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Identification and characterization of two novel antimicrobial peptides from Japanese sea bass (*Lateolabrax japonicus*) with antimicrobial activity and MO/MΦ activation capability

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ABSTRACT

Piscidins participate in the innate immune response of fish, which aims to eliminate recognized foreign microbes and restore the homeostasis of immune system. We characterized two piscidin-like antimicrobial peptides (LjPL-3 and LjPL-2) isolated from Japanese sea bass (*Lateolabrax japonicus*). *LjPL-3* and *LjPL-2* showed different expression patterns in tissues. After *Vibrio harveyi* infection, the mRNA expression of *LjPL-3* and *LjPL-2* was upregulated in the liver, spleen, head kidney, and trunk kidney. The synthetic mature peptides LjPL-3 and LjPL-2 exhibited different antimicrobial spectra. Furthermore, LjPL-3 and LjPL-2 treatments decreased inflammatory cytokine production while promoting chemotaxis and phagocytosis in monocytes/macrophages (MO/MΦ). LjPL-2, but not LjPL-3, displayed bacterial killing capability in MO/MΦ. LjPL-3 and LjPL-2 administration increased Japanese sea bass survival after *V. harveyi* challenge, which was accompanied by a decline in bacterial burden. These data suggested that LjPL-3 and LjPL-2 participate in immune response through direct bacterial killing and MO/MΦ activation.

1. Introduction

Antimicrobial peptides (AMPs) are evolutionarily ancient molecules, which serve as therapeutic options against bacteria, fungi, and viruses (Chee et al., 2019; Das et al., 2022; Lazzaro et al., 2020). The AMP family is broadly classified into four groups based on structural characteristics: α-helical (piscidins, bicarinalins, and aureins), β-sheets (hepcidins, β-defensins, and lactophoricins), β-hairpin (capitellacins, thanatins, and protegrins), and non-αβ peptides (indolicidins and andricins) (Feng et al., 2022; Katzenback, 2015; León et al., 2020). Piscidins exhibit similar amphipathic and α-helical structures, but share little sequence homology (Barroso et al., 2020; Zaccone et al., 2022). Members of the piscidin family include piscidins, moronecidins, dicentracins, pleurocidins, epinecidins, and ceratotoxins (Katzenback, 2015; Sun et al., 2007). Piscidins are firstly found in the hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Silphaduang and Noga, 2001). Later, another amphipathic α-helical peptide is also found in the hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) and named moronecidin (Lauth et al., 2002). Furthermore, the piscidin family member genes have been identified in the winter flounder (*Pleuronectes americanus*) (Cole et al., 1997), Nile tilapia (*Oreochromis niloticus*) (Lin et al., 2022), large yellow croaker (*Larimichthys crocea*) (Zhou et al., 2014), Japanese medaka (*Oryzias latipes*) (Wang et al., 2015), and greater amberjack (*Seriola dumerili*) (Milne et al., 2019).

Due to α-helix amphipathic structures with positive charges, mature piscidin peptides play an antimicrobial role through direct antimicrobial effects and the innate immune system (McMillan and Coombs, 2021; Zaccone et al., 2022). With their cationic structure, piscidins specifically bind to the negatively charged bacterial membrane, which is an antimicrobial mechanism of piscidins (Lin et al., 2022; Portelinha et al.,

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2021). Subsequently, piscidins dose-dependently induce membrane permeabilization and cause leakage of the cellular contents (Hazam et al., 2023; Portelinha et al., 2021). Two piscidin isoforms from hybrid striped bass that bind phosphate-containing targets in an amphipathic α-helical conformation, disrupt bacterial membranes of target cells (Hayden et al., 2015). Piscidin 1 exhibits greater disruption of bacterial model membranes compared to piscidin 3, while piscidin 3 demonstrates stronger Cu-dependent DNA cleavage (Hayden et al., 2015; Libardo et al., 2017). In addition, the amino acids at positions 8 and 13 of piscidin 1 are essential for its antibacterial activity and bacterial cell selectivity (Lee et al., 2007). The substitution of lysine residues in the hydrophobic face also influences the antimicrobial activity of piscidin 1 (Jiang et al., 2014). AMPs are natural components of the immune system that activate immune cells against pathogens (Neumann et al., 2001; Zhang et al., 2017, 2022). Piscidins are expressed in immune cells to regulate their functions (Silphaduang and Noga, 2001; Zaccone et al., 2022). Piscidins from Nile tilapia have been found to reprogram macrophages and decrease pro-inflammatory mediators (Liu et al., 2021). Piscidins from acidophilic granulocytes are released into the phagosome, which contributes to the killing of ingested pathogenic bacteria (Mulero et al., 2008). Previous research has recognized that piscidins affect the expression of pro-inflammatory cytokines, such as interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α (Su and Chen, 2020). Piscidins also activate formyl peptide receptor (FPR)-mediated chemotaxis in the immune cells (Kim et al., 2018). However, AMPs are not only involved in immune regulation, but also exhibit other biological functions. It has been reported that piscidin 1 from hybrid striped bass exhibits anticancer activity (Lin et al., 2012). Tilapia piscidin 4 (TP4) from Nile tilapia also has anticancer activity by upregulating a specific activator protein-1 (Ting et al., 2016). TP4 promotes wound healing by regulating the expression of epidermal growth factor (EGF), transforming growth factor (TGF)-β, and vascular endothelial growth factor (VEGF) (Huang et al., 2015).

The Japanese sea bass (*lateolabrax japonicus*), an eurythermal and carnivorous species, has become a major marine fish farmed in Asia. However, with exposure to pathogens in seawater, marine fishes are always infected by microbes, especially *Vibrio harveyi* (Chen et al., 2020). This study aimed to determine the functions of two Japanese sea bass piscidin-like peptides *in vitro* and *in vivo*. We identified the cDNA sequences of two piscidin-like AMPs from Japanese sea bass (*LjPL-3* and *LjPL-2*) and analyzed their mRNA expression in tissues. Antimicrobial assays were performed to test the bactericidal activities of LjPL-3 and LjPL-2. Moreover, we evaluated the effects of the two AMPs on the function of monocytes/macrophages (MO/MΦ) during infection. In addition, we investigated the effects of LjPL-3 and LjPL-2 on survival rates and bacterial burden in tissues.

2. Methods

2.1. Fish

All fish (weighed 100–120 g) used in the present study were purchased from an aquatic product in Xiangshan County, Ningbo City, China. The fish were maintaining in the artificial seawater (20‰ of salinity) at 27–28 °C and managed normally for two weeks prior to experimental use. This experiment was conducted following the Animal Ethics Committee of Hangzhou Normal University and adhere to accordance with the Experimental Animal Management Law of China.

2.2. Cloning and sequence analyses of LjPL-3 and LjPL-2

Parts of the *LjPL-3* and *LjPL-2* cDNA sequences were obtained from the transcriptome of Japanese sea bass tissue-mixed samples. The corresponding full-length cDNA sequences were generated by 5′ rapid amplification of cDNA ends (RACE) and 3′ RACE using a SMART RACE cDNA amplification kit (Takara, Dalian, China). Polymerase chain reaction (PCR), cloning and sequencing were performed to confirm the authenticity of *LjPL-3* and *LjPL-2* cDNA. Primers used in this study are listed in Table S1. The open reading frame was predicted using the Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The cleavage sites of the signal peptides were predicted using the SignalP5.0 program (https://services.healthtech.dtu.dk/service.php?SignalP-5.0). Sequence identities were analyzed by using the BioEdit version 7.1. Multiple sequence alignments were conducted by the ClustalW program (http://clustalw.ddbj.nig.ac.jp/). Phylogenetic analyses were accomplished using the neighbor-joining method in MEGA 7.0 software. The information of sequences used in this study are listed in Table S2. The molecular weight and isoelectric point (pI) values were predicted using the ProtParam tool (https://web.expasy.org/protparam/).

