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ORIGINAL RESEARCH

Comparative analysis of potential effects of three phage endolysins against antibiotic-resistant bacteria from the genus *Aeromonas*

Serik Bakiyev . Izat Smekenov . Amangeldy Bissenbaev

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Abstract *Aeromonas* species exhibit high antibiotic resistance and—as pathogenic bacteria—are a cause of massive mortality in farmed fish. This emerging increasingly important phenomenon requires new strategies to develop novel antimicrobials. Here, we conducted a comparative study on antimicrobial efficacy of three endolysins (LysPA26, Gp110, and OBPgp279) against antibiotic-resistant bacteria from the genus *Aeromonas*. Each gene was cloned and expressed with an N-terminal 6×His tag, and the proteins were purified. The purified endolysins manifested antibacterial activities toward *Aeromonas* species including *A. hydrophila*, *A. salmonicida*, and *A. veronii* without pretreatment with an outer-membrane permeabilizer. The most pronounced effect was exerted by Gp110. The results obtained *in vitro* were confirmed by *in vivo* assays because the survival of infected *Oreochromis niloticus* was better when *O. niloticus* individuals were treated with endolysin Gp110 in comparison with the other endolysins. Furthermore, effects of intramuscular injection of Gp110 on wound-healing progression were evaluated in *Acipenser baerii* naturally affected by aeromonosis. The percentage of wound closure in the fish treated with Gp110 was 41.8% on the 6th day, 79% on the 12th day, and 95.7% on the 25th day. Thus, Gp110 is a promising candidate for the development of therapeutics against *Aeromonas* infections in aquaculture.

Keywords *Acipenser baerii* . *Aeromonas hydrophila* . Endolysin . Gp110 . OBPgp279 . LysPA26

Introduction

Aquaculture is the fastest-growing food production sector and contributes substantially to the global food supply. According to a report from the Food and Agriculture Organization of the United Nations (FAO), global fishery and aquaculture output in 2020 was 177.8 million tons, of which 87.5 million tons resulted from aquaculture production (FAO 2022). In addition, aquaculture output is expected to increase to 106 million tons in 2030, with a total increase of 22% as compared to 2020 (FAO 2022). Nevertheless, the rapid development of aquaculture has been accompanied by outbreaks of diseases caused by bacterial infections (primarily due to unsanitary and often stressful conditions of cultivation) that lead to high mortality of the animals and catastrophic economic losses in aquaculture.

Aeromonas species are rod-shaped, gram-negative, non–spore forming, facultative anaerobic, opportunistic bacteria (Gonçalves et al. 2019). Almost all species belonging to this genus are pathogenic, including *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Aeromonas veronii*. They are also primary pathogens in farmed fish (Reith et al. 2008). The severest bacterial diseases in farmed fish are *A. hydrophila* infections (Hossain et al. 2014). More recently, however, *A. salmonicida* and *A. veronii* were increasingly infecting

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fish, with clinical symptoms similar to those of fish infected with *A. hydrophila* (Chen et al. 2019; Hussain et al. 2021). Lately, due to the widespread and often uncontrolled use of antibiotics, the number of antibiotic-resistant bacterial strains has been increasing dramatically, and these strains represent a leading cause of morbidity and mortality of fish (Sherif and Kassab 2023). This phenomenon may not only lead to a failure of antimicrobial therapy but also raise safety concerns about fish products (Cabello 2006). For this reason, novel strategies to combat these multidrug-resistant pathogens are urgently needed. Bacteriophage therapy is considered one of such alternatives. Phage therapy experiments have shown promising results in the eradication of several pathogenic bacteria in aquaculture (Almeida et al. 2019). Nevertheless, the success of phage therapy in aquaculture may also be influenced by environmental factors such as salt concentrations, pH, and temperature (Principi et al. 2019). Moreover, bacteriophage technology can sometimes induce resistance, which is why phage cocktails must be continuously tested and updated. Endolysins are phage-encoded enzymes that have peptidoglycan hydrolase activity and are therefore able to degrade the bacterial cell wall, thereby allowing the progeny virions to escape the host cell after replication (Gerstmans et al. 2018). Initial *in vitro* and *in vivo* data are very promising, indicating impressive efficacy in the lysing of bacterial cells including multidrug-resistant ones. As opposed to antibiotics and bacteriophages, bacterial strains do not develop resistance to endolysins. Compared to phages with narrow host spectra, endolysins are generally broad-spectrum antibacterial substances, offering a better strategy for infection control (Rahman et al. 2021). To date, however, few studies have focused endolysins' potential in the aquaculture field, although cultured fish, just as other animals and humans, are constantly threatened by microbial outbreaks. There is only one report that directly proves the effectiveness of the PlyD4 endolysin in protecting against *A. hydrophila* infection in a zebrafish model (Wang et al. 2022). It has been shown that the effect of PlyD4 on bacteria is substantially improved by the addition of ethylenediaminetetraacetic acid (EDTA). Although EDTA has long been regarded as a substance of low toxicity, it salts can have serious, even life-threatening side effects. It has been demonstrated that acute exposure to EDTA may cause kidney injury, gastrointestinal upset, transient bone marrow depression, and muscle cramps (Oviedo and Rodríguez 2003; El-Naggar et al. 2020). Furthermore, EDTA may induce DNA damage or chromosomal breaks in humans and mice (Hagiwara et al. 2006). Indeed, most studies indicate that after pretreatment with an outer-membrane permeabilizer (OMP), such as EDTA, gram-negative bacteria can be destroyed by lysins (Gontijo et al. 2021). Nevertheless, some published data have shown that certain natural lysins have strong antibacterial activity against gram-negative bacteria in the absence of an OMP, for example, endolysins LysAB21 and PlyF307 from an *Acinetobacter baumannii* phage (Lai et al. 2011; Lood et al. 2015), endolysin OBPgp279 from *Pseudomonas fluorescens* phage OBP (Walmagh et al. 2012), and LysPA26 from *Pseudomonas aeruginosa* (Guo et al. 2017). *Salmonella* bacteriophage modular endolysin Gp110 (DUF3380; pfam11860) possesses higher specific activity than that of 14 previously characterized endolysins active against peptidoglycan from gram-negative bacteria (corresponding to 1.7- to 364-fold higher activity) (Rodríguez-Rubio et al. 2016). It is reported that the above endolysins are highly bactericidal against gram-negative bacteria, such as *P. aeruginosa*. On the other hand, the antibacterial activity of these previously characterized endolysins has not been evaluated against *A. hydrophila*.

