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Antimicrobial activity and mechanisms of multiple antimicrobial peptides isolated from rockfish *Sebastiscus marmoratus*

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ABSTRACT

Pathogenic disease is a major factor affecting the aquaculture of the rockfish *Sebastiscus marmoratus,* an important commercial species inhabiting the nearshore waters of the Western Pacific Ocean. Antimicrobial peptides (AMPs), as critical components of innate immunity, have been considered as promising antibiotic substitutes. The aims of this study were 1) to identify major AMPs in the rockfish, 2) to assess their antimicrobial activity and 3) to evaluate their potential therapeutic application. Six AMPs were identified, Hepcidin 1, liver-expressed antimicrobial peptide 2 (LEAP-2), Piscidin, Moronecidin, NK-lysin and β-defensin through analysis of the liver transcriptome of *S. marmoratus*. The transcriptional expression profiles of these AMPs were investigated by real-time quantitative PCR (RT-qPCR). These AMPs showed tissue-specific distribution patterns, and *S. marmoratus* displays a time-, dose- and tissue-dependent expression of AMPs in response to lipopolysaccharide (LPS) challenge. While the synthetic peptides of LEAP-2 and Moronecidin exerted broad-spectrum antimicrobial activity against important aquatic pathogens *in vitro* by directly disrupting microbial membrane, and no cytotoxicity against murine hepatic cells was observed at the effective concentrations from 5 μM to 40 μM. The existence of multiple AMPs and their distinct tissue distribution patterns and inducible expression patterns suggests a sophisticated, highly redundant, and multilevel network of antimicrobial defensive mechanisms of *S. marmoratus*. Therefore, *S. marmoratus*-derived AMPs appear to be potential therapeutic applications against pathogen infections in aquaculture.

1. Introduction

Pathogens are omnipresent and inevitable for all beings, leading to the evolution of varying immune responses and adaptations [1]. The aquatic environment presents a high level of exposure to numerous pathogens. Fish have developed a strong and effective innate immune system to respond to pathogenic challenges from the aquatic environment [2]. Antimicrobial peptides (AMPs), a type of small molecule produced by all life forms are an essential part of the innate immune response. AMPs refer to a large number of small molecule peptides first characterized on the basis of their antibiotic and antifungal activities [3]. AMPs possess broad-spectrum potent antimicrobial [4], antiviral [5], antiparasitic [6,7] and anticancer activities [8]. Additionally, AMPs prevent excessive inflammatory responses [9,10]. Thus these small bioactive peptides act not only as a fast response to invasive pathogens, but also have an immune regulatory function [11].

Many AMPs have been identified from organisms in the past few decades. Generally, AMPs exhibit distinct molecular sizes, secondary structures and net charges, which allow a diversity in their mechanisms [12]. AMPs might translocate in the cytoplasm and cause cell death by inducing apoptosis [13], inhibiting protein synthesis processes [14,15] or interfering cell wall formation, etc. [16,17]. Most AMPs act non-specifically and exhibit their inhibitory effects by disruptive cytolytic or pore-forming actions [18]. The non-specific antimicrobial mechanism of AMPs makes them the ideal alternative to antibiotics as in theory it would be difficult for microbes to develop resistance [19,20]. In recent years, this promising therapeutic application of AMPs has been intensively reviewed and discussed for their potential in human drug

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design and pharmaceutical treatments [21,22]. Fish express and secrete multiple AMPs to resist latent pathogens [23]. Therefore, fish-derived AMPs could be potential candidate for future application in aquaculture, therapeutics or host defense modulation in fish.

The rockfish *Sebastiscus marmoratus* is an ovoviviparous teleost, which mainly inhabits nearshore waters of the West Pacific Ocean. Rockfish are an economically important species for farming and sport fisheries in Asian countries, including China, Japan and Korea. Various experiments have been carried out using *S. marmoratus* for physiological, ecotoxicological and ecological purposes, which are often focused on reproductive toxicology caused by marine environmental contaminants [24,25]. However, concerning the rockfish immune response against pathogenic infections only a few lines of evidence exist [26].

Over past decades, *S. marmoratus* farming has rapidly increased in China, and diseases caused by infective pathogens have caused huge financial losses in rockfish aquaculture [27,28]. Antibiotics have been applied extensively for disease control in aquatic animals. However, antibiotics could accumulate in fish at high concentrations and eventually affect human health [29,30]. An additional risk is horizontal transfer of resistance genes among bacterial species, and thus, antimicrobial resistance genes from fish bacteria may move to human-pathogenic bacteria – particularly for bacterial groups like *Aeromonadaceae*, which contain both fish- and human pathogens. Therefore, seeking eco-friendly and effective antibiotic alternatives to prevent and treat pathogenic diseases is the top priority in the aquaculture industry. Pertaining to solutions within the problem itself, the natural AMPs invoked by immune system upon pathogenic infection might provide an answer to the puzzle [23,31].

Taking the above into account the aims of this study were 1) to identify major AMPs in the rockfish, 2) to assess their antimicrobial activity and 3) to evaluate their potential therapeutic application. After identifying the coding sequences of the candidate AMPs, the tissue distribution patterns in the liver, gonad, mid-intestine, gill and spleen tissues in healthy fish were analysed and the transcriptional expression patterns in the liver, intestine and spleen were further investigated upon lipopolysaccharide (LPS) challenge. Finally, antimicrobial activity of the AMPs was assessed using the chemically synthetic peptides and the possible modes of action were assessed by scanning electron microscope (SEM). A cytotoxicity assay was performed on both murine and human hepatic cell lines to confirm whether the AMPs could be safely used as antimicrobial agents in future industrial applications.

2. Materials and methods

2.1. Fish and microbial strains

Juvenile *S. marmoratus* were obtained from Dongshan fish hatchery, Fujian Province, China. The average weight and length of the fish upon arrival was 26.13 ± 0.34 g and 11.65 ± 0.07 cm (Mean ± S.E). The fish were acclimatized in a flow-through system with constant aeration at a temperature of 20 ± 1 °C, salinity of 30 ± 1‰ and pH 8.0 ± 0.1 for 2 weeks prior to the experiment in a 3000 L cement tank containing seawater treated with sand filtration, kept on a natural daylight cycle and fed with commercial fish bait at 1% of body weight (BW) daily during the acclimation period. All experiments were carried out in accordance with the Animal Care and Use Guidelines of the China Council on Animal Care.

Microbial strains were purchased from the China General Microbiological Culture Collection Center (CGMCCC), and 15 microbial strains used in the present study are shown in Table 1. The bacteria were cultured overnight at the appropriate temperature (28 °C or 37 °C) either on Muller-Hinton agar or marine agar 2216 (Difco). Yeast strains were grown on YPG agar (yeast extract 1%, peptone 1%, and glucose 2%) at 28 °C for 2 d for the antimicrobial assay (See 2.7). Murine hepatocyte AML12 cell line and human hepatocyte L02 cell line were purchased from Cell Bank, Chinese Academy of Science.

2.2. Tissue distribution of AMP expression

Ten healthy fish (n = 10) were randomly sampled from the acclimated fish to determine the tissue distribution pattern of the AMPs. Liver, gonad, mid-intestine, gill and spleen tissues were collected and immediately snap-frozen in liquid nitrogen and stored at −80 °C for future RNA extraction and real-time quantitative PCR (RT-qPCR) (see 2.5).

