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The protective effect of fish-derived cathelicidins on bacterial infections in zebrafish, *Danio rerio*

Chen Chen[a], Aili Wang[d], Fen Zhang[b], Minghui Zhang[b], Huaixin Yang[c], Jianan Li[a], Pengchao Su[a], Yan Chen[b], Haining Yu[c,*], Yipeng Wang[b,**]

[a] Chinese-German Joint Laboratory for Natural Product Research, College of Biological Science and Engineering, Shaanxi University of Technology, Hanzhong, Shaanxi, 723000, China
[b] Department of Pharmaceutical Sciences, College of Pharmaceutical Sciences, Soochow University, Suzhou, Jiangsu, 215123, China
[c] Department of Bioscience and Biotechnology, Dalian University of Technology, Dalian, Liaoning, 116023, China
[d] Weifang University of Science and Technology, Shouguang, Shandong, 262700, China

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ABSTRACT

Antibiotic-resistant bacteria are severe threats to aquaculture industry. Boosting and modulating host immune responses has been proved to be an effective strategy to combat with bacterial infections and there is an urgent need for novel immunomodulators. Cathelicidins is an important family of host defense peptides (HDPs) that possess direct antimicrobial activities and potent immunomodulatory properties. Several cathelicidins have been identified and characterized from diverse fish species. Considering the relatively conserved immune systems between different fish species, it is reasonable to speculate that cathelicidins from different fish species possess immunomodulating functions on the other fish species. In the present study, two fish-derived cathelicidins (CATH_BRALE and codCath1) were selected to investigate their protective effect on zebrafish with bacterial infections. They exhibited potent and broad-spectrum antimicrobial activities against the tested aquatic Gram-positive and Gram-negative pathogenic bacteria, with MIC values ranging 2.34–18.75 μg/ml for CATH_BRALE and 2.34–37.5 μg/ml for codCath1. And their antimicrobial effect is so rapid that they killed the bacteria within 60 min. Unlike conventional antibiotics, they kill bacteria by inducing bacterial membrane permeabilization and cell disruption. Besides direct antimicrobial activity, CATH_BRALE and codCath1 exhibited potent immunomodulatory functions by both inhibiting bacteria induced zebrafish pro-inflammatory cytokine gene (TNF-α, IL-1β, and IL-6) expression and stimulating zebrafish chemokine gene IL-8 expression. *In vivo* challenge test proved that they could significantly decrease the bacterial numbers and enhance the survival rates of zebrafish. All the results above imply the great potential of CATH_BRALE and codCath1 as novel peptide immunomodulators in fish aquaculture industry.

1. Introduction

To satisfy human's increasing requirement for protein, the world aquaculture has been experiencing rapid expansion [1]. The intensive culture of aquatic animals brings along more and more severe diseases that cause economical and welfare issues [2]. Among them, bacterial infections represent one kind of common diseases that cause severe economic losses for the aquaculture industry. In present, the treatment of bacterial infectious diseases with conventional antibiotics is still the most favorable strategy [3]. On the other hand, the increasing spread of antibiotic-resistant bacteria become a severe threat to aquaculture industry. Therefore, there is an urgent need for developing novel strategies to control the spread of antibiotic-resistant bacteria induced diseases in aquaculture industry. In the past decades, prophylactic approaches are regarded as another effective means for overcoming bacterial diseases in aquaculture [1,4,5]. Past experiences have proved that boosting host's immune responses to pathogens is an attractive strategy. Therefore, development of novel immunomodulators as adjuvants in vaccines and/or immunostimulants in feed were major research hotspots in the recent decades [6–8]. Being highly heterogeneous group of compounds, adjuvants and immunostimulants have one thing in common: the ability to enhance the immune responses of aquatic

* Corresponding author. Department of Bioscience and Biotechnology, Dalian University of Technology, No. 2 Linggong Road, Dalian, 116023, China.
** Corresponding author. Department of Pharmaceutical Sciences, College of Pharmaceutical Sciences, Soochow University, No. 199 Renai Road, Suzhou, 215123, China.
E-mail addresses: yuhaining@dlut.edu.cn (H. Yu), yipengwang@suda.edu.cn (Y. Wang).

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animals.

Host defense (also referred to as “antimicrobial”) peptides are a class of gene-encoded peptides that broadly distribute in nearly all sorts of multi-organisms [9]. They are key players of innate immune system and play critical roles in host immune responses against microbial invasions [10,11]. HDPs possess direct antimicrobial activities against nearly all sorts of microorganisms, such as bacteria, fungi, viruses, and even parasites [12]. Therefore, they usually are considered having potential as new broad-spectrum antimicrobial agents [12]. Besides direct antimicrobial function, most HDPs also broadly participate in boosting and modulating host immune responses, which gains more and more research interests [10,11,13].

In the present study, the immuno-protective effect of two fish-derived HDPs (CATH_BRALE and codCath1) on zebrafish *Danio rerio* was studied. The direct antimicrobial activity and antimicrobial mechanism of CATH_BRALE and codCath1 against aquatic bacteria were investigated. The immunomodulatory and *in vivo* protective effects of CATH_BRALE and codCath1 on zebrafish were determined. The present study provides theoretical support for application of CATH_BRALE and codCath1 as potential immunomodulators in fish aquaculture industry.

2. Materials and methods

2.1. Zebrafish, bacteria and peptides

Zebrafish wild type AB strain (weighing 0.3 ± 0.1 g, 3-5 month-old) were purchased from a zebrafish breeding corporation in Shanghai, China. The zebrafish were maintained in circulating filtered water tanks at 20–25 °C. Fish were fed twice a day and maintained under conditions described upon for two weeks before experiment. The animal experimental protocols in the present study were approved by the Animal Care and Use Ethics Committee of Dalian University of Technology.

