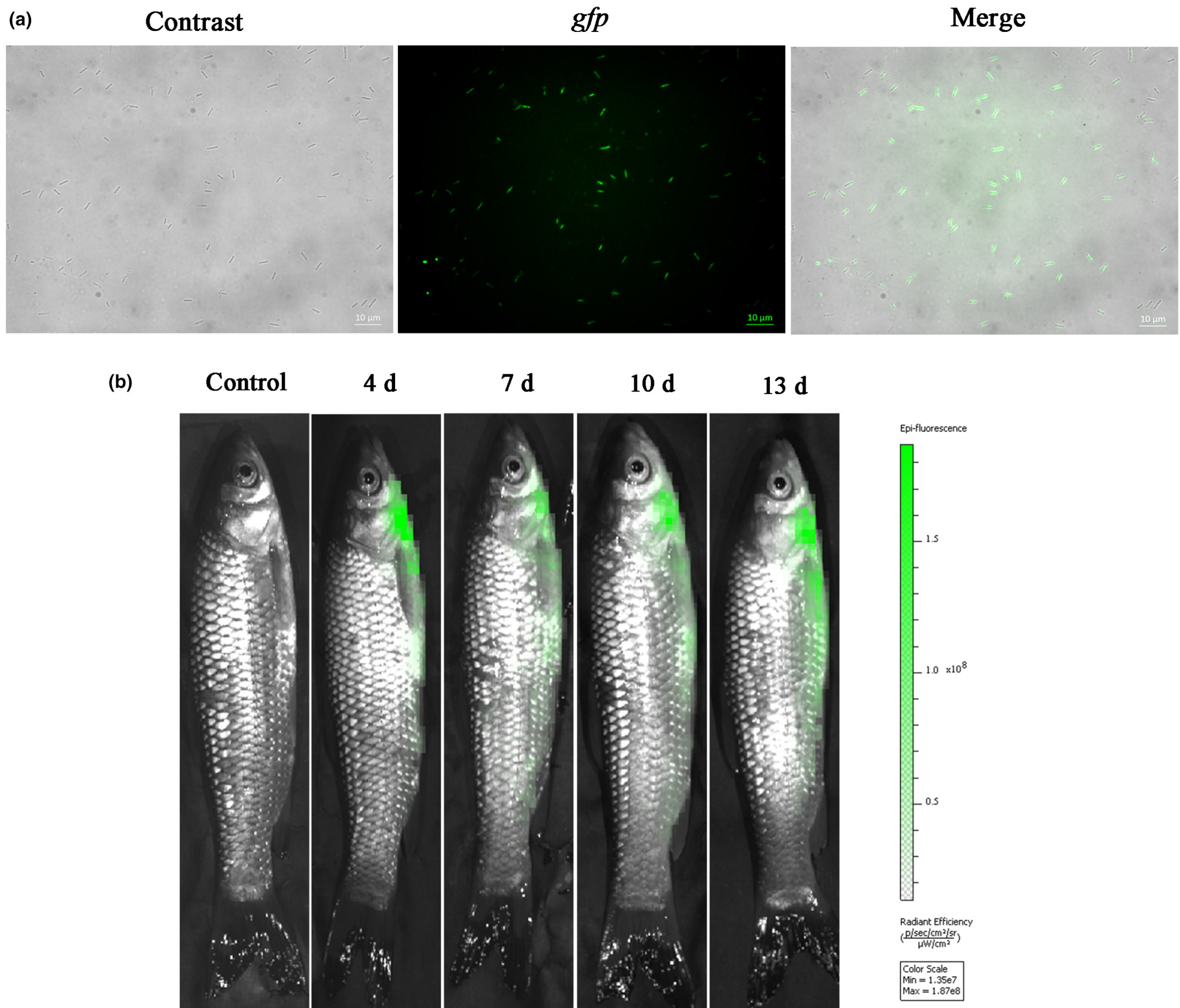

FIGURE 5 Fluorescence labelling of strain S3 and its distribution in grass carp. Strain $S3^{GFP}$ observed under the fluorescence microscope (b) Observation of colonization of strain $S3^{GFP}$ in grass carp using IVIS.

