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The *Bacillus velezensis* CYS06 Strain Exhibits Promising Applications in Fighting Grass Carp Bacterial Diseases

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Abstract: *Aeromonas* septicemia and columnaris disease are major bacterial diseases in grass carp; however, the drugs currently used to control these diseases pose environmental and health risks. This study aimed to screen for a probiotic *Bacillus* strain with antagonistic activity to prevent and control bacterial diseases in grass carp and to evaluate the antimicrobial activities, biosafety, and biocontrol effects of this strain. A *Bacillus* strain with antagonistic activity against *Aeromonas hydrophila*, obtained from grass carp intestines, was screened, and the isolate CYS06 was identified by analyzing the 16S rRNA and *gyrA* gene sequences. The antimicrobial spectrum of the strain CYS06 was determined, and the activities of amylase, cellulase, protease, and lipase of the strain CYS06 were determined. The whole genome of the strain CYS06 was sequenced using the nanopore sequencing technology platform, followed by the analysis of the antagonistic substance synthesis gene clusters and CAZy enzyme gene families. The biosafety of the strain CYS06 was evaluated via intraperitoneal injection into healthy grass carp. After the strain CYS06 was fed to the grass carp, its biological control effect on this fish was evaluated through artificial infection experiments. The strain CYS06 was identified as *Bacillus velezensis*, based on molecular identification, which shows broad antimicrobial activity against various fish pathogens. The strain CYS06 secretes amylase, cellulase, protease, and lipase. The genome size of the strain CYS06 is 3,914,159 bp, and it contains eight antagonistic substance synthesis gene clusters and many CAZy enzymes. The strain CYS06 exhibits high biological safety for grass carp, based on the challenge test. Feeding grass carp with the strain CYS06 for 4 weeks significantly enhanced the resistance of the fish to *A. hydrophila*. Strain CYS06 could inhibit the growth of *Flavobacterium columnare* under co-culture and reduce the amount of *F. columnare* adherence on the gills of grass carp, indicating that CYS06 has good potential for the prevention and control of columnaris disease. In conclusion, we isolated an antagonistic probiotic strain, CYS06, which exhibits a biological control effect on septicemia and columnaris disease caused by *Aeromonas* spp. and *F. columnare* in grass carp, respectively. This strain contains many antagonistic substance synthesis-related gene clusters and holds the potential to degrade various types of carbohydrates. As a biological control agent, the strain CYS06 exhibits significant potential for the prevention and control of bacterial diseases in grass carp.

Keywords: *Bacillus velezensis*; grass carp; biological control; genome

Key Contribution: This study provides a probiotic CYS06 strain with highly antagonistic activity which exhibits excellent application potential in the prevention and control of Motile Aeromonas Septicemia and columnaris disease in grass carp aquaculture.

Citation: Liu, L.; Jiang, D.; Ren, Y.; Shi, C.; Wang, Y.; Yin, J.; Wang, Q.; Zhang, D. The *Bacillus velezensis* CYS06 Strain Exhibits Promising Applications in Fighting Grass Carp Bacterial Diseases. *Fishes* **2024**, *9*, 7. https://doi.org/10.3390/fishes9010007

Academic Editor: Bernardo Baldisserotto

Received: 3 November 2023
Revised: 21 December 2023
Accepted: 21 December 2023
Published: 22 December 2023

1. Introduction

The grass carp (*Ctenopharyngodon idella*) is the most widespread freshwater fish species in China, with an annual yield exceeding 5.5 million tons, accounting for approximately 20% of the country's total freshwater fish production [1]. It is widely consumed as a good source of high-quality proteins. Grass carp is an herbivorous fish that has been widely introduced throughout the world, and it consumes up to 27.6 kg of vegetation per kg of fish every year [2]. The grass carp is not only widely farmed in ponds, but it also widely distributed in streams, rivers, reservoirs, lakes, and wetlands to regulate water quality [1]. However, the grass carp, especially in ponds, is susceptible to various diseases, including bacterial septicemia caused by *Aeromonas* spp., viral hemorrhagic disease caused by grass carp reovirus (GCRV), and columnaris disease caused by *Flavobacterium columnare* [3–5]. Although GCRV severely affects grass carp aquaculture, vaccination is widely used in China to prevent and control GCRV. Bacterial diseases are common in grass carp aquaculture, and the main pathogens are *F. columnare* [3], *Aeromonas hydrophila* [4], *Acinetobacter lwoffii* [5], *Vibrio mimicus* [6], and *Vibrio vulnificus* [7]. Compared with the other freshwater fish species (e.g. crucian carp and bighead carp), the grass carp is more susceptible to *F. columnare* and *Aeromonas* sp. in China [3,8]. *F. columnare* causes serious clinical symptoms, including gill lesions, skin lesions, and fin erosions, resulting in severe economic losses relative to grass carp [3].

Recently, bacterial diseases in grass carp in China have been prevented and controlled using antibiotics and disinfectants. The large-scale and repeated application of these chemical drugs can cause a series of problems, including drug residues, bacterial resistance, and environmental pollution [9]. Thus, biological control using probiotics, which causes less contamination and has a low risk of resistance, is an alternative method for preventing diseases. *Bacillus* is ubiquitous in water, soil, air, animals, and plants and is an important biocontrol bacterium in crops, livestock, animals, and fish. *Bacillus* probiotics have been extensively used in aquaculture as alternatives to antibiotics for fish farming [10]. *Bacillus velezensis* exhibits broad-spectrum antibacterial activity, making it a vital biocontrol agent against various plant and animal diseases [11]. For instance, *B. velezensis* demonstrates potent biocontrol effects on plant diseases including potato scab [12], lotus root rot [13], and wheat *Fusarium* head blight [14], as well as on infections in crucian carp (*Carassius auratus*) caused by *A. hydrophila* [15], those in Nile tilapia (*Oreochromis niloticus*) caused by *Streptococcus agalactiae* [16], in hybrid grouper resulting from *Vibrio harveyi* [17], and in grass carp from *A. hydrophila* [18]. *B. velezensis* FZB42 strain is successfully used in agriculture to biocontrol rhizobacteria and to promote plant growth [11]. Additionally, *B. velezensis* CPA1-1 is a potential probiotic for inhibiting non-O1 *Vibrio cholerae* and improving host immunity in oriental river prawn (*Macrobrachium nipponense*) [19]. *B. velezensis* Bs916 is used as a potential probiotic for the biological control of white spot disease in crayfish [20].

B. velezensis could produce anti-microbial active compounds, such as bacilysin, bacillibactin, bacillaene, fengycin, marcolactin H, and surfactin, exhibiting a strong antagonistic action against animal and plant pathogens [21]. These secondary metabolites also induce systemic resistance in plants. Genome sequence analysis indicated that *B. velezensis* harbors many gene clusters encoding secondary metabolites, i.e., *B. velezensis* VJH504 contains several gene clusters encoding NRPS, transAT-PKS, T3PKS, and PKS-like types of secondary metabolites [22]; and *B. velezensis* HNA3 possesses 12 gene clusters related to 14 secondary metabolites with bioactive compounds [23]. Furthermore, *B. velezensis* shows high hydrolase activity due to the presence of protease, chitinase, cellulase, and glucanase, which is associated with carbon source and cellulose utilization [24].