2.3. Bacterial challenges

Logarithmic growth phase *V. harveyi* strain (ATCC 33866) was chosen to infect fish. *V. harveyi* was cultured with tryptic soy broth at 28 °C, and used in the further experiment. To determine the impact of stimuli on LjPL-3 and LjPL-2 mRNA expression *in vivo*, Japanese sea bass were injected intraperitoneally (i.p.) with *V. harveyi* (1.2×10^4 CFU/fish in 100 μL phosphate buffer saline [PBS]) or PBS as control. After injecting, fish were sacrificed at 4, 8, 12, 24 and 48 h. Then the liver, spleen, head kidney, and trunk kidney were harvested. Snap-frozen tissues were stored at −80 °C for subsequent mRNA extraction.

As for *in vitro* experiment, MO/MΦ were isolated and cultured at a concentration of 2×10^6 cells/mL. *V. harveyi* was co-cultured with MO/MΦ at a multiplicity of infection (MOI) of 2. After treatments, cells were collected for further analysis.

2.4. Peptide synthesis and purification

Peptides were synthesized by the Fmoc solid-phase method at GL Biochem (Shanghai, China). Peptides were extracted, lyophilized, and purified by reverse-phase high-performance liquid chromatography (HPLC). The mobile phase contains 0.1% (v/v) trifluoroacetic acid (TFA, Sigma-Aldrich, Steinheim, Germany). The molecular masses and purities of the purified peptides were respectively verified by mass spectroscopy and HPLC (>95% purity, Fig. S1). The peptides were obtained as TFA salts. And the TFA salts of the peptides were used for final testing. The LjPL-2 sequence was FLKSIWRAAKGAIRGAKSGWRA, and the molecular weight of LjPL-2 mature peptide was 2431.09 Da. The LjPL-3 sequence was FFGMLIHGAIHAGKVIHGLIHG, and the molecular weight of LjPL-3 mature peptide was 2326.12 Da. Synthetic peptides were reconstituted in phosphate-buffered saline (PBS; pH 7.4) for the experiments.

2.5. Antimicrobial assays

To measure the antimicrobial activities of synthetic LjPL-3 and LjPL-2 (GL Biochem), various pathogenic strains were divided into several groups based on their characteristics. The minimum inhibitory concentration (MIC) was measured as mentioned above (Chen et al., 2016). Briefly, LjPL-3 and LjPL-2 were added to the culture media of *V. harveyi* (ATCC 33866), *Vibrio anguillarum* (ATCC 19264), *Vibrio alginolyticus* (ATCC 19264), *Escherichia coli* BL21 (ATCC BAA-1025), *Staphylococcus aureus* (ATCC 6538), *Streptococcus iniae* (ATCC 29178), *Aeromonas hydrophila* (ATCC 7966), *Pseudomonas aeruginosa* (ATCC 9027), *Edwardsiella tarda* (ATCC 15947), and *Saccharomyces cerevisiae* (ATCC 9080) in 96-well plates (Corning Inc., Corning, NY, USA). The final concentrations of LjPL-3 and LjPL-2 were 100.0, 50.00, 25.00, 12.50, 6.250, 3.125, and 1.563 μg/mL for each treatment. After incubating for 24 h at the appropriate temperature, the absorbance at 600 nm was examined to determine the MIC.

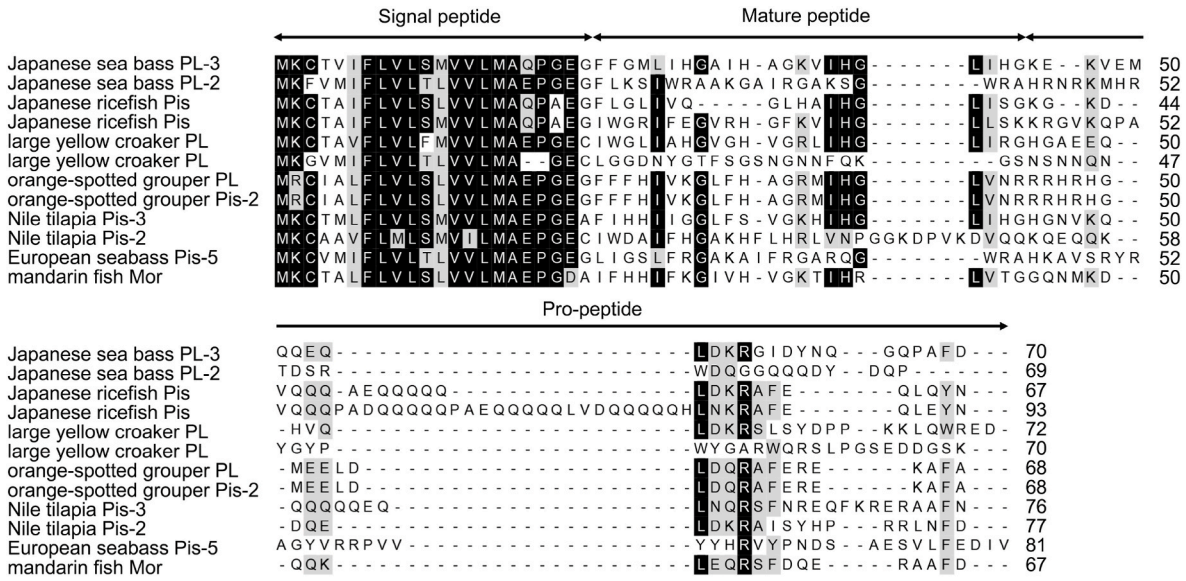

Fig. 1. Multiple sequence alignment of LjPL-3 and LjPL-2 amino acid sequences. Similar residues are shaded in gray, identical residues are shaded in black, and alignment gaps are marked as "–". The threshold for shading is 60%. The cleavage site for signal peptide, mature peptide and pro-peptide are indicated by "→" and "←". The signal peptide, mature peptide and pro-peptide sequence are indicated above the corresponding sequences. Pis: Piscidin; PL: Piscidin-like; Mor: Moronecidin. GenBank accession numbers of the amino acid sequences are listed in Table S2.

2.6. Real-time quantitative PCR (RT-qPCR)

Total cellular RNA was obtained from Japanese sea bass tissues and isolated MO/MΦ cells using Trizol (Invitrogen, Burlington, Canada). After treatment with DNase I (Thermo Fisher Scientific, Waltham, MA, USA), complementary DNA (cDNA) was synthesized using AMV reverse transcriptase (Takara). For RT-qPCR, cDNA was conducted on the Roche Light Cycler 480 real-time PCR System using SYBR Premix Ex Taq II (Takara). The primers for detection and quantification of fish genes used in this study are listed in Table S1. The RT-qPCR reaction system contained cDNA, primers, SYBR Premix Ex Taq II, and the protocol. The RT-qPCR reaction system was running 40 cycles (30 s at 95 °C, 30 s at 55 °C, and 30 S at 72 °C). Data were analyzed using the comparative ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001).

2.7. Primary culture of MO/MΦ

The Japanese sea bass head kidney-derived MO/MΦ were isolated and cultured as previously described (Zhan et al., 2022). Briefly, fish were sacrificed under anesthetic (eugenol, 1:10000; Shanghai Reagent, Shanghai, China). The head kidney was rapidly taken and cleaned up, and cells were dispersed using a 100 μm nylon cell strainer (Corning Inc.). Head kidney leukocytes were enriched though Ficoll density gradient separation (Ficoll-Paque, GE Healthcare, Shanghai, China). Cells were cultured with 2×10^7 cells/mL density at 24 °C with 5% CO_2. Afterwards, adherent cells were incubated in a complete medium after removing nonadherent cells. Over 95% of the adherent cells were MO/MΦ based on the morphological characteristics according to Wright–Giemsa staining (Baso Diagnostics Inc., Zhuhai, China) results.