TABLE 1 Influence of strain S3 on growth performance of grass carp

	W_0 (g)	W_t (g)	WGR (%)	SGR (%)	SR (%)
Control	30.18 ± 3.31	32.78 ± 3.61	8.61 ± 0.41	0.28 ± 0.011	100
Oral	30.00 ± 2.03	34.80 ± 3.77	16.01 ± 0.69***	0.50 ± 0.033***	100
Immersion	30.54 ± 2.29	35.56 ± 4.07	16.44 ± 0.45***	0.51 ± 0.029***	100

Abbreviations: SGR, specified growth rate; SR, survival rate; WGR, weight gain rate.
***Indicated $p < .001$

significantly different in the kidney of the immersion group and the spleen of the oral group ($p > .001$), and its expression was 8.75 and 2.16 times higher than that of the control group, respectively. Overall, compared with the control group, the immune-related genes in liver, kidney and spleen of grass carp treated with strain S3 were upregulated, indicating that strain S3 can improve the immunity of grass carp.

3.9 | Protective effect of strain S3 treatment on grass carp

After 30 days of the experiment, 40 experimental grass carp were collected from each group. Each grass carp was injected with 200 µl of 1×10^6 cfu/ml Ah X040 for continuous observation for 15 days. The challenge test showed that all grass carp in the control group

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TABLE 2 Statistics of gene clusters of secondary metabolites in the genome of strain S3

Cluster	Type	Most similar known cluster	Similarity(%)
Cluster 1.1	Betalactone	Anabaenopeptin NZ857/ Nostamide A	100
Cluster 1.2	Cyclic-lactone-autoinducer	—	—
Cluster 1.3	transAT-PKS, NRPS, T3PKS, PKS-like	Aurantinin B/ Aurantinin C/ Aurantinin D	35
Cluster 1.4	Ranthipeptide	—	—
Cluster 1.5	NRPS	Polymyxin	100
Cluster 1.6	NRPS	Fusaricidin B	100
Cluster 1.7	Lanthipeptide-class-i, cyclic-lactone-autoinducer	Paenibacillin	100
Cluster 1.8	transAT-PKS, NRPS	—	—
Cluster 1.9	Proteusin	—	—
Cluster 1.10	NRPS, transAT-PKS	—	—
Cluster 1.11	Lassopeptide	Paeninodin	40
Cluster 1.12	Cyclic-lactone-autoinducer	—	—
Cluster 1.13	NRPS	Marthiapeptide A	41
Cluster 1.14	Lanthipeptide-class-i	Paenilan	100
Cluster 1.15	NRPS-like, cyclic-lactone-autoinducer	—	—
Cluster 1.16	NRPS	Tridecaptin	100

died within 10 days after infection with Ah X040, while the survival rate of grass carp in the oral group was 80% on the 10th day and 51% on the 15th day. The survival rate of grass carp in the immersion group was 85% on the 10th day and 58% on the 15th day. These results indicate that strain S3 can significantly improve the survival rate of susceptible grass carp (Figure 8).

4 | DISCUSSION

The use of probiotics can effectively prevent and control the spread of bacterial diseases in fish, and screening potential probiotics is the most important step in this strategy. Probiotics can be obtained from natural environments or from organs of fish. Gong et al. (2019) screened a strain of *Pediococcus pentosaceus* SL001 from soil and found that SL001 can make grass carp intestinal villi longer. Thankappan et al. (2015) screened three *Bacillus* strains with antibacterial activity against *Aeromonas hydrophila* from the intestine of *Labeo rohita* that can be used as probiotics in the aquaculture. Fish probiotics have been studied by many scholars, and some important results have been achieved. However, the natural microbial resources are very rich, and new types of fish probiotics need to be discovered and developed.