2.3. LPS challenge

Acclimatized rockfish were challenged with LPS (*E.coli* 055:B5) (Sigma-Aldrich, USA). In short, fish were euthanized with 250 mg/L MS222 (Sigma Aldrich, USA), and LPS was intraperitoneally injected at doses of 0.5, 5.0 and 25.0 mg/kg body weight in 100 μL of sterile physiological saline solution. The selected doses were chosen according to our previous experiment [32]. The solvent control fish received the same volume of sterile physiological saline solution but LPS-free. All the treatments were performed in duplicate. Sampling was performed at 6, 12, 24 and 48 h (n = 10 each) post LPS challenge or saline-injection. Liver, spleen and intestine were sampled from each fish to determine the mRNA expression levels of the AMPs by means of RT-qPCR.

2.4. Full-length cDNA cloning of the AMPs genes and bioinformatic analysis

To isolate the full-length cDNA sequence of the *S. marmoratus* AMPs genes, 5'-/3′-Rapid Amplification of cDNA Ends (RACE) was performed. Specific primers for RACE are shown in Supplementary Table 1 (Table S1), which was based on the obtained partial cDNA sequences from our transcriptome data of the *S. marmoratus* livers (unpublished data). The procedures of RACE were performed as described previously [33]. The gene and deduced amino acid sequences were analysed by ClustalX 1.83 software. Homology searches were performed using BLASTn and BLASTp (http://www.ncbi.nlm.nih.gov/). The pairwise sequence alignment was performed through online software (https://www.ebi.ac.uk/Tools/psa/). The various physiochemical parameters of the AMPs were determined by the protein analysis tool of ProtParam (https://web.expasy.org/protparam/). The information obtained from the sequence analysis was used for the design of the primers for RT-qPCR (see below).

2.5. RT-qPCR analysis of AMPs expression

Tissue distribution and induced expression patterns of AMPs in *S. marmoratus* stimulation with LPS were determined by RT-qPCR. RNA was extracted from the individual fish tissues (liver, gonad, intestine, gill and spleen) using the TRIzol method and then reverse transcribed into cDNA using the One-Step TaKaRa Primescript™ RT Reagent Kit according to the manufacture's guidelines (TaKaRa, Japan). The RT-qPCR assay was carried out as previously described [34]. The seven pairs of gene-specific primers for qPCR are listed in Table S2 β-Actin was used as the reference gene. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [35].

2.6. Synthesis of peptides

All peptides were synthesized by a bio-company (Glory Chemistry Co., China) using solid phase peptides synthesis method, and the molecular mass and purity of the purified peptides were verified by mass spectroscopy and high-performance liquid chromatography, respectively (Fig. S1 and Table S3). The purity of all the AMPs was over 95%, except β-defensin (93.5%). The peptides were stored in −80 °C and dissolved in sterile water before use.

Table 1
Antimicrobial activity of the AMPs excavated from *S. marmoratus*.

Peptides	Hepcidin 1		LEAP-2		NK-lysin		Piscidin		Moronecidin		β-Defensin	
Strains	MIC[a] (μM)	MBC[b] (μM)	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)
Gram-positive bacteria												
Bacillus cereus	> 50	NT	> 50	NT	> 50	NT	> 50	NT	12.5–25	25–50	NT	NT
Bacillus subtilis	6.25–12.5	12.5	6.25–12.5	< 12.5	> 50	NT	25–50	< 50	6.25–12.5	< 12.5	> 50	NT
Staphylococcus aureus	> 50	NT	> 50	NT	> 50	NT	> 50	NT	3.125–6.25	12.5–25	> 50	NT
Corynebacterium glutamicum	6.25–12.5	12.5	1.56–3.125	< 3.125	6.25–12.5	25–50	NT	NT	1.56–3.125	< 3.125	> 50	NT
Staphylococcus epidermidis	> 50	NT	12.5–25	< 25	> 50	NT	> 50	NT	12.5–25	> 50	> 50	NT
Gram-negative bacteria												
Pseudomonas aeruginosa	25–50	< 50	25–50	> 50	> 50	NT	> 50	NT	25–50	< 50	> 50	NT
Pseudomonas fluorescens	12.5–25	< 25	12.5–25	25–50	NT	NT	> 50	NT	6.25–12.5	25–50	> 50	NT
Pseudomonas stutzeri	6.25–12.5	25–50	3.125–6.25	< 6.25	6.25–12.5	< 12.5	6.25–12.5	12.5–25	3.125–6.25	< 6.25	> 50	NT
Aeromonas hydrophila	> 50	NT	> 50	NT	> 50	> 50	> 50	NT	> 50	NT	> 50	NT
Escherichia coli	> 50	NT	25–50	> 50	25–50	> 50	> 50	NT	12.5–25	> 50	> 50	NT
Shigella flexneri	> 50	NT	12.5–25	< 25	> 50	NT	> 50	NT	6.25–12.5	> 50	> 50	NT
Vibrio parahaemolyticus	> 50	NT	> 50	NT	> 50	NT	> 50	NT	> 50	NT	> 50	NT
Vibrio alginolyticus	> 50	NT	> 50	NT	> 50	NT	> 50	NT	> 50	NT	> 50	NT
Fungi												
Candida albicans	> 50	NT	> 50	NT	> 50	NT	> 50	NT	12.5–25	< 25	> 50	NT
Cryptococcus neoformans	> 50	NT	3.125–6.25	< 6.25	> 50	NT	12.5–25	> 50	1.56–3.125	< 3.125	> 50	NT

NT means not test.
[a] Minimum inhibitory concentration (MIC) was determined as the lowest AMP protein concentration that prevented visible microbial growth.
[b] Minimum bactericidal concentration (MBC) was defined as the concentration that prevents microbial growth of more than 99.9%. All assays (MIC and MBC) were performed in triplicate.

2.7. Antimicrobial assay

To assess the antimicrobial activity of the AMPs, synthetic peptides were tested against various fish pathogenic strains (*Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Corynebacterium glutamicum, Staphylococcus epidermidis, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas stutzeri, Aeromonas hydrophila, Escherichia coli, Shigella flexneri, Vibrio parahaemolyticus, Vibrio alginolyticus, Candida albicans* and *Cryptococcus neoformans*). Nutrition broth (NB), Mueller-Hinton broth (MH broth), Difco marine broth and YPG medium were used for cultivation of bacteria, marine bacteria and fungi. The antimicrobial assay was performed using liquid growth inhibition methods with slight modifications. Mid-logarithmic phase cultures of microbes were centrifuged at 3000 g at room temperature for 10 min to harvest the microbial cells, which were then re-suspended in corresponding culture medium. 10^4 cfu/well of bacterium and 10^3 cfu/well of fungus were incubated with serially diluted AMPs in a 96-well flat-bottom tissue culture plate. The microbes were incubated for 24 or 48 h at their optimal temperatures. Minimum inhibitory concentration (MIC) was determined as the lowest AMP protein concentration that prevented visible microbial growth. After observation of MIC, cultures without visible growth were mixed by multiple pipetting and inoculated agar plates of the corresponding media. Plates were incubated at the optimal growth temperature for 24 or 48 h, and minimum bactericidal concentration (MBC) was defined as the concentration that prevents microbial growth of more than 99.9%. All assays were performed in triplicate.

