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Antimicrobial peptides of an anti-lipopolysaccharide factor, epinecidin-1, and hepcidin reduce the lethality of *Riemerella anatipestifer* sepsis in ducks

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ABSTRACT

Antimicrobial peptides (AMPs) are effective against a wide range of microbes, but still no research results have reported their use in duck disease therapy. *Riemerella anatipestifer* (RA) is a Gram-negative bacterium which infects ducks and causes very significant economic losses. The minimum inhibitory concentrations (MICs) of epinecidin-1 for the tested RA strains ranged 6.25–50 μg/ml, those of the SALF55–76 cyclic peptide ranged 12.5–25 μg/ml, those of the SALF55–76 linear peptide ranged 6.25–25 μg/ml, those of hepcidin TH1–5 ranged 25–400 μg/ml, and those of hepcidin TH2–3 ranged 100–400 μg/ml. The antimicrobial activities of these peptides were confirmed by transmission electron microscopy which showed that RA disruption of the outer membrane brought about cell death. In addition, pretreatment, co-treatment, and post-treatment with peptides were all effective in promoting a significant decrease in duck mortality and decreasing the number of infectious bacteria. A quantitative RT-PCR was performed to survey levels of gene expressions of Mn superoxide dismutase in the brain, lipoprotein lipase in the liver, and H5 histone in the spleen induced in response to bacterial infection and an injection of the AMPs in experiments with the duck, *Cairina moschata*. Our results indicated that the rescue of ducks by the peptides and the behavior of the peptides, which was like an enhancer in immunology, may involve regulation of the expressions of these genes. Collectively, these peptides reduced the mortality in ducks during bacterial challenge, suggesting that AMPs have the potential to serve as therapeutic drugs for use against bacterial infectious diseases in ducks.

1. Introduction

The duck-raising industry has been severely hampered by outbreaks of *Riemerella anatipestifer* (RA) worldwide [31]. Primary outbreaks in young, growing ducks are characterized by high mortality, septicemia, and polyserositis [36]. Infection by RA occurs not only in ducks but also in other birds, including turkeys, chickens, quail, pheasants, whistling swans, geese, and other waterfowl [33]. RA has caused large economic losses, and so far, no adequate treatment is available except for antibiotics such as ceftiofur [6]. Different serotypes of RA from field cases were reported [17,21], and serotyping is used to discriminate isotypes of infectious RA strains. Although extensive studies on the pathogenesis and vaccine development have played roles in reducing RA infection and genotypic variations have been used to produce different vaccines, a lack of cross-protection among different serotypes of RA limits the usefulness of vaccines in controlling this disease. As a result, most farms continue to administer antibiotics to treat RA disease.

Even though RA is usually susceptible to a wide range of antibiotics [1,9], the excessive and inappropriate use of antibiotics has resulted in the production of resistant bacterial strains. At the same time, new antibacterial therapies have been developed that use antimicrobial peptides (AMPs) [25]. AMPs are widely distributed intrinsic host defense molecules that are produced by plants, vertebrates, and invertebrates [40]. Many AMPs contain cationic amino acid residues and can form amphiphilic α-helices that are well separated in space from hydrophobic residues [10,24]. Fish and shrimp have evolved to thrive in aqueous environments that contain rich microbial floras, and their innate immune systems act as the first line of defense against microbial invasion. Many AMPs from different fish species including those living in fresh water and seawater show broad spectra of antimicrobial activities against Gram-positive and -negative

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bacteria [16]. These peptides provide fish protection against pathogenic infections and have been shown to play important roles in fish innate immunity.

Recently, three AMPs, epinecidin-1, anti-lipopolysaccharide (LPS) factor, and hepcidin, were, respectively, identified in grouper (*Epinephelus coioides*), shrimp (*Penaeus monodon*), and tilapia (*Oreochromis mossambicus*) [14,27,28]. Epinecidin-1 is able to rescue infected fish and inhibit LPS-induced tumor necrosis factor (TNF)-α expression [28], while the anti-LPS factor reduces the lethality of *Pseudomonas aeruginosa* sepsis in mice [28]. Therefore, because of the different performances of epinecidin-1, hepcidin, and the anti-LPS factor published before and on the basis of their suggested sequences, charges, structures, or activities, we thought that they might be able to ameliorate the course of sepsis due to RA infection in ducks. In this study, we investigated the effects of synthesized epinecidin-1, anti-LPS factor, and hepcidin peptides on RA-induced sepsis in ducks. Our results show that epinecidin-1, hepcidin, and the anti-LPS factor are attractive candidates for development of therapeutic drugs for use against RA infection in ducks.

2. Materials and methods

2.1. Synthesis of epinecidin-1, anti-LPS factor, and hepcidin

Peptides were synthesized by GL Biochem (Shanghai, China) using a solid-phase procedure of Fmoc/tBu chemistry. Crude peptides were extracted, lyophilized, and then purified by reverse-phase high-performance liquid chromatography (RP-HPLC). The molecular masses and purities of the purified peptides were verified by mass spectrum (MS) analysis and HPLC, respectively. Synthetic peptides were reconstituted in phosphate-buffered saline (PBS; pH 7.4) for the experiments and are shown in Table 1. The C-terminals of these synthetic peptides were amidated.

2.2. Antimicrobial testing

The minimal inhibitory concentration (MIC) was measured for RA (strains A2, B2, T6, RA16, and T10 were obtained from Dr. Chang-You Yu's laboratory at the Department of Veterinary Medicine, National Chiayi University, Chiayi, Taiwan; strains CFC27, CFC437, CFC363, RA3, and RA9 were obtained from Dr. Chao-Fu Chang at the Institute of Veterinary Medicine, National Taiwan University, Taipei, Taiwan; strain MRS was isolated from a Cherry Valley duck on a private duck farm at Jiaushi, Taiwan). The MICs were determined by a microdilution broth method according to a previous publication [37]. Ampicillin (Amp) and kanamycin (Kana) were used as controls.

2.3. Transmission electron microscopy (TEM)

The TEM procedures followed the protocols of a previous publication with no modifications [28]. Briefly, bacteria were concentrated by centrifugation (Beckman Coulter, Avanti J-20XP, JA-25.50, Palo Alto, CA, USA) at 3000 rpm for 5 min at 4 °C. The pellet was resuspended in PBS to a final OD 550 nm of 1.0. The bacterial suspension (5 ml) was mixed with a freshly prepared AMP solution in PBS to a final concentration of 100 μg/ml of EPI, CA, RA, and TH1–5 for Ra (strains T6, MRS, and CFC27). The pathogens were treated for 16 h. The control group consisted of only a bacterial suspension in PBS of the RA strain in culture medium only. After sectioning, images of specimens were observed by TEM (Hitachi, H-7000, Tokyo, Japan) at 75 kV following a previous publication [28].