In this study, a strain S3 with antagonistic effects on 11 major fish pathogens (especially *Aeromonas hydrophila*), but had no toxicity to grass carp was screened from the sediment of fishponds. Morphological observation shows that the colony of the strain S3 is large, moist, white and opaque, and the bacterial structure is long rod-shaped. Through physiological and biochemical identification, it was found that strain S3 could ferment most sugars, was positive for contact enzyme reaction and could survive in an anaerobic environment. Lu et al. (2007) identified a new cellulase-producing strain of *Paenibacillus polymyxa* EJS-3, and the physiological and biochemical properties of strain S3 were consistent with it. By sequencing the 16S rRNA of strain S3 and constructing a phylogenetic tree, it was found that the homology of strain S3 with *Paenibacillus polymyxa* CF05 reached 99.59%. However, genome collinearity analysis showed that the two strains had some regions with poor collinearity. Sequence homology analysis showed that there were 3215 genes in strain S3, and their sequences were different from those of strain CF05. Moreover, 486 genes were unique to strain S3. Based on the above results, strain S3 was identified as a new strain of *Paenibacillus polymyxa*.

Aeromonas hydrophila is a pathogen causing zoonosis, which can cause cell necrosis and cause host disease. The growth curve of strain S3 and AhX040 showed that strain S3 could inhibit the growth of AhX040 in aquaculture water. Cytotoxicity test showed that strain S3 was safe to grass carp hepatocytes, indicating that strain S3 as a probiotic meets the basic requirements. The colonization of probiotics in host is the key to its probiotic function. (Wang et al., 2016) proved that *Bacillus coagulans* and *Lactobacillus plantarum* colonize the intestines of *zebrafish* to protect them from *Aeromonas hydrophila* infection. Fluorescence signals of strain S3 with green fluorescent plasmid appeared in gill and abdomen of grass carp, indicating that strain S3 could colonize grass carp in vivo. Whether oral or immersion with strain S3, the WGR and SGR

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of grass carp were significantly higher than those of the control group, indicating that strain S3 could promote the growth of grass carp (Hu et al., 2021), the results of challenge test showed that strain S3 significantly improved the survival rate of grass carp invaded by AhX040.

The activities of ACP, AKP, CAT and GSH-Px in serum are important indices to measure the non-specific immunity of grass carp. ACP and AKP are important phosphotransferases that are closely related to the internalization of nutrients and the removal of harmful substances in organisms. The enhanced activities of ACP and AKP indicate that the metabolism of organisms is fast. CAT can decompose hydrogen peroxide into oxygen and water, remove free radicals in organisms and protect organisms from free radicals. GSH-Px can transform toxic substances into non-toxic substances, maintaining the normal physiological activities of organisms (Lin et al., 2018). The activities of ACP, AKP, CAT and GSH-Px in the oral group and immersion group were increased, indicating that strain S3 could improve the non-specific immunity of grass carp.

The *Keap1/Nrf2* pathway is an important antioxidant pathway(Yu & Xiao, 2021), which can remove reactive oxygen species caused by oxidative stress over time, so as to maintain the health of fish. After strain S3 treatment, the expression level of *Keap1* in grass carp was significantly upregulated in the liver and kidney, and the expression