Bacteria used in the present study (including 10 Gram-negative bacteria: *Aeromonas sobria, Aeromonas hydrophila, Aeromonas veronii, Vibrio harveyi, Vibrio parahaemotyticus, Vibrio anguillarum, Vibrio vulnificus, Vibrio splendidus,* and *Vibrio cholera*, and 4 Gram-positive bacteria: *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Nocardia asteroides*) were collected by our group and stored in our laboratory. The bacteria were cultured in nutrient broth (Oxoid, UK) at 37 °C.

CATH_BRALE and codCath1 were synthesized by GL Biochem Ltd. (Shanghai, China). The crude peptides were purified by RP-HPLC and analyzed by MALDI-TOF MS. Purity of the synthetic peptides was confirmed to be higher than 95%. The physicochemical parameters of CATH_BRALE and codCath1 were shown in Table 1.

2.2. Antimicrobial assay

A standard two-fold broth microdilution method described in the previous studies was used in the present study to determine the antimicrobial activity of CATH_BRALE and codCath1 against the selected aquatic pathogenic bacteria [14,15]. Briefly, bacteria were cultured in nutrient broth (Oxoid, UK) at 37 °C to exponential phase and diluted with fresh nutrient broth to 10^6 CFU/ml. Serial dilutions of CATH_BRALE and codCath1 were prepared in 96-well microtiter plates and mixed with equal volume of the prepared bacteria inoculum to an ultimate volume of 100 μl per well. The plates were incubated at 37 °C for 18 h and the minimal concentrations at which no visible bacterial growth occurred were recorded as MIC values. The conventional antibiotic ampicillin and terramycin were used as positive control.

2.3. Bacterial killing kinetic assay

Bacterial killing kinetic assay was conducted according to the method described previously with minor modifications to examine the antimicrobial speed of CATH_BRALE and codCath1 against aquatic pathogenic bacteria [14,15]. In brief, *V. parahaemotyticus* was cultured

Table 1
Physicochemical parameters of CATH_BRALE and codCath1.

Peptide	Sequence	Length	Net charge	Theoretical pI	Mw	Source
CATH_BRALE	RRSKARGGSRGSKMGRKDSKGGSRGRPGSGSRPGGGSSIAGASRGDRGGTRNA	53	+13	12.43	5200.7	*Brachymystax lenok* [2]
codCath1	RRSRSGRGSGKGGRGGSRGSSGSRGSKGPSGSRGSSGSRGSKGSRGGRSGRGSTIAGNGNRNNGGTRTA	69	+17	13.12	6550.9	*Gadus morhua* [17]

in nutrient broth to exponential phase at 37 °C and diluted to 10^6 CFU/ml. CATH_BRALE and codCath1 were added to a final concentration of 5 × MIC (11.7 and 46.9 μg/ml, respectively). The bacteria were incubated at 37 °C for 0, 10, 20, 30, 45, 60, 90, 120, and 180 min. At each time point, bacterial solutions (50 μl) were extracted and diluted 1000 times. 50 μl of the dilutions were coated on nutrient broth agar plates and incubated for 12 h at 37 °C. The viable colonies grown on the plates were recorded. Ampicillin was used as positive control and sterile deionized water was used as blank control.

2.4. Outer membrane permeabilization assay

Gram-negative bacterial outer membrane permeabilization assay was conducted according to the method described by Wang et al. [16]. *V. parahaemotyticus* and *A. veronii* were incubated in nutrient broth to exponential phase and diluted with 5 mM N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid (HEPES) buffer (pH 7.4, containing 5 mM glucose) to 2 × 10^8 CFU/ml. Bacteria were incubated with N-phenyl-1-naphthylamine (10 μM) for 30 min, and the background fluorescence was recorded for subtraction (excitation 350 nm, emission 420 nm) with Tecan Infinite M1000 PRO microplate reader (Tecan, Switzerland). 100 μl of bacterial suspension was mixed with equal volumes of peptide solution at concentrations ranging from 1 × MIC to 16 × MIC in a sterile 96-well black microtiter plate. The fluorescence was recorded over time until no further increase detected. The results were converted into percentage of NPN uptake using the following equation:

$$\text{NPN uptake (\%)} = (F_{obs} - F_{buffer}) / (F_{positive} - F_{buffer}) \times 100\%$$

Where F_{obs} is the observed fluorescence absorbance of peptides at a given concentration, F_{buffer} is the fluorescence of buffer, and $F_{positive}$ is the fluorescence of 10 μg/ml polymyxin B (Sigma-Aldrich, USA) which was known for its strong outer-membrane permeabilizing property and therefore was used as positive control in the present experiment.

2.5. Cytoplasmic membrane permeabilization assay

Cytoplasmic membrane permeabilization assay was conducted using the method described previously with minor modifications [15]. *V. parahaemotyticus* and *A. veronii* were cultured in nutrient broth to exponential phase and washed with sterile PBS buffer three times. The bacteria were stained with calcein-AM (5 μM in PBS, Yeasen, Shanghai, China) for 90 min at 37 °C and washed with PBS three times to remove the excessive stain. The bacterial pellets were resuspended with PBS (1 × 10^8 CFU/ml) and peptides were added to final concentrations ranging from 1 × MIC to 16 × MIC. The mixtures were incubated at 37 °C for 2 h, and centrifuged at 6000 rpm for 10 min. The calcein fluorescence leakage in the supernatant was recorded using a Tecan Infinite M1000 PRO microplate reader (Tecan, Switzerland) (excitation at 490 nm and emission at 515 nm). 100% calcein leakage was recorded by incubating bacteria with 1% Triton X-100 (v/v).