2.8. Phagocytosis assays

In order to acquire DH5α-FITC, *E. coli* DH5α cells were incubated with fluorescein isothiocyanate (FITC, Sigma-Aldrich) according to the manufacturer's instruction. Japanese sea bass MO/MΦ (2×10^6 cells/mL in 2 mL) were treated with 1.25, 2.5, 5, and 10 μg/mL LjPL-3 or LjPL-2 for 8 h, PBS for control. DH5α-FITC was then added at an MOI of 10 and incubated for 30 min. The cells were washed with PBS. The fluorescence that adhered to the cell surface was quenched with 10 μL trypan blue (0.4%; Sangon Biotech, Shanghai, China). Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) and FlowJo software (Treestar Inc., Ashland, OR, USA) were used to analyze bacterial engulfment. Relative mean fluorescence intensity (MFI) of LjPL-3 and LjPL-2-treated group was performed as a fold-change relative to the negative group (without bacteria treated). And the MFI of PBS-treated group was defined as a value unit of 100. Five independent experiments were performed.

2.9. Bacterial killing assays

Bacterial killing assays were performed as previously described (Dai et al., 2021). After incubation with 1.25, 2.5, 5, and 10 μg/mL LjPL-3 or LjPL-2 for 8 h and PBS for control, Japanese sea bass MO/MΦ (2×10^6 cells/mL) were infected with *V. harveyi* at an MOI of 10 for 30 min at 24 °C. Gentamicin (50 μg/mL; Aladdin, shanghai, China) was used to eliminate the remaining bacteria outside (Gao et al., 2021). Meantime, the MO/MΦ were washed thrice with sterile PBS. One set of samples (the "uptake" group) was collected and lysed in 1% Triton X-100 (Sigma-Aldrich) to determine the bacterial contents in cells. Corresponding to the "uptake" group, another set of samples (the "kill" group) was incubated for 1.5 h to facilitate bacterial killing prior to cell lysis. The lysis supernatant from both groups was serially diluted, and then plated onto Thiosulfate Citrate Bile Salts (TCBS) agar (Solarbio, Beijing, China) medium at 28 °C for 24 h to assess the *V. harveyi* CFUs. The bacterial survival rate was determined by dividing the number of CFUs in the kill group by those in the uptake group.

2.10. Migration assays

For *in vitro* chemotaxis assays, cells were cultured in a 24-well transwell chamber (Corning Inc.) as previously reported (Gao et al., 2020). Four concentrations of LjPL-3 and LjPL-2 (1.25, 2.5, 5, and 10 μg/mL) were added to the lower chambers in a volume of 600 μL. PBS was used as a control. Japanese sea bass MO/MΦ (2×10^6 in 100 μL) were added to the upper chambers. The chambers were incubated at 24 °C with 5% CO_2 for 4 h. Cells that migrated to the lower chamber were stained with Wright–Giemsa staining and counted using a microscope.

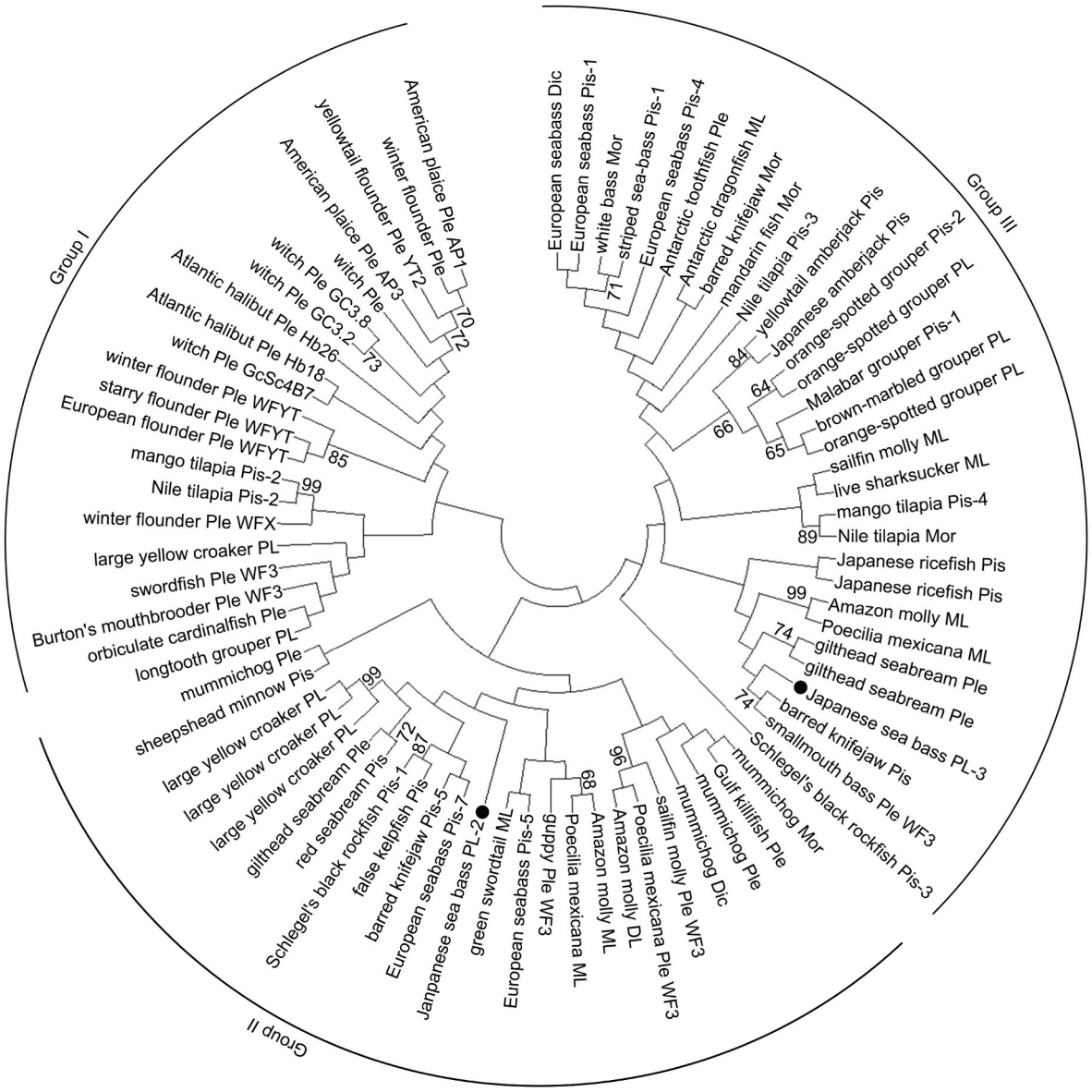

Fig. 2. Phylogenetic tree analysis of LjPL-3 and LjPL-2 amino acid sequences and other related piscidins. The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1,000 replicates; shown only when >60%). The scale bar represents the number of substitutions per base position. Ple: Pleurocidin; Pis: Piscidin; PL: Piscidin-like; Mor: Moronecidin, ML: Moronecidin-like, Dic: Dicentracin, DL: Dicentracin-like. GenBank accession numbers of sequences used are listed in Table S2.

Migration assays *in vivo* were conducted according to the method previously described (Zhang et al., 2021). Briefly, Japanese sea bass were i.p. injected with 1.25, 2.5, 5, and 10 μg/g LjPL-3 or LjPL-2 (in 100 μL PBS), whereas the control group was injected with the same volume of PBS. After treatments for 24 h, fish were sacrificed, and the peritoneal fluid was harvested by rinsing with a sterilized syringe containing 2 mL PBS. The cell pellets were retained by centrifugation at 2000 rpm for 8 min and resuspended in 1 mL of PBS. Migrated cells were counted and observed using a hemocytometer and microscopically using Wright–Giemsa staining.

2.11. Fish survival and bacterial burden assays

Fish were divided into five groups (each containing 32 fish) for survival assays. Fish were i.p. injected with 1.2×10^4 CFU/fish live *V. harveyi*. After 30 min, fish received i.p. injections of 1.25, 2.5, 5, and 10 μg/g LjPL-3 or LjPL-2 of fish weight, whereas the control group received PBS. Fish were observed every 24 h for death or moribund state for seven days.