The aim of this study is to isolate probiotics that can control aquaculturally important pathogens found in the intestines of the healthy grass carp and to evaluate the biocontrol potential and safety of the strain. In this study, a representative strain CYS06 of *Bacillus*, with antagonistic activity, was isolated from the intestines of healthy grass carp. The broad-spectrum bacteriostatic activity, extracellular enzyme activity, environmental adaptability, and biocontrol efficacy of this strain were analyzed. The whole genome of this strain

was sequenced, and key functional and biosafety-related genes were analyzed. The strain CYS06 is an excellent candidate for commercialization as a biocontrol agent, especially in grass carp farming.

2. Materials and Methods

2.1. Fish, Bacterial Strains, and Culture Conditions

Grass carp were obtained from an aquaculture farm in Nansha District, Guangzhou City, China. The livers, spleens, and kidneys of ten healthy grass carp were selected for specific PCR to confirm that the fish did not harbor GCRV and *Aeromonas* [25,26]. The intestine (without the contents) of healthy grass carp were obtained and rinsed thrice with sterile phosphate-buffered saline (PBS) buffer. A total of 2 g of the intestinal tissue was obtained, homogenized, and added to 10 mL of sterile PBS, followed by heating at 80 °C for 10 min to kill non-spore strains. A total of 100 µL of the intestinal homogenates, along with 10-fold and 100-fold dilutions, were spread onto LB agar plates. The plates were then incubated at 30 °C for 24–36 h. Colonies similar to those of *Bacillus* were obtained to determine their antagonistic activity. Briefly, the indicator strain *A. hydrophila* GYK1 (GenBank accession no. CP016392) was adjusted to 10^7 CFU/mL (colony formation unit, CFU), and then 100 µL of the strain was spread on LB agar plates, four wells (diameter = 6 mm) were made in each LB plate, and each of the isolates (OD_{600} = 0.5, 50 µL) was added into the well. The plates were incubated at 30 °C for 24 h, and the diameters of the inhibition zone were measured. Additionally, the isolates with antimicrobial activity were inoculated onto sheep blood agar plates to determine the hemolytic activity.

2.2. Antagonistic Activity

The strain CYS06, which exhibited the largest inhibition zone against the *A. hydrophila* GYK1 strain, was selected. In addition, this strain showed non-hemolytic activity. This strain was tested against common bacterial pathogens of freshwater fish using the well-diffusion agar assay method, according to the method previously described in Ref. [16]. The indicator strains of fish pathogens are listed in Table 1. First, 100 µL of the overnight culture of the indicator bacteria solution (10^7 CFU/mL) was spread on brain heart infusion (BHI) agar plates, and three wells with a diameter of 6 mm were created on each plate. Second, in each well, 50 µL of strain CYS06 solution in logarithmic growth phase (OD_{600} = 0.8) was added. The plates were incubated in an incubator at 30 °C for 24 h, and the diameter of the inhibition zone was measured. Shieh agar plates were used to determine the inhibitory effect of strain CYS06 on *F. columnare*, and the plates were incubated at 28 °C for 36 h. The experiments were performed in triplicate, and statistical analyses were performed using SPSS 22.0. The *Escherichia coli* strain ATCC 25,922 was used as the control strain.

Table 1. The information on the indicator strains isolated from aquatic animals.

Strain	Species	Host
MaY12106	*Aeromonas hydrophila*	*Megalobrama amblycephala*
GYK1	*Aeromonas hydrophila*	Mandarin fish (*Siniperca chuatsi*)
Ip092	*Aeromonas hydrophila*	Channel catfish (*Ictalurus punctatus*)
Ci001	*Aeromonas hydrophila*	Grass carp (*C. idella*)
Hm092	*Aeromonas hydrophila*	Silver carp (*Hypophthalmichthys molitrix*)
Hm0910	*Aeromonas hydrophila*	Silver carp (*H. molitrix*)
Ca1701	*Aeromonas hydrophila*	Crucian carp (*C. auratus*)
Hn091	*Aeromonas hydrophila*	Bighead carp (*Hypopthalmichthys nobilis*)
Hn092	*Aeromonas veronii*	Bighead carp (*H. nobilis*)
Ci091	*Aeromonas veronii*	Grass carp (*C. idella*)
Ci1273	*Aeromonas jandaei*	Grass carp (*C. idella*)
Ip121	*Aeromonas jandaei*	Channel catfish (*I. punctatus*)
WL1483	*Aeromonas schubertii*	Hybrid snakehead (*Channa argus* × *C. maculate*)
WL23	*Aeromonas schubertii*	Hybrid snakehead (*C. argus* × *C. maculate*)
So1382	*Aeromonas aquariorum*	Red drum (*Sciaenops ocellatus*)

Table 1. *Cont.*

Strain	Species	Host
So1383	*Aeromonas aquariorum*	Red drum (*S. ocellatus*)
Pef1401	*Edwardsiella ictaluri*	Yellow catfish (*Tachysurus fulvidraco*)
Fc001	*Flavobacterium columnare*	Grass carp (*C. idella*)
Sn03	*Streptococcus iniae*	Nile tilapia (*O. niloticus*)

2.3. Identification of Antagonistic Strains

The genomic DNA of strain CYS06 was extracted using a bacterial genomic DNA extraction kit (Guangzhou Magen Biotechnology, Co., Ltd., Guangzhou, China). To identify the strain CYS06, the 16S rRNA and *gyrA* genes were amplified using the primer sets 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′), and gyrA-F (5′-ATTCACGCTATCACTGACTTATTC-3′) and gyrA-R (5′-ATGGGAGACAA AGTAGAACCGAG-3′), respectively. The PCR products were sequenced. A similarity analysis of the 16S rRNA and *gyrA* gene sequences was conducted using online Blastn to initially determine the taxonomic relationships of the strain CYS06. The phylogenetic trees were constructed using MEGA 7.05 software using the neighbor-joining method based on the 16S rRNA and *gyrA* genes, respectively.

2.4. Extracellular Enzyme Activity

The extracellular enzyme activities of the amylase, cellulase, protease, and lipase of strain CYS06 were determined using the agar plate method, as previously described in Ref. [27]. Briefly, The LB agar plates containing 1.5% soluble starch, 1% carboxymethylcellulose, 1.5% non-fat milk powder, or 1% triglyceride tributyrate were prepared, a well with a diameter of 6 mm was made on each plate, and the strain CYS06 (50 µL) was added to each well. The experiments were performed in triplicate. The plates were held at 30 °C for 24 h. To determine the cellulase activity of the strain CYS06, 1% Congo red dye solution was added to the LB agar plate containing 1% sodium carboxymethylcellulose after the plates had been incubated for 24 h; the dye solution was removed after 30 min, and 1 mol/L NaCl solution was added for distaining. The cellulase activity was determined based on the diameter of the transparent halos. To determine the amylase activity of the strain CYS06, iodine solution was added to the plates containing 1.5% soluble starch after the plates were cultured for 24 h; the iodine solution was removed after 10 min, and the diameter of the transparent halos was measured. The lipase and protease activities were determined based on the diameter of the transparent halos. The experiments were performed in triplicate.