In this study, recombinant endolysins LysPA26, OBPgp279, and Gp110 were compared in terms of protective effects of against *A. hydrophila* infection in fish. Our results showed that Gp110 has a significant *in vitro* and *in vivo* antimicrobial activity against *A. hydrophila*. Additionally, intramuscular injection of Gp110 resulted in a reduction in the wound surface area, the formation of granulation tissue, epithelization, and wound contraction in naturally infected Siberian sturgeons (*Acipenser baerii*).

Materials and methods

Constructs, plasmids and strains

Nucleotide sequences of a *P. fluorescens* phage OBP endolysin (OBPgp279; NCBI accession YP_004958186) (Cornelissen et al. 2012), a *P. aeruginosa* phage endolysin (LysPA26; ARB16052.1) (Guo et al. 2017), and a *Salmonella* bacteriophage endolysin (Gp110; KU705467) (Rodríguez-Rubio et al. 2016) were synthesized (GeneCust Europe, Dudelange – Luxembourg). Each sequence was inserted at *Eco*RI and *Nde*I restriction sites into the pET28c vector, thereby adding 6×His tag–coding sequences to the 5′ end of each gene

(yielding N-terminally 6×His-tagged proteins). All strains used in this work were grown in Luria–Bertani (LB) broth at 37 °C with shaking at 180 rpm.

Protein expression and purification

The recombinant proteins OBPgp279, LysPA26, and Gp110 were expressed in and purified from the *Escherichia coli* Rosetta 2 (DE3) pLysS strain. Briefly, *E. coli* cells were transformed separately with plasmids harboring a gene encoding 6×His-tagged recombinant protein OBPgp279, LysPA26, or Gp110, the resulting kanamycin-resistant transformants were grown to OD_{600} (optical density at 600 nm) of 0.6 at 37 °C, and protein overproduction was then induced by means of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 30 °C. All purification procedures were carried out at 4 °C.

Bacteria were harvested by centrifugation, and cell pellets were lysed using a French press at 18,000 psi in Lysis buffer [50 mM Tris-HCl (pH 9.0), 50 mM NaCl, 20 mM imidazole, 1 mM EDTA, 5 mM β-mercaptoethanol, 1 mM DTT, 2% of Triton X-100, and 5% of glycerol)] supplemented with the Complete Protease Inhibitor Cocktail (Roche Diagnostics, Switzerland). The lysates were cleared by centrifugation at 40,000 × *g* for 30 min at 4 °C, and composition of the resulting supernatant was adjusted to 500 mM NaCl and 20 mM imidazole. The supernatant was loaded onto a HiTrap Chelating HP column (GE Healthcare) charged with Ni^{2+}. The bound proteins were eluted in a linear gradient from 10 to 500 mM imidazole. The purified protein samples were stored at −20 °C in 50% glycerol. The homogeneity of the proteins was verified by SDS-PAGE.

Western blotting analysis

Two micrograms of each purified protein was separated by SDS-PAGE in a 15% gel. The gel was then electroblotted onto a PVDF membrane in a Bio-Rad Mini-transblot Cell according to the manufacturer's instructions. After that, the membrane was gently shaken in a blocking solution consisting of 5% milk and 0.1% Tween 20 in 1 × TBS (Tris-buffered saline: 50 mM Tris-HCl pH 7.5, 20 mM NaCl) for 1.5 h at room temperature. After the removal of the blocking solution, the membrane was incubated in 10 mL of a solution of the monoclonal anti-His antibody (1:5,000 dilution in the blocking solution with 0.1% of Tween 20; Santa Cruz Biotechnology) overnight at 4 °C. The membrane was washed five times in 10 mL of wash buffer (1 × TBS with 0.1% of Tween 20), for 5 min each time. After that, the membrane was incubated in 10 mL of a solution of an anti-rabbit IgG antibody (secondary antibody; 1:10,000 dilution in the blocking solution with 0.1% of Tween 20) for 1 h at room temperature. Then, the membrane was washed five times in 10 mL of wash buffer, for 5 min each time. A substrate working solution was prepared by mixing equal volumes of an H_2O_2 solution and luminol/enhancer solution and was used at 0.1 mL per cm^2 of the blot area. The membrane was incubated in the working solution for 2 min in darkness, and Kodak X-Omat was exposed to the membrane.

Zymogram assay

For this purpose, the zymogram analysis was performed by a previously described method (Hong et al. 2022), with a modification. An overnight culture of the *A. hydrophila* AB005 strain (Bakiyev et al. 2022) was centrifuged (Eppendorf Centrifuge 5804R) at 6,000 rpm for 5 min. The supernatant was discarded, and the resultant pellet was washed with 1 × phosphate-buffered saline (PBS; pH 7.5). After that, the cell suspension was centrifuged at 4,000 × *g* for 15 min, and the pellet was resuspended in 3 mL of deionized water. The cells were autoclaved, and the resulting protein extracts were mixed with 2 × sample buffer (0.5 mM Tris-HCl, pH 6.8, 20% of glycerol, and 0.2% of bromophenol blue). Next, the proteins were separated by SDS-PAGE in 15% gels. After that, each gel was washed with deionized water for 1 h and incubated in renaturation buffer (1% of Triton X-100, 20 mM Tris-HCl, pH 7.5) at room temperature at 37 °C overnight. Then, the gel was washed once with distilled water and stained with 0.5% methylene blue in 0.01% KOH for 3 h. The enzymatic activity of the endolysins was registered as clear zones on a gel containing an *A. hydrophila* AB005 lysate.

Antibacterial activity

The antimicrobial activity of endolysins OBPgp279, LysPA26, and Gp110 against *A. hydrophila* AB005 was determined by a colony-forming unit (CFU) reduction assay as previously described (Rodriguez et al. 2011). Bacteria were grown to the exponential phase (OD_{600} = 0.65), and collected by centrifugation at 6,000 rpm for 5 min. Then, the pellets were washed three times with reaction buffer (20 mM Tris-HCl, pH 7.5) and diluted with reaction buffer to approximately 10^9 cells/mL. Next, 150 µL of the bacterial suspension was mixed with 150 µL of each purified endolysin to a final concentration of 1 µg/µL, unless specified otherwise. After 1 h incubation at 20 °C, the mixture was diluted by 10-fold and plated on LB agar plates. After overnight incubation at 37 °C, bacterial colonies were counted to determine the viable-cell number. Factors affecting endolysins' antimicrobial activity were analyzed as described above under different reaction conditions, including different concentrations of the recombinant endolysins (0–1.5 µg/µL).