2.8. Bactericidal kinetic curves of the synthesized AMP

Based on the results from the antimicrobial assay of sm-AMPs, susceptible microbes (*P. stutzeri* and *C. neoformans*) were selected as representative strains to further investigate the time-killing kinetics of sm-Moronecidin and sm-LEAP-2 (sm-liver-expressed antimicrobial peptide 2). Mid-logarithmic phase cultures of *P. stutzeri* and *C. neoformans* were prepared, diluted and incubated with AMPs at different concentrations as described in antimicrobial assay. At various intervals, 4 μL of the cultures were diluted in 10 mM NaPB and plated on NB agar plates (*P. stutzeri*) and YPG agar plates (*C. neoformans*), respectively. Plates were grown for 24–48 h before the numbers of CFU were calculated. Assays were carried out in triplicates on separate occasions. The percentage of CFU was defined relative to the CFU obtained at the beginning (100% CFU at 0 min).

2.9. Morphological observation by SEM

To get an intuitive understanding of the modes of action of AMPs to microbes, SEM was applied to observe the morphological changes of microbes after AMP treatments. *P. stutzeri* and *C. neoformans* grew to mid-logarithmic phase, harvested by centrifugation and re-suspended in the freshly prepared media. Aliquots of microbial suspension (10^8 CFU/mL) were supplemented with NaPB (blank) or AMPs at supra-MBC. Microbes grew at 30 °C for 30 min for sm-LEAP-2 treatment and 10 min for sm-Moronecidin treatment. The microbes were then fixed with 2.5% glutaraldehyde for 2 h and washed three times with 50 mM NaPB (pH 7.4), and then the cells were immobilized on a poly-L-lysin coated glass slide at 4 °C for 3 h and subsequently dehydrated with graded ethanol. Specimens were further dehydrated with tert-butyl alcohol and lyophilized using critical point method. The specimens were spray-gold and examined with an XL-30 Environmental Scanning Electron Microscope (FEI, Netherlands).

2.10. Cytotoxicity assay

To evaluate whether the AMPs could be safely used as antimicrobial agents in future industrial applications, that is whether the synthesized AMPs significantly suppressed cell growth under certain concentrations, mouse hepatocytes AML12 and human hepatocyte cell line L02 were selected for cytotoxicity assay. Cell cytotoxicity was determined on AML12 and L02 cell lines using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) according to the instruction of CellTiter 96®AQueous Non-Radioactive Cell Proliferation assay (Promega, USA). In brief, AML12 and L02 cells were cultured in recommended media before disassociation and re-suspended in freshly prepared culture media. 2×10^3 cells/well was incubated with serially diluted AMPs in a 96-well flat-bottom tissue culture plate. Subsequently, 20 μL MTS solution was added to each well after 48 h-incubation, and cells were incubated at 37 °C for 2–4 h

followed by absorbance readings at 492 nm.

2.11. Statistical analysis

The results were expressed as the mean ± standard error (S.E.). The data were first tested for normality and homogeneity using Bartlett's test. Then the one-way analysis of variance (ANOVA) followed by Tukey's HSD test to identify significant differences between the treatment groups and control groups. Significant differences were accepted at * = $p < 0.05$, ** = $p < 0.01$. Statistical analysis was performed using SPSS 16.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Characterization of the AMP genes

The full-length cDNA sequences of the selected genes were obtained by 5′- and 3′-RACE technique. In total, six genes encoding AMPs were identified in *S. marmoratus* for the first time. The GenBank accession numbers for the six genes are as follows: Hepcidin 1 (MK913630), liver-expressed antimicrobial peptide 2 (LEAP-2) (MK913631), Piscidin (MK9136332), Moronecidin (MK387855), NK-lysin (MK913633) and β-defensin (MK913634). The characteristics (number of nucleotides, number of encoding amino acid, molecular formula, molecular weight, theoretical pI, instability index, grand average of hydropathicity and GenBank accession number) of these AMPs nucleic acid and amino acid were shown in Table S4. The theoretical pI of the six AMPs varied from 6.13 to 10.08, and the instability index were from 32.05 to 96.73. The values of grand average of hydropathicity varied from −0.645 to 0.122.

The full-length cDNA sequence of sm-Hepcidin 1 was composed of 789 bp in size, encoded 90 amino acids. A sequence homolog to sm-Hepcidin 1 was also cloned and classified as sm-LEAP-2, which encoded 79 amino acids with a 988 bp cDNA sequence. The identity, similarity and gaps of the amino acid sequences between sm-Hepcidin 1 and sm-LEAP-2 was 19.6%, 29.5% and 49.1%, respectively. The full-length cDNA sequence of sm-Piscidin contained an open reading frame (ORF) of 234 bp that encoded a predicted peptide of 77 amino acid residues. The sm-Moronecidin cDNA was 524 bp in length and contained a 183 bp ORF that encodes a product of 60 amino acid residues. SignalP 4.1 predicted that the first 22 amino acid residues would form a signal peptide with a putative cleavage site between alanine and phenylalanine residues. The predicted mature peptide (38-amino acid residues) of sm-Moronecidin had a calculated molecular weight of 4555.12 Da and theoretical isoelectric point (pI) of 8.74. Sequence analysis of mature sm-Moronecidin revealed the presence of six arginine residues representing 15% of the whole sequence. The net charge of sm-Moronecidin in solution (pH 7.0) was +1, suggesting that it was a cationic antimicrobial peptide. Three-dimension modeling by SWISS-MODEL revealed that the mature peptide of sm-Moronecidin formed α-helices but no β-sheets. A 736 bp cDNA sequence encoding 150 amino acid residues of sm-NK-lysin was obtained. The partial sm-β-Defensin cDNA was composed of 39 amino acid residues that encoding by a 408 bp cDNA sequence.

3.2. Tissue distribution profiles of AMP expression

The gene expression profiles of AMPs in the liver, gonad, intestine, gill and spleen of *S. marmoratus* are shown in Fig. 1. Both sm-Hepcidin 1 and sm-LEAP-2 showed tissue-specific expression patterns with sm-Hepcidin 1 specifically detected in liver while sm-LEAP-2 was abundant in the intestine relative to the liver, gonad, gill and spleen. Sm-Piscidin expression was detected in all tested tissues. The expression level of sm-Moronecidin was the highest in gill compared to the other four tissues. Sm-NK-lysin showed a high expression in spleen, and sm-β-Defensin was highly presented in intestine relative to the other four tissues.

The liver, intestine and spleen were the three main distribution sites of AMPs expression in healthy *S. marmoratus* (Fig. 1), thus we further investigated AMPs expression profiles in these organs in fish after challenge by LPS. In the liver of *S. marmoratus*, an upregulation of sm-Hepcidin 1 was observed at 6 h post injection (hpi) with 5.0 mg/kg and 25.0 mg/kg of LPS, and it was induced to 18-fold and 17-fold in comparison with the physical saline treatment group (Fig. 2A). However, sm-Hepcidin 1 slightly decreased at 12 hpi at the dose of 5.0 mg/kg. Sm-LEAP-2 showed a remarkable increase at 6 hpi in fish challenged with 25.0 mg/kg LPS. The expression level of the sm-Piscidin was markedly increased at 12 hpi and the level was 3.0-fold and 4.2-fold in fish injected LPS with 5.0 and 25.0 mg/kg, and 4.1-fold and 3.6-fold induction were observed at 24 hpi when fish challenged with 0.5 mg/kg and 5.0 mg/kg LPS. Notably, sm-Moronecidin showed a similar expression pattern with that of sm-NK-lysin at 25.0 mg/kg LPS challenge. As to sm-NK-lysin, both 5.0 mg/kg and 25.0 mg/kg LPS challenge elevated its expression level at 24 hpi. Whereas, sm-β-Defensin showed no significant response to any of the three doses of LPS. All the six AMPs expression decreased to the level of the control at 48 hpi. Overall, there was a dose- and time-dependency profile for all the AMPs mRNA expression (except β-defensin).