2.4. Duck infection and rescue models

Ducks (*C. moschata* and Cherry Valley ducks) were obtained from a commercial hatchery at about 1 day after hatching, and after 2 days after hatching began to feed on commercial fodder and autoclaved water. Ducks were housed in isolated rooms in cages, and standard industry guidelines were followed in maintaining the temperature in the isolation rooms. In experiments to study the efficacies of EPI, CA, LA, TH2–3, and TH1–5 in protecting ducks against RA (strain T6 for *C. moschata* and strain MRS for Cherry Valley ducks) by injection into 4-day-old chicks after hatching, RA and the peptides were directly injected into a duck's abdominal cavity (peritoneum) using a syringe (29 G × 1/2, Terumo Medical, Elkton, MD, USA). Control ducks were only injected with PBS buffer. The first trial of every group used 30 Cherry Valley ducks: (a) group 1 was only injected with RA (1×10^8 colony-forming units (cfu)/duck); (b) group 2 was only injected with PBS; (c) in group 3, RA (1×10^8 cfu/duck) was mixed with each peptide (at 100 μg/duck) for 20 min at room temperature and then injected into ducks, and 336 h after the first injection, the surviving ducks from the first co-treatment were again injected with RA (1×10^6 cfu/duck). In the second challenge for the RA-injected only group (control group), ducks which had not been treated with RA or peptide and had similar body weights and ages were chosen. The mortality rate was recorded every 24 h (supplementary Fig. 1a–d).

For the third trial, each group contained 20 ducks, and each duck was injected with the same concentration of each peptide (100 μg/duck), and after 2 h, RA (1×10^8 cfu/duck) was injected. The mortality rate was recorded every 24 h. For the fourth trial, each group contained 20 ducks, and each duck was injected with RA (1×10^8 cfu/duck), and after 2 h, the peptides (100 μg/duck) were injected. The mortality rate was recorded every 24 h. The trial for RA challenge used 20 *C. moschata* ducks which were treated the same as the Cherry Valley duck experiments described above. But the RA strain used in *C. moschata* ducks was the T6 strain, and the RA strain used in Cherry Valley ducks was the MRS strain. Peptides were pretreated and post-treated in Cherry Valley ducks with a 2-h time gap, while in *C. moschata*, there was a 24-h time gap before or after the injection. The time gap difference between Cherry Valley ducks and *C. moschata* was due to differences in the RA strains. The peptides used in the *C. moschata* experiments were EPI, CA, LA, TH1–5, and TH2–3, and those used in the Cherry Valley duck experiments were EPI, CA, LA, and TH1–5 (supplementary Fig. 1a and b). Multiple group comparisons were tested using analysis of variance (ANOVA) in SPSS software (SPSS, Chicago, IL, USA). Differences were defined as significant at $p < 0.05$ and <0.01. Different letters shown in the figures indicate significant

Table 1
Information on the peptides used in this paper.

Peptide name (abbreviation name)	Sequences	Theoretical pI/Mw	References
Epinecidin-1 (EPI)	GFIFHIIKGLFHAGKMIHGLV	10.00/2335.88	[27]
Cyclic anti-lipopolysaccharide factor peptide (CA)	ECKFTVKPYLKRFQVYYKGRMWCP	9.70/3071.71	[28]
Linear anti-lipopolysaccharide factor peptide (LA)	ECKFTVKPYLKRFQVYYKGRMWCP	9.70/3071.71	[28]
Hepcidin TH1–5 (TH1–5)	GIKCRFCCGCCTPGICGVCCRF	8.54/2329.91	[14]
Hepcidin TH2–2 (TH2–2)	GIKCCFCCGCCNSGVCELCCRF	7.67/2351.88	[14]
Hepcidin TH2–3 (TH2–3)	QSHLSLCRWCCNCCRSNKGC	8.70/2301.70	[14]

differences between groups, while the same letter indicates no difference between groups.

2.5. Evaluation of the antibacterial activity of ducks treated with synthesized epinecidin-1, the anti-LPS factor, and hepcidin

Bacteria were isolated from infected ducks and the rescue models described above. One group was first injected with the RA T6 strain (1×10^8 cfu/duck), then 24 h later, injected with the peptide (100 μg/duck); while another group was first injected with the peptide (100 μg/duck), and 24 h later, was injected with the RA T6 strain (1×10^8 cfu/duck). The third group of *C. moschata* was simultaneously co-treated with the RA T6 strain (1×10^8 cfu/duck) and peptide (100 μg/duck). Liver samples were obtained from each group of *C. moschata* after treatment for 1, 3, 5, and 7 days. The liver tissue sample was homogenized and placed in PBS buffer before being spread on a sheep blood agar plate. The plates were incubated at 37 °C for 24 h, and the number of bacterial colonies was counted. Significance was calculated by the SPSS statistical program and was set to $p < 0.05$.

2.6. Determination of differentially expressed genes by quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the duck (*C. moschata*) infection and rescue models described above; other groups included one group injected with only RA (1×10^8 cfu/duck), one group injected with only the AMP (100 μg/duck), one group injected with only PBS, and one group injected with a mixture of RA (1×10^8 cfu/duck) and an AMP (100 μg/duck). Samples were collected to extract RNA after treatment for 1 and 7 days following a previous publication [28]. Levels of duck total RNA from the brain, liver, and spleen were determined by a comparative real-time qRT-PCR analysis after each experimental test. For the real-time qRT-PCR experiments, the 12S ribosomal (r)RNA (abbreviated 12S) (GenBank accession no. AF173705), lipoprotein lipase (abbreviated LPL) (GenBank accession no. EU598459), Mn superoxide dismutase (abbreviated MnSOD) (GenBank accession no. EU598450), and H5 histone (abbreviated H5) (GenBank accession no. X01065) were detected. Messenger (m)RNA was transcribed to first-strand complementary (c)DNA, and single-stranded cDNA was used in the real-time comparative qPCR to detect the relative expression levels of each gene. Primers were designed by ABI protocols (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA) and are shown in Table 2. All samples were examined in triplicate, the sample size was three ducks, and the experimental procedures followed those of a previous publication [14]. The reaction contained 5 μg of total RNA for gene expression. The comparative qRT-PCR Mastermix for the SYBR Green kit (Applied Biosystems, Perkin-Elmer) was used for quantification. The total reaction volume was 25 μl. The reactions were run in 96-well plates and consisted of 40 cycles of 15 s at 96 °C and 60 s at 60 °C. A dissociation thermal start temperature of 65 °C was added to each experiment to analyze the melting peaks of the PCR products generated. Data from each experiment were expressed as the detected gene expression/12S ribosomal RNA expression. Statistical analysis was performed using *t*-test to compare two groups. Multiple group comparisons were tested using ANOVA in the SPSS software. Differences were defined as significant at $p < 0.05$ and <0.01. The different letters shown in the figures indicate a significant difference between groups, while the same letter indicates no difference between two groups.

Table 2
Primers used in this paper.