To test the influence of EDTA on endolysin activity, 150 µL of a cell suspension (*A. hydrophila* strain AB005 at an OD_{600} of 0.65) pretreated with 1 mM EDTA, after washing three times with reaction buffer (20 mM Tris-HCl, pH 7.5), was mixed with 150 µL of an endolysin (2 µg/µL) to a final concentration of 1 µg/µL and incubated for 1 h at 20 °C. After overnight incubation at 37 °C, bacterial colonies were counted. The strains used in this study are listed in Table 1. All assays were performed in triplicate. The values are the means and standard deviations from triplicate assays.

To investigate the effect of temperature on the activity of the recombinant endolysins, 150 µL of each endolysin (2 µg/µL) was separately added to 150 µL of the *A. hydrophila* AB005 suspension, with a final cell concentration of approximately 10^9 CFU/mL, and the mixture was incubated for 60 min at different temperatures. To evaluate the effect of pH on bactericidal activity, 150 µL of each endolysin (2 µg/µL) was separately incubated for 60 min with 150 µL of the cell suspension in buffers having different pH ranges (50 mM sodium acetate for pH 6.0- and 20-mM Tris-HCl for pH 7.0–9.0). The preincubated endolysins were subjected to the CFU reduction assay as described above. A relative lysis activity was calculated as a proportion of the highest activity.

Spot dilution growth assay

A. hydrophila AB005 cells from overnight culture in the liquid LB medium were harvested and washed three times with reaction buffer (20 mM Tris-HCl, pH 7.5). The concentration of resuspended cells was adjusted to an OD_{600} of 0.65. Next, 150 µL of the *A. hydrophila* AB005 cell suspension with the OD_{600} of 0.65 was mixed with 150 µl of Gp110 (2 µg/µL) to a final concentration of 1 µg/µL and incubated for 1 h at 20 °C. After that, serial 10-fold dilutions of the suspension (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) were prepared, and 25 µL of each diluted suspension was then spotted onto agar plates.

Protection from *A. hydrophila* infection in a Nile tilapia model

Oreochromis niloticus was selected as a model organism for testing the efficacy of Gp110 endolysin therapy against *A. hydrophila* infections. Due to its success at infecting *O. niloticus* in previous experiments (Bakiyev et al. 2022), *A. hydrophila* strain AB005 was chosen as a candidate pathogen for infecting *O.*

Table 1 Multiresistant bacterial strains used in the antibacterial-activity assays of endolysins

Bacteria	Strain	Source	NCBI accession number	Antibiotic resistance	Reference
A. hydrophila	AB005	*A. baerii* (our lab)	OK634406	oxacillin, penicillin, ampicillin, amoxicillin, cefazolin, erythromycin, lincomycin, rifampicin, novobiocin	(Bakiyev et al. 2022)
A. salmonicida	AB001	*A. baerii* (our lab)	OK634025	oxacillin, penicillin, ampicillin, amoxicillin, cefazolin, lincomycin, rifampicin, novobiocin	(Bakiyev et al. 2023)
A. veronii	AB003	*A. baerii* (our lab)	OK634393	oxacillin, penicillin, ampicillin, amoxicillin, cefazolin, oxytetracycline, tetracycline, lincomycin, rifampicin, novobiocin, trimethoprim + sulfamethoxazole	-
S. aureus	ATCC 6538	Human lesion (ATCC)	MT573388	penicillin, ampicillin, ampicillin/sulbactam, methicillin, cefazolin, ceftazidime, ceftriaxone, cefaclor, imipenem, meropenem, vancomycin, bacitracin, amikacin, gentamicin, kanamycin, streptomycin, spectinomycin, erythromycin, azithromycin, clindamycin, sulfadiazine, trimethoprim, rifampicin	(Uymaz et al. 2009)

niloticus. Fifty apparently healthy *O. niloticus* individuals (with average length 14.2 ± 0.9 cm; mean ± standard deviation) were inoculated intraperitoneally with 0.5 mL of 10^7 CFU/mL bacterial suspensions of *A. hydrophila* strain AB005. Then, the *O. niloticus* individuals were randomly distributed into five groups with each containing 10 fish. Fish of three groups were intraperitoneally injected with 0.2 mL (150 µg) of Gp110, OBPgp279, or LysPA26 without EDTA. One control group received PBS. To test the safety of the recombinant endolysins, 10 additional *O. niloticus* fish were intraperitoneally injected with 0.2 mL (150 µg) of a purified endolysin without EDTA as another control group. All fish were reared in 200 L aquaria containing 100 L of static water with aeration at 25–27 °C.

The survival rate and clinical signs in each group were monitored every 24 h up to 4 days post-infection. For the intraperitoneal injection, the nickel column–purified endolysin proteins were dialyzed gradually against PBS.

Wound-healing assay

To study wound healing, we used fish naturally affected by aeromonosis on a sturgeon fish farm located in Western Kazakhstan (Uralsk) [LLP Educational and Scientific Complex of Pilot Industrial Production of Aquaculture]. Clinical signs associated with the aeromonad infection observed in this work consisted of a single or multiple skin ulcers penetrating deep into muscle along the edges on the dorsal, abdominal, or tail parts of the fish. Sturgeon individuals with pronounced clinical signs of a bacterial disease were identified during examination. A total of 15 diseased *A. baerii* were collected and were kept in quarantine pools. The diseased fishes' body weight was 1260 ± 456 g, and total length ranged from 62 to 84 cm. During the experiment, the temperature in the quarantine pools was maintained at an optimal level of 20–22 °C. The treatment groups were (i) Gp110 32.1 µg/kg (n = 5) and (ii) cefazolin 42.3 µg/kg, (n = 5). The treatment was applied once every day for 6 days through intramuscular injection. The healing process was observed by taking photographs, starting from the day the experiments began. The wound healing was evaluated clinically (the state of a wound, a reduction in the wound surface area, the formation of granulation tissue, epithelization, and wound contraction). Wounds were considered healed if the colliquative granulation tissue was no longer visible and the wound was covered with a new epithelium. Wound healing was calculated according to the following formula (Rameshbabu et al. 2018):

$$\text{Rate of wound closure (\%)} = ((A_0 - A_t)/A_0) \times 100,$$

where A_0 is the initial area of the wound, and A_t is the area of the wound at a certain time point.