The expression level of β-Defensin mRNA was very low in the intestines and spleens of fish stimulated with LPS (data were not shown), therefore we show only the results for the other five AMPs genes in the two tissues (Fig. 2B and 2C). A rapid induced of Hepcidin 1 mRNA was detected in the intestines at 6, 12 and 24 hpi when fish challenged with 5.0 mg LPS, and it was induced to 22.8-fold, 6.1-fold and 7.2-fold, respectively. It was significantly induced to 27.5-fold, 6.8-fold and 38.4-fold at 6, 12 and 48 h of fish injected 25.0 mg/kg LPS (Fig. 2B). LEAP-2 mRNA expression was downregulated at 6 hpi and then it was induced at 48 hpi. Moronecidin expression was remarkably decreased at 6 hpi of fish challenged with 5.0 mg/kg LPS, and it was significantly induced at 48 hpi with 25.0 mg/kg LPS. The expression trend of NK-lysin was similar to that of moronecidin. No significant change of Piscidin gene expression was observed within 48 h.

The gene expression profile of AMPs in the spleen is shown in Fig. 2C. For Hepcidin 1 mRNA, significant induction was detected at 6 hpi for all the three doses of LPS (13.2-fold, 10.8-fold and 11.1-fold, respectively). Hepicidin was up-regulated in fish injected with 5.0 and 25.0 mg/kg LPS at 24 h, and it was still inducible at 48 h for the highest dose of LPS challenge. LEAP-2 mRNA expression was strongly expressed at 6 hpi and the expression was significantly decreased at 48 hpi for all the doses of LPS injection. Piscidin mRNA expression was inhibited at 6 and 48 hpi, and Moronecidin mRNA was inhibited at 6 and 12 hpi. NK-lysin mRNA expression was inhibited at 6 hpi and strongly up-regulated at 24 hpi with 5.0 and 25.0 mg/kg LPS challenge.

3.3. Antimicrobial activity of the AMPs

The MIC and MBC values of the synthetic peptides are presented in Table 1. Sm-NK-lysin and sm-Piscidin only inhibited the growth of few tested strains, and sm-β-Defensin had no effects on all tested strains. Sm-Hepcidin 1 showed moderate inhibitory effect on both Gram-positive and Gram-negative bacteria. Sm-LEAP-2 possessed a broader antimicrobial spectrum compare to sm-Hepcidin 1, moreover, sm-LEAP-2 exerted a fungicidal effect against *C. neoformans*. Notably, sm-Moronecidin displayed potent and broad-spectrum antimicrobial activity against the majority of the tested microbes, but no antimicrobial activity was observed against *V. alginolyticus*, *V. parahaemolyticus* and *A. hydrophila* (MIC > 50 μM).

Based on the results obtained from the above antimicrobial assay, the two AMPs, sm-LEAP-2 and sm-Moronecidin, which showed potent broad-spectrum antimicrobial activity (Table 1), were selected for a further investigation of their antimicrobial mechanisms. The bactericidal and fungicidal kinetic of the both AMPs were assessed using two

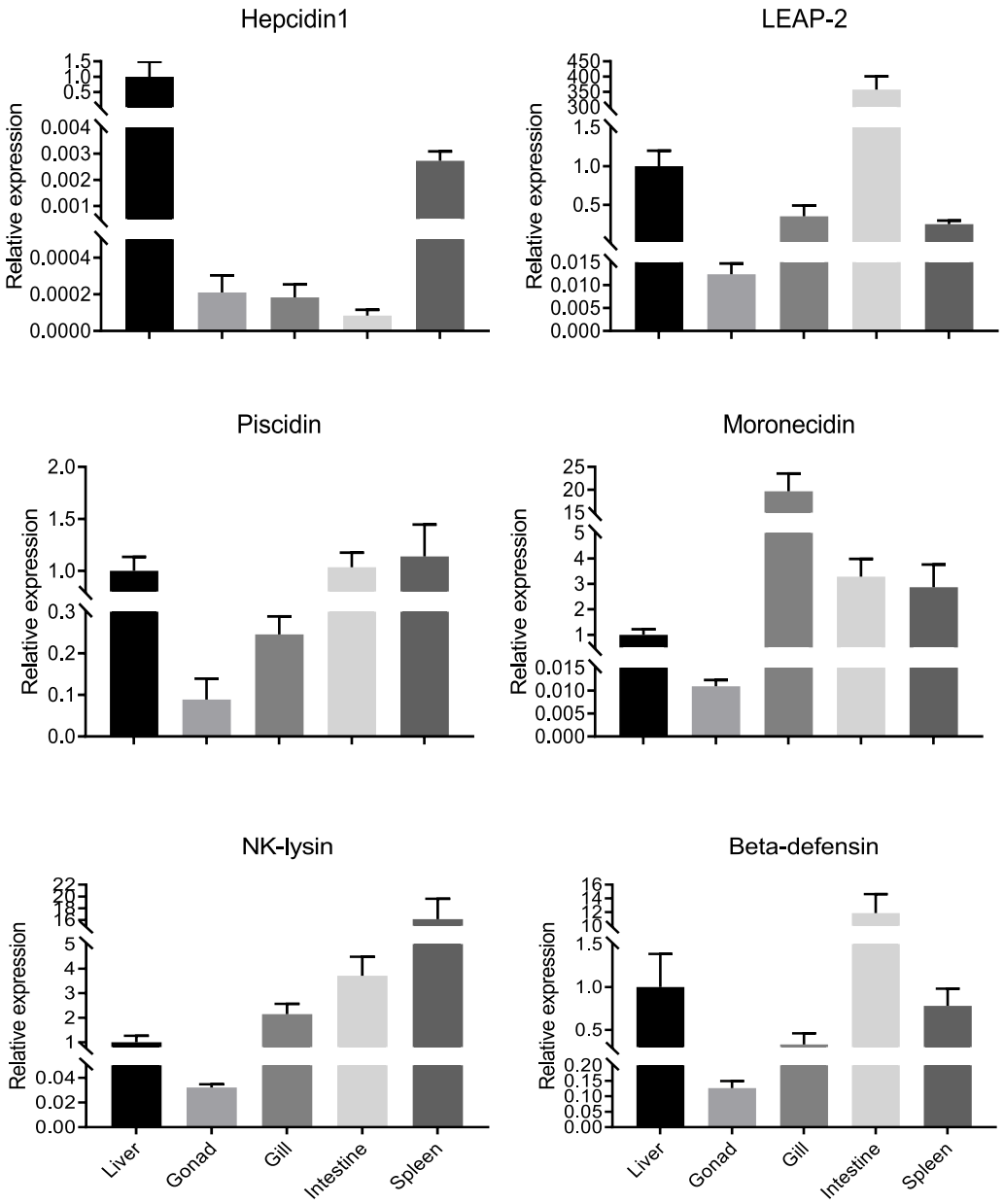

Fig. 1. Tissue expression patterns of the AMPs genes in *S. marmoratus*. The relative expression was the target gene mRNA over reference gene (*β-actin*) mRNA. Data are expressed as mean ± S.E. (n = 10).

species of fish pathogens *P. stutzeri* and *C. neoformans*. As shown in Fig. 3, sm-LEAP-2 (2 × MBC) eliminated more than half of the *P. stutzeri* after 1 h incubation, and it killed all bacterial cells around 3 h. When sm-LEAP-2 was incubated with *C. neoformans* at 2 × MBC, it killed 90% of the fungi after 5 h and destroyed all the fungi around post 8 h. The fungicidal effect of sm-Moronecidin was similar to that of sm-LEAP-2. Notably, the results showed that sm-Moronecidin exerted rapid bactericidal activity by eliminating all *P. stutzeri* within 20 min.