The bacterial burden assays were conducted as previously reported (Zhang et al., 2021). In this experiment, fish were divided into five groups of five fish each. Each fish was i.p. injected with 1.2×10^4 CFUs of *V. harveyi*. After 30 min, each fish was injected with 1.25, 2.5, 5, and 10 μg/g LjPL-3 or LjPL-2. Subsequently, the liver, spleen, head kidney, and blood were aseptically collected at 24 h post infection (hpi) for bacterial burden assays. Fresh tissue (0.1 g) and blood (0.1 mL) samples were homogenized in 1 mL PBS. The homogenates were serially diluted in sterile PBS, plated on TCBS agar plates, and incubated for 18–24 h at 28 °C. Colonies were counted. CFUs were calculated by multiplying the number of colonies on the plate by the dilution factor and normalized to 0.1 g tissue weight or 0.1 mL blood volume.

2.12. Statistical analysis

After confirming normality, data were showed as the mean ± standard error (SEM). The biological repeats were indicated by 'n'. Sample

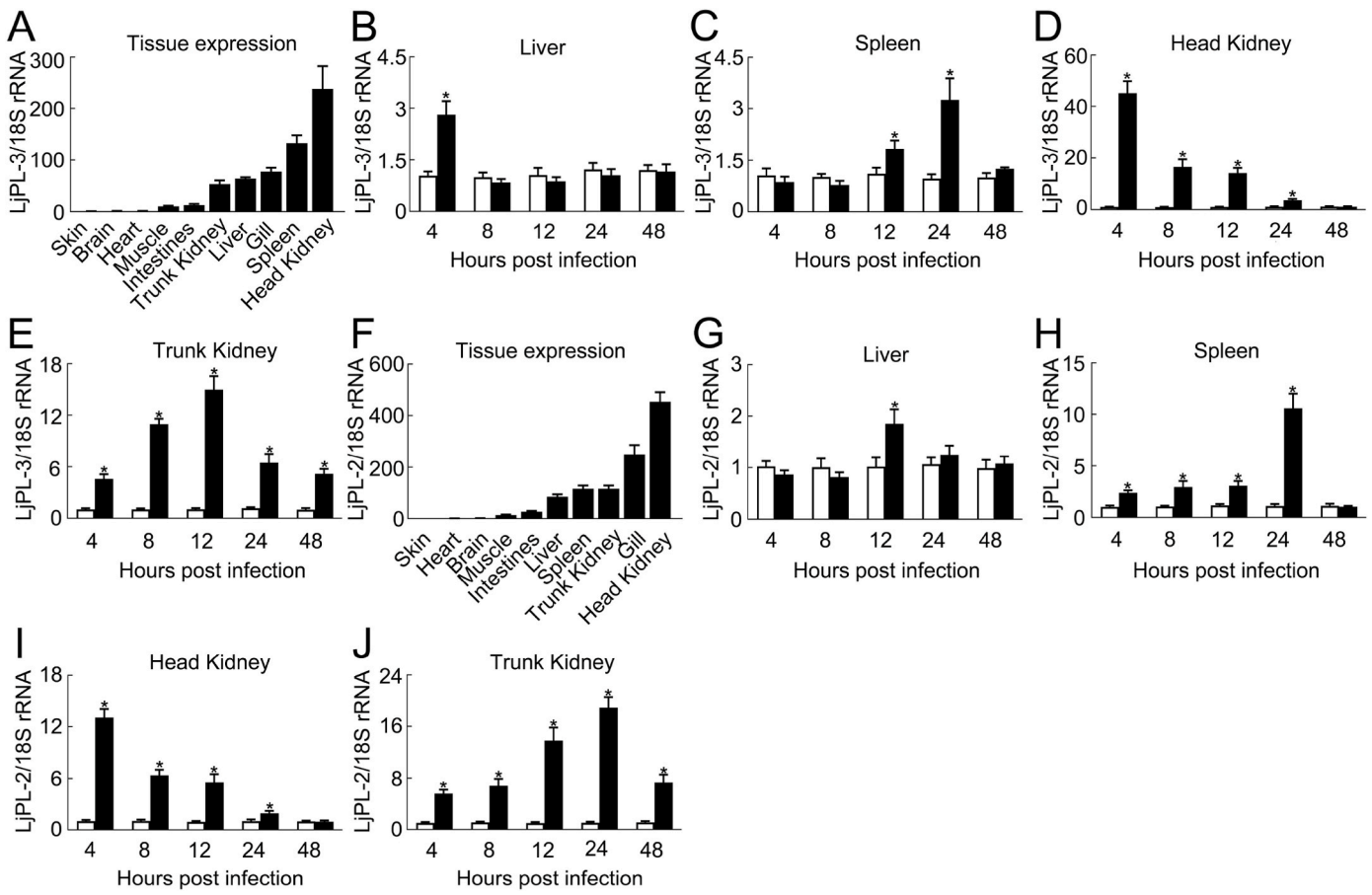

Fig. 3. RT-qPCR analysis of LjPL-3 and LjPL-2 mRNA expression in Japanese sea bass tissues. (A) LjPL-3 mRNA expression in different tissues. Gene expression is reported as the fold change of different tissues relative to the amount of the skin. (B–E) LjPL-3 mRNA expression in Japanese sea bass tissues after infection. LjPL-3 expression is normalized to that of 18S rRNA. (F) LjPL-2 mRNA expression in different tissues. Gene expression is reported as the fold change of different tissues relative to the amount of the skin. (G–J) LjPL-2 mRNA expression in Japanese sea bass tissues after infection. LjPL-2 expression is normalized to that of 18S rRNA. Data are expressed as the mean ± SEM. n = 5. $^*P < 0.05$.

Table 1
MIC values of LjPL-3 and LjPL-2 against microbes.

Microorganisms	Isolates/ strains	LjPL-3 MIC (μg/ mL)	LjPL-2 MIC (μg/ mL)
Gram-negative bacteria			
Escherichia coli	BAA-1025	25.00	50.00
Pseudomonas aeruginosa	ATCC9027	25.00	NT
Aeromonas hydrophila	ATCC7966	50.00	NT
Edwardsiella tarda	ATCC15947	NT	50.00
Vibrio anguillarum	ATCC19264	50.00	3.125
Vibrio harveyi	ATCC33866	3.125	25.00
Vibrio alginolyticus	ATCC19264	NT	25.00
Gram-positive bacteria			
Streptococcus iniae	ATCC29178	25.00	100.0
Staphylococcus aureus	ATCC6538	NT	3.125
Fungi			
Saccharomyces cerevisiae	ATCC9080	25.00	50.00

size was chosen based on preliminary data and observed effect sizes. Besides, all results were analyzed with one-way ANOVA followed by Duncan's multiple range test using SPSS statistical software (version 13.0; Chicago, IL, USA). Finally, GraphPad Prism software version 8.3.0 was used to perform the results of statistical analyses. P values < 0.05 were taken to be statistically significant.

3. Results

3.1. Sequence analysis and prediction of LjPL-3 and LjPL-2

The sequences of *LjPL-3* and *LjPL-2* determined in this study have been deposited in the GenBank Data Library with accession numbers OQ338172 and OQ338173, respectively. The full-length cDNA sequence of *LjPL-3* contained an open reading frame of 213 base pairs (bp) that encoded a 70 amino acids (aa) protein. The full-length cDNA sequence of *LjPL-2* contained an open reading frame of 210 bp, which encoded a 69 aa protein. The signal peptide (aa 1–22) was conserved in LjPL-3 and LjPL-2 (Fig. 1A). The molecular weight and pI of the full-length LjPL-3 peptide were 7.75 kDa and 6.38, respectively, and the molecular weight and pI of the full-length LjPL-2 peptide were 7.97 kDa and 10.58, respectively. Removal of the signal peptide and pro-peptide domain resulted in the formation of a 22 aa active peptide. The mature LjPL-3 peptide is predicted to have a net positive charge of +1, while LjPL-2 is predicted to have a net charge of +6 at pH 7.0. LjPL-3 active peptide has a molecular weight of 2.33 kDa and a pI of 8.78, and LjPL-2 active peptide has a molecular weight of 2.43 kDa and a pI of 12.31.