Statistical analyses

In all experiments, data are presented as the mean ± standard deviation. Results were subjected to one-way analysis of variance (ANOVA) and post hoc Tukey's test using online software.

Ethics approval

The ethics governing the use and conduct of experiments on animals were strictly observed, and the experimental protocol was approved by the Local Ethics Committee of the Medical Faculty, Higher School of Public Health at al-Farabi Kazakh National University (#20136/12, 12 July 2020; Almaty, Kazakhstan).

Results

Purification and peptidoglycan hydrolytic activity of Gp110, OBPgp279, and LysPA26 against *A. hydrophila*

The three recombinant endolysins were expressed in *E. coli* with N-terminal 6×His tags and purified by nickel affinity chromatography, yielding protein samples of 95% purity as demonstrated by SDS-PAGE (Fig. 1A). OBPgp279, and LysPA26 migrated as a single band having a molecular weight similar to their

predicted molecular weights (38.7, and 18.8-kDa, respectively). Gp110 showed the most intense band near its predicted molecular mass and contained a weak band (that migrated slightly below a 70-kDa protein marker). The presence of the correct recombinant proteins was confirmed by western blotting with anti-His antibodies (Fig. 1B).

The western blotting analysis of His-tagged recombinant proteins yielded clear-cut protein bands that closely matched the positions of Gp110, OBPgp279, and LysPA26 on an SDS-polyacrylamide gel.

To examine the enzymatic activity, we conducted an in-gel zymography assay using cell walls of *A. hydrophila* strain AB005 as a substrate. In all purified enzymes, the zymogram revealed antibacterial activity against *A. hydrophila* as apparent from clear zones of lysis within the opaque gel containing AB005 strain cells (Fig. 1C). Nonetheless, the intensity of the clear zones differed among the enzymes: OBPgp279 produced barely visible bands, whereas LysPA26 yielded the most intense bands, wherein minor contaminating bands (as seen on the polyacrylamide gel) contributed no activity. In the zymogram, Gp110 displayed a broad band corresponding to its predicted molecular weight, accompanied by a smaller band closer to 70-kDa. Both protein bands exhibited peptidoglycan hydrolase activity against the *A. hydrophila* strain AB005, suggesting that Gp110 might undergo dimerization (Fig. 1C).

The comparative analysis of antimicrobial activity of endolysins Gp110, OBPgp279, and LysPA26 against *A. hydrophila*

This analysis was performed as the CFU reduction assay after each phage endolysin was added at a final concentration of 1 µg/µL to a bacterial cell suspension (OD_{600} = 0.65). As expected, antimicrobial activity of OBPgp279, LysPA26, and Gp110 increased with incubation time. OBPgp279, LysPA26, and Gp110 had an efficient bactericidal effect on *A. hydrophila* within 1 h. Nonetheless, there were differences in bacteriolytic activity between the three endolysins. Gp110 manifested more efficient bacteriolytic activity than the phage endolysins OBPgp279 and LysPA26. It is noteworthy that OBPgp279 and LysPA26, a well-characterized endolysins, showed only moderate activity. In contrast, Gp110 was clearly the most active enzyme against the *A. hydrophila* AB005 strain. Besides, different concentrations of phage endolysins were tested. The addition of 1µg/mL OBPgp279, LysPA26, or Gp110 reduced numbers of viable *A. hydrophila* AB005 cells by 2.46, 2.74, and 4.04 log units, respectively, during 60-min incubation at 20 °C (Fig. 2). The lytic capacity of all endolysins did not increase significantly when the concentration exceeded 1 µg/mL. Therefore, the endolysin concentration of 1 µg/mL was chosen for the following assays. Although endolysins exerted better lytic action against *A. hydrophila* AB005 without EDTA pretreatment, we decided to experiment with an EDTA-pretreated cell suspension as a substrate.

As depicted in Fig. 3, EDTA alone had no antibacterial activity against *A. hydrophila*. In the presence of EDTA, Gp110 again showed better lytic performance when compared with OBPgp279 and LysPA26, because Gp110 led to a reduction in the viable-cell number by nearly 4.35 log units, whereas OBPgp279 and LysPA26 by only 2.97 and 3.2 log units, respectively.

Analysis of the activity across a range of pH by means of the *A. hydrophila* AB005 strain as an experi-

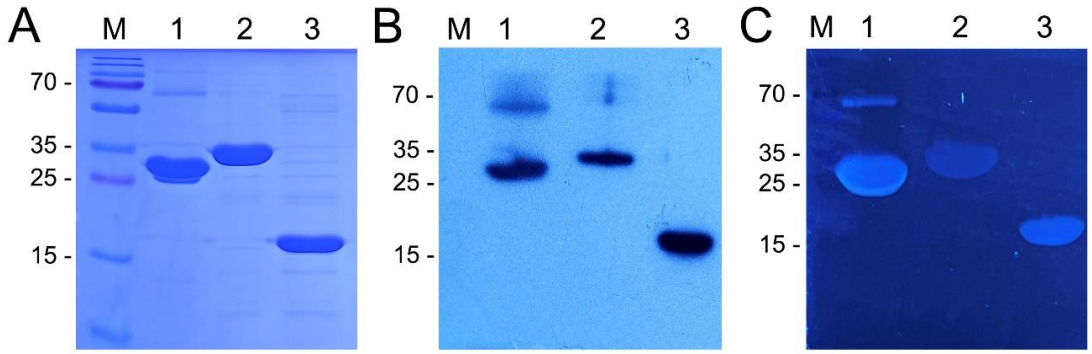

Fig. 1 Gp110, OBPgp279, and LysPA26 exert a peptidoglycan hydrolase action against the *A. hydrophila* AB005 strain. The purified proteins were resolved by SDS-PAGE followed by A) Coomassie blue staining, B) western blot analysis of the purified proteins, and C) zymogram analysis involving *A. hydrophila* AB005 peptidoglycan as a substrate. Lane M: standard protein molecular weight markers, lane 1: the purified Gp110 protein, lane 2: the purified OBPgp279 protein, lane 3: the purified LysPA26 protein.

mental model showed that all three endolysins were barely active at pH 6.0 but exerted a maximum activity at pH 7.5, with a similar activity at pH 8.0 (Fig. 4A). To determine the optimal temperature of the endolysins, endolysin aliquots were incubated at different temperatures (10, 20, 30, or 40 °C) for 1 h with an *A.*