3.4. Sm-moronecidin and sm-LEAP-2 induced morphological changes in microbes

In the control groups (NaPB), *C. neoformans* and *P. stutzeri* cells presented a smooth and organized surface (Fig. 4). After sm-LEAP-2 treatment, *P. stutzeri* showed a rougher and tattered surface, obvious membrane damage, even the leakage of cytoplasm and collapse of architecture. The similar phenomena were observed in sm-LEAP-2-treated *C. neoformans*. Moreover, sm-LEAP-2 treatment induced the craters formation on the surface of *C. neoformans*, which ultimately led to the release of cytoplasm and cytoclasis. Sm-Moronecidin treatment induced microbial cell surface breakdown to a lesser degree compared to sm-LEAP-2. Nevertheless, *C. neoformans* after sm-Moronecidin incubation resulted in craters in various sizes on the surface and nest-like substances in extracellular space.

3.5. Cytotoxicity of sm-Moronecidin and sm-LEAP-2

The cytotoxicity of sm-Moronecidin and sm-LEAP-2 against murine and mammalian cells was presented in Fig. 5. In general, sm-Moronecidin and sm-LEAP-2 showed no cytotoxicity to AML12 cells. The higher concentrations of sm-Moronecidin (20 and 40 μM) inhibited the growth of L02 cells, while sm-LEAP-2 suppressed cell growth at low concentration (5 μM).

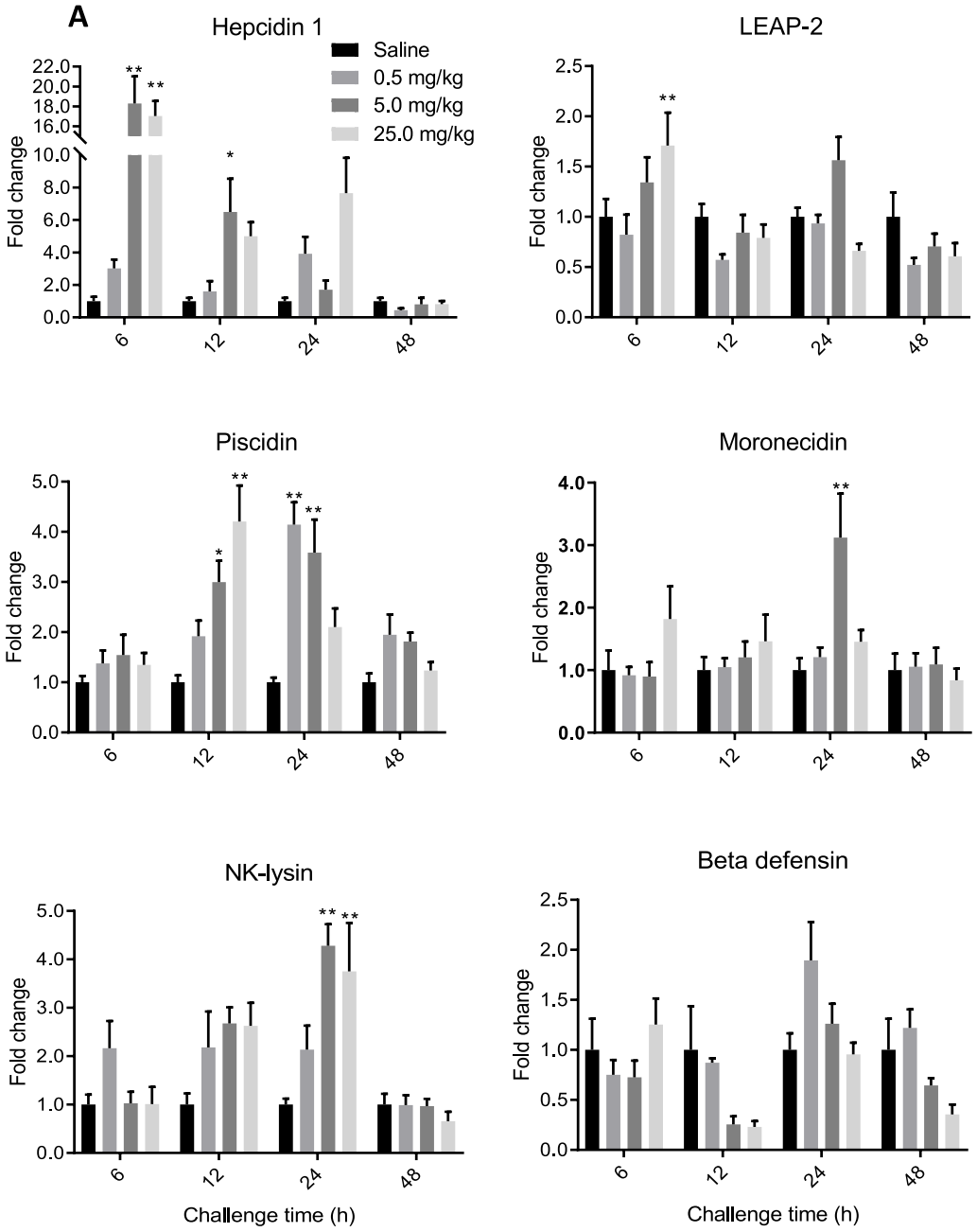

Fig. 2. AMPs genes expression patterns in the liver (A), intestine (B) and spleen (C) of *S. marmoratus* challenged with LPS. Data are expressed as mean ± S.E. (n = 10). Asterisks indicate significant difference between the LPS challenged group and the saline injected group at each time point (*$p < 0.05$, **$p < 0.01$).

4. Discussion

In the present study, six AMPs genes were identified in *S. marmoratus* for the first time. These AMPs had distinct tissue distribution patterns, and the significant induction displayed rapid responses upon LPS challenge. Among all the six AMPs, sm-Moronecidin and sm-LEAP-2 showed a potent broad-spectrum *in vitro* antimicrobial activity and rapid time-killing kinetic by directly interacting and damaging microbial cell membrane, suggesting that they might participate in pathogen clearance as the first line of defense. In addition, synthetic AMPs showed no cytotoxicity against murine and human cells within a certain concentration range. The empirical evidence generated in this study sheds light on the disease resistant mechanisms of *S. marmoratus*. Our findings provide the experimental basis for future application of *S. marmoratus*-derived AMPs as bioactive products in aquaculture.

Several studies suggest that fish hepcidins have antimicrobial [36,37], antiviral [38] and antifungal properties [39]. Moreover, fish hepcidins play a role in the control of iron homeostasis [40]. In the present study, sm-Hepcidin 1 expression was liver-specific and showed rapid induction upon LPS challenge. Additionally, the synthetic mature peptide of sm-Hepcidin 1 exerted a moderate inhibitory effect against a few tested bacteria. Therefore, Sm-Hepcidin 1 might participate in defense of invasive bacteria with a higher biological activity since the AMPs synthesized by chemical method may lack functional structures of the native protein or may be misfolded.