3.2. Multiple alignments and phylogenetic analysis of LjPL-3 and LjPL-2

To examine the evolutionary relationships between LjPL-3, LjPL-2, and similar piscidins in other species, we used the neighbor-joining method to construct a phylogenetic tree (Fig. 2). As shown in Fig. 2, all piscidins in the tree were divided into three distinct clusters. LjPL-3

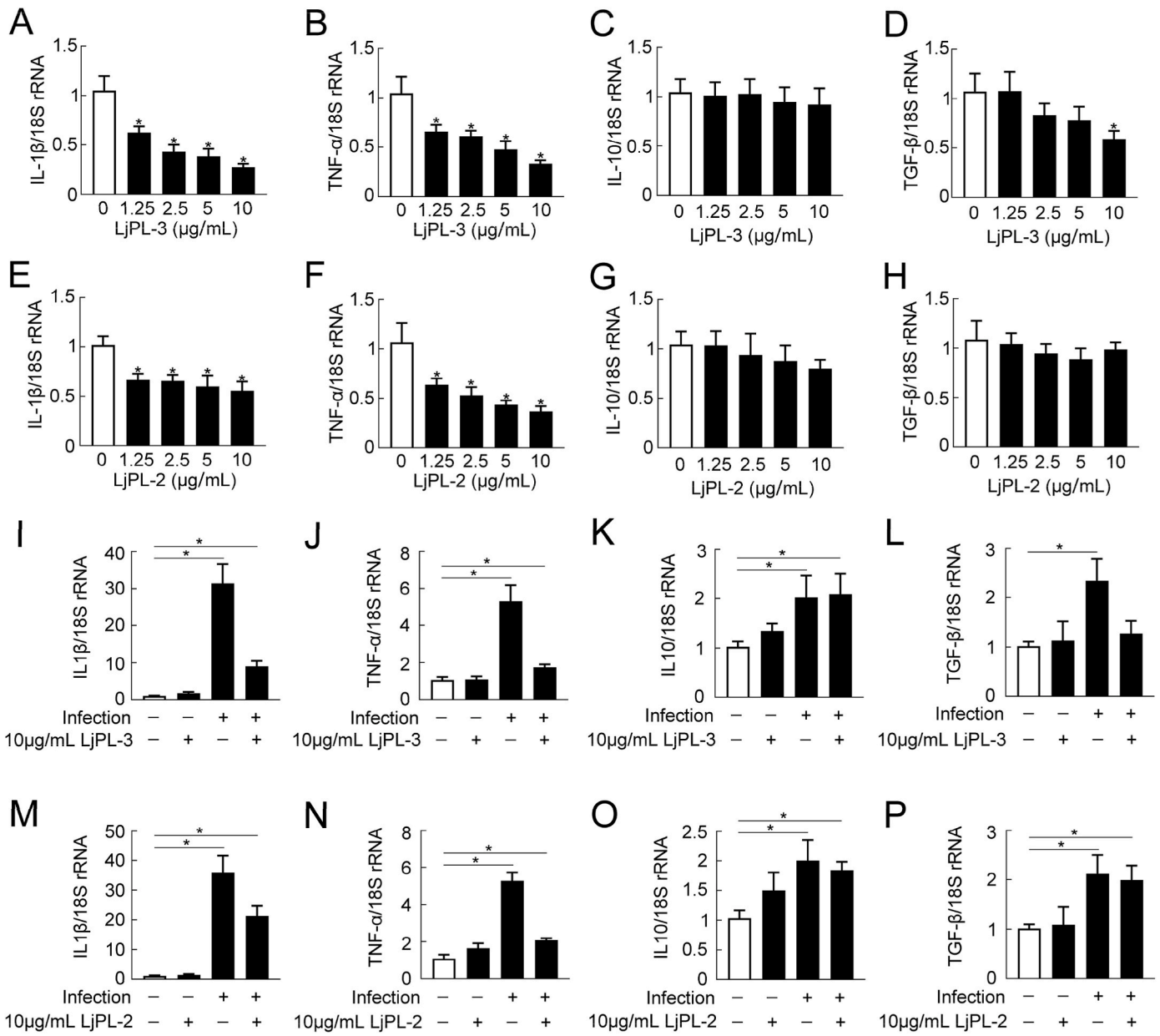

Fig. 4. Effects of LjPL-3 and LjPL-2 on the mRNA levels of cytokines in Japanese sea bass MO/MΦ. (A–H) Effects of LjPL-3 and LjPL-2 on the mRNA levels of cytokines. MO/MΦ were pretreated with 0, 1.25, 2.5, 5, and 10 μg/mL LjPL-3 or LjPL-2. Then, MO/MΦ were infected with *V. harveyi* for 12 h. (I–P) Effects of single treatments with infection, LjPL-3, and LjPL-2 on the mRNA levels of cytokines. Cytokine mRNA levels are normalized to those of 18S rRNA. Gene expression is presented as the fold changes relative to levels in the control group. Data are presented as the means ± SEM. n = 5. $^*P < 0.05$.

was clustered with barred knifejaw piscidin and smallmouth bass pleurocidin WF3 in group 3, while LjPL-2 was clustered in group 2.

3.3. mRNA distribution of LjPL-3 and LjPL-2

To further illustrate the tissue distribution of *LjPL-3* and *LjPL-2*, RT-qPCR was performed to analyze the levels of *LjPL-3* and *LjPL-2* mRNAs in normal and infected tissues. *LjPL-3* and *LjPL-2* mRNAs were detected in all healthy tissues, with the highest expression detected in the head kidney (Fig. 3A and F). Next, we analyzed *LjPL-3* and *LjPL-2* mRNA levels in immune tissues following infection with *V. harveyi*. *LjPL-3* and *LjPL-2* mRNA levels were upregulated in the liver, spleen, head kidney, and trunk kidney at different time points (Fig. 3B–E and G-J).

3.4. LjPL-3 and LjPL-2 possess direct antimicrobial activity

To investigate the antimicrobial activity of LjPL-3 and LjPL-2 against gram-positive and gram-negative bacteria and fungi, the MIC was determined. The results showed that two piscidin-like peptides from the Japanese sea bass exhibited distinct antimicrobial activity. The synthesized mature LjPL-3 peptide showed broad-spectrum antimicrobial activity against *E. coli*, *P. aeruginosa*, *A. hydrophila*, *V. anguillarum*, *V. harveyi*, *S. iniae*, and *S. cerevisiae* (Table 1). LjPL-3 exhibited the highest antimicrobial activity against *V. harveyi* (MIC = 3.125 μg/mL). The synthesized mature LjPL-2 peptide showed broad-spectrum antimicrobial activity against *E. coli*, *E. tarda*, *V. anguillarum*, *V. harveyi*, *V. alginolyticus*, *S. iniae*, *S. aureus*, and *S. cerevisiae* (Table 1). LjPL-2 showed the highest antimicrobial activity against *V. anguillarum* and *S. aureus* (MIC = 3.125 μg/mL).