2.5. Genome Sequencing and Analysis

2.5.1. Genome Sequencing and Functional Annotation

The genomic DNA of the strain CYS06 was extracted for whole genome sequencing analysis. Whole genome sequencing was performed using the nanopore sequencing technology platform by Biomarker Technologies Co., Ltd., Beijing, China. The sequences of the strain CYS06 were assembled using Canu v1.5 software [28], and sequence correction was performed using Pilon v1.22 software [29] to obtain the complete genome sequence. Encoding genes were predicted using Prodigal v2.6.3 software [30], non-coding genes such as tRNA were predicted using tRNAscan-SE, and other ncRNAs in the genome except tRNA and rRNA were predicted using Infernal 1.1 [31] based on the Rfam database [32]. Pseudogenes in the genome were identified using GenBlastA v1.0.4 software [33] and GeneWise v2.2.0 software [34]. The predicted gene sequences were blasted and analyzed using functional databases such as COG, KEGG, Swiss-Prot, TrEMBL, and Nr to obtain gene function annotation results. In addition, COG, KEGG metabolic pathway enrichment, and GO functional enrichment analysis were performed. The predicted gene protein sequences were blasted and analyzed using functional databases such as the Transporter Classification

Database (TCDB), the Antibiotic Resistance Gene Database (CRDB), and the Virulence Factor Database (VFDB).

2.5.2. Genome Evolution Analysis

The use of the average nucleotide identity (ANI) is a useful method to verify species identification in prokaryotic genomes. The value of 95% ANI is proposed for delineating prokaryotic species in the previous report [35]. The taxonomic status of the strain CYS06 was further determined using online ANI analysis (https://www.ezbiocloud.net/tools/ani (accessed on 27 June 2023)). Furthermore, DNA-DNA hybridization (DDH) is an important tool for microbial species delineation [36]. The taxonomic status of the strain CYS06 was further determined using online DDH (http://ggdc.dsmz.de/ggdc.php (accessed on 28 June 2023)) analysis.

2.5.3. Functional Gene

The antagonistic substance synthesis gene cluster of the strain CYS06 was analyzed online using antiSMASH v5.1.2 software (https://antismash.secondarymetabolites.org/#!/start (accessed on 11 May 2022)). Based on the results of the analysis, a schematic diagram of the antagonistic substance synthesis-related gene cluster in the strain CYS06 was constructed. Based on the predicted antagonistic substance synthesis-related gene sequences, the proportion of the antagonistic substance gene cluster in relation to the total length of the genome sequence was analyzed.

Carbohydrate-active enzymes can be classified according to their functions and include glycoside hydrolases, glycosyltransferases, polysaccharide lyases, carbohydrate esterases, auxiliary oxidoreductases, and carbohydrate-binding modules that have no catalytic activity. The carbohydrate enzyme gene family of the strain CYS06 was annotated based on the carbohydrate-active enzyme database (CAZyme).

2.5.4. Risk-Associated Genes

The risk-associated genes in the genome of the strain CYS06, including biogenic amine encoding genes, enterotoxin genes, and antibiotic resistance genes, were analyzed based on gene similarity. The biogenic amine encoding the related genes of tyrosine decarboxylase, agmatine deiminase, arginine decarboxylase, arginine deiminase, histidine decarboxylase, putrescine carbamoyltransferase, and ornithine carbamoyltransferase were searched in the genome of strain CYS06. The enterotoxin genes, including haemolysin BL (*hblC*, *hblD*, *hblA*, and *hblB*), non-hemolytic enterotoxin NHE (*nheA*, *nheB*, and *nheC*), enterotoxin T (*bacT*), cytotoxin K (*cytK*), and cereulide (*cesA*, *cesH*, *cesP*, *cesT*, *cesB*, *cesC*, and *cesD*), were screened by searching the strain CYS06 genome. The antibiotic resistance genes were predicted using the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/analyze/rgi (accessed on 5 October 2022)) with online RGI (Resistance Gene Identifier).

2.6. Challenge Test

The pathogenicity of strain CYS06 was evaluated using a challenge test. The CYS06 was grown in LB broth and incubated at 37 °C for 12 h and collected by centrifugation (2292× *g*, 10 min); the strain was then re-suspended in sterile PBS to obtain a concentration of 1.0×10^9 CFU/mL. A total of 60 healthy juvenile grass carp (11.82 ± 3.45 g) were randomly divided into two groups (the control group and the experimental group) and further divided into three tanks in each group, with 10 fish in each tank. Each fish in the experimental group was injected intraperitoneally with 0.1 mL of strain CYS06 at a dose of 1.0×10^9 CFU/mL, and each fish in the control group was injected intraperitoneally with 0.1 mL of sterile PBS. The clinical signs and mortality of the challenged fish were monitored for 2 weeks. At the end of the experiment, the liver, spleen, and kidney tissues of the grass carp in both the experimental and the control groups were collected under sterile conditions, homogenized, and spread on LB agar plates to isolate the CYS06 strain.

All experimental procedures were conducted according to the guidelines of the Laboratory Animal Ethics Committee, Pearl River Fisheries Research Institute, CAFS (ID Number: LAEC-PRFRI-2022-03-48).

2.7. Biological Control

2.7.1. Resistance against *A. hydrophila*

A total of 900 healthy grass carp were randomly divided into three groups, with three tanks per group and 100 fish per tank. The experimental group 1 fish was fed a commercialized puffed diet, supplemented with strain CYS06 (10^7 CFU/g), in the amount of 3% of the experimental fish body weight daily; experimental group 2 was fed a commercialized puffed diet, supplemented with strain CYS06 (10^6 CFU/g), in the amount of 3% of experimental fish body weight daily; and the control group (group 3) was fed a commercialized puffed diet in the amount of 3% of the experimental fish body weight daily.

After 4 weeks of feeding, 45 grass carp were randomly selected from the experimental and control groups, with each group consisting of three biological replicates, with 15 grass carp in each replicate. The infected strain, *A. hydrophila* GYK1, was collected during the logarithmic growth period and adjusted to a concentration of 5.88×10^7 CFU/mL with sterile PBS. Each grass carp in the experimental groups and the control group was intraperitoneally injected with 100 µL of the GYK1 strain. The negative control group (from the control group) was injected with an equal volume of sterile saline. The water temperature was 30 ± 1 °C during the experiment, and one-quarter of the water was replaced daily. Clinical signs and the death of the challenged fish were observed and recorded. The relative percentage of survival (RPS) was calculated as follows: RPS (%) = (1 − mortality of feeding fish/mortality of control fish) × 100.

2.7.2. Resistance against *F. columnare*

To further evaluate the antagonistic effect of strain CYS06 against *F. columnare* Fc001, a co-culture assay was performed. The CYS06, *Bacillus subtilis* Bs168 (as positive control strain), and Fc001 strains were inoculated in Shieh medium (100 mL) to adjust the final concentrations of 5×10^6 CFU/mL, respectively. In the experiment group, the CYS06 and Fc001 strains were inoculated in Shieh medium (100 mL) for co-culture; in the positive control group, the Bs168 and Fc001 strains were inoculated in Shieh medium (100 mL) for co-culture. Then, the co-cultured media was incubated for 48 h under shaking (180 r/min) at 28 °C. The strains of *Bacillus* and *F. columnare* were isolated, and colonies of viable bacterial cell were counted at 12 h, 24 h, 36 h, and 48 h post-culture, respectively.