2.6. Cytotoxic assay

The cytotoxicity of CATH_BRALE and codCath1 was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, Sigma) method. Two fish cell lines: epithelioma papulosum cyprinid (EPC) carp cells and fathead minnow (FHM) muscle cells were used and the experiment was carried out according to our previous paper [14]. Briefly, the cells were cultured in M199 medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum in a humidified 5% CO_2 atmosphere at 25 °C. After digestion, the cells were diluted with M199 medium to approximately 2 × 10^5 cells/ml, seeded in 96-well plates and cultured overnight until adhesion. CATH_BRALE and codCath1 dissolved in serum-free M199 was added to wells and the plates were incubated at 25 °C for 48 h. The subsequent procedures followed the standard MTT method. Cell death induced by CATH_BRALE and codCath1 was calculated as the percentage of the negative control group, which was regarded as 100%.

2.7. Quantitative real-time PCR

V. parahaemotyticus was cultured in nutrient broth to exponential phase and washed with sterile PBS buffer three times. Pellet was resuspended with sterile PBS and adjusted to 5 × 10^7 CFU/ml. Adult zebrafish were injected with 10 μl of bacterial solutions to induce intraperitoneal infections. CATH_BRALE and codCath1 were dissolved in PBS buffer and sterilized by filtration. 10 mg/kg peptides were intraperitoneally injected into zebrafish immediately after bacterial challenge. Spleen and kidney tissues were collected at 0, 6, 12, and 24 h after infection from a total of five zebrafish of each group. All samples were stored at −80 °C until use.

Total RNA of zebrafish spleen and kidney tissues was isolated using TRIzol reagent (Life Technology, USA). PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan) was used to produce the 1st strand cDNA. SYBR Premix Ex Taq™ II (Tli RNaseH Plus) two-step qRT-PCR kit (Takara, Japan) was used to perform the qRT-PCR experiment on an ABI Prism 7000 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). 2-△△CT method was used to analyze the expression level of each target gene. The cycle counts of the target genes were normalized to β-actin gene, and accordingly the fold changes of the target genes were calculated. The primers used for qRT-PCR were listed in Table 2.

2.8. Challenge test

V. parahaemotyticus was incubated in nutrient broth to exponential phase, washed with sterile PBS buffer three times and resuspended in PBS to 5 × 10^7 CFU/ml. Zebrafish were randomly divided into four groups (20 per group): vehicle, bacteria only, bacteria + CATH_BRALE (10 mg/kg), and bacteria + codCath1 (10 mg/kg). Bacteria (10 μl, 5 × 10^7 CFU/ml) were injected into the peritoneal cavity of zebrafish, and then peptides were injected. After bacterial challenge, survival rates of zebrafish were recorded daily for 96 h.

2.9. In vivo bactericidal assay

V. parahaemotyticus was incubated in nutrient broth to exponential phase and washed with sterile PBS buffer three times. Zebrafish were randomly divided into three groups (10 per group): PBS group, CATH_BRALE group and codCath1 group. Zebrafish were intraperitoneally injected with *V. parahaemotyticus* (10 μl, 2 × 10^7 CFU/ml). After bacterial injection, zebrafish were immediately intraperitoneally injected with peptides (10 mg/kg) or equal volume of PBS (PBS group). 24 h later, the zebrafish were anesthetized in 0.2% tricaine and then euthanized via incubation in ice water for 15 min. Zebrafish were washed with sterile PBS buffer three times and cut into pieces. 10 ml of PBS was added and the zebrafish bodies were homogenized for 20 min. The homogenate solution was diluted with sterile PBS buffer 1000 times, 50 μl of the dilutions were plated on nutrient broth agar and incubated at 37 °C for 18 h. The number of viable bacterial colonies grown on the plates was recorded.

Table 2
Primers of zebrafish immune-related genes for qRT-PCR.

Name	Forward (5′-3′)	Reverse (3′-5′)
TNF-α	CTGCTTCACGCTCCATAAGA	CTGGTCCTGGTCATCTCTCC
IL-1β	TGGACTTCGCAGCACAAAATG	GTTCACTTCACGCTCTTGGATG
IL-6	AGACCGCTGCCTGTCTAAAA	TTTGATGTCGTTCACCAGGA
IL-8	GTCGCTGCATTGAAACAGAA	CTTAACCCATGGAGCAGAGG
β-actin	ATGGATGAGGAAATCGCTGC	ATGCCAACCATCACTCCCTG

Table 3
Antimicrobial activity of CATH_BRALE and codCath1.

Microorganisms	MIC (μg/ml)			
	CATH_BRALE	codCath1	Ampicillin	Terramycin
Gram-negative bacteria				
Aeromonas sobria	18.75 (3.6 μM)	18.75 (2.86 μM)	> 200	37.5 (81.45 μM)
Aeromonas hydrophila	9.38 (1.8 μM)	37.5 (5.72 μM)	> 200	> 200
Aeromonas veronii	9.38 (1.8 μM)	4.69 (0.72 μM)	> 200	37.5 (81.45 μM)
Vibrio harveyi	4.69 (0.9 μM)	18.75 (2.86 μM)	37.50 (100.9 μM)	75 (160.89 μM)
Vibrio parahaemotyticus	2.34 (0.45 μM)	9.38 (1.43 μM)	18.75 (50.49 μM)	18.75 (40.72 μM)
Vibrio anguillarum	9.38 (1.8 μM)	9.38 (1.43 μM)	> 200	75 (160.89 μM)
Vibrio vulnificus	2.34 (0.45 μM)	37.5 (5.72 μM)	> 200	> 200
Vibrio splendidus 1	18.75 (3.6 μM)	2.34 (0.36 μM)	> 200	150 (325.78 μM)
Vibrio splendidus 2	4.69 (0.9 μM)	4.69 (0.72 μM)	> 200	75 (160.89 μM)
Vibrio cholerae	4.69 (0.9 μM)	18.75 (2.86 μM)	> 200	> 200
Gram-positive bacteria				
Staphylococcus aureus 1	9.38 (1.8 μM)	2.34 (0.36 μM)	18.75 (50.49 μM)	37.5 (81.45 μM)
Staphylococcus aureus 2	18.75 (3.6 μM)	18.75 (3.6 μM)	> 200	> 200
Streptococcus agalactiae	9.38 (1.8 μM)	4.69 (0.72 μM)	37.50 (100.9 μM)	75 (160.89 μM)
Nocardia asteroides	4.69 (0.9 μM)	9.38 (1.8 μM)	18.75 (50.49 μM)	> 200

MIC, minimal inhibitory concentration. The results represent mean values of three independent experiments.