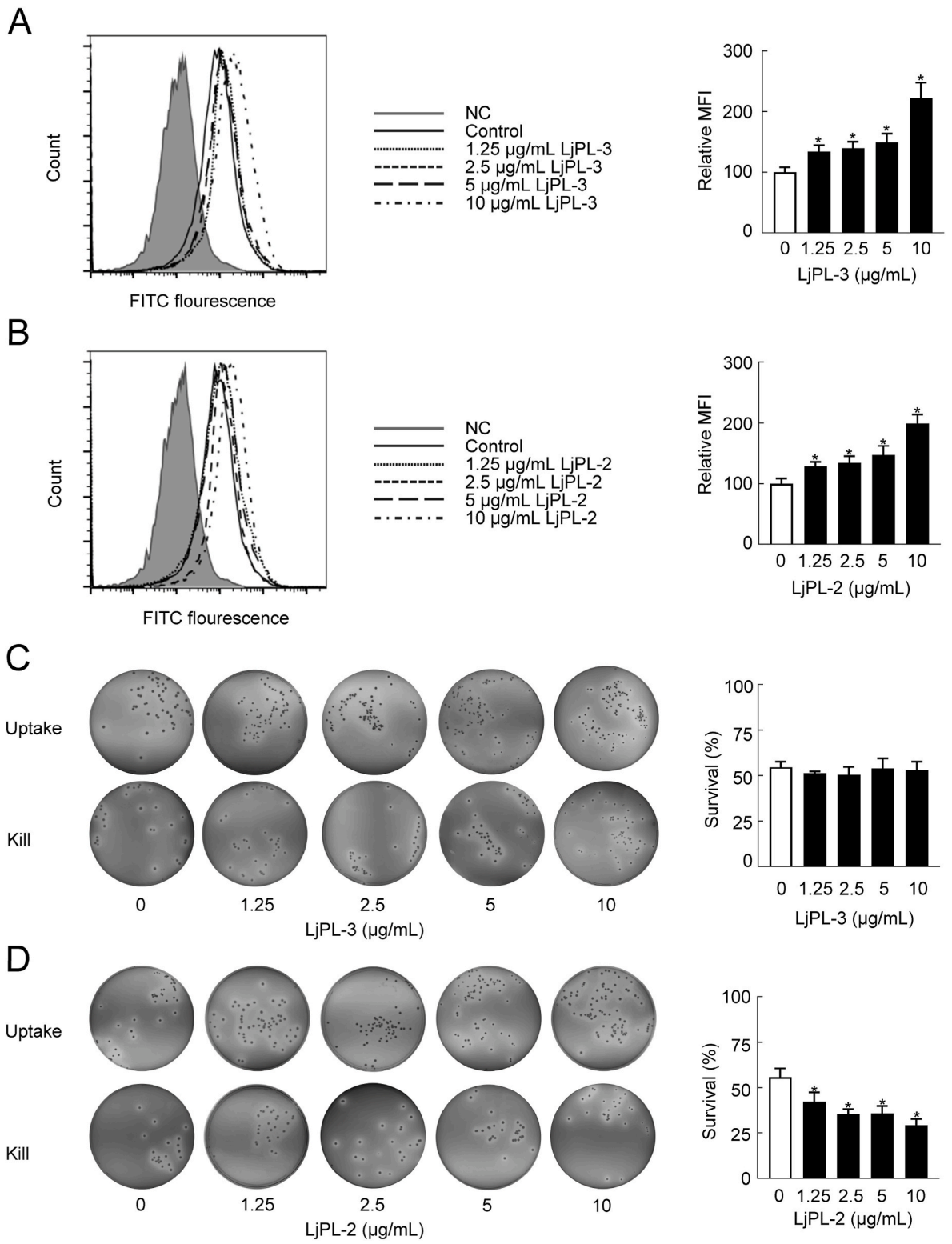

Fig. 5. Effects of LjPL-3 and LjPL-2 on phagocytosis and bacterial killing of Japanese sea bass MO/MΦ. (A and B) The phagocytosis of FITC-DH5α by MO/MΦ treated with LjPL-3 or LjPL-2. The MFI is presented as fold-change relative to the PBS-treated group, which is assigned an arbitrary value of 100. (C and D) The effect of LjPL-3 or LjPL-2 on bacterial killing of MO/MΦ. Data are expressed as mean ± SEM. n = 5. *$P < 0.05$.

3.5. *Effect of LjPL-3 and LjPL-2 on cytokine expression in MO/MΦ*

LjPL-3 and LjPL-2 were incubated with different concentrations of MO/MΦ from Japanese sea bass. Next, we analyzed the effects of LjPL-3 and LjPL-2 on the expression of tumor necrosis factor-α (LjTNF-α), interleukin-1β (LjIL-1β), transforming growth factor-β (LjTGF-β), and interleukin-10 (LjIL-10) after *V. harveyi* infection in Japanese sea bass. LjPL-3 treatment decreased the expression of LjIL-1β, LjTNF-α, and LjTGF-β in *V. harveyi* infected MO/MΦ (Fig. 4A–D). However, there was no significant change in the LjIL-10 mRNA levels in MO/MΦ after

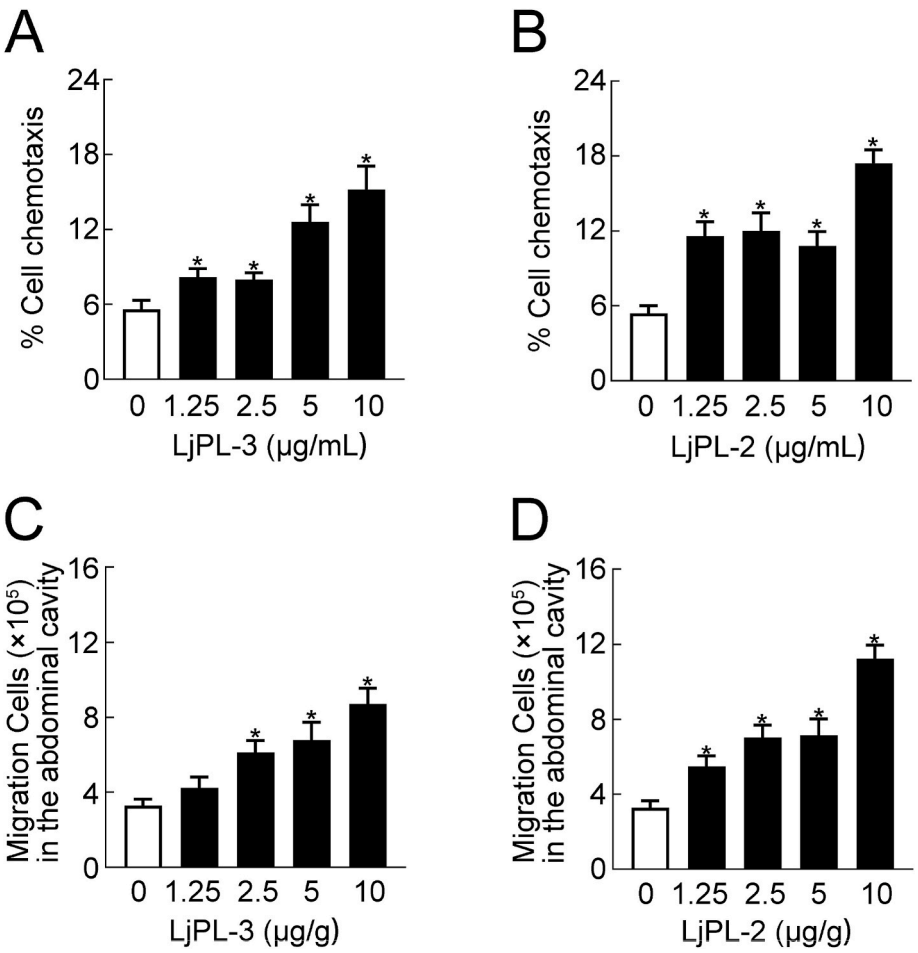

Fig. 6. Effects of LjPL-3 and LjPL-2 on the migration of MO/MΦ. (A and B) The chemotaxis percentage of MO/MΦ *in vitro*. (C and D) The number of MO/MΦ in the abdominal cavity *in vivo*. Data are expressed as the mean ± SEM. n = 5. *$P < 0.05$.

treatment with LjPL-3 (Fig. 4C). According to qPCR data, LjPL-2 treatment inhibited *V. harveyi*-induced mRNA expression of LjIL-1β and LjTNF-α in MO/MΦ at 12 hpi (Fig. 4E–H). Treatment with LjPL-3 and LjPL-2 alone did not regulate the expression of LjIL-1β, LjTNF-α, LjIL-10, and LjTGF-β (Fig. 4I-P).