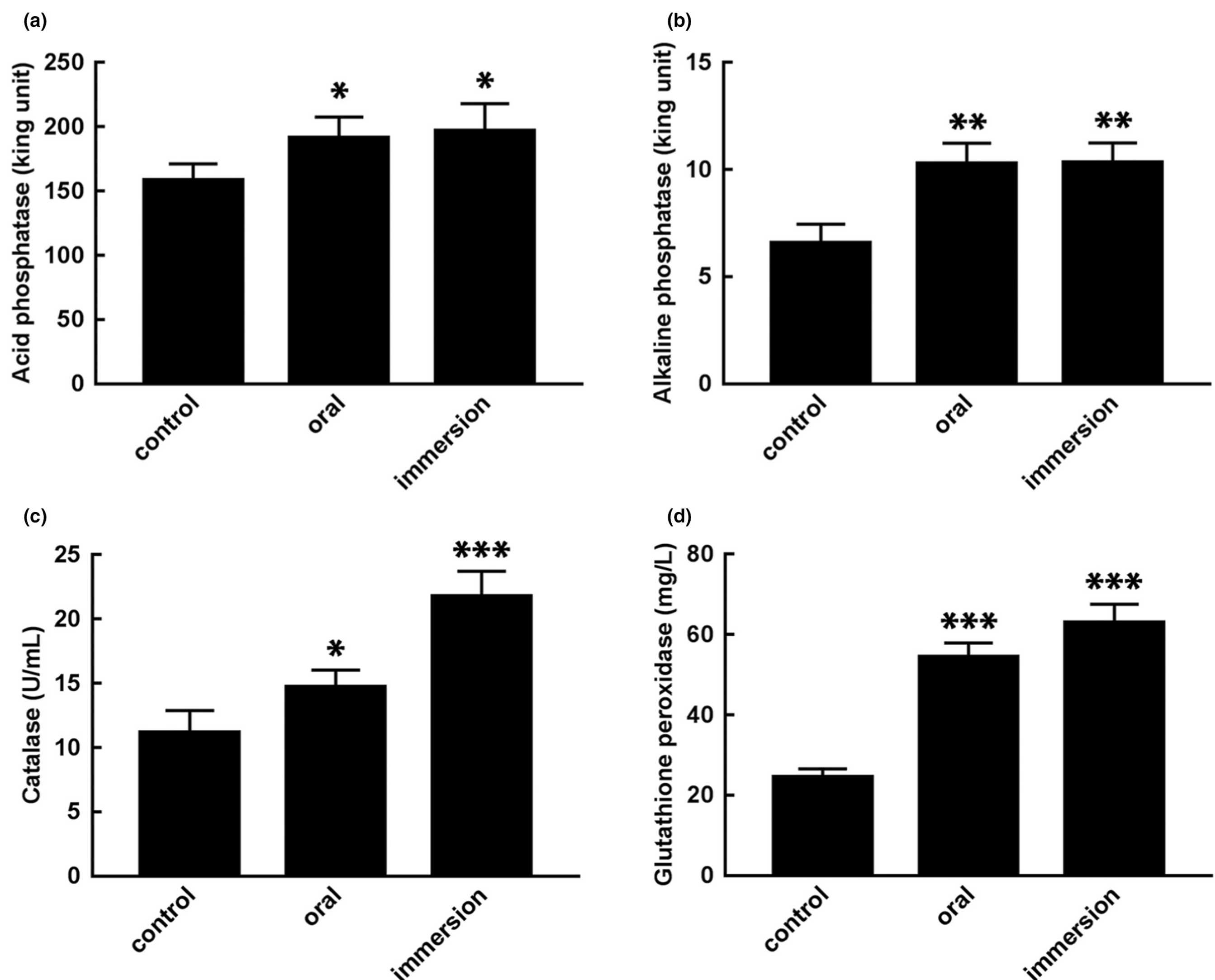

FIGURE 6 Effect of strain S3 treatment on immune-related enzymes in grass carp serum. (a) Acid phosphatase activity; (b) alkaline phosphatase activity; (c) catalase activity; (d) glutathione peroxidase activity.

FIGURE 7 Effect of strain S3 treatment on the expression of antioxidant-related genes and immune-related genes in grass carp tissues. (a) Antioxidant-related genes in liver; (b) antioxidant-related genes in kidney; (c) antioxidant-related genes in spleen; (d) antioxidant-related genes in intestine; (e) immune-related gene in liver; (f) immune-related gene in kidney; (g) immune-related gene in spleen; (h) immune-related gene in intestine.

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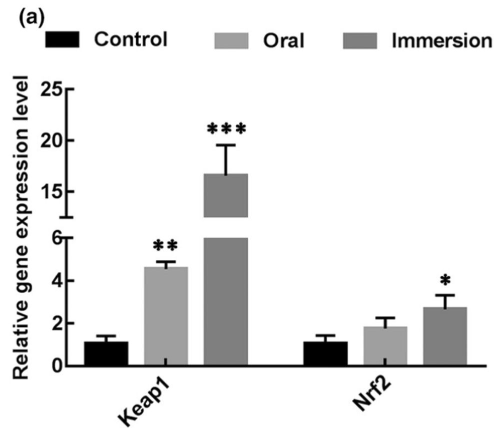