2.10. Statistical analysis

Each experiment was performed in triplicate for three times. The data was analyzed by one-way analysis of variance (ANOVA) test with a Bonferroni post test using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered highly significant.

3. Results

3.1. In vitro antimicrobial activities of CATH_BRALE and codCath1 against aquatic pathogens

CATH_BRALE and codCath1 were chemically synthesized and their *in vitro* antimicrobial activities against common aquatic bacterial pathogens were first determined. The results were listed in Table 3. Both CATH_BRALE and codCath1 exhibited potent and broad-spectrum antimicrobial activities against the tested Gram-positive and Gram-negative aquatic bacteria, with MIC values ranging from 2.34 to 18.75 μg/ml for CATH_BRALE and 2.34–37.5 μg/ml for codCath1. Among the 14 tested bacteria, *Vibrio parahaemotyticus* and *Vibrio vulnificus* showed the highest sensitivity toward CATH_BRALE with MIC value of 2.34 μg/ml, while *Vibrio splendidus* 1 showed the highest sensitivity toward codCath1 with the same MIC value of 2.34 μg/ml. Moreover, 8 strains of the 14 tested bacteria were ampicillin-resistant (MIC values > 200 μg/ml) and 5 of them were terramycin-resistant (MIC values > 200 μg/ml), which imply the potential of CATH_BRALE and codCath1 in the treatment of aquatic animal infections induced by drug-resistant pathogens.

3.2. Bacterial killing kinetics of CATH_BRALE and codCath1

To determine the antimicrobial speed of CATH_BRALE and codCath1 against aquatic pathogens, a standard bacterial killing kinetic assay was performed. As illustrated in Table 4, CATH_BRALE and codCath1 exhibited a rapid killing effect toward the tested *V. parahaemotyticus*. At a concentration of 5 × MIC, CATH_BRALE and codCath1 killed the bacteria within 60 min, while the positive control ampicillin needed at least 180 min. Besides, it can be observed that when the incubation time extended to 180 min, the colony forming units (CFUs) in CATH_BRALE and codCath1 treated groups remained zero, which implied that the antimicrobial effect of CATH_BRALE and codCath1 was bactericidal rather than bacteriostatic.

3.3. Antimicrobial mechanism of CATH_BRALE and codCath1

Previous studies about CATH_BRALE and codCath1 didn't examine their antimicrobial mechanisms [2,17]. In the present study, outer membrane permeabilization and cytoplasmic membrane permeabilization assays were carried out to study the possible effect of CATH_BRALE and codCath1 on bacterial outer and cytoplasmic membrane integrity. As shown in Fig. 1A, both CATH_BRALE and codCath1 showed a dose-dependent permeabilization effect on the outer membrane of *V. parahaemotyticus*. At a concentration of 16 × MIC (7.2 μM for CATH_BRALE, 22.88 μM for codCath1), CATH_BRALE could induce the outer membrane permeabilization of 95.06%, while codCath1 induced 91.64% permeabilization. Similarly, at the same concentration, CATH_BRALE and codCath1 induced *A. veronii* outer membrane permeabilization of 94.69% and 89.12%, respectively (Fig. 1B). Next, the cytoplasmic membrane permeabilization assay was carried out using the fluorescent dye calcein-AM to determine the effect of peptides on the integrity of bacterial cytoplasmic membrane. As shown in Fig. 1C and D, CATH_BRALE and codCath1 significantly induced the release of fluorescent dye calcein from *V. parahaemotyticus* and *A. veronii* cells in a dose-dependent manner. At a concentration of 16 × MIC (7.2 μM for CATH_BRALE, 22.88 μM for codCath1), CATH_BRALE induced 71.8% calcein release from *V. parahaemotyticus* and 71.54% calcein release from *A. veronii*, while codCath1 induced 68.54% release from *V. parahaemotyticus* and 67.91% release from *A. veronii*. The results above indicate that CATH_BRALE and codCath1 induce microbial membrane permeabilization and cell disruption, which ultimately result in cell death.

3.4. Cytotoxicity of CATH_BRALE and codCath1

Using MTT method, the cytotoxicity of CATH_BRALE and codCath1 toward fish cells was determined. Both CATH_BRALE and codCath1 exhibited very low cytotoxic effect on the tested EPC and FHM cells. At a concentration of 200 μg/ml, CATH_BRALE induced cell death rates of 6.4% and 8.5% for EPC and FHM, respectively. At the same concentration, codCath1 induced 7.6% and 9.7% cell death for EPC and FHM, respectively.

3.5. Effect of CATH_BRALE and codCath1 on immune-related gene expression in zebrafish

To examine whether CATH_BRALE and codCath1 function as immunomodulatory factors in zebrafish, the mRNA expression patterns of

Table 4
Killing kinetics of CATH_BRALE and codCath1 against *Vibrio parahaemotyticus*.