A hepcidin-homologous protein containing two disulfide linkages, sm-LEAP-2, was identified in our study. While LEAP-2 is constitutively expressed in most fish tissues, it is predominantly expressed in the liver

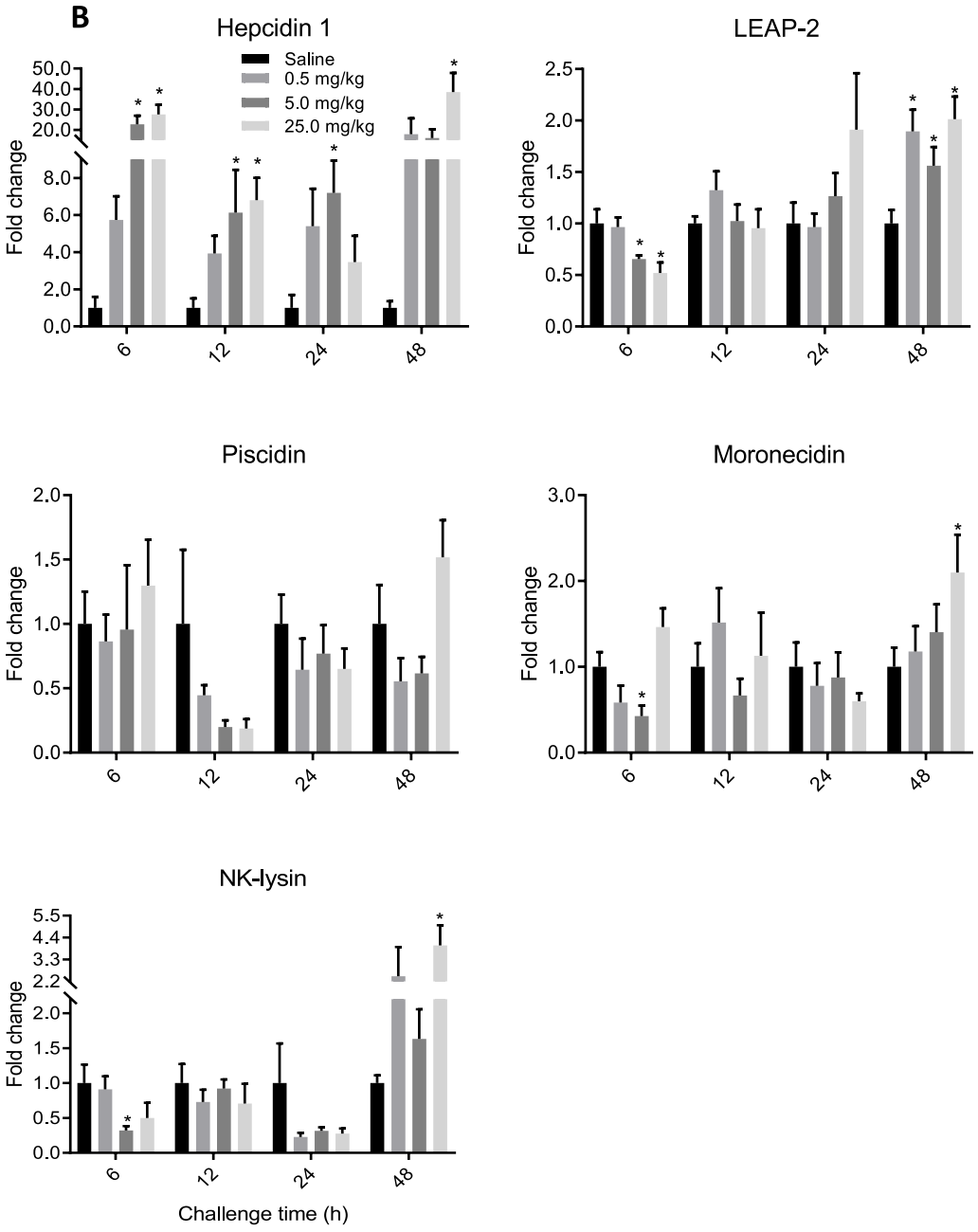

Fig. 2. (*continued*)

[41,42]. However, LEAP-2 transcript expression in channel catfish *Ictalurus punctatus* was the lowest in the liver among the 11 tested tissues (head kidney, intestine, liver, spleen, trunk kidney, brain, gill, muscle, ovary, skin and stomach) [43]. In our study, LEAP-2 transcripts were highly expressed in the intestine of *S. marmoratus*, moderately expressed in the liver and spleen. Thus this differentially expressed pattern of LEAP-2 in different organisms may be species-specific in fish [43,44].

The different types of AMPs in *S. marmoratus* challenged with LPS exhibited different transcript patterns within the same tissue, and the expression pattern was also distinct for specific AMPs gene in different tissues in this study. Hepcidin 1 mRNA was strongly inducible in liver, intestine and spleen of *S. marmoratus* challenged with LPS, while the transcriptional expression for LEAP-2, Piscidin, Moronecidin and NK-lysin genes was significantly downregulated at certain points in the intestine and spleen (Fig. 2). Campoverde et al. [45] found that the significant downregulation of AMPs (Beta-defensin, Hepcidin and Piscidin) in several tissues (spleen, intestine and gill) in meagre (*Argyrosomus regius*) stimulated with LPS. It was reported that LEAP-2 transcript in the intestine of Ayu, *Plecoglossus altivelis* was downregulated after the bacterial infection [41]. The negative regulation of AMPs gene expression was also observed in *Drosophila* treated with peptidoglycan, a major cell wall component of most bacteria, which is considered to play an important role to reach an equilibrium between the efficient clearance of pathogens and the preservation of microorganisms beneficial to the host [46]. In our study, the regulation of AMPs included rapid release and arrest through which the host might combat infections and maintain homeostasis. However, the downregulation mechanism of AMPs in *S. marmoratus* remains to be elucidated.

Among the synthesized AMPs, sm-Moronecidin displayed the strongest broad-spectrum antimicrobial activity. This alpha-helical AMP was strongly expressed in gill of *S. marmoratus* and also showed inducible expression in liver post LPS injection. Previous studies