Immersion experiment: A total of 180 healthy grass carp (average weight: 14.78 g per fish) were randomly divided into three groups, with three tanks per group and 20 fish per tank (100 L of water). Strain Fc001, at the final concentrations of 5×10^6 CFU/mL, respectively, was added to the tanks in experiment group 1. Strains Fc001 and CYS06, at the final concentrations of 5×10^6 CFU/mL, respectively, were added to the tanks in experiment group 2. Strains Fc001 and Bs168 (as the positive control strain), at the final concentrations of 5×10^6 CFU/mL, respectively, were added to the tanks in experiment group 3. The water temperature was 28 ± 1 °C during the experiment. The gill tissues (three fish per tank) of fish in the three groups were collected. Then, the gill tissues were homogenized, and streaked onto Shieh agar plates, and the colonies of viable bacterial cell were counted at 12 h, 24 h, 36 h, and 48 h post-immersion, respectively.

One-way analysis of variance was used to determine significant differences in strain CYS06 against *F. columnare*.

3. Results

3.1. Antagonistic Strain Screening and Identification

A total of 112 strains were isolated from the grass carp intestines, and 9 of them show an antagonistic activity against indicator GYK1. Among these strains, CYS06 exhibits the strongest antibacterial activity, as well as non-hemolytic activity. The strain CYS06

16S rRNA gene sequence showed the highest similarity to that of the *B. velezensis* strain GH1-13 (GenBank accession no. CP019040.1), with 100% similarity. Moreover, the *gyrA* gene sequence was found to share 99.50% similarity with strain GH1-13. The phylogenetic tree based on the 16S rRNA gene showed that strain CYS06 clustered with *B. velezensis* strains GH1-13, str.FZB42, CC09, and G341 (Figure 1A). The phylogenetic tree based on the *gyrA* gene showed that strain CYS06 clustered with *B. velezensis* (Figure 1B). These results suggested that strain CYS06 belongs to *B. velezensis*.

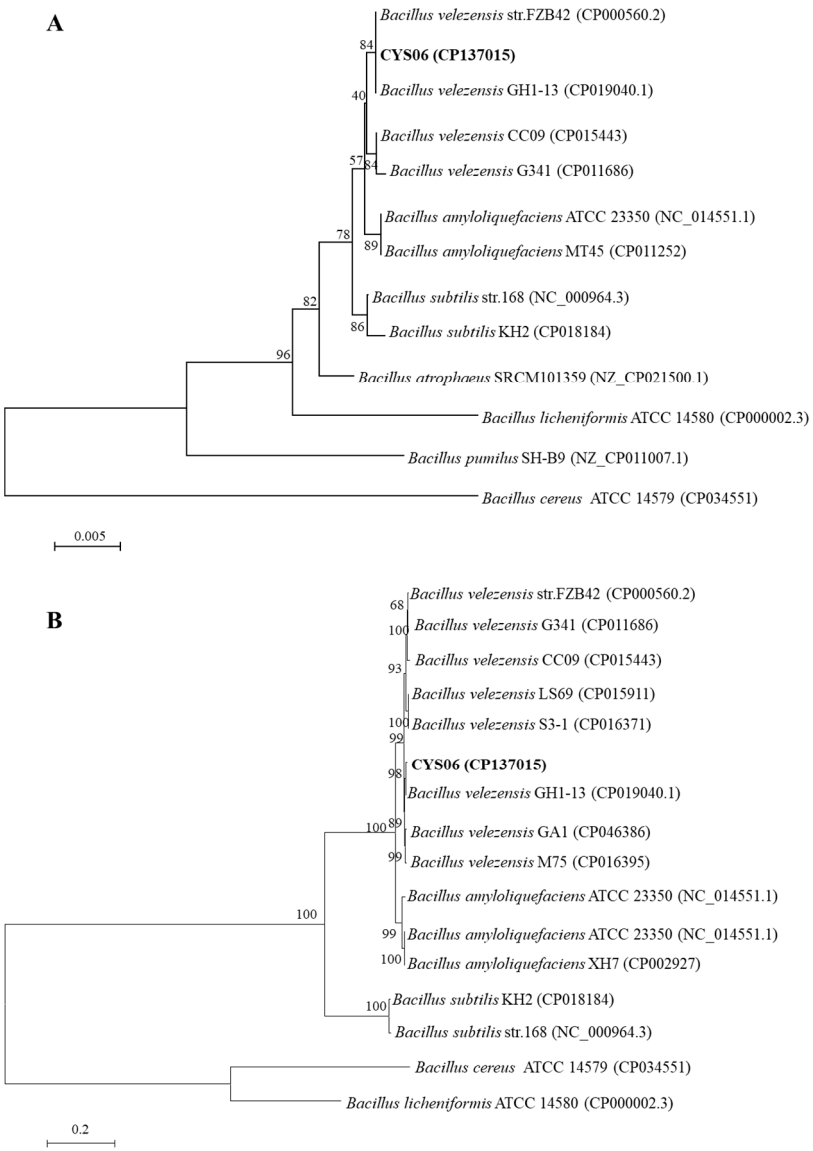

Figure 1. Phylogenetic trees of strain CYS06 based on 16S rRNA (**A**) and *gyrA* (**B**) genes.

3.2. Extracellular Enzyme Activity

The results of the enzyme activity test of strain CYS06 showed that this strain can form a hydrolysis ring in the corresponding agar plates (Figure 2), indicating that it has the ability to hydrolyze starch, protein, cellulose, and tributyrin. The results indicated that strain CYS06 has the ability to secrete amylase, protease, cellulase, and lipase.

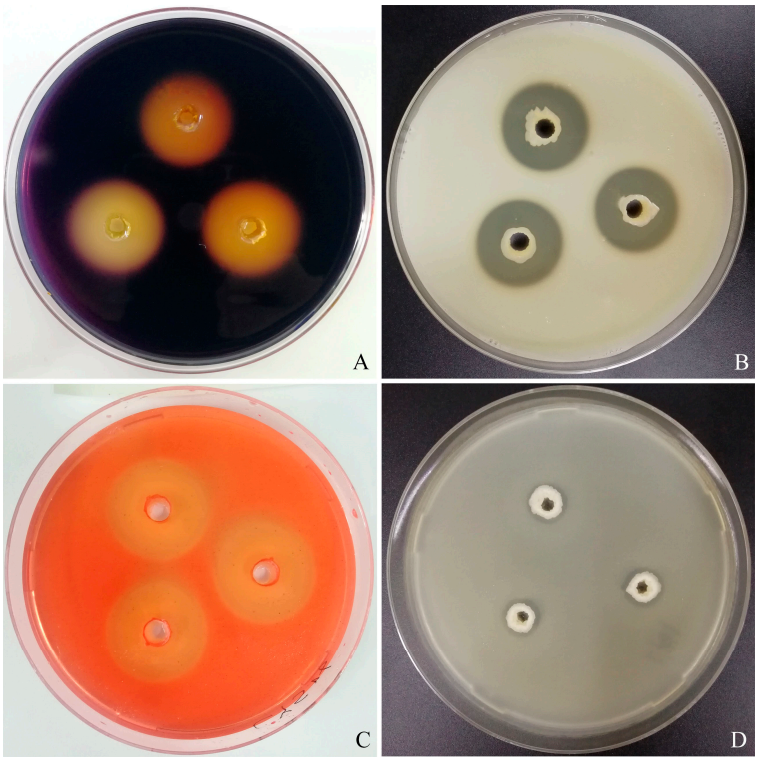

Figure 2. The hydrolysis activities of starch (**A**), casein (**B**), cellulose (**C**), and fat (**D**) from the strain CYS06.