Time	Colony Forming Units ($\times 10^3$, CFUs/ml)								
	0 min	10 min	20 min	30 min	45 min	60 min	90 min	120 min	180 min
CATH_BRALE	76 ± 8.2	55 ± 5.6	65 ± 12.2	58 ± 6.2	14 ± 2.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
codCath1	55 ± 8.6	59 ± 9.2	65 ± 5.9	44 ± 8.2	8 ± 1.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Ampicillin	65 ± 11.5	73 ± 8.8	79 ± 12.8	64 ± 8.8	54 ± 10.7	25 ± 4.7	14 ± 3.2	8 ± 2.4	0 ± 0.0
Sterile H_2O	69 ± 9.6	66 ± 7.9	55 ± 12.9	68 ± 14.8	97 ± 15.6	138 ± 28.2	188 ± 43.4	347 ± 48.5	1172 ± 354.8

Vibrio parahaemotyticus was mixed with CATH_BRALE and codCath1 at concentration of 5 × MIC for 0, 10, 20, 30, 45, 60, 90, 120 and 180 min. The MICs of CATH_BRALE, codCath and ampicillin against *Vibrio parahaemotyticus* are 2.34, 9.38, and 18.75 μg/ml, respectively. The results represent mean values of three independent experiments.

four immune-related genes in different tissues with or without peptides treatment were studied using quantitative Real-time PCR. The pro-inflammatory cytokine genes TNF-α, IL-1β, and IL-6, as well as the chemokine gene IL-8 were studied.

As shown in Fig. 2, in zebrafish spleen, *V. parahaemotyticus* infection significantly increased the mRNA expression level of TNF-α, IL-1β, IL-6, and IL-8 in a time-dependent manner. At the time point of 12 h post-infection, expression of the four genes reached the highest level. After that, the expression levels started to decline and at 24 h post-infection the levels reached nearly their background values at 0 h. 10 mg/kg of CATH_BRALE and codCath1 significantly inhibited the expression of pro-inflammatory cytokine genes TNF-α, IL-1β, and IL-6 within 12 h post-infection (Fig. 2A–C). However, it is interesting to observe that CATH_BRALE and codCath1 treatment could significantly increase the expression level of chemokine gene IL-8 in peptide-treated groups compared with the bacteria-only group (Fig. 2D).

Significantly inhibitory effects of CATH_BRALE and codCath1 on pro-inflammatory cytokine gene expression and stimulatory effect on chemokine gene expression also could be observed in zebrafish kidney (Fig. 3). The difference was that compared with the reference gene β-actin, the expression level of the four tested genes was higher in zebrafish spleen than in kidney. All the results above indicated that CATH_BRALE and codCath1 could serve as effective immunomodulatory factors in zebrafish by inhibiting bacteria-induced inflammation and stimulating phagocytic cell-based innate immune responses.

3.6. CATH_BRALE and codCath1 enhance zebrafish survival rate in bacterial infection

After bacterial challenge and peptide treatment, the survival rate of zebrafish was recorded every 12 h for 96 h. As shown in Fig. 4, the survival rate of zebrafish in PBS group remained 100% in the 96 h. For bacteria only group, the survival rate decreased from 100% at 0 h to 36.7% at 96 h post-infection. 10 mg/kg of CATH_BRALE and codCath1 could significantly enhance the survival rate of zebrafish. The survival rate in CATH_BRALE treated group was 68.3% at 96 h post-infection ($P < 0.05$), while in codCath1 treated group the value was 60% ($P < 0.05$).

3.7. CATH_BRALE and codCath1 decrease bacterial numbers in zebrafish

To determine the *in vivo* antimicrobial activities of CATH_BRALE and codCath1, zebrafish were infected with *V. parahaemotyticus* and the bacterial numbers in zebrafish after peptides treatment were recorded. As shown in Fig. 5, compared with PBS group, the bacterial numbers in CATH_BRALE and codCath1 groups were significantly lower ($P < 0.01$), which apparently indicated that CATH_BRALE and codCath1 could significantly enhance the zebrafish's anti-infective ability and decrease the bacterial numbers in face of bacterial infections.

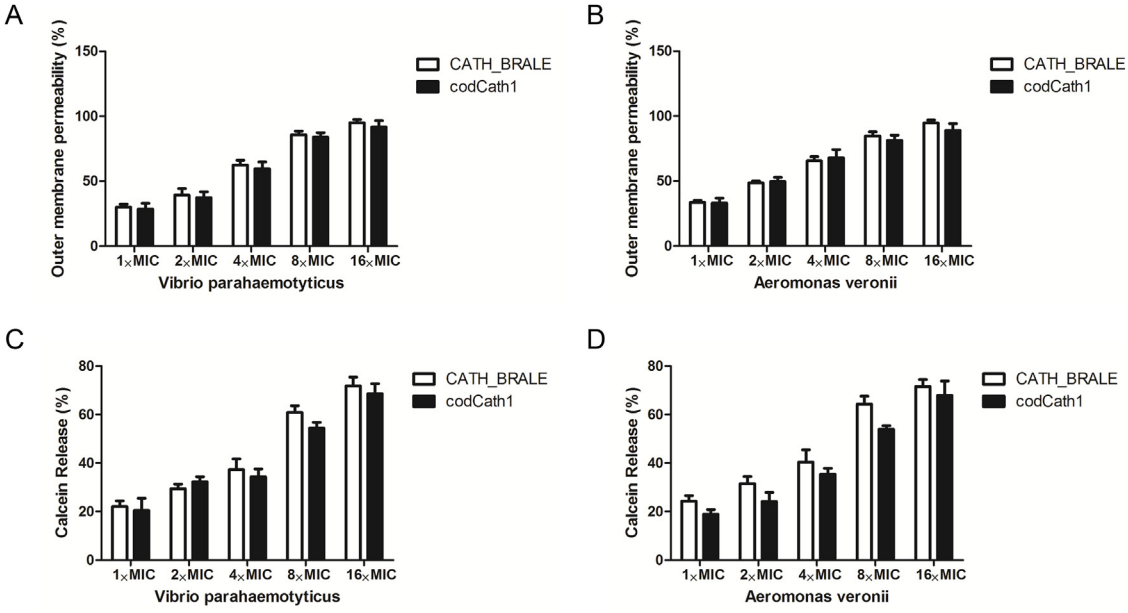

Fig. 1. Bacterial outer membrane and cytoplasmic membrane permeabilization induced by CATH_BRALE and codCath1. (A) *V. parahaemotyticus* outer membrane permeabilization; (B) *A. veronii* outer membrane permeabilization; (C) Calcein release from *V. parahaemotyticus*; (D) Calcein release from *A. veronii*.