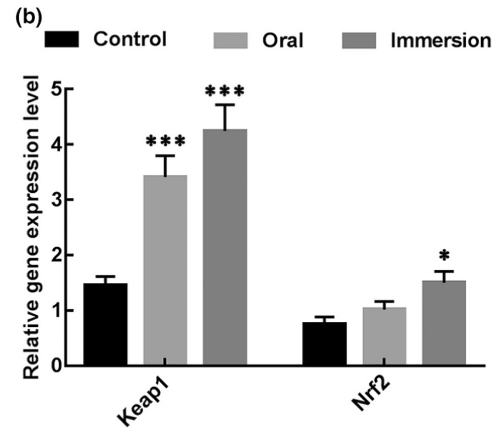

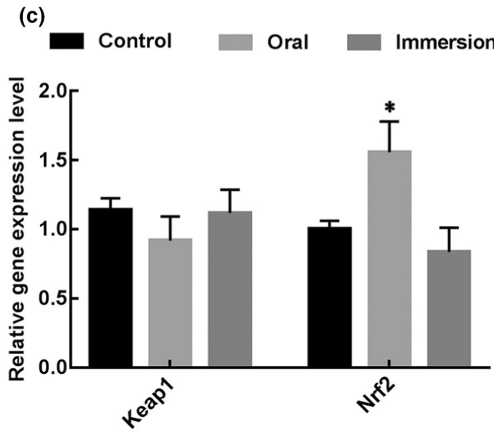

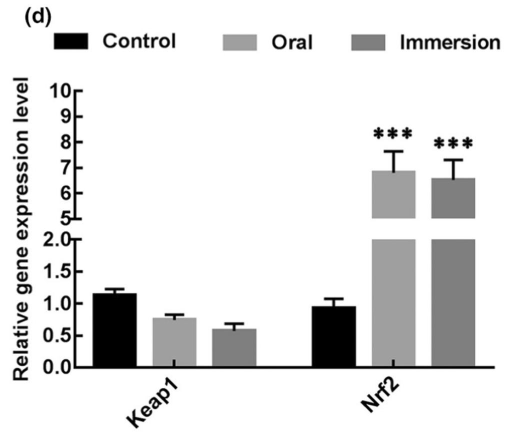

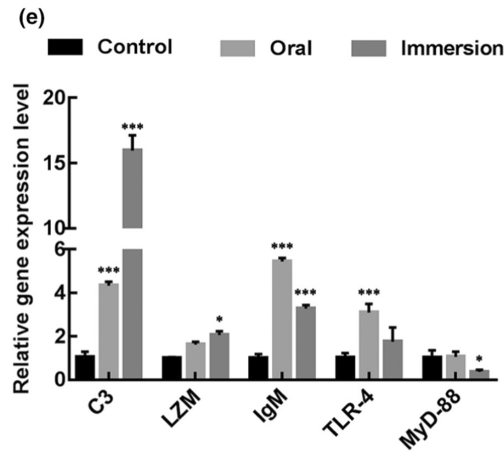

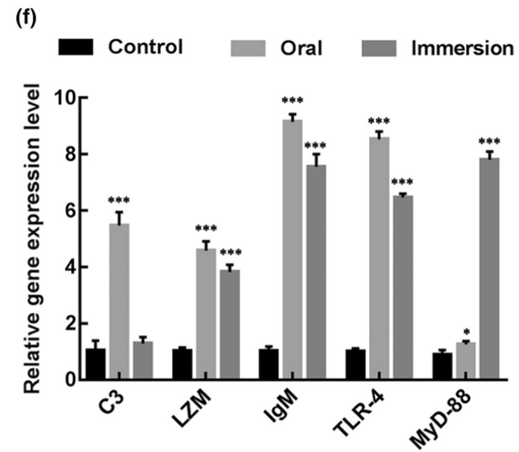

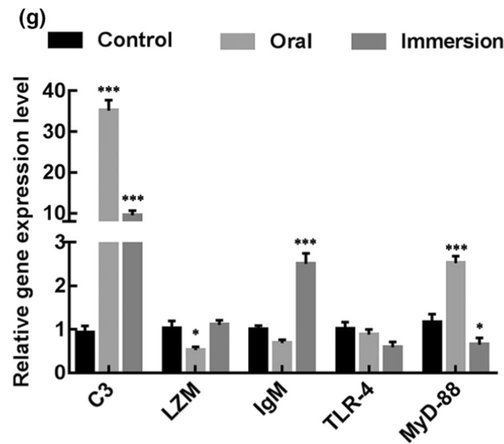

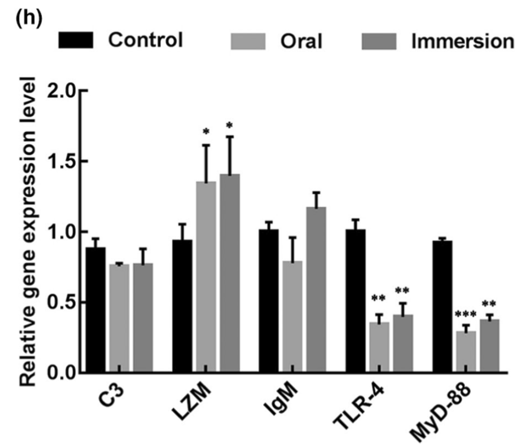

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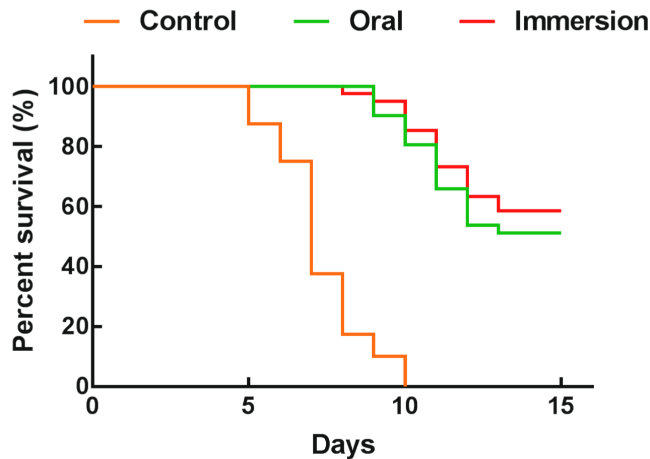

FIGURE 8 Survival curve of grass carp challenged with Ah X040.

level of *Nrf2* in grass carp was significantly upregulated in the liver, spleen and intestine. The strain S3 could alleviate the damage to grass carp caused by oxidative stress. *C3* is a member of the complement system. In bony fish, *C3* can mediate the surface conformational changes in phagocytes to eliminate pathogens(Anastasiou et al., 2011). The strain S3 could significantly increase the expression level of *C3* in liver and kidney of grass carp, which provided theoretical support for strain S3 to enhance the resistance of grass carp. *IgM* is an immunoglobulin. When antigens invade fish, *IgM* combines with antigens to activate the complement pathway and play an immune function(Bilal et al., 2021). In this study, the expression levels of *IgM* in the oral group and the immersion group were significantly upregulated in their livers and kidneys. When pathogens invade fish, lysozyme expressed by *LZM* can rapidly hydrolyse antigenic structure of pathogens (Song et al., 2021). Strain S3 could improve the expression level of *LZM* in the kidney and intestine of grass carp. Meanwhile, strain S3 significantly increased the expression levels of *TLR-4* and *MyD-88* in the kidney of grass carp. *TLR-4* is located on the surface of