Primer name	Primer sequence
Duck MHCII-b-F	CCCGAAATACTGGAGCAGAGA
Duck MHCII-b-R	CACCTTGGGCTCAACTTTCC
Duck CD4-F	GCCAGGCTTTGTTTGAGATACC
Duck CD4-R	CAGCAGCTGGCACGTGTACT
Duck CD8-F	GCTGGGACCCTTCACTTCATC
Duck CD8-R	CACTCTTTGACACCCCATAACG
Duck H5-F	AGTCGCTGAAGACATCCAAGGT
Duck H5-R	CCTCCAGACTTCTCACTTCTTTTTG
Duck 12S-F	AACCACCCCTTGCCAAAAC
Duck 12S-R	GCTGTTGCGCTCACTGTTGT
Duck GADD-F	GCGCTGCAGATCCACTTCA
Duck GADD-R	CGGGCCGGGTTGCT
Duck SODMn-F	GAGCCTAAAGGAGAACTGATGGAA
Duck SODMn-R	ACAGCTGTTAGCTTCTCCTTGAAGT
Duck LPL-F	GATCCATCCACCTCTTCATCGA
Duck LPL-R	GGCCTCCTTCGTGTTGCA
Duck GK-F	TCACACGCATCCACTTCCA
Duck GK-R	CACGGCAGCAACCTGGTT

3. Results

3.1. Antimicrobial activities of EPI, CA, LA, TH2–3, TH2–2, and TH1–5 against different RA strains

The antimicrobial activities of these AMPs against different strains of RA are shown in Table 3. CA and LA exhibited antimicrobial activities against several RA strains compared to the other AMPs. The MIC values of Amp and Kana were both <1.5 μg/ml. TH2–2 showed no antibacterial activities.

3.2. Effects of EPI, CA, LA, and TH1–5 on the ultrastructure of RA

Our experiments showed that EPI, CA, LA, and TH1–5 had antimicrobial abilities following bacteriostatic activity identified

Table 3
Activities of antimicrobial peptides against the duck pathogen, *Riemerella anatipestifer* (RA). MIC, minimal inhibitory concentration; Amp, ampicillin; Kana, kanamycin. The RA strains were isolated or obtained from various institutes as gifts as described in Section 2. ND, lack of activity of the corresponding peptide. The abbreviated names of the peptides are as given in Table 1.

Microorganisms (clinical isolate)	CA MIC (μg/ml)	LA MIC (μg/ml)	EPI MIC (μg/ml)	TH1–5 MIC (μg/ml)	TH2–2 MIC (μg/ml)	TH2–3 (μg/ml)	Amp MIC (μg/ml)	Kana MIC (μg/ml)
Riemerella anatipestifer (CFC 437)	25	12.5	25	50	ND	ND	<1.5	<1.5
Riemerella anatipestifer (CFC 363)	12.5	6.25	50	50	ND	100	<1.5	<1.5
Riemerella anatipestifer (RA 3)	12.5	12.5	25	25	ND	ND	<1.5	<1.5
Riemerella anatipestifer (RA 9)	12.5	12.5	50	50	ND	100	<1.5	<1.5
Riemerella anatipestifer (CFC 27)	25	25	25	100	ND	100	<1.5	<1.5
Riemerella anatipestifer (RA 16)	12.5	12.5	25	25	ND	100	<1.5	<1.5
Riemerella anatipestifer (T 10)	12.5	12.5	25	50	ND	ND	<1.5	<1.5
Riemerella anatipestifer (B 2)	12.5	12.5	6.25	25	ND	ND	<1.5	<1.5
Riemerella anatipestifer (T 6)	12.5	12.5	25	400	ND	450	<1.5	<1.5
Riemerella anatipestifer (A 2)	25	12.5	25	50	ND	ND	<1.5	<1.5
Riemerella anatipestifer (MRS)	25	25	25	50	ND	ND	ND	<1.5

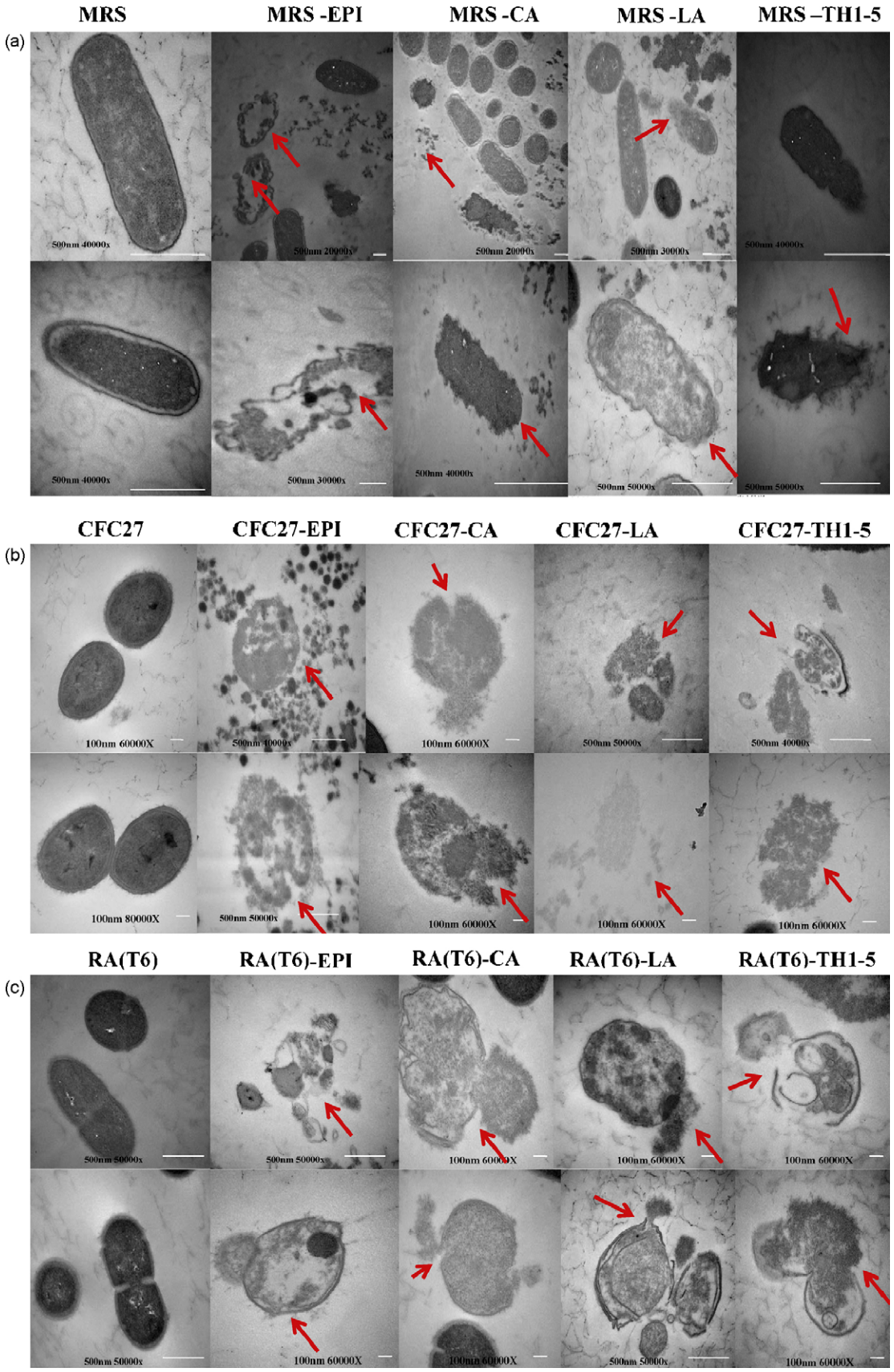

Fig. 1. Transmission electron micrographs of different *Riemerella anatipestifer* (RA) strains of MRS (a), CFC27 (b), and RA (T6) (c) either untreated (as indicated by only the strain names) or treated with epinecidin-1 (EPI), the SALF55–76 cyclic peptide (CA), SALF55–76 linear peptide (LA), and hepcidin TH1–5 (TH1–5). The red arrow indicates damage to the plasma membrane of many cells. The upper and lower pictures are of the same conditions and show duplicate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