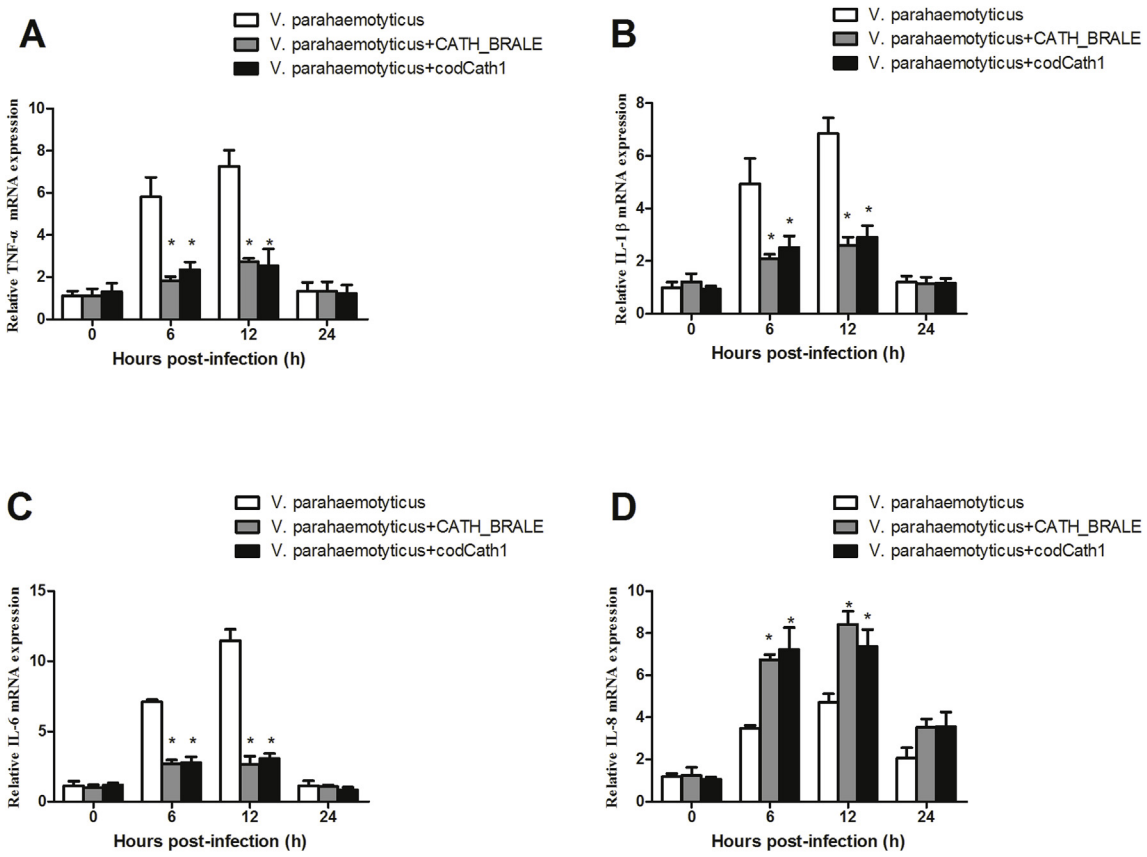

Fig. 2. Effect of CATH_BRALE and codCath1 on expression profiles of the immune-related cytokine genes in zebrafish spleen after *V. parahaemotyticus* challenge. (A) TNF-α; (B) IL-1β; (C) IL-6; (D) IL-8. Data are mean ± SEM values of three separate experiments. *P < 0.05 significantly different from the bacteria-only group.

4. Discussion

In recent years, there has been an increasing understanding of diversity of vertebrate HDPs in general. There are more and more kinds of HDPs being identified and characterized. For example, currently at least seven families of HDPs has been described in humans. They are α-defensins with six members, β-defensins with four members, cathelicidin with a single member LL-37, histatins with three members, hepcidin with one member, dermcidin with one member, and two recently described thrombin-induced platelet thrombocidins [18–20]. Among the identified vertebrate HDPs, cathelicidins represent an important family that ubiquitously distributed in all vertebrate species. The structure of cathelicidins precursors comprise three parts: an N-terminal signal peptide (30 residues), a highly conserved cathelin domain (99–114 residues) and a heterogenic C-terminal mature peptide (12–100 residues) [21,22]. The mature peptides of cathelicidins are released from precursors after cleavage by different proteases and start to exert their functions [21,23]. Most cathelicidins possess potent, broad-spectrum and rapid antimicrobial properties against a variety of microorganisms, including a large number of drug-resistant pathogens [15,22,24]. Therefore, they are regarded as a potential kind of next generation antimicrobial agents. Currently, four cathelicidin analogs are undergoing clinical trials as novel anti-infective drugs, including cattle indolicidin analogue omiganan (MBI-226, CPI226), cattle indolicidin analogue MX-594AN, pig protegrin-1 analogue iseganan (IB-367), and snake cathelicidin BF-30 [24–27]. Besides antimicrobial activity, many cathelicidins possess other functions, such as immunomodulating activity, immune cell chemoattractant activity, wound healing activity, pro-angiogenic activity, pro-apoptotic activity, and adjuvant activity [13,21,28]. The anti-infective functions of several cathelicidins have been proved in *in vivo* animal models, showing that they could effectively rescue animals from severe infections, and the results of mechanism study revealed that immunomodulation instead of direct microbial killing activity is more important for their *in vivo* anti-infective function [15,29–31]. To date, several cathelicidins have been identified and characterized from different fish species, including cathelicidins from *Acipenser dabryanus* [32], *Brachymystax lenok* [2], *Gadus morhua* [33,34], *Plecoglossus altivelis* [35], *Salmo trutta fario* [36], *Salvelinus fontinalis* [36], *Thymallus thymallus* [36], *Salvelinus alpinus* [17], *Oncorhynchus mykiss* [37,38], *Salmo salar* [37], and *Myxine glutinosa* [39]. However, the immunomodulatory function of these fish-derived cathelicidins on host immune system has not been well studied. Zebrafish is a kind of widely used animal model due to its small size, rapid life cycle, and huge offspring [40]. The objective of the present study is to evaluate the immuno-protective effect of two fish-derived HDPs (CATH_BRALE and codCath1) on zebrafish, which are challenged with fatal bacterial pathogens. By this study, we hope to provide novel immunomodulators for aquaculture industry to combat with severe bacterial infections.