macrophages, presenting antigens and activating downstream pathways. *MyD-88* is an important linker signalling molecule in the pathway. They cooperate with other immune factors to remove the antigens in organisms (Shen et al., 2021). Based on the above analysis, strain S3 could upregulate the expression of immune-related genes in grass carp tissues, thereby improving the immunity of grass carp.

However, further research is needed on the identification of bacteriostatic active substances, the location of coding genes or gene clusters in the genome and the preparation of probiotics.

5 | CONCLUSIONS

In this study, the probiotic strain S3 was screened from a sediment of a fishpond and found to have antagonistic effects on 11 major fish pathogens including *Aeromonas hydrophila* and *Aeromonas salmonicida*. Strain S3 was identified as a new strain of *Paenibacillus polymyxa*. Cytotoxicity indicated that strain S3 was safe for grass carp liver cells. Strain S3^{GFP} was constructed, which indicates that strain S3 could colonize grass carp. Bioassay results showed that strain S3 could promote the growth of grass carp and improve its antioxidant capacity and immunity. The whole genome sequencing analysis results of strain S3 showed that it had abundant secondary metabolites, which suggest the probiotic function of strain S3 at the molecular level. In summary, strain S3 can be prepared into microecological preparations and has potential application value in aquaculture.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest in this work.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supplementary Material of this article.

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