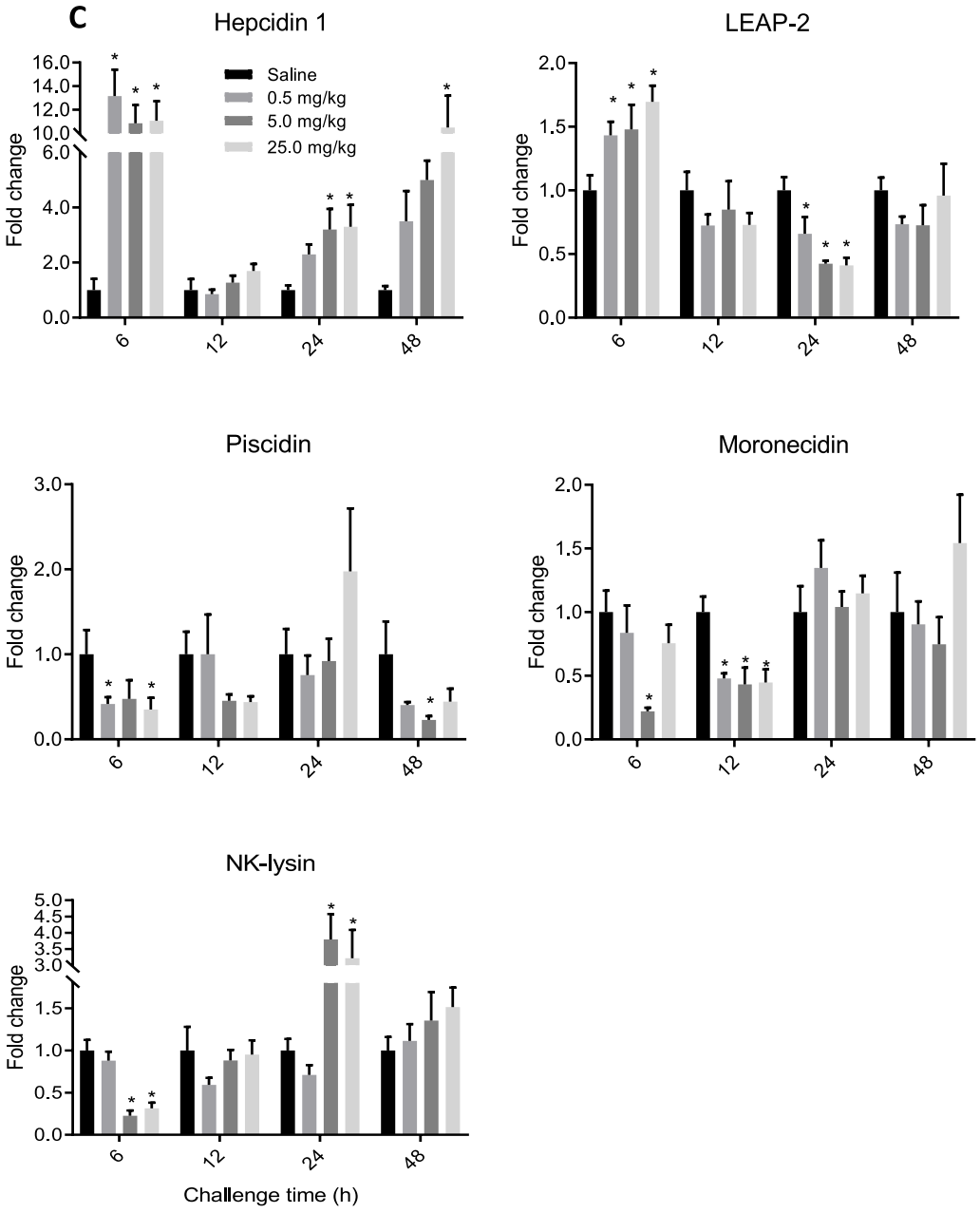

Fig. 2. (*continued*)

showed that broad-spectrum antimicrobial activity of white bass (*Morone chrysops*) moronecidin was retained at high salt concentration [47]. It is noteworthy that sm-Moronecidin killed invasive bacteria in a short period through directly disrupting bacterial membrane. Thus, the biological characteristics of sm-Moronecidin suggest that it might be the first responder to bacterial infections up-taken through the gills. The intriguing nature of this defensive process merits further study. Although the synthetic products of other identified AMPs only displayed limited inhibitory effects against the tested pathogenic strains, their roles in immune regulation upon infections and potential as molecular scaffolds for designing novel drugs are undeniable.

To explore the potential therapeutic applications of AMPs in protecting fish against pathogen infections, we tested two AMPs which showed particularly strong and broad-scaled antimicrobial activities in the *in vitro* bactericidal assays. Sm-Moronecidin mature peptide formed α-helices and carried a net positive charge in both a seawater and physiological environment. Sm-Moronecidin was mainly expressed in the gills, which is similar to their homologous proteins from other fish species [47,48]. As confirmed by the time-killing kinetic assessment and SEM observation, sm-Moronecidin treatment could induce morphological changes of microbial surface and eliminate 99.99% bacteria within 20 min. Therefore, upon bacterial invasion, cationic sm-Moronecidin expression in the gills of *S. marmoratus* would bind to the negatively charged bacterial cell surface, disrupting the microbial cell membrane and consequently causing the leakage of cytoplasm and cell death. As the first-line of defense, the instant bactericidal responses of sm-Moronecidin avoided infection from pathogenic bacteria in seawater and ensured fish health. Sm-LEAP-2 was mainly expressed in intestine. Our *in vitro* assay results suggest that sm-LEAP-2 exerted a narrower antimicrobial spectrum against gram-positive bacteria. In physiological condition of organisms, four cysteines residues of LEAP-2 naturally form two pairs of disulfide bonds [49,50], and the synthetic peptide is absent of post-translational modifications, thus the bactericidal activity might be affected. Therefore, it is speculated that sm-LEAP-2 might be more active under natural physiological conditions.

Besides antibacterial activity, sm-LEAP-2 and sm-Moronecidin both

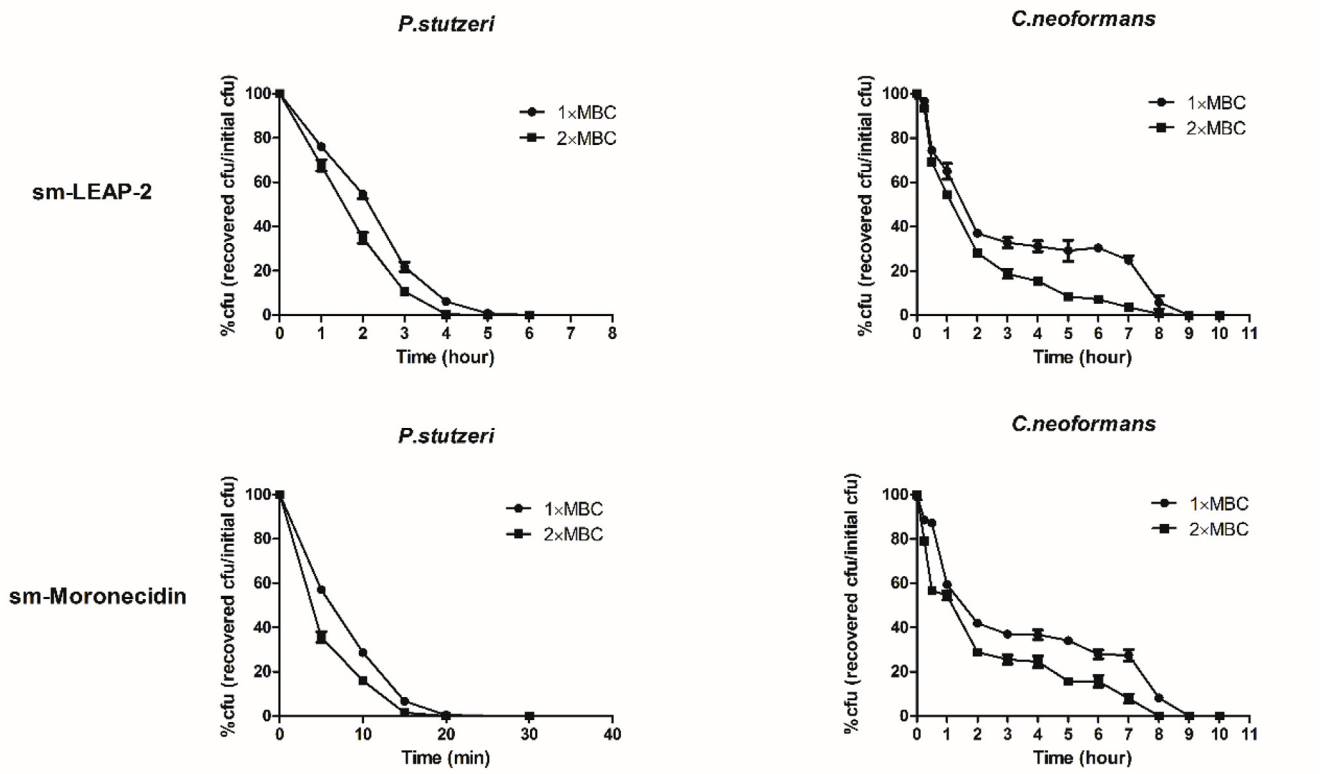

Fig. 3. Bactericidal kinetic curves of synthesized sm-LEAP-2 and sm-Moronecidin. *P. stutzeri* and *C. neoformans* were used. The percentage of CFU is defined relative to the CFU obtained in the control (100% CFU at 0 min). Each bar represents the means ± S.E. of three determinations (n = 3).