by the MIC method. To understand the functions of EPI, CA, LA, and TH1–5 against bacterial plasma membranes, RA strains of MRS, CFC27, and T6 were separately incubated with 100 μg/ml each of EPI, CA, LA, and TH1–5 for 16 h and analyzed by TEM. TEM clearly showed differences in morphology between the untreated and peptide-treated bacteria for the MRS (Fig. 1a), CFC27 (Fig. 1b), and T6 (Fig. 1c) strains. There were breaks in the plasma membranes of peptide-treated bacteria, and cellular inclusions were found to have effluxed extracellularly.

3.3. Activities of EPI, CA, LA, TH1–5, and TH2–3 in models of infection

We established a duck model of aggressive bacterial infection, widely used to assess AMP efficacy, with the RA strains of MRS and T6, major causes of duck septicemia. EPI, CA, LA, TH1–5, and TH2–3 were given by different routes after RA intraperitoneal challenge. It is difficult to completely eradicate bacteria, and this was rarely seen in any of the AMP rescue models, but EPI, CA, LA, and TH1–5 significantly decreased mortality by the MRS strain in Cherry Valley ducks, and EPI, CA, LA, TH1–5, and TH2–3 significantly decreased the mortality of the T6 strain in *C. moschata* ducks. EPI, CA, LA, and TH1–5 were co-treated with the MRS RA strain, then injected into Cherry Valley ducks which showed significantly decreased mortality over the 14-day experimental period (Fig. 2a). As shown in Fig. 2a, treatment of Cherry Valley ducks with bacteria (1×10^8 cfu/duck) resulted in high mortality within 14 days (23 of 30 ducks died within 10 days). When using co-treatment of Cherry Valley ducks with the peptide (100 μg/duck) and bacteria (1×10^8 cfu/duck), a 93.3% survival rate was obtained for LA and TH1–5 treatments after 14 days (2 of 30 ducks died within 3 days). However, 76.7% and 73.3% survival rates were obtained for EPI (7 of 30 ducks died within 9 days) and CA (8 of 30 ducks died within 9 days) treatment after 14 days, which showed a significant difference compared to the RA-treated group only. During re-challenge with the MRS RA strain at 14 days after the first challenge described above, Cherry Valley ducks exhibited 47.8%, 63.6%, 71.4%, 75%, and 16.7% survival rates with EPI, CA, LA, TH1–5, and RA treatment (Fig. 2b). The lower mortality in the group receiving the peptide combined with RA than in the group receiving RA alone was significant ($p < 0.05$). AMPs were given either 2 h prior to (Fig. 2d) or 2 h after the bacterial challenge (Fig. 2c), and similar efficacies were demonstrated in all cases for Cherry Valley ducks (Fig. 2c and d). Our results indicate the potential of using EPI, CA, LA, and TH1–5 therapy alone which was adequate.

To understand the effects of AMPs on different serotypes of RA in different kinds of duck, we repeated the experiments and used the Ra T6 strain in *C. moschata*. The survival rate in the RA control group (into which only 1×10^8 cfu/duck of the T6 bacterial strain