CATH_BRALE and codCath1 were two novel cathelicidins identified from *Brachymystax lenok* and *Gadus morhua*, respectively [2,17]. Previous studies have indicated that CATH_BRALE and codCath1 possess potent antimicrobial activity against several kinds of microorganisms [2,33]. In our preliminary experiments, we synthesized most fish-derived cathelicidins identified to date, and examined their antimicrobial and immunomodulatory activities. Among the selected fish-derived cathelicidins, CATH_BRALE and codCath1 exhibited relatively the most potent antimicrobial and immunomodulatory functions (data not shown). Therefore, in the present study we selected CATH_BRALE and codCath1 for further study to evaluate their protective effects on bacterial infections in zebrafish. First, we found that both CATH_BRALE and codCath1 possess potent and broad-spectrum antimicrobial activity

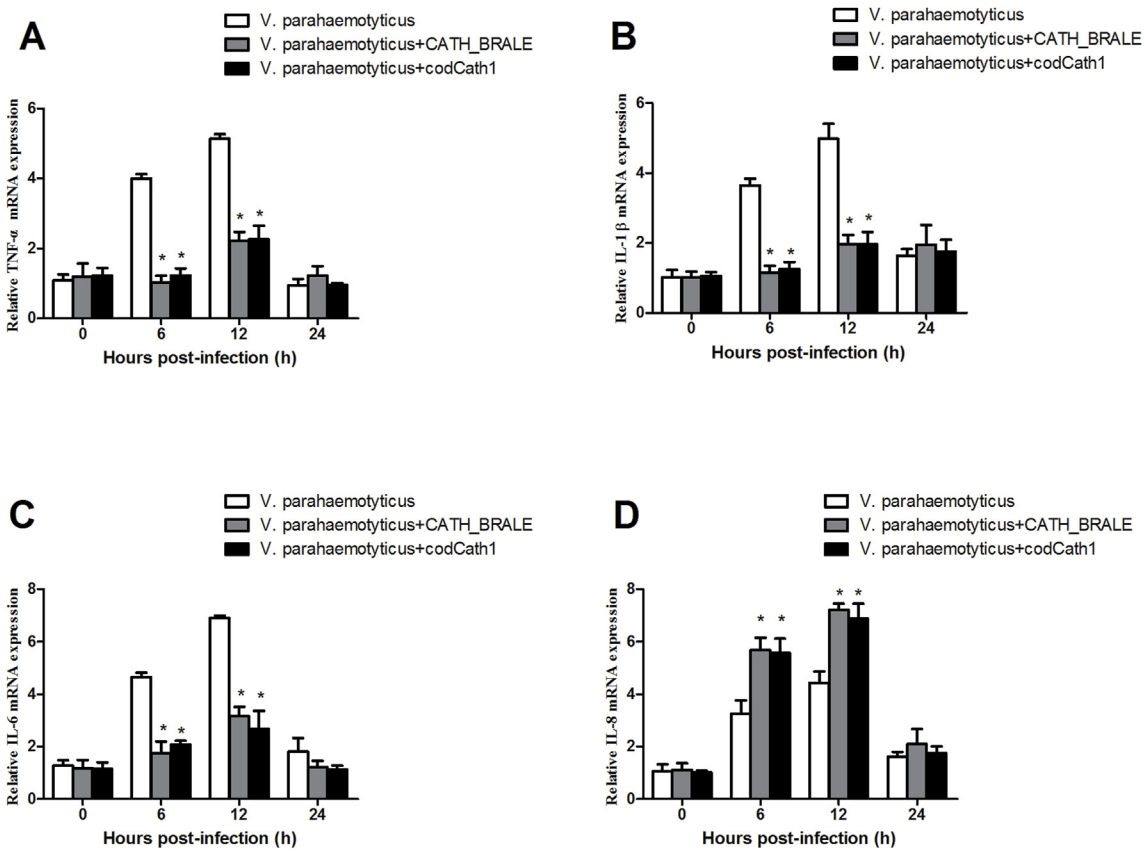

Fig. 3. Effect of CATH_BRALE and codCath1 on expression profiles of the immune-related cytokine genes in zebrafish kidney after *V. parahaemotyticus* challenge. (A) TNF-α; (B) IL-1β; (C) IL-6; (D) IL-8. Data are mean ± SEM values of three separate experiments. *P < 0.05 significantly different from the bacteria-only group.