3.6. Effect of LjPL-3 and LjPL-2 on phagocytosis and bacterial killing of Japanese sea bass MO/MΦ

As LjPL-3 and LjPL-2 impacted the expression of cytokine mRNAs, we further investigated the effects of LjPL-3 and LjPL-2 on phagocytosis and bacterial killing of MO/MΦ. Treatments with LjPL-3 and LjPL-2 improved the phagocytotic ability of MO/MΦ (Fig. 5A and B). In addition, the plate counting method was performed to measure bacterial killing of MO/MΦ (Fig. 5C and D). We found that the survival rate of *V. harveyi* in LjPL-2-treated Japanese sea bass MO/MΦ was lower than that in PBS-treatment (Fig. 5D). The survival rate of *V. harveyi* was not significantly different between the LjPL-3-treated and control groups (Fig. 5C and D).

3.7. In vitro and in vivo effect of LjPL-3 and LjPL-2 on MO/MΦ migration

An *in vitro* Transwell chemotaxis assay was conducted to test the chemotactic activity of LjPL-3 and LjPL-2. Dose-dependent migration of MO/MΦ was observed following LjPL-3 and LjPL-2 treatments (Fig. 6A and B). The treatments of 1.25, 2.5, 5, and 10 μg/mL LjPL-3 induced the chemotaxis of MO/MΦ at a level of 8.20 ± 0.66%, 8.00 ± 0.55%, 12.60 ± 1.36%, and 15.20 ± 1.85%, respectively, while that of the control was 5.60 ± 0.75% (Fig. 6A). The treatments of 1.25, 2.5, 5, and 10 μg/mL LjPL-2 induced the chemotaxis of MO/MΦ at a level of 11.60 ± 1.12%, 12.00 ± 1.45%, 10.80 ± 1.16%, and 17.40 ± 1.08%, while that of the control was 5.40 ± 0.60% (Fig. 6B).

An *in vivo* cell migration assay was conducted to count the number of migrated MO/MΦ in the abdominal cavity of Japanese sea bass. Administration of 2.5, 5 and 10 μg/g LjPL-3 increased the number of MO/MΦ in the abdominal cavity of fish (Fig. 6C). LjPL-2 increased the number of MO/MΦ in the abdominal cavity of fish (Fig. 6D). However, 1.25 μg/mL LjPL-3 treatment showed no significant change in the migration of MO/MΦ *in vivo*.

3.8. Effect of LjPL-3 and LjPL-2 on survival rate and bacterial burden of V. harveyi-infection

We investigated the effects of the synthesized mature peptides *in vivo* on host defense by monitoring the survival of Japanese sea bass infected with *V. harveyi*. Treatments with LjPL-3 and LjPL-2 decreased the mortality rate of Japanese sea bass (Fig. 7A and B). Seven days after *V. harveyi* infection, the survival rates were 18.75%, 28.13%, 34.38%, and 40.63% for Japanese sea bass treated with 1.25, 2.5, 5, and 10 μg/g LjPL-3, respectively (Fig. 7A). The survival rates of Japanese sea bass treated with 1.25, 2.5, 5, and 10 μg/g LjPL-2 were 31.25%, 34.38%, 31.25%, and 59.38%, respectively (Fig. 7B).

Bacterial loads were quantitated in the liver, spleen, head kidney, and blood following i.p. injection of LjPL-3 and LjPL-2 in *V. harveyi*

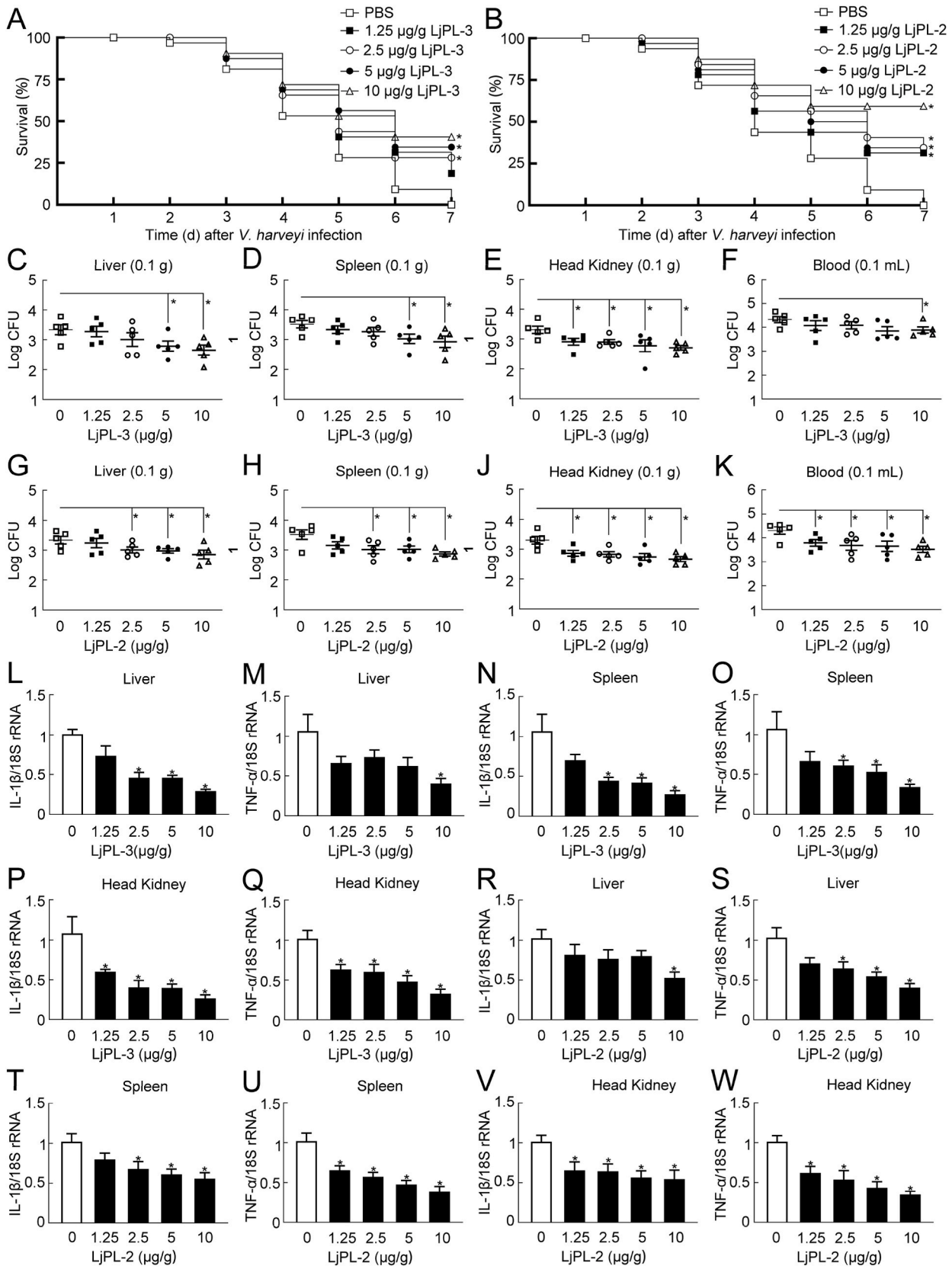

Fig. 7. LjPL-3 and LjPL-2 improve outcomes in infected Japanese sea bass. (A and B) The survival rates of LjPL-3 and LjPL-2 treated Japanese sea bass. Experiments are representative of 32 fish per group. $^*P < 0.05$. (C–K) Effects of LjPL-3 and LjPL-2 on bacterial burden in the liver, spleen, head kidney, and blood. Tissues were collected at 24 h after *V. harveyi* challenge. CFUs were normalized to 0.1 g of tissue weight or 0.1 mL of blood. n = 5. (L–W) Effect of LjPL-3 and LjPL-2 on mRNA levels of LjIL-1β and LjTNF-α. Tissues were collected at 12 h after *V. harveyi* challenge. LjIL-1β and LjTNF-α transcript levels are normalized to 18S rRNA. n = 5. Data are expressed as mean ± SEM. $^*P < 0.05$.

challenged fish. Fish treated with LjPL-3 and LjPL-2 showed a reduction in *V. harveyi* load in the liver, spleen, head kidney, and blood after *V. harveyi* challenge (Fig. 7C–K).