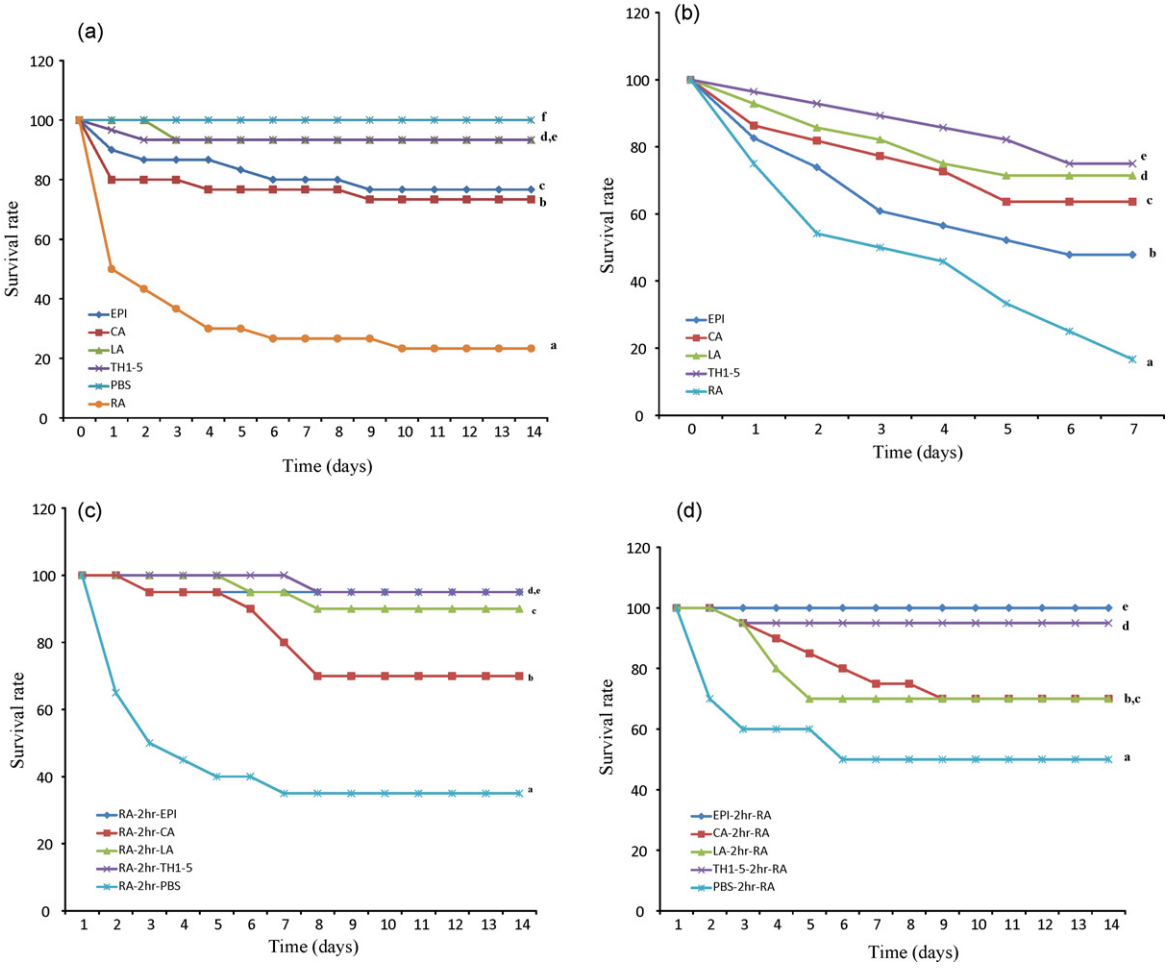

Fig. 2. The ability of the synthesized antimicrobial peptides to protect Cherry Valley ducks from a lethal challenge by *Riemerella anatipestifer* (RA) (MRS strain; 1×10^8 colony-forming units (cfu)/duck). (a) There were six groups in this experiment: PBS, injected with PBS-only; RA, injected with RA only (1×10^8 cfu/duck); and a mixture of RA (1×10^8 cfu/duck) and each peptide (at 100 μg/duck) injected together. The mortality rate was recorded every 24 h. (b) Survival rates after re-challenge of the survivors from the first trial described above with RA (1×10^8 cfu/duck) at 14 days. (c) Time-dependent effects of the pretreatment with RA (1×10^8 cfu/duck) and then after 2 h, an injection of the different antimicrobial peptides or PBS (given as a control). (d) Pretreatment with the antimicrobial peptides or PBS (as a control) and after 2 h, injection of RA (1×10^8 cfu/duck). The survival rate was recorded every 24 h. Data with different letters significantly differ ($p < 0.05$) among treatments.

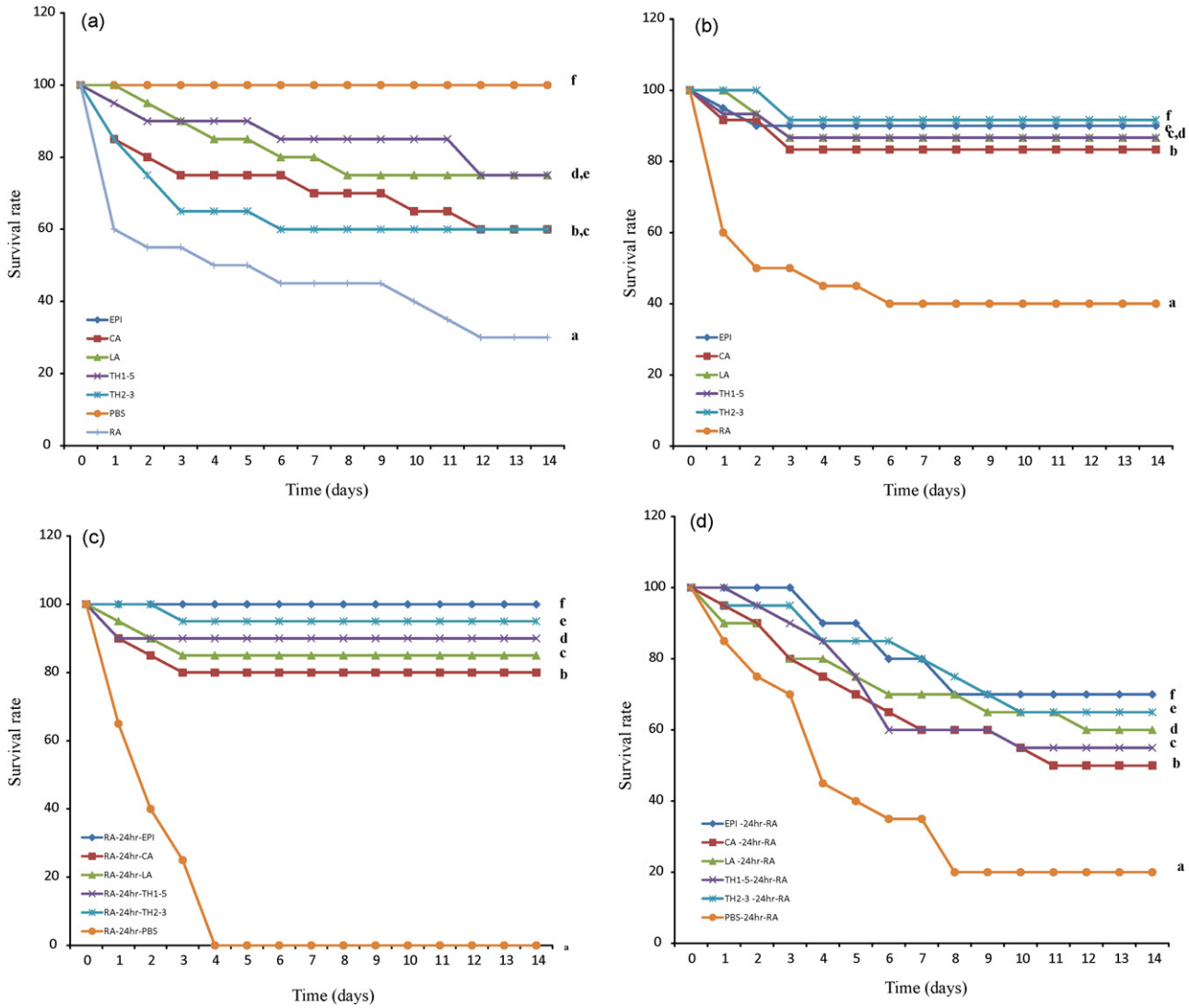

Fig. 3. The ability of the synthesized antimicrobial peptides to protect *Cairina moschata* ducks from a lethal challenge of *Riemerella anatipestifer* (RA) (T6 strain; 1×10^8 colony-forming units (cfu)/duck). All experimental conditions were the same as those described in Fig. 2, except that 24 h was used between the pre- and post-treatment. The survival rate was recorded every 24 h. Data with different letters significantly differ ($p < 0.05$) among treatments.

was administered) was 30%, and that of the PBS group (into which only PBS was administered) was 100% within 14 days (Fig. 3a). EPI, CA, LA, TH1–5, and TH2–3 co-treatment with the RA T6 strain led to decreased mortality ($p < 0.05$). Specifically, at 14 days, survival rates of 100%, 75%, 75%, 60%, and 60% were observed for groups treated with EPI, TH1–5, LA, TH2–3, and CA, respectively (Fig. 3a). Re-challenge with the RA T6 strain after 14 days showed that *C. moschata* exhibited 91.7%, 90%, 86.7%, 86.7%, and 83.3% survival rates for the TH2–3, EPI, TH1–5, LA, and CA for RA T6 treatments (Fig. 3b). The higher survival rates in the groups receiving peptides co-treated with RA T6 and challenged with RA T6 again than in the control group receiving RA T6 (with a survival rate of 40%) alone showed significant differences ($p < 0.05$). These results showed that TH2–3, EPI, TH1–5, LA, and CA can function as immune-related stimulators to reduce RA's infective ability and suggest that these peptides disrupt the bacterial membrane.