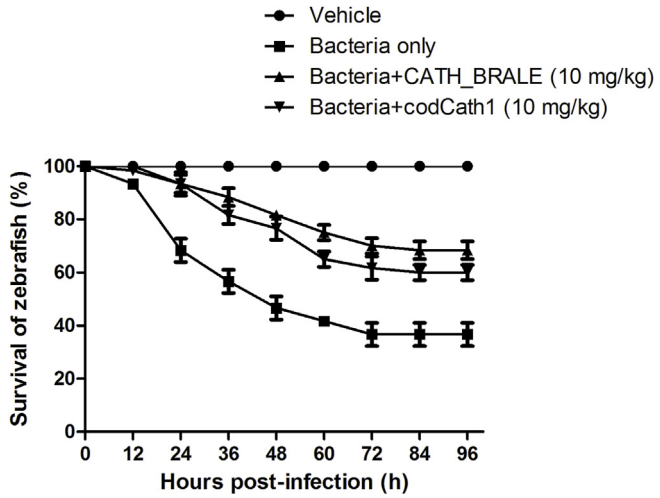

Fig. 4. Effect of CATH_BRALE and codCath1 on survival rate of zebrafish infected with *V. parahaemotyticus*. Peptides (10 mg/kg) and bacteria were simultaneously injected into zebrafish. Survival rates were recorded every 12 h for up to 96 h after bacterial challenge. Survival curves were significantly different (P < 0.01) between bacteria-only and peptide-treated groups.

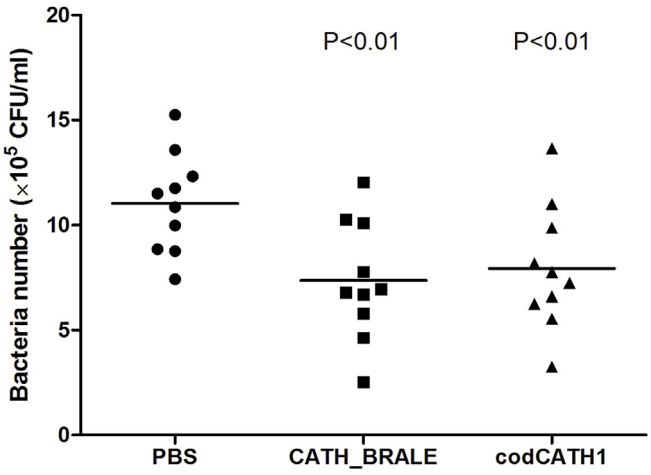

Fig. 5. *In vivo* antimicrobial effect of CATH_BRALE and codCath1 on *V. parahaemotyticus* growth in zebrafish. Peptides (10 mg/kg) and bacteria were simultaneously injected into zebrafish. 24 h later, the zebrafish were killed and homogenized, and the bacterial counts were determined.

against common Gram-positive and Gram-negative aquatic bacterial pathogens with MICs ranging in 2.34–37.5 μg/ml (Table 3). The killing kinetic property of CATH_BRALE and codCath1 against *V. parahaemotyticus* was then determined. The result showed that compared to the conventional antibiotic ampicillin (within 180 min), CATH_BRALE and codCath1 exhibited far more rapid bactericidal effect (within 60 min) (Table 4). Generally speaking, the more rapid of antimicrobial effect, the less likely for antimicrobial agents to induce bacteria resistance. The antimicrobial mechanisms of CATH_BRALE and codCath1 against aquatic bacteria were also investigated. CATH_BRALE and codCath1 could obviously induce the outer membrane and cytoplasmic membrane permeabilization of Gram-negative bacteria, which indicate that CATH_BRALE and codCath1 target on bacterial cell membrane, induce cell disruption and death (Fig. 1). The different antimicrobial mechanisms of CATH_BRALE and codCath1 from most broadly used

conventional antibiotics in aquaculture industry provide a chance for combined use of them, which may significantly reduce the use of conventional antibiotics and thereby decrease the possibility of bacterial resistance appearance. On the other hand, CATH_BRALE and codCath1 exhibited very low cytotoxicity toward fish cells. The good selectivity of CATH_BRALE and codCath1 to prokaryotic cells and low cytotoxicity to eukaryotic cells are great advantages for their application in fish aquaculture industry.

Zebrafish comprise innate and adaptive immune systems, and their first line immune defenses involve various cytokine production and phagocytic cell function [41]. We found CATH_BRALE and codCath1 could significantly inhibit the bacteria-induced transcription of zebrafish pro-inflammatory cytokine genes TNF-α, IL-1β, and IL-6 in spleen and kidney (Fig. 2A–C). This imply that CATH_BRALE and codCath1 may possess the ability to inhibit the bacteria-induced hyperactivation of zebrafish innate immune system and excessive production of pro-inflammatory cytokines, which can significantly reduce the mortality rate of severe bacterial infections. More importantly, CATH_BRALE and codCath1 also possess the ability to increase the expression level of IL-8 (Fig. 2D). IL-8 is an important kind of chemokine that is expressed in several cell types such as macrophages and epithelial cells [42]. IL-8 possesses the ability to induce the chemotaxis of many immune cells, such as neutrophils, monocytes, macrophages, and NK cells [42]. By stimulating IL-8 production, CATH_BRALE and codCath1 may possess the function to induce the recruitment of monocytes, macrophages, and neutrophils to the infection site and kill the invasive bacteria by phagocytosis. Subsequent *in vivo* experiments in the present study proved that CATH_BRALE and codCath1 could significantly enhance the survival rate of zebrafish and decrease the bacterial numbers in zebrafish (Figs. 4 and 5).

In conclusion, CATH_BRALE and codCath1, two fish-derived cathelicidins, possess potent, broad-spectrum, and rapid antimicrobial activities against aquatic pathogenic bacteria. They kill bacterial cells by inducing membrane permeabilization and cell disruption. Besides direct antimicrobial activity, they also possess potent immunomodulatory function. They could significantly decrease the bacterial number in zebrafish and enhance the survival rate of zebrafish. All the results above imply the great potential of CATH_BRALE and codCath1 as novel peptide immunomodulators in fish aquaculture industry.

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