exerted antifungal and fungicidal activity against *C. neoformans*. SEM observation revealed that sm-LEAP-2 and sm-Moronecidin induced different morphological changes to conduct their fungicidal activity. Numerous models have been built to explain action modes of membrane-targeted AMPs, such as barrel-stave model, carpet model and toroidal-pore model [51,52]. The varied spatial structures and concentration of AMPs, together with the property and fluidity of phospholipid membrane, determine the optimum action mechanisms to some extent [18]. Notably, one particular AMP might act through different mechanisms to conduct their function against different microbes, meanwhile different AMPs might adopt distinct action modes against the same microbe [13,53]. Similar to sm-Moronecidin treated bacteria, sm-LEAP-2 treated *C. neoformans* showed clearly rougher cell surface and leakage of cytoplasm. *C. neoformans* treated with sm-Moronecidin showed a smooth surface with craters formed at the fungal cells. These empirical findings indicate that sm-LEAP-2 and sm-Moronecidin might conduct distinct membrane-associated mechanisms to fulfil their fungicidal activity. However, the exact mechanism and whether sm-LEAP-2 and sm-Moronecrone would act through other non-membrane dependent ways to accomplish its antimicrobial activity requires future research.

In this study, non-cancer murine and mammalian hepatic cell lines were utilized to test the cytotoxicity of both sm-LEAP-2 and sm-Moronecidin. At effective concentrations, sm-Moronecidin showed no cytotoxicity against murine and human hepatic cells, nevertheless, high concentration would inhibit cell proliferation of human hepatic cells. These results suggested that sm-Moronecidin could be further applied for therapeutic use within the safe concentration range. Although it

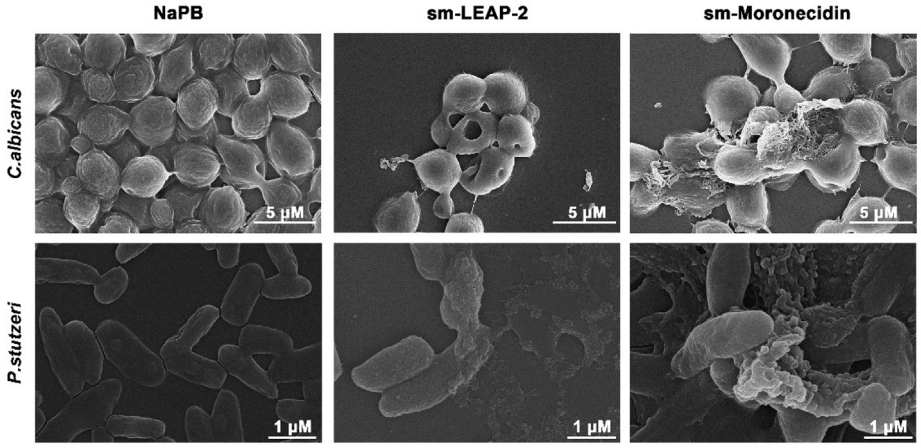

Fig. 4. Effects of sm-LEAP-2 and sm-Moronecidin on the morphology of *P. stutzeri* and *C. albicans* by SEM.

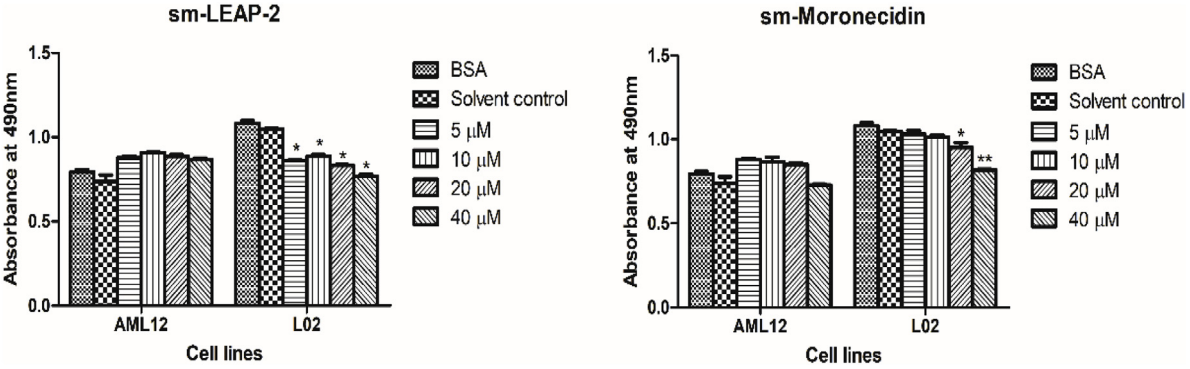

Fig. 5. Cytotoxicity effects of sm-LEAP-2 and sm-Moronecidin on murine and human hepatic cells. Cells were incubated with synthesis sm-LEAP-2 or sm-Moronecidin for 48 h before determined their viability using MTS reagents, all the experiments were carried out by triplicates. Asterisks indicate significant difference between the AMPs treatment group and the solvent control group (*$p < 0.05$, **$p < 0.01$).

showed no cytotoxicity to murine hepatic cells, sm-LEAP-2 significantly inhibited growth of human hepatic cells at all trail concentrations. In consideration of the fact that the highest gene expression level of sm-LEAP-2 was detected in the intestine, the cytotoxicity assay should be carried out on more cell lines to comprehensively evaluate its cytotoxicity. Still, sm-LEAP-2 could serve as a potential template for drug design and development, for example by excavating functional domains and amino acid substitution of sm-LEAP-2 and sm-Moronecidin, smaller peptides with lower cytotoxicity and better antimicrobial activity at lower costs might be expected.

Collectively our results provide evidence that *S. marmoratus* displays a time-, dose- and tissue-dependent expression of AMPs in response to LPS challenge. The results of antimicrobial experiments suggest that this represents a coping strategy to deal with the diversity of microbial infection. The existence of multiple AMPs and their distinct tissue distribution patterns and inducible expression patterns suggests a sophisticated, highly redundant, and multilevel network of antimicrobial defensive mechanisms of *S. marmoratus*. The synthetic peptides of LEAP-2 and Moronecidin exerted broad-spectrum antimicrobial activity by directly disrupting the microbial membrane, and no cytotoxicity against murine hepatic cells was observed at the effective concentrations. Therefore, *S. marmoratus*-derived AMPs appear to be potential therapeutic applications against pathogen infections in aquaculture.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.054.

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