In the present study, *C. moschata* ducks were treated with the RA T6 strain (1×10^8 cfu/duck) 24 h before receiving an injection of 100 μg of each peptide to evaluate their mortality during septic events. As shown in Fig. 3c, injection of bacteria into untreated *C. moschata* (given only PBS) resulted in high mortality within 14 days (20 of 20 *C. moschata* died within 4 days). With a pretreatment injection of the RA T6 strain and after 24 h, injection of EPI, TH2–3, TH1–5, LA, or CA, 100%, 95%, 90%, 85%, and 80% survival rates were, respectively, obtained at the end of the 14-day experimental period (Fig. 3c). As shown in Fig. 3d, the pretreated groups were given a single injection of 100 μl of each peptide, and after 24 h, the RA T6 strain (1×10^8 cfu/duck) was injected; the mortality was recorded every 24 h. The results for the group injected with only PBS showed that 80% of *C. moschata* died during the 14-day trial. In contrast, administration of the different peptides before the bacterial challenge had an impact on the lethality rates. The results showed that after EPI, CA, LA, TH1–5, and TH2–3 treatment, higher survival rates (35%, 50%, 60%, 55%, and 65%, respectively) were obtained compared to PBS-only treatment (Fig. 3d). Our results indicate that the AMPs have strong antiseptic activities in both Cherry Valley and *C. moschata* ducks. These results suggest that EPI, CA, LA, TH1–5, and TH2–3 played significant roles in protecting ducks from RA-induced septic death.

3.4. In vivo bactericidal activities of EPI, CA, LA, TH1–5, and TH2–3 in the duck liver

The in vivo antibacterial activities of EPI, CA, LA, TH1–5, and TH2–3 were evaluated in three duck models of RA infection. The effects of treatment on bactericidal activities were compared

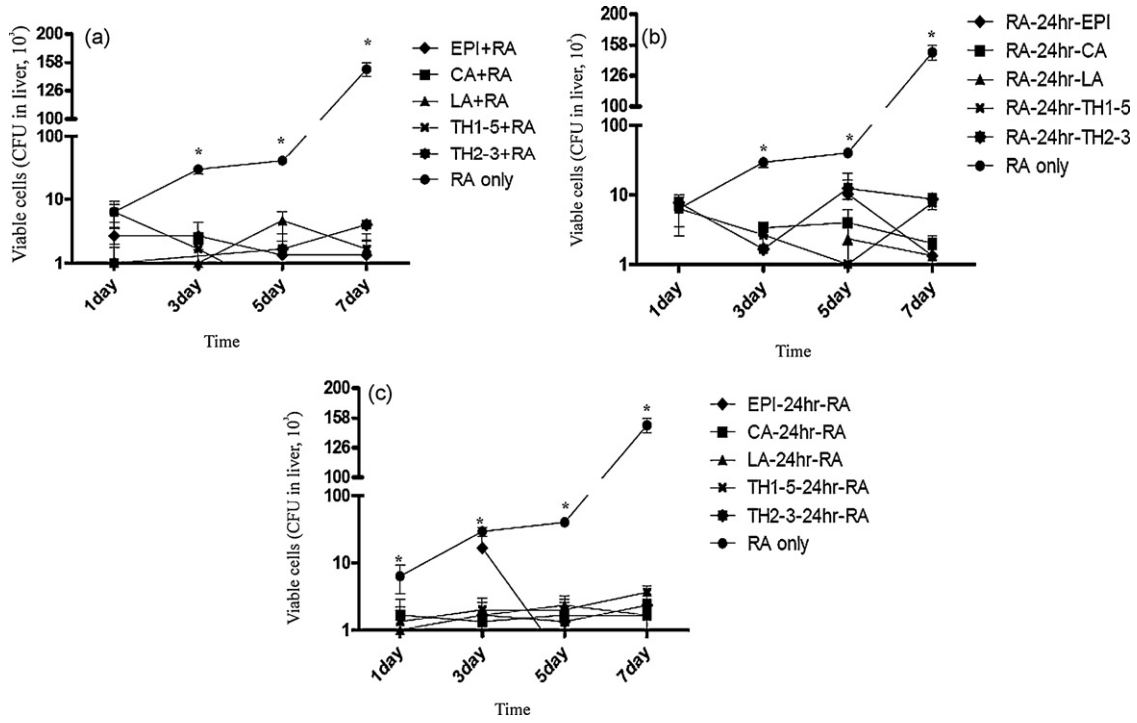

Fig. 4. In vivo bacteriostatic properties of the different injected antimicrobial peptides in a *Cairina moschata* model of peritonitis induced by an intraperitoneal injection of *Riemerella anatipestifer* (RA) (T6 strain; 1×10^8 colony-forming units (cfu)/duck). *Cairina moschata* ducks were co-treated with antimicrobial peptides and RA (a), or post-treated with antimicrobial peptides (b), or pretreated with antimicrobial peptides (c). Livers was obtained from *C. moschata* on days 1, 3, 5, and 7 after treatment. Colony counts are shown as the mean and standard error of the mean. A significant difference ($^*p < 0.05$) was determined by comparing treated groups.

between co-treatment experiments and RA treatment only. Fig. 4a shows the results of a study in ducks co-treated with the RA T6 strain (1×10^8 cfu/duck) and peptides. Liver bacterial counts were performed at 1, 3, 5, and 7 days. We observed substantial protection when the peptide was co-treated (Fig. 4a), post-treated (Fig. 4b), or pretreated (Fig. 4c) with an i.p. injection. The complete eradication of bacteria is rarely seen in any animal model, but EPI, CA, LA, TH1–5, and TH2–3 produced significantly ($p < 0.05$) decreased bacterial counts in our results after the 7-day trail.

3.5. Analysis of differentially expressed genes by qPCR

As shown in Fig. 5, a significant increase was seen in MnSOD gene expression (Fig. 5a) in brain tissue after 7 days with an RA injection only, an EPI injection only, and an EPI + RA injection. In liver tissue, expression of LPL was induced by only an Ra injection after 7 days (Fig. 5b), but induction by an EPI or EPI + RA injection did not statistically differ from the PBS-only treatment group after either 1 or 7 days of treatment. The H5 histone gene in the spleen was downregulated in the RA-, CA-, LA-, EPI-, and EPI + RA-injected groups (Supplementary Fig. 2). As to the tissue expression patterns of other genes, all of those genes were either up- or down-regulated in the RA-injected group, but expression levels also changed in the PBS-injected group between 1 and 7 days (data not shown). Variations in expression levels of the PBS group between 1 and 7 days are not discussed in the text, but expression levels of other genes after treatment for 1 and 7 days are discussed.