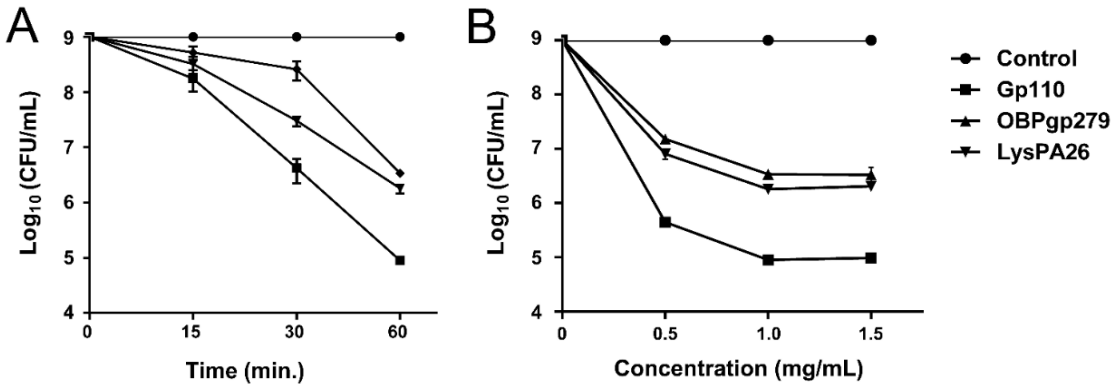

Fig. 2 *In vitro* efficacy of the three endolysins. A) The time-dependent curve of endolysin activity at 1 μg/μL against exponentially growing *A. hydrophila* AB005 cells, and B) the bactericidal effect at different concentrations (mg/mL) of Gp110, OBPgp279, or LysPA26 against exponentially growing *A. hydrophila* AB005 cells. Error bars represent standard deviations of three independent assays.

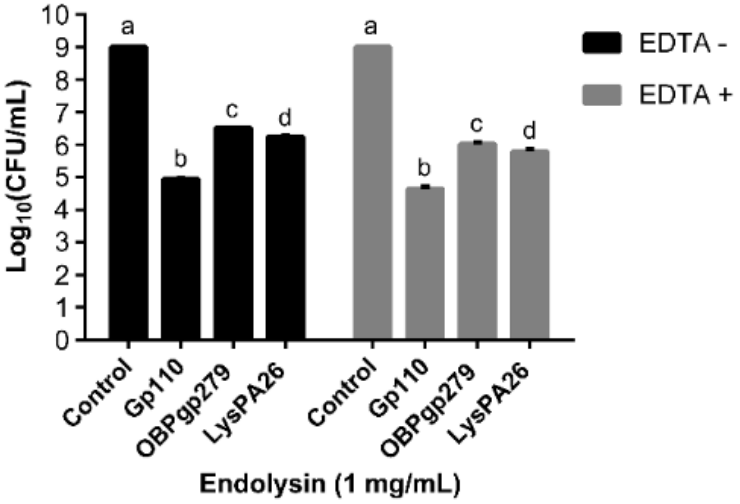

Fig. 3 Activity of endolysins against exponentially growing cells of *A. hydrophila* AB005 treated or untreated with 1 mM EDTA. The error bars show the standard deviation, and the black horizontal line marks the limit of detection. Different lowercase letters (a, b, c, and d) indicate significant differences between treatments ($p < 0.05$).

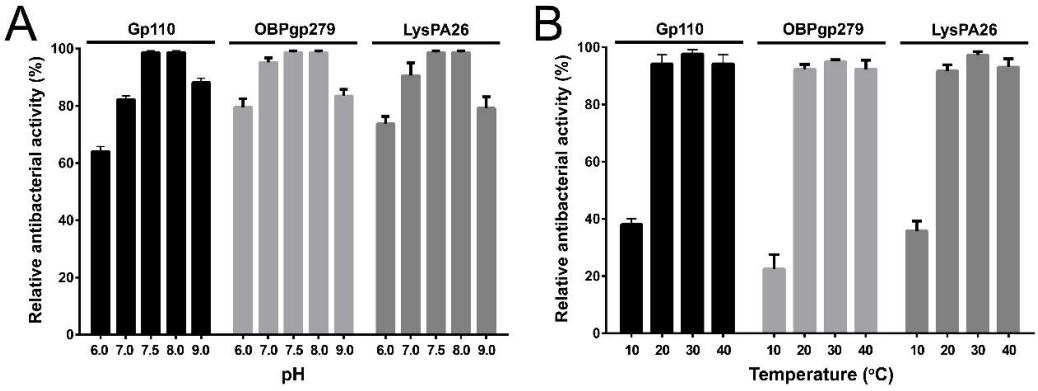

Fig. 4 The influence of pH and temperature on the bactericidal activity of Gp110, OBPgp279, and LysPA26. A) The effect of pH on the lytic activity, B) the effect of temperature on the lytic activity. Data are means ± standard deviations of three replicates. Error bars represent standard deviations of three independent assays.

hydrophila AB005 cell suspension. Elevated temperatures up to 40 °C did not affect the enzyme function and did not hinder bacterial growth when endolysins were absent. All three endolysins had substantial bactericidal activity between 20 and 40 °C, with noticeably lower activity observed at 10 °C (Fig. 4B).

It is known that the specificity and antibacterial activity of endolysins toward a host pathogen can notably vary against various bacterial genera and species (Farkasovska et al. 2003; Loessner 2005). Therefore, in subsequent experiments, we conducted a comparative analysis of the effect of endolysins on some species belonging to the genus *Aeromonas*, such as *A. hydrophila* (OK634406), *A. salmonicida* (OK634025), and *A. veronii* (OK634393) (Table 1). We also included a gram-positive strain of *Staphylococcus aureus*. The results of the antimicrobial assays of the three endolysins toward these bacterial species are presented in Fig. 5. All the enzymes manifested a wide but diverse range of bactericidal activities depending on the endolysin and the bacterial species used. All three endolysins showed enhanced activity against *A. veronii*. By contrast, reduced activity was observed for all enzymes against *A. salmonicida*. No activity against gram-positive staphylococcal strains was detectable (Fig. 5). According to these findings, Gp110 could be suggested as an antimicrobial agent against *Aeromonas* species. Representative pictures of cell viability

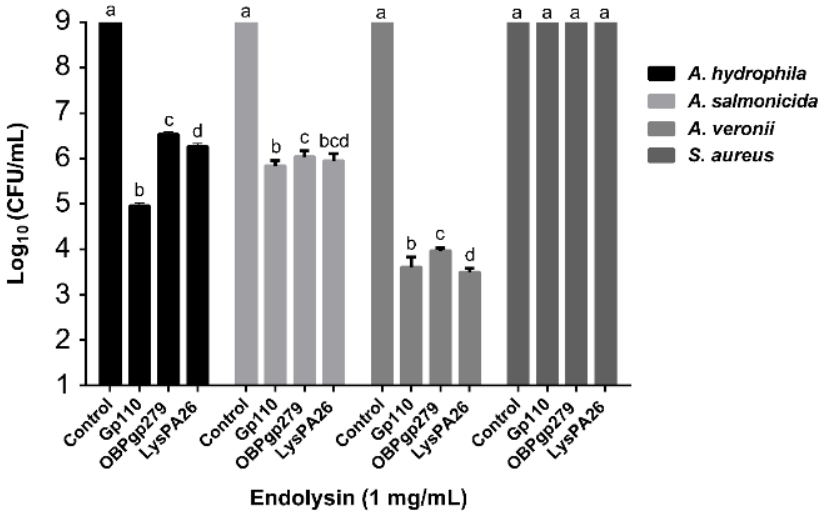

Fig. 5 Endolysin activity against exponentially growing *Aeromonas* species. *A. hydrophila* AB005, *A. salmonicida* AB001, *A. veronii* AB003, and *S. aureus* (ATCC) were treated with Gp110, OBPgp279, or LysPA26, and the colonies were counted. The error bars are standard deviation, and the black horizontal line denotes the limit of detection. Different lowercase letters (a, b, c, and d) indicate significant differences between treatments ($p < 0.05$).

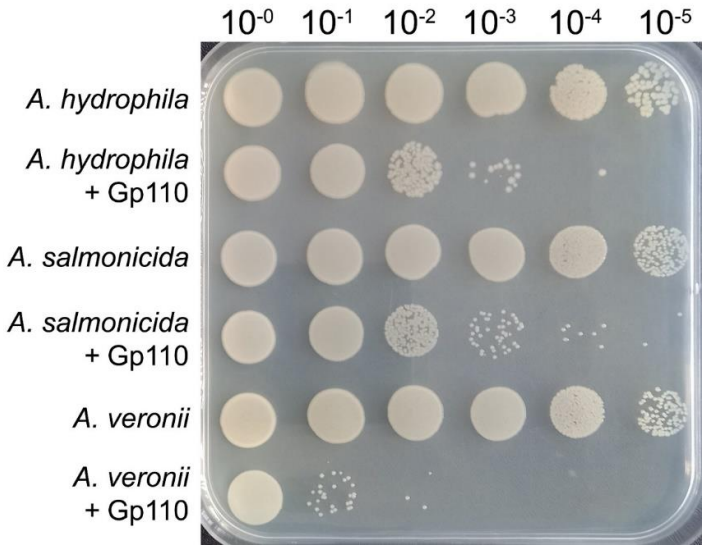

Fig. 6 Representative pictures of cell viability before and after Gp110 treatment. To measure viability, 10-fold dilutions of exponentially growing *A. hydrophila* AB005, *A. salmonicida* AB001, and *A. veronii* AB003 cells and Gp110-treated cells were spotted onto agar plates.

before and after Gp110 treatment are given in Fig. 6.

Therapeutic effects of endolysins in a Nile tilapia model

In vivo efficacy tests were performed for the three endolysins using *O. niloticus* as an animal model (Fig. 7). *O. niloticus* individuals were infected with *A. hydrophila* AB005 and treated with one of the endolysins, followed by examination of survival for 96 h. In this experiment, an infection dose of 10^7 CFU/mL caused 80% mortality of *O. niloticus* within 96 h after intraperitoneal injection. Moreover, *O. niloticus* challenged with strain AB005 manifested fading of body pigmentation, a distended abdomen, and extensive skin hemorrhages including the base of opercula and fins. Internally, the infected fish showed massive hemorrhaging of internal organs and branchial ischemia (Fig. 8). As displayed in Fig. 7, intraperitoneal injection with 150 µg/fish Gp110 significantly increased survival by 96 h. It was found that a single injection of Gp110 can rescue 100% of the fish. Besides, most of the above-mentioned pathological signs were markedly alleviated

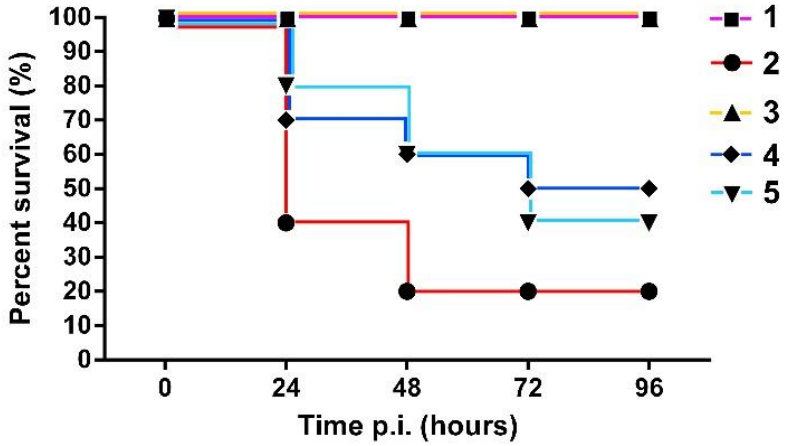

Fig. 7 *In vivo* efficacy of each endolysin in the *O. niloticus* model of infection. The animals were divided into five groups: 1) group "PBS," i.e., fish injected with PBS without infection, 2) group "*A. hydrophila* AB005," i.e., fish infected with the bacteria (10^7 CFU/mL), 3) group "Gp110," meaning those infected with the bacteria and treated with the Gp110 endolysin (150 µg/fish), 4) group "LysPA26," i.e., those infected with the bacteria and treated with LysPA26 endolysin (150 µg/fish), and 5) group "OBPgp279," representing those infected with the bacteria and treated with endolysin OBPgp279 (150 µg/fish).

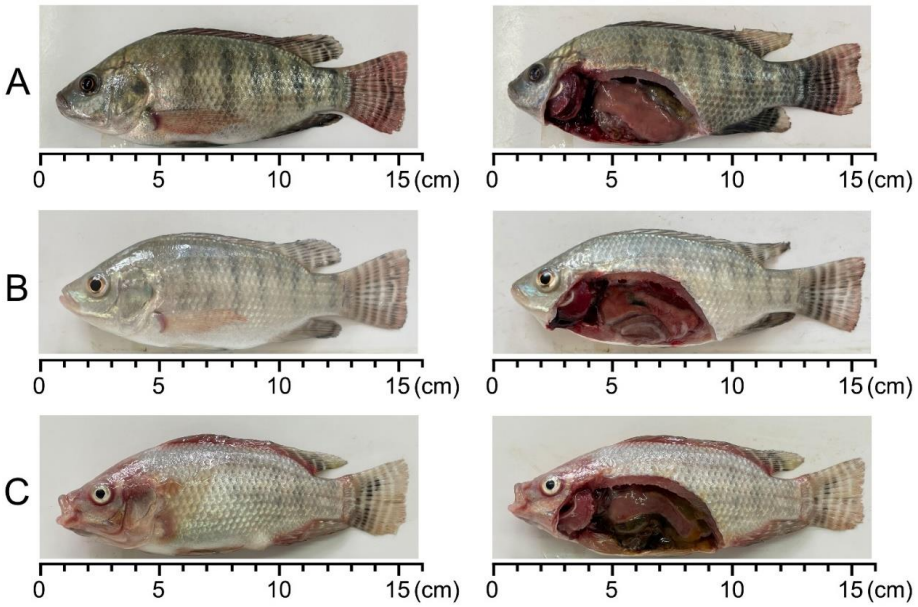

Fig. 8 *In vivo* therapeutic effects of endolysins in the Nile tilapia model. A) control fish, B) fish treated with Gp110, and C) fish injected with *A. hydrophila* AB005.

in the organs of the Gp110-treated group (Fig. 8B), indicating that Gp110 had a protective effect against *A. hydrophila* infection in *O. niloticus*. Endolysins LysPA26 and OBPgp279 were also effective but not to the extent of Gp110. The survival rate of *A. hydrophila* AB005–infected *O. niloticus* treated with LysPA26 or OBPgp279 reached 50% and 40%, respectively. It should be pointed out that fish injected with each endolysin only remained alive for 96 h (data not shown).

A potential ability of endolysin therapy to cure skin lesions of the diseased *A. baerii*

To assess wound healing, we used fish naturally affected by aeromonosis (Bakiyev et al. 2022), with moderate disease severity. Gp110 was chosen for this experiment because this enzyme showed greater *in vitro* and *in vivo* lytic activity against *A. hydrophila* than other endolysins did. Representative images of an ulcer area reduction in an experimental group are presented in Figure 9. In the first group (Gp110), at the beginning of the experiment, the wound penetrated deep into the muscle layer, and pronounced necrosis of muscle tissue cells was also visible. Even so, the development of granulation tissue and subsequent epithelialization of defective skin areas began 12 days after the first injection of Gp110. The extent (percentage) of wound closure in the fish treated with Gp110 was 41.8% on the 6th day, 79% on the 12th day, and 95.7% on the 25th day, respectively (Fig. 9). The influence of cefazolin on the healing process was similar to that seen in the Gp110-treated group. In this case, ulcers decreased by nearly 96% in size at the final evaluation. Nevertheless, in this group, one fish died, and all fish survived in the Gp110-treated group. In the control group, all five individuals of *A. baerii* died within less than 18 days after the beginning of the experiment.

Discussion

In the case of aquaculture, even though several antibiotics have been approved by the Food and Drug Administration (FDA), the mortality rate of *Aeromonas* infections is still high (Figueras et al. 2016; Nicholson et al. 2020; Rasmussen-Ivey et al. 2016; Shameena et al. 2020; Yardimci and Turgay 2020). Furthermore, the vast majority of these pathogens in aquaculture are resistant to multiple antibiotics (Sherif and Kassab 2023). The emergence and dissemination of antibiotic resistance among the aeromonads of fish have been indicative of the abuse of antibiotics in aquaculture. This emerging increasingly important phenomenon requires new strategies for the development of novel antimicrobials. Endolysins are promising alternatives to antibiotics, and in contrast to antibiotics and phage therapy, there are no reports of bacteria developing resistance to endolysins (Rahman et al. 2021). There has been much research on phage lysins, which have been shown to successfully kill bacteria *in vitro* and *in vivo* (Schmelcher et al. 2012; Schuch et al. 2002).

A total of 1956 bacteriophage genomes are currently available in the NCBI genome database [National Center for Biotechnology Information (NCBI)], of which 19 are genome sequences of phages infecting *Aeromonas* (Kazimierczak et al. 2019). Besides, in GenBank, there are currently thousands of genes encoding putative endolysins. The majority do not have intrinsic antibacterial activities when tested as recombinant proteins (Jeong et al. 2023). Accordingly, the discovery of a potent endolysin is still challenging because the entire process takes a lot of time and is labor-intensive, and it is difficult to obtain a soluble form

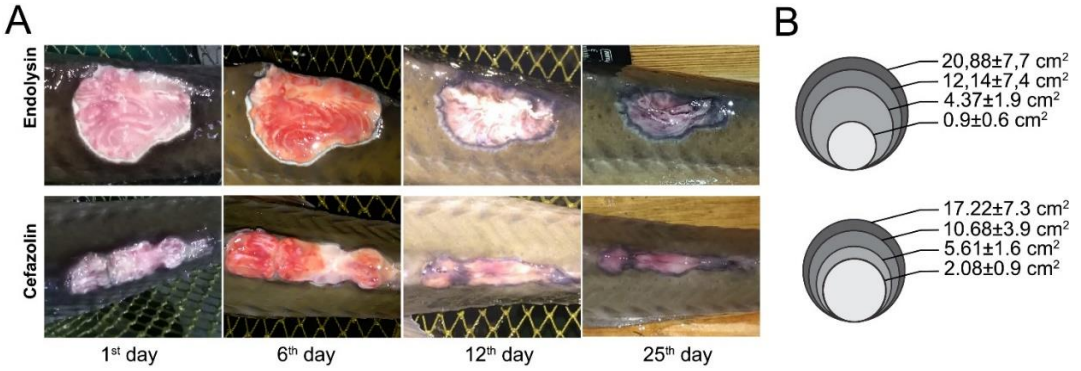

Fig. 9 Effects of intramuscular injection of Gp110 on wound-healing progression in *A. baerii* naturally affected by aeromonosis. A) representative images of an ulcer area reduction, B) dynamics of a wound average area reduction.

of an endolysin with high lytic activity. In this regard, evaluating the effectiveness of known endolysins against antibiotic-resistant *Aeromonas* species may be a promising approach to the search for effective lytic enzymes for the treatment of diseases caused by these pathogens.

In this work, potential benefits of three endolysins (LysPA26, Gp110, and OBPgp279) are investigated in terms of combatting antibiotic-resistant bacteria from the genus *Aeromonas*. Gp110 has modular structure consisting of an N-terminal cell wall–binding domain (CBD) and a C-terminal catalytic domain (CD) (DUF3380; pfam11860). It has been demonstrated that Gp110 has N-acetylmuramidase (lysozyme) activity, specifically cleaving the β-(1,4)-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine residues in the sugar backbone of peptidoglycan (Rodríguez-Rubio et al. 2016). On the other hand, OBPgp279 is also a modular endolysin, comprising two N-terminal CBDs and one C-terminal CD. OBPgp279 is predicted to be a member of glycoside hydrolase family 19 (GH19) owing to the presence of a conserved sequence motif in its catalytic domain (Walmagh et al. 2012). In contrast to the above proteins, LysPA26 is a single-domain endolysin. It has been identified as a member of the lysozyme-like domain family within the cd00442 superfamily (Guo et al. 2017). All the three endolysins are already known to lyse gram-negative bacteria such as *P. aeruginosa*.

Here we show that some bacteria from the genus *Aeromonas*, such as *A. hydrophila*, *A. salmonicida*, and *A. veronii* can now be added to this list. *A. hydrophila* has been described as the dominant infectious agent responsible for disease outbreaks in farmed freshwater fish all over the world (Rasmussen-Ivey et al. 2016). *A. hydrophila* has been associated with epizootic ulcerative syndrome (EUS), which is the cause of mass mortality in farmed fish (Iqbal et al. 1999). Previously, we have isolated the *A. hydrophila* AB005 strain from diseased *A. baerii* (Bakiyev et al. 2022). It was demonstrated in that study that the *A. hydrophila* AB005 strain has considerable virulence and leads to acute infection in fish. Furthermore, drug sensitivity testing there revealed that this isolate is sensitive to quinolones, aminoglycosides, nitrofurans, amphenicols, and tetracyclines.

Consistent with previous reports, OBPgp279 reduced the number of cells counts of *P. aeruginosa* PAO1 in 1 log unit without pretreatment with an outer membrane permeabilizer (OMP) (Walmagh et al. 2012). A higher antimicrobial activity was observed with the endolysin LysPA26, which could eliminate up to 4 log units of an exponential growing suspension of *P. aeruginosa* cells without EDTA pretreatment (Guo et al. 2017). In another paper, antibacterial activity of 2.5 μM Gp110 without EDTA was found to be very low, whereas in combination with EDTA, it reduced the number of *P. aeruginosa* PAO1 cells by roughly 3 log units but only by 0.38 log units in the case of *Salmonella Typhimurium* LT2 cells (Rodríguez-Rubio et al. 2016). By contrast, in our work, 1 μg/μL Gp110 reduced the number of viable *A. hydrophila* AB005 cells by over 4 log units without the EDTA pretreatment. Increasing the duration of incubation with the bacterial suspension (to 60 min versus 30 min) and raising the concentration of Gp110 (to 32 μM versus 2.5 μM) seemed to be associated with a significant enhancement of the antibacterial activity against *A. hydrophila* AB005 without the EDTA pretreatment in our study. This outcome was anticipated because Lim et al. (2014) also noticed substantial lytic activity of the SPN9CC endolysin against *E. coli* even in the absence of EDTA pretreatment, especially when both the endolysin quantity and incubation duration were increased.

To the best of our knowledge, this is the first report of significant antibacterial activity of endolysins OBPgp279, LysPA26, and Gp110 against *Aeromonas* species. The only endolysin with a reported ability to kill *A. hydrophila* is PlyD4 (encoded by the phAhD4 prophage), whose lytic activity is substantially improved by the addition of EDTA (5 mmol/L) (Wang et al. 2022). EDTA is well known to be toxic, and accordingly, its application in aquaculture may lead to undesirable effects on fish health and on the environment (Hagiwara et al. 2006). Therefore, our findings suggest that endolysins OBPgp279, LysPA26, and Gp110 may be promising antimicrobial candidates for the prevention and/or control of *Aeromonas* pathogens in aquaculture owing to the observed robust bactericidal activity against of *Aeromonas* pathogens in the absence of EDTA. Nevertheless, differences in bacteriolytic activity among the three endolysins were observed: Gp110 has higher bacteriolytic activity than the others do.

The results obtained *in vitro* were confirmed by *in vivo* assays because the survival of the infected *O. niloticus* individuals was better when the *O. niloticus* fishes were treated with endolysin Gp110 than when treated with OBPgp279 or LysPA26 alone (Fig. 7). The largest amount of sturgeon products in Kazakhstan is produced in the Uralsk region on a full-system sturgeon farm called the LLP Educational and Scientific Complex of Pilot Industrial Production of Aquaculture (Uralsk, Kazakhstan). This enterprise maintains the

largest sturgeon brood stock in Kazakhstan. Lately, diseases with characteristic symptoms of generalized bacterial hemorrhagic septicemia and skin ulceration have been occurring among cultured *A. baerii* fishes on this farm (Nurzhanova et al. 2021, Sergaliev et al. 2021). Similar clinical signs, such as petechial hemorrhages and skin ulcers penetrating deep into muscle, have been observed in the common carp *Cyprinus carpio* L., rainbow trout *Oncorhynchus mykiss*, and largemouth bass *Micropterus salmoides* infected with *A. hydrophila*, especially when the fish are under stress (Huizinga et al. 1979; Kozińska and Pękala 2012). Healing in fish has mainly been researched in artificially modeled wounds or mechanical injuries (Roy et al. 2019; Sveen et al. 2020; Yun et al. 2021). The impact of endolysins on the healing of wounds from pathogenic microorganisms has not been well studied. As shown in Fig. 9, significant wound healing activity was observed in animals treated with Gp110. It should be noted that the effect of cefazolin on the healing process was similar to the wound healing seen in the Gp110-treated group. These data indicate potential utility of endolysins as an alternative