We further investigated the effects of LjPL-3 and LjPL-2 on inflammatory gene expression *in vivo*. The mRNA levels of the inflammatory cytokines LjIL-1β and LjTNF-α were evaluated in tissues after LjPL-3, LjPL-2, and PBS treatment following infection with *V. harveyi* for 12 h. According to the qPCR data, LjPL-3 and LjPL-2 treatments inhibited the expression of LjIL-1β and LjTNF-α in the liver, spleen, and head kidney (Fig. 7L-W).

4. Discussion

The piscidin family comprises fish-specific AMPs (Bhat et al., 2022). In the present study, LjPL-3 and LjPL-2 were identified and characterized in the Japanese sea bass. We observed that both LjPL-3 and LjPL-2 reduced inflammatory cytokine production after infection, while promoting chemotaxis and phagocytosis in MO/MΦ. Only LjPL-2 displayed bacterial killing capability in MO/MΦ. In Nile tilapia, piscidin also regulates pro-inflammatory activity in macrophages (Liu et al., 2021), but the regulatory activity of piscidins in the immune cells of other fish is unclear. Our study showed that two piscidin-like peptides from Japanese sea bass have different regulatory functions in MO/MΦ.

Transcripts of piscidin genes are widely distributed in various tissues (Neshani et al., 2019; Serna-Duque et al., 2022). In seabream specimens, piscidin-1 is highly expressed in the thymus of healthy fish, while piscidin-2 is mostly expressed in the head kidney (Serna-Duque et al., 2022). Piscidin 5-like type 4 from the large yellow croaker is abundant in the head kidney, spleen, gill, and liver (Zheng et al., 2021). In this study, the LjPL-3 transcript was highly expressed in the head kidney, spleen, and gill of Japanese sea bass, and the LjPL-2 transcript was highly expressed in the head kidney, gill, and trunk kidney, suggesting that piscidin genes have a variety of tissue expression patterns. After bacterial challenge, the expressions of *Micropterus salmoides* Piscidin-1, -2, and -3 are upregulated in the skin, gill, spleen, head kidney, intestine, and liver (Duan et al., 2022). Piscidin mRNA expression is significantly up-regulated in the liver, intestine, and spleen of rockfish after lipopolysaccharide (LPS) challenge (Bo et al., 2019). In the current study, LjPL-3 and LjPL-2 mRNA levels were upregulated in the trunk kidney, head kidney, spleen, and liver at different time points after *V. harveyi* infection. These results indicate that the mRNA expression of piscidins in fish is upregulated after microbial pathogen infection.

Piscidins usually exhibit antimicrobial activity against both gram-positive and gram-negative bacteria. In previous studies on fish AMPs, *Micropterus salmoides* Piscidin-2 and Piscidin-3 showed strong antimicrobial capacities against *V. harveyi*, *V. alginolyticus*, and *V. anguillarum* (Duan et al., 2022). The synthetic piscidin-like cerocin peptide exhibits direct antibacterial activity against *V. harveyi* (MIC 24–48 μM) (Qiao et al., 2021). Tilapia piscidin 3 (TP3) and TP4 present excellent antimicrobial activity against *V. vulnificus* 204 and *V. alginolyticus* than other tilapia piscidins (Peng et al., 2012). LjPL-3 and LjPL-2 also showed direct antimicrobial activity against *V. harveyi* (MIC 3.125 and 25.00 μg/mL). Comparison of our results with TP3 and TP4, we found that LjPL-3 and -2 exhibited similar antimicrobial activity against *Vibrio* species (Peng et al., 2012). This similarity may be attributed to the fact that both mature proteins have a pI value greater than 8.00. Despite having a higher pI and net charge than LjPL-3, LjPL-2 demonstrated lower antimicrobial activity against *V. harveyi*. We inferred that other factors may contribute to its bioactivity. And they showed different antimicrobial activities against bacteria and fungi.

In addition to their direct antimicrobial effects, several AMPs also possess immunomodulatory functions (Luo and Song, 2021; Weiss and Schaible, 2015; Zaccone et al., 2022). The fish immune response is a cascade of diverse reactions that aims to eliminate recognized foreign microbes and restore homeostasis (Johnstone and Chaves-Pozo, 2022; Sayyaf Dezfuli et al., 2021; Zhang et al., 2017). LjPL-3 and LjPL-2 decreased mortality after *V. harveyi* challenge, which was accompanied by lower bacterial burdens. Cytokines (e.g., IL-1β, TNF-α, and TGF-β) usually play central roles in the early inflammatory response, and the altered expression of cytokines in MO/MΦ reflects cell activation (Grayfer et al., 2018; Mu et al., 2019; Yang et al., 2023). Epinecidin-1 in orange-spotted grouper suppresses the production of pro-inflammatory factors in macrophages (Su and Chen, 2020). Grass carp β-defensin 1 inhibits LPS-induced TNF-α secretion in head kidney MO/MΦ (Yang et al., 2019). TP4 decreases pro-inflammatory mediators in macrophages (Liu et al., 2021). In the present study, both LjPL-3 and LjPL-2 treatments decreased *V. harveyi*-induced LjIL-1β and LjTNF-α mRNA expression in MO/MΦ.

In both mammals and fish, MO/MΦ are important components of the innate immune system (Bai et al., 2022; Lu and Chen, 2019; Shwartz et al., 2019). AMPs have been found to regulate MO/MΦ activity in other teleost species. For example, mudskipper liver-expressed antimicrobial peptide 2 (LEAP-2) promotes MO/MΦ chemotaxis and bacterial killing activity via mediation by *Boleophthalmus pectinirostris* motile sperm domain-containing protein 2 (BpMOSPD2) (Li et al., 2020). Large yellow croaker β-defensin enhances the phagocytosis of MO/MΦ (Li et al., 2021). In this study, we demonstrated that LjPL-3 and LjPL-2 promoted chemotaxis and phagocytosis in Japanese sea bass MO/MΦ. Only LjPL-2 promoted bacterial killing in MO/MΦ. These data suggested that LjPL-3 and LjPL-2 activated MO/MΦ to participate in host defenses. The activation of phagocytic ability in MO/MΦ is influenced by pro-inflammatory cytokines and nuclear factor kappa-B (NF-κB) pathway (Grayfer et al., 2018; Zou and Secombes, 2016; Dorrington and Fraser, 2019; Mussbacher et al., 2023). In grass carp, IL-1β interacts with AMPs to enhance the phagocytic activity of head kidney macrophages by activating the NF-κB pathway (Guo et al., 2022). NF-κB subunit-deficient mice have impaired innate immune responses to bacterial sepsis, with macrophages showing deficiencies in phagocytosis, bacterial killing, and antimicrobial peptide production (Courtine et al., 2012). These results suggest that LjPL-3 and LjPL-2 not only have direct antimicrobial activity, but also modulate the functions of MO/MΦ in Japanese sea bass.

The synthesized LjPL-2 and LjPL-3 peptides displayed similar structural properties such as a potential amphipathic α-helical structure and cationic features. But they have different MICs and biological activities, the reasons may be related to conformational differences and biological function. LjPL-2 has higher pI value and net charge than LjPL-3. Amino acid changes affect the bacterial cell selectivity, as shown by hybrid striped bass piscidin 1 with a glycine replacing alanine at position 8 (Lee et al., 2007). LjPL-3 has alanine at position 8, while LjPL-2 has glycine at position 8, which influences their antimicrobial activity. Further experiments are needed to elucidate the mechanism behind the different antimicrobial spectra of LjPL-3 and LjPL-2.

In conclusion, this study identifies the differential antimicrobial activities of LjPL-3 and LjPL-2, which play important roles in host defenses in Japanese sea bass. Our results provide new information on piscidin-like peptides with immunomodulatory properties and set the stage for future studies on disease prevention and treatments in Japanese sea bass.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2023.104726.

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