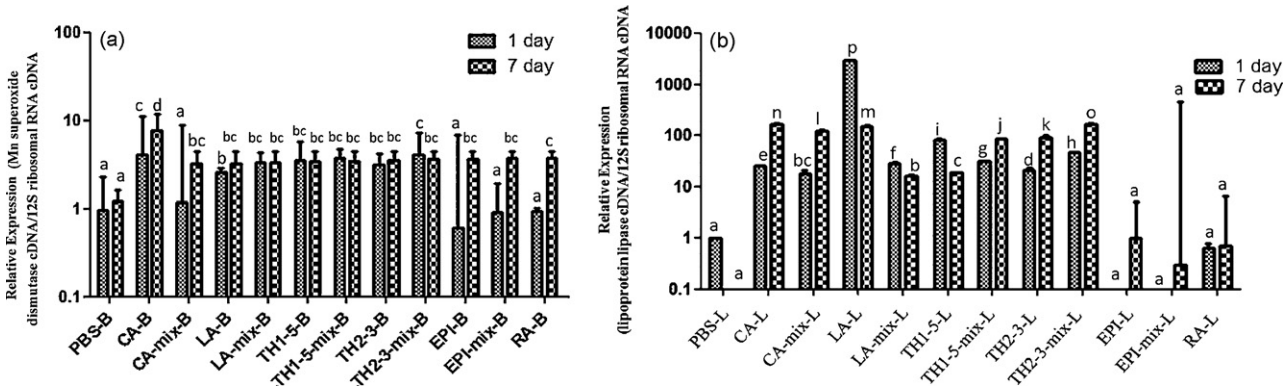

Fig. 5. Comparative RT-PCR analysis of mRNA gene expression levels of the Mn superoxide dismutase in the brain (a) and lipoprotein lipase in the liver (b) after different experimental conditions as described in Fig. 2. Each bar represents the mean value from three determinations with the standard error. Data (mean ± S.E.) with different lowercase letters significantly differ ($p < 0.05$) among treatments: B, brain; L, liver; PBS-B, injected with only PBS and gene expression detected in brain tissue; RA-B, injected with only *Riemerella anatipestifer* (RA) and gene expression detected in brain tissue; CA-B, injected with only CA and gene expression detected in brain tissue; CA-mix-B, injected with CA co-treated with RA and gene expression detected in brain tissue. Other peptide abbreviations are as given in Table 1.

4. Discussion

RA is an important disease in waterfowl, especially ducks. The precise route of infection is still controversial, and several reports focused on the transmission of RA but did not obtain standard rules to explain RA's epidemiology in ducks [2,33]. Clinically, movements of ducklings become severely repressed and uncoordinated, with diarrhea, and tremors of the head and neck. Ducklings younger than 35 days usually die within 24–48 h after clinical signs appear. RA infection usually primarily breaks out in young, growing ducks accompanied by high mortality, septicemia, and polyserositis [35]. Several studies indicated that clinical outbreaks can be controlled with ceftriofur, enrofloxacin, rofenaid, or a vaccination against RA infection [6,13,23,36]. However, the reasons for the rapid emergence and dissemination of drug-resistant bacteria in duck farms are multifactorial and may include a higher resistance of RA to certain antimicrobial agents. This increasing resistance may further drive increased consumption of several so-called "last-line" antimicrobial agents, such that many new antibiotics for multidrug-resistant organisms need to be developed [8]. A few years ago, AMPs were published and seriously considered as potential therapeutic sources. There are hundreds of naturally occurring peptides which have been examined for their utility in therapeutics, but only a few display significant activity in animal models [5,26,27], and some have already entered clinical trials [3,12,38]. Duck research has not focused on employing AMPs in therapy against RA infection. But, this ideally can be achieved by adopting a high standard of farm hygiene to avoid exposure, and by using immunoprophylaxis with AMPs which can be made available.

We identified the EPI, CA, LA, TH1–5, and TH2–3 AMPs from fish and shrimp [14,27,28], which can selectively modulate innate immune responses, thereby providing prophylaxis or treatment of a broad spectrum of infections. Our previous results suggested that EPI, CA, LA, TH1–5, and TH2–3 may have antimicrobial activities against RA bacteria and can provide valuable guidance in the choice of chemotherapy. Therefore our results indicated that all isolates were inhibited by EPI, CA, LA, TH1–5, and TH2–3 with MIC values in a range of 6.25–450 μg/ml as shown in Table 2. In contrast, all isolates from our results were inhibited by <1.5 μg/ml kanamycin and ampicillin. A previous report showed that 5 strains of RA isolated from US ducks could grow in Mueller–Hinton medium containing 532.8 μg/ml kanamycin. The difference in antimicrobial activities against various isolates might be attributed to serotype differences and geographic separation [6]. Indeed, we demonstrated that concentrations of 12.5, 12.5, 25, 400, and 450 μg/ml of medium containing CA, LA, EPI, TH1–5, or TH2–3 could restrict the growth of the T6 strain of RA according to the MIC method, and 25, 25, 25, and 50 μg/ml of CA, LA, EPI, or TH1–5 in the medium could restrict the growth of the MRS strain of RA according to the MIC method. After in vitro demonstration of a clear antimicrobial effect for CA, LA, EPI, TH1–5, and TH2–3 against RA, we investigated whether they also had similar effects in vivo. An i.p. injection of RA (T6) (1×10^8 cfu/duck) into *C. moschata* or an injection of RA (MRS) (1×10^8 cfu/duck) into Cherry Valley ducks which were pretreated, co-treated, or post-treated with the AMPs had beneficial effects in this acute duck model (Figs. 2 and 3). Indeed, co-treatment in these duck experiments showed that CA, LA, EPI, TH1–5, and TH2–3 can serve as adjuvants. The AMPs used in this paper exhibit antimicrobial activity by either interacting with the microorganism or by affecting events in target cells. Multiple mechanisms of inhibition have not yet been described in duck research. Our results show that RA (MRS strain) mixed with CA, LA, EPI, and TH1–5, then injected into Cherry Valley ducks produced >75% survival in 14-day trials. CA, LA, EPI, TH1–5, and TH2–3 mixed with RA (T6 strain) then injected into *C. moschata* produced >60% survival in 14-day trials. Thus, it is very possible that a high concentration of AMPs (100 μg/duck) can cause more RA to be damaged and bring about higher survival. This means that the amount of live RA inoculated into ducks at the beginning was not really equal compared to the different co-treatment with peptides, in that each peptide had different antimicrobial activities for killing RA. Therefore, the high survival rates observed with the different AMPs might have been due at least partly to smaller amounts of RA at the initial infection due to the different AMP's antimicrobial activity. Moreover, it is also possible that dead RA after co-treatment with AMPs may have stimulated the host to produce neutralizing antibodies against RA or may have induced immune-related gene responses in sera (similar to dead RA vaccine inoculation), which needs to be determined in further studies. However, we used 100 μg/ml of each peptide in the in vivo trails. Under this test condition, we used the same concentrations of each peptide for the TEM study.