3.3. Antagonistic Activity

The CYS06 strain exhibited antimicrobial activity against 19 strains of aquatic animal pathogens, including *A. hydrophila*, *A. veronii*, *A. schubertii*, *A. jandaei*, *A. aquariorum*, *E. ictaluri*, and *F. columnare*. Among them, strain CYS06 showed good antibacterial activity on pathogens of the genus *Aeromonas*, but also showed significant differences in antibacterial effects on pathogens of the same species from different host sources (Figure 3).

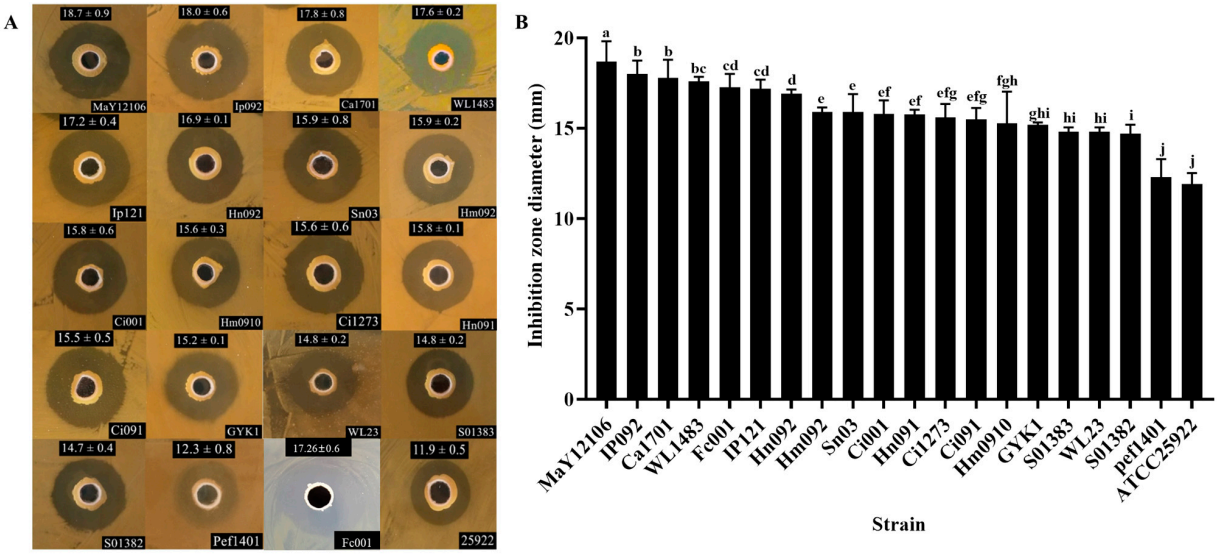

Figure 3. Antagonistic activity of the strain CYS06 against fish pathogens. (**A**) Antagonistic activities of the strain CYS06 against various fish pathogens. (**B**) Comparative analysis of the antagonistic effect. Different letters indicate a significant difference ($p < 0.05$).

3.4. Genome Sequence Annotation and Functional Analysis

3.4.1. Sequence Assembly and Annotation

The whole-genome sequence of strain CYS06 was 3,914,159 bp, with a G + C content of 46.59%. It contains 3692 coding genes, 7 pseudogenes, 27 ribosomal RNAs (rRNAs), 86 transfer RNAs (tRNAs), and 42 other non-coding RNAs (ncRNAs) (Table 2). A total of 2845 genes in the genome of strain CYS06 were annotated in the COG database. Genes related to amino acid transport and metabolism, transcription, carbohydrate transport and metabolism, inorganic ion transport and metabolism, cell wall/membrane and envelope biogenesis, ribosome structure, and biogenesis were the most abundant. Four genes were annotated into the ARDB database, including *bacA* (bacitracin resistance gene), *fosB* (fosfomycin resistance gene), *lmrB* (lincomycin resistance gene), and *tetL* (tetracycline resistance gene). However, strain CYS06 was sensitive to various drugs (e.g., tetracycline, MIC = 1 μg/mL), and moderately sensitive or resistant to fosfomycin (MIC = 128 μg/mL), bacitracin (MIC = 128 μg/mL), and lincomycin (MIC = 32 μg/mL). This could be due to the fact that these are not key resistance genes. The genome sequence of strain CYS06 was deposited in the NCBI GenBank with accession number CP137015.

Table 2. General features of the genome of strain CYS06.

Type	Number/Copy
Genome size (bp)	3,914,159
GC content (%)	46.59
Coding sequences	3692
16S rRNA	9
23S rRNA	9
5S rRNA	9
tRNA	86
ncRNA	42
pseudogene	7
CRISPR	9
Genomic island	7
Prophage	2

3.4.2. Taxonomic Status

The ANI values for strain CYS06 compared with those for *B. velezensis* strains LG37, WLYS23, LS69, FZB42, G341, M75, BvL03, and GH1-13 were 97.63%, 97.64%, 97.69%, 97.73%, 97.81%, 98.68%, 99.13%, and 99.46% (Table 3), respectively, with the ANI values greater than the threshold (95%) for species delineation. In contrast, the ANI values for *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, and *B. cereus* is ≤94.17%, which are below the species classification threshold of 95%. Likewise, the DDH values for strain CYS06 compared with those for the *B. velezensis* strains LG37, WLYS23, LS69, FZB42, G341, M75, BvL03, and GH1-13 were 79.80%, 79.80%, 79.80%, 80.90%, 81.40%, 88.80%, 93.10%, and 96.00% (Table 3), respectively, with the DDH values greater than the defined threshold (70%) of the recommended species. However, the DDH values between strain CYS06 and *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, and *B. cereus* range from 19.40~56.00%, with all below 70%. Therefore, the strain CYS06 was identified as *B. velezensis*, based on the ANI and DDH values.

Table 3. The values of ANI and DDH for the strain CYS06 and its related species.

Strain (GenBank Accession No.)	CYS06 Strain	
	ANI (%)	DDH (%)
Bacillus velezensis GH1-13 (CP019040)	99.46	96.00
Bacillus velezensis BvL03 (CP041192)	99.13	93.10
Bacillus velezensis M75 (CP016395)	98.68	88.80

Table 3. *Cont.*

Strain (GenBank Accession No.)	CYS06 Strain	
	ANI (%)	DDH (%)
Bacillus velezensis G341 (CP011686)	97.81	81.40
Bacillus velezensis FZB42 (CP000560)	97.73	80.90
Bacillus velezensis LS69 (CP015911)	97.69	79.80
Bacillus velezensis WLYS23 (CP055160)	97.64	79.80
Bacillus velezensis LG37 (CP023341)	97.63	79.80
Bacillus amyloliquefaciens ATCC 23350 (NC_014551)	94.17	56.00
Bacillus amyloliquefaciens MT45 (CP011252)	94.02	55.60
Bacillus subtilis str. 168 (NC_000964)	76.89	20.90
Bacillus licheniformis ATCC 14,580 (NC_006270)	72.76	20.20
Bacillus cereus ATCC 14,579 (CP034551)	68.28	34.00
Bacillus pumilus SH-B9 (CP011007)	70.51	19.40

3.4.3. Antagonistic Substance Gene Clusters

Online analysis using antiSMASH v5.1.2 revealed that the CYS06 genome contained bacilysin, mersacidin, bacillibactin, surfactin, fengycin, macrolactin H, difficidin, and bacillaene antimicrobial substance synthesis-related gene clusters. Further alignment analysis of these antagonistic substance genes showed that the CYS06 genome contained the aforementioned antimicrobial substance gene clusters (Figure 4). The core gene fragments of these gene clusters are approximately 323.1 kb in size, accounting for approximately 8.25% of the total genome length.

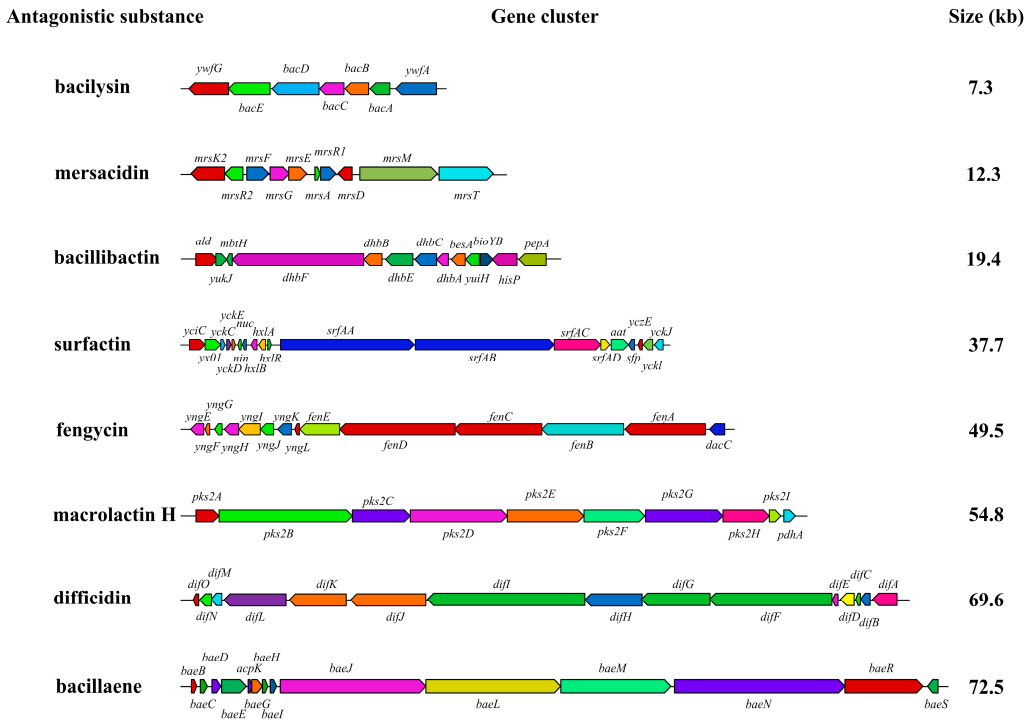

Figure 4. The gene clusters of the strain CYS06 for the biosynthesis of nine secondary metabolites.

3.4.4. CAZy Enzymes

A total of 156 CAZy enzyme genes are encoded by strain CYS06, including the GH (43), GT (39), CE (32), PL (4), AA (7), and CBM (31) gene families (Table 4). The GH family was the most common, accounting for 27.56% of the entire gene family, followed by the GT family, which accounted for approximately 25.00%. Among these gene families, those mainly associated with cellulose and hemicellulose degradation included GH5, GH11,

GH26, GH43, GH51, and GH53; those related to starch hydrolysis primarily included GH13 and GH126; those related to chitin degradation included GH18, GH23, CE9, and CBM50; those related to xylan degradation included GH43, CE1, CE3, CE4, CE6, and CE7; those related to pectin degradation primarily included GH43, CE1, PL1, and PL9; those related to peptidoglycan degradation included GH23 and GH73; and those related to the glucan enzyme included GH3, GH16, and GH30. This suggests that the strain CYS06 has the potential to degrade substances such as cellulose, hemicellulose, starch, chitin, pectin, peptidoglycan, and glucan.

Table 4. CAZymes families predicted in the genome of *B. velezensis* CYS06.

CAZymes Families	Gene Subfamilies (Number)
Glycoside Hydrolases, GHs	GH1 (3); GH3 (1); GH4 (4); GH5 (1); GH11 (1); GH13 (4); GH16 (1); GH18 (2); GH23 (3); GH26 (1); GH30 (2); GH32 (3); GH43 (4); GH46 (1); GH51 (2); GH53 (1); GH68 (1); GH73 (2); GH76 (1); GH109 (4); GH126 (1)
Glycosyl Transferases, GTs	GT1 (3); GT2 (14); GT4 (8); GT8 (1); GT19 (1); GT26 (1); GT28 (3); GT46 (2); GT51 (4); GT83 (2)
Carbohydrate Esterases, CEs	CE1 (8); CE3 (2); CE4 (7); CE6 (1); CE7 (2); CE9 (3); CE10 (4); CE12 (2); CE14 (4)
Polysaccharide Lyases, PLs	PL1 (2); PL9 (1)
Auxiliary Activities, AAs	AA4 (1); AA6 (1); AA7 (4); AA10 (1)
Carbohydrate-Binding Modules, CBMs	CBM2 (1); CBM3 (1); CBM6 (1); CBM12 (1); CBM26 (1); CBM37 (1); CBM50 (25)

3.4.5. Biogenic Amine and Toxin Encoding Genes

Biogenic amines: The capacity for biogenic amine production of strain CYS06 was investigated by a search for the biogenic amine encoding genes. The results suggested that the major encoding genes related to tyramine, histamine, and putrescine were absent from the genome (Table 5), indicating that strain CYS06 did not have the ability to synthesize these biogenic amines. However, the genes related to spermidine synthase and S-adenosylmethionine decarboxylase were present in the genome of strain CYS06, revealing that this strain had the potential to synthesize spermidine.

Table 5. Major biogenic amine encoding genes predicted in the genome of the strain CYS06.

Enzymes of Biogenic Amine	Biogenic Amine	GenBank Accession No. [a]	CYS06
Tyrosine decarboxylase	Tyramine	JH792376	Negative
Histidine decarboxylase	Histamine	AB553281.1	Negative
Agmatine deiminase	Putrescine	NZ_GL635753.1	Negative
Arginine decarboxylase	Putrescine	CP010005.1	Negative
Arginine deiminase	Putrescine	CP009651.1	Negative
Putrescine carbamoyltransferase	Putrescine	NZ_CAPG01000089.1	Negative
N-carbamoylputrescine amidase	Putrescine	CP002394	Negative
Ornithine carbamoyltransferase	Putrescine	CP000764.1	Negative
Spermidine synthase	Spermidine	CP010052.1	Positive
S-adenosylmethionine decarboxylase	Spermidine	CP010052.1	Positive

[a] GenBank accession number of each reference gene.

The key toxin-encoding genes, including *hblC*, *hblD*, *hblA*, *hblB*, *nheA*, *nheB*, *nheC*, *bacT*, *cytK*, *cesA*, *cesH*, *cesP*, *cesT*, *cesB*, *cesC*, and *cesD*, were absent in the genome of strain CYS06 (Table 6), indicating that this strain could not produce cereulide, enterotoxin T, hemolysin BL, non-hemolytic enterotoxin, or cytotoxin K.

Table 6. Toxin encoding genes screened in the genome of strain CYS06.

Enterotoxin	Major Encoding Genes	Reference [a]	CYS06
Cereulide	*cesA*, *cesH*, *cesP*, *cesB*, *cesC*, *cesD*	DQ360825.1	Negative
Enterotoxin T	*bacT*	D17312.1	Negative
Hemolysin BL	*hbA*, *hblB*, *hblC*, *hblD*	AJ007794.1	Negative
Non-hemolytic enterotoxin	*nheA*, *nheB*, *nheC*	Y19005.2	Negative
Cytotoxin K	*cytK*	AJ277962.1	Negative

[a] GenBank accession number of the reference genes.

Antibiotic resistance genes were predicted in the genome of strain CYS06 using the CARD database, and the *bacA*, *fosb*, *lmrb*, and *tetL* genes were identified, which were associated with resistance to bacitracin, fosfomycin, lincomycin, and tetracycline. However, the strain CYS06 was not resistant to these antimicrobial agents. Furthermore, the mobile genetic elements were not detected at the up- and down-stream of the resistance genes.

3.5. Challenge Test

The biosafety of strain CYS06 in grass carp was tested via intraperitoneal injection. The challenge test results showed that there were no deaths or clinical signs in the fish during the experimental period; moreover, no bacillus strain was isolated from the experimental fish, indicating that the strain CYS06 has good biosafety for grass carp.

3.6. Biocontrol Efficacy

3.6.1. Resistance against *A. hydrophila*

An *A. hydrophila* infection experiment was carried out on grass carp fed with *B. velezensis*. The cumulative mortality rate of grass carp fed the strain CYS06 at the dose of 10^7 CFU/g was 26.67%, whereas the cumulative mortality rate of grass carp fed the strain CYS06 at the dose of 10^6 CFU/g was 53.33%. The cumulative mortality rate of grass carp in the control group was 73.33%. The RPSs of the strain CYS06 at the dosages of 10^7 CFU/g and 10^6 CFU/g were 63.63% and 27.27%, respectively. This study suggested that the addition of *B. velezensis* could improve the survival rate of grass carp against *A. hydrophila*. The results revealed that strain CYS06 as an additive could improve bacterial disease resistance in grass carp.

3.6.2. Resistance against *F. columnare*

Compared with *B. subtilis* Bs168, *B. velezensis* CYS06 could significantly inhibit the growth of strain Fc001. No viable Fc001 was detected within 12 h when the CYS06 and Fc001 strains were co-cultured, indicating that strain CYS06 could inhibit the growth of strain Fc001 and kill it in a relatively short time (Figure 5). For the strain Bs168 without antagonistic activity, the Fc001 grew rapidly in the early stage (within 24 h), and approximately 10^4 CFU/mL of Fc001 were detected at the end of the experiment. The growth rate of strain Fc001 was not significantly inhibited by hte Bs168 strain, but was inhibited by the CYS06 strain, indicating that strain CYS06 is a potential biocontrol agent for the control of *F. columnare*.

The CFUs of strain Fc001 on the gills were significantly lower in experiment group 2 than those in the experiment group 1 and experiment group 3, especially in the early stage within 36 h (Figure 6), indicating that strain CYS06 could inhibit the *F. columnare* on the gills of grass carp. However, there was no significant difference in the CFUs of strain Fc001 on the gills of grass carp between experiment group 1 and experiment group 3 (Figure 6), indicating that strain Bs168 could not significantly inhibit *F. columnare* on the gills of grass carp.

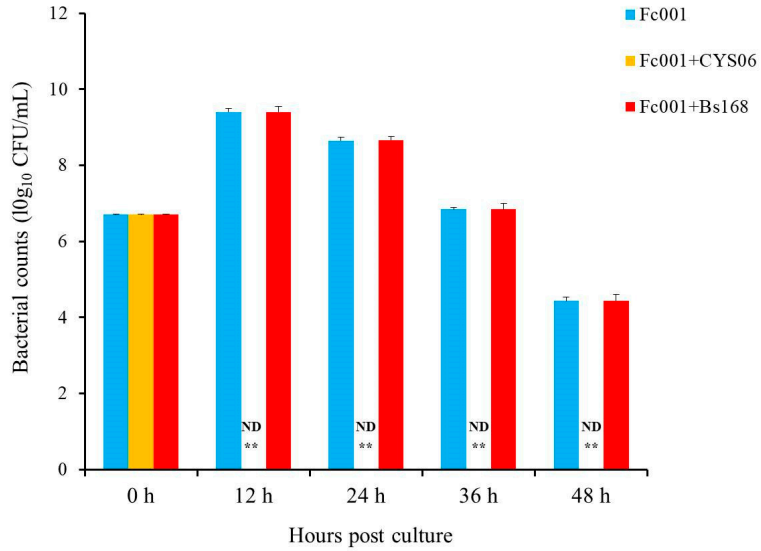

Figure 5. The *F. columnare* growth of strain Fc001 co-cultured with CYS06 and Bs168 strains. ** $p < 0.01$.

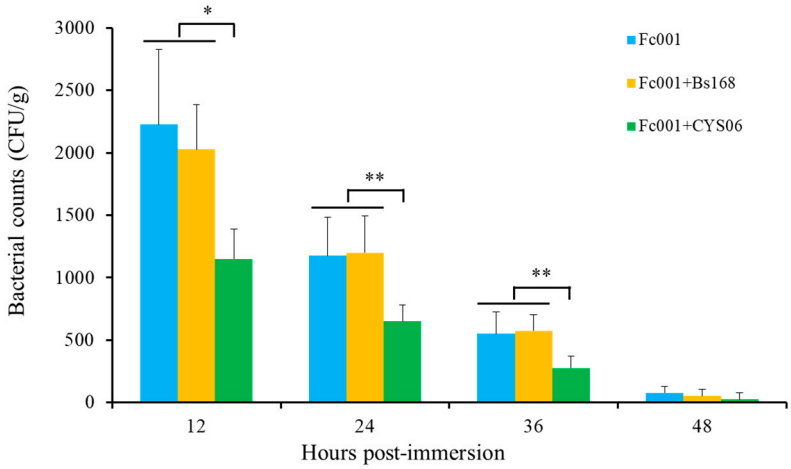

Figure 6. Bacterial counts of the *F. columnare* strain on the gill tissues of grass carp in different immersion groups. *, $p < 0.05$; **, $p < 0.01$.

4. Discussion

Probiotics, including bacteria, yeast, and actinomycetes, are considered to be environment-friendly biological control agents for the prevention and control of fish diseases. Antagonistic probiotics are commonly used to biocontrol aquatic animals pathogens [17,19,37], including *A. hydrophila*, *Aeromonas salmonicida*, *S. agalactiae*, *V. harveyi*, and *V. cholerae*. *B. velezensis* exhibits highly antagonistic effects against various aquatic animals pathogens, representing a new research hotspot for the biological control of bacterial diseases in aquatic animals [21]. However, limited probiotics are available for the prevention and control of grass carp bacterial diseases. Given that *Bacillus* could form endospores to protect it from extreme stresses, the *Bacillus* strain with antagonistic activity against *Aeromonas* sp. and *F. columnare* was screened to control fish bacterial diseases. Among 112 strains of *Bacillus*, 9 strains showed antagonistic activity, and strain CYS06 exhibits the strongest antagonistic activity. The strain CYS06, exhibiting several highly antagonistic fish pathogens, was isolated from the intestine of healthy grass carp, and this strain was identified as *B. velezensis*, based on molecular identification, including sequence analysis of the 16S rRNA, *gyrA*, ANI, and DDH.

Previous reports showed that *B. velezensis* possessed anti-microbial active compounds against a broad range of fish pathogens, including the bacteria belonging to the genus of *Aeromonas*, *Edwardsiella*, *Streptococcus*, *Vibrio*, *Nocardia*, and *Salmonella* [10,16,27]. In this

study, *B. velezensis* CYS06 exhibited broad-spectrum antagonistic activity against *Aermonas* spp., *E. ictaluri*, and *F. columnare*. Specifically, the *A. schubertii* WL-23 strain showed multiple resistance characteristics, such as resistance to macrolides, tetracyclines, aminoglycosides, β-lactams, chloramphenicols, lincomycin, sulfonamides, and rifampicin (data published in Chinese). The strain CYS06 can significantly inhibit the WL-23 strain, indicating a difference in the antibacterial mechanism between CYS06 and common antibiotics. These results suggest that the antibacterial substances secreted by the strain CYS06 can be used for screening new drugs. *B. velezensis* could produce antimicrobial substances such as polyketides, lipopeptides, and peptides, which inhibit bacteria and fungi [21]. The secondary metabolites produced by *B. velezensis* involve bacillibactin, fengycin, bacilysin, surfactin, difficidin, and mersacidin, which exhibit broad antagonistic activities against pathogenic bacteria or fungi [22]. For example, anti-microbial substances such as bacillibactin chelated iron, fengycin altered cell wall permeability, bacilysin hindered glucosamine synthesis, surfactin destroyed cell membrane, and difficidin inhibited the protein biosynthesis of bacteria [23]. Mersacidin belongs to the type B lantibiotics, which can inhibit bacteria by two methods: the inhibition of cell wall biosynthesis by scavenging the peptidoglycan precursor lipid II, and lysis of the cell membrane by forming pores [38]. In this study, eight biosynthesis gene clusters related to bacilysin, mersacidin, bacillibactin, surfactin, fengycin, macrolactin H, difficidin, and bacillaene in the genome of strain CYS06 were predicted using antiSMASH software, indicating that strain CYS06 has the ability to produce a wealth of secondary metabolites with antagonistic activity. Moreover, the antimicrobial substances of *B. velezensis* showed high thermal stability, broad pH tolerance, and resistant to enzyme digestion in the previously described study [27,39]. Thus, we deduce that the potential antimicrobial substances produced by CYS06 could exert antibacterial effects in various environments, indicating that this strain has promising applications. The application of strain CYS06 or its secondary metabolites could reduce the use of antibiotics in aquaculture.

B. velezensis could secrete hydrolases, such as protease, chitinase, cellulase, and glucanase, which could significantly control plant diseases and promote plant growth [24,40]. The strain CYS06 possesses high protease activity, which can promote its antibacterial properties; moreover, the protease, amylase, cellulase, and lipase secreted by CYS06 could promote the digestion and utilization of nutrient substances in the fish gastrointestinal tract, if this strain is used as a feed additive. In this study, the genes related to protease, α-amylase, cellulase, and lipase were confirmed by genome CAZymes analysis. The gene families related to cellulase and hemi-cellulase (GH5, GH11, GH26, GH43, GH51, and GH53), starch hydrolases (GH13 and GH126), and chitin degradation (GH18, GH23, CE9, and CBM50) were found in the genome of strain CYS06, indicating that it has a broad range of applications in agriculture and industry.

The pathogenicity of the strain CYS06 was evaluated, suggesting that this strain is safe for grass carp. Additionally, the presence of risk associated genes, including antibiotic resistance genes, and biogenic amine and enterotoxin genes in the CYS06 genome, were evaluated. The results showed that the major biogenic amine encoding genes and enterotoxin genes were absent in the genome of strain CYS06. Furthermore, there was a low associated risk of resistance gene lateral transfer due to the absence of mobile elements within the gene vicinity. In the previous report, most *B. velezensis* strains (96.7%) possessed potential tetracycline resistance gene (*tetL*), but no strains harbored any acquired antimicrobial resistance genes, indicating that *B. velezensis* possesses a low risk in terms of antibiotic resistance [41]. These results showed that the strain CYS06 has good biosafety.

The commercial puffed diet supplemented with strain CYS06 significantly enhanced the ability of grass carp to fight *A. hydrophila* infection compared with that noted for the control diet, and the RPS was 63.63% at the concentration of 10^7 CFU/g, indicating that dietary supplementation with strain CYS06 enhances disease resistance. In addition, co-cultivation of the CYS06 and Fc001 strains in vitro resulted in the death of *F. columnare* within 12 h; moreover, the immersion experiment showed that strain CYS06 could inhibit the growth of *F. columnare* Fc001 on the gills of grass carp, indicating that strain CYS06 has

the ability to prevent and control columnaris disease caused by *F. columnare*. Therefore, strain CYS06 can be used as a promising biocontrol agent for bacterial disease control in grass carp aquaculture.

5. Conclusions

Strain CYS06, with antagonistic activity and hydrolase activity, was isolated from the intestines of healthy grass carp. The genome of this strain was sequenced, and its genome was found to contain many gene clusters encoding for antagonistic metabolites and extracellular enzymes (protease, amylase, cellulase, and lipase). Importantly, the risk-associated genes, such as transferrable ARGs, biogenic amine producing genes, and enterotoxin genes, were absent in its genome, indicating that strain CYS06 is safe. Feeding grass carp diets supplement with strain CYS06 could improve grass carp resistance to *A. hydrophila* infection. Additionally, strain CYS06 could inhibit *F. columnare*, based on the co-cultivation test and the immersion experiment. Strain CYS06 can be used as a promising biocontrol agent to improve bacterial disease control in grass carp aquaculture.

Author Contributions: Conceptualization, D.Z. and C.S.; methodology, L.L. and D.J.; validation, Y.R. and J.Y.; formal analysis, D.Z. and Y.R.; investigation, Y.W. and Y.R.; resources, C.S. and D.Z.; writing—original draft preparation, D.Z. and L.L.; writing—review and editing, Q.W. and C.S.; visualization, L.L. and D.J.; supervision, Q.W.; project administration, Q.W.; funding acquisition, C.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by Earmarked Fund for China Agriculture Research System (CARS-45), the Guangdong Provincial Special Fund for Modern Agriculture Industry Technology Innovation Teams (2023KJ150), and the Central Public-Interest Scientific Institution Basal Research Fund, CAFS (2023ZX06).

Institutional Review Board Statement: The study was approved by the Laboratory Animal Ethics Committee, Pearl River Fisheries Research Institute (Approval Code: CAFS LAEC-PRFRI-2022-03-48).

Data Availability Statement: Data are contained within the article.

Acknowledgments: We are grateful to Yanxia Gao and Xiuling Xiong for preparing a part of the experimental materials.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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