As is known, vaccinations against RA infection utilize bacterins and are usually made from formalin-killed preparations of the organism [30,32]. This method is not efficacious due to unacceptably toxicity, incomplete protection, and its short-lived nature. A more-advanced method for effectively protecting ducks uses vaccines produced by live, attenuated organisms [20,32]. But live vaccines are not acceptable to authorities responsible for vaccine licensing, and RA recovered from ducks can adversely affect growth, although live attenuated vaccines stimulate protracted cell-mediated immunity and antibody responses [13]. In the present study, after treatment with CA, LA, EPI, TH1–5, and TH2–3, the RA outer membrane was uneven and disrupted as shown in Fig. 1 which led to leakage of the intracellular contents precipitating cell death. Bacterial debris may stimulate a duck antibody response as antigen production and protect ducks from a second RA challenge (Figs. 2b and 3b). However, another report on a mouse cathelin-related AMP which can be a chemoattractant for leukocytes using formyl peptide receptor-like 1/mouse formyl peptide receptor-like 2 as the receptor found that it acts like an immune adjuvant [18]. It is generally believed that the peptide KLKL(5)KLK combination of a oligodeoxynucleotide named IC31 represents a promising novel adjuvant [34]. The comparative data described above suggest that AMP functions include adjuvant activity in an adaptive response which not only kills microorganisms but also has a multiplicity of roles in modulating innate immunity to suppress harmful inflammatory or septic responses [4]. Similar results indicate that CA, LA, EPI, TH1–5, and TH2–3 are small host defense peptides related to innate immunity which are reported to have antimicrobial activity from our previous publications and which may also orchestrate chemotaxis and activate effector immune cells in ducks.

The prophylactic or post-treatment administration of CA, LA, EPI, TH1–5, and TH2–3 protected ducks against serious sepsis (Fig. 2c and d and Fig. 3c and d). Septic shock was induced by the intraperitoneal administration of RA, and the presence of RA infection is a more-realistic sepsis model in clinical situations with pronounced purulent inflammation of the upper respiratory tract and central nervous system disorders accompanied by high mortality [35]. Hence, our artificial infection provided a valid model to test the efficacies of CA, LA, EPI, TH1–5, and TH2–3. The outcomes of those trials were in line with the efficacies obtained by an injection of an aquatic AMP at a concentration of 100 μg/duck in two kinds of ducks affected by RA. Wherever clinical outbreaks of duck septicemia are frequent, ceftiofur, enrofloxacin, novobiocin, lincomycin, and sulphonamides are used in the fodder or water to control mortality and morbidity [6,23,36]. These antibiotics have side effects, and drug resistance may develop by certain RA bacterial species, which awaits further studies [6]. But from a human food standpoint, if we eat ducks treated with antibiotics,

drug-resistant bacteria may develop in the human body. One hypothesis explored if one of the antibiotics (fluoroquinolone) used in poultry gives rise to fluoroquinolone-resistant campylobacters that can enter the food chain [15]. In contrast, naturally occurring AMPs are widely distributed among evolutionarily divergent organisms including mammals, amphibians, insects, birds, and plants, and play significant roles in innate immunity [39]. This study demonstrates that therapy or prophylaxis with CA, LA, EPI, TH1–5, and TH2–3 against RA infection is an alternative to existing antibacterial medications.

Recent data from different inoculation routes and percentages of bacterial recovery of RA from various cultured tissues indicated that the liver should be cultured for diagnostic purposes; it also appears that the liver, heart, and spleen are the best organs to routinely culture for RA infection. We chose the liver to detect bacterial numbers to test the efficiency of AMPs against RA. Our results indicate that the antimicrobial abilities of CA, LA, EPI, TH1–5, and TH2–3 were similar to those of other peptides or antibiotics which reduced bacterial loads in vivo (Fig. 4). This is reminiscent of our previous study on *Vibrio vulnificus* injected by an intraperitoneal route, similar to what was used here to inhibit bacterial growth by EPI [28]. The data suggest that these AMPs are sufficient to prevent the RA strain from multiplying in the presence of the peptides, with any type of experiment using pre-, co-, or post-treatment of the AMPs. This is opposite to the situation found with antibiotics such as fluoroquinolones, the use of which allowed resistant strains to proliferate and predominate [11,15]. These resistant isolates were isolated from the feces of some commercial poultry flocks up to the point of slaughter, which may have ramifications for public health. Our data support the AMPs examined in this paper being used as prophylactic drugs, therapeutic drugs, or adjuvants which can inhibit bacterial growth.

To increase our knowledge of duck responses to RA infections, we analyzed the immune-related gene expressions of a duck (*C. moschata*) after injections with RA, AMPs only, and RA mixed with AMPs. One hypothesis is that the strong early antibacterial response induced by the injected AMPs plays a role in enhancing long-term immunity. This study focused on defining the immune-related genes underlying the early antibacterial phase by qRT-PCR. Thus, to protect against bacterial challenge, antibacterial effector molecules would need to be up- or down-regulated by RA, such as in RA-infected tissues. Examination of these tissues after injection of RA, AMPs only, and RA mixed with AMPs by qRT-PCR revealed that the ducks injected with CA, EPI, and CA mixed with RA, or EPI mixed with RA produced upregulated expression of the MnSOD gene in brain tissue. It should be noted that MnSOD gene mRNA expression in response to bacterial infection of *Hemibarbus mylodon* suggested that MnSOD plays an important role in the host defense against oxidative damage caused by infection-mediated inflammation [7]. One prominent feature of SOD 1-deficient mice is that they produce fewer caspase-1-dependent cytokines and are less susceptible to LPS-induced septic shock, suggesting that SOD 1 regulates caspase-1 and endotoxic shock [22]. The detailed mechanism of the direct effects of MnSOD in brain tissue on RA infection requires further investigation.

According to the qRT-PCR analysis, the LPL gene was upregulated in liver tissues 7 days after the RA infection. However, according to the qRT-PCR assay, the LPL gene with CA mixed with RA, LA mixed with RA, TH1–5 only, and TH2–3 mixed with RA was downregulated in liver tissue 7 days after the injection. LPL was identified in septic rats due to Gram-negative sepsis suppressing LPL activity in adipose tissues and altering the clearance of triglycerides [29]. Hypertriglyceridemia of sepsis is associated with suppressed tissue LPL activities [19], and our data demonstrated that CA, LA, and TH2–3 downregulated LPL gene expression in the liver 7 days after injection of the peptide. These peptides regulating LPL in liver tissues may involve posttranslational mechanisms which need to be identified. Upon treatment, we observed a substantial and statistically significant ($p < 0.01$) decrease in H5 mRNA expression in the duck spleen 7 days after RA was injected. Intriguingly, we did not find a significant increase in the EPI-injected group but a significant decrease in the EPI + RA treatment group 7 days after treatment. On the contrary, animals that received CA mixed with RA, LA mixed with RA, TH1–5 mixed with RA, and TH2–3 mixed with RA exhibited >2-fold increases in spleen tissue. Therefore, we concluded that exposure of ducks to the RA pathogen results in increased levels of DNA damage in this organ.

Our study is the first to show that AMPs from fish and shrimp, regardless of the species from which they originate, can lead to distinct molecular and morphological changes in bacterial organisms. Previous studies only showed that the proliferation of RA can be inhibited by antibiotics, and it is difficult to treat it with drugs or vaccines. This study may therefore serve as a roadmap for further applications of AMPs to utilize their antibacterial effects against RA infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2010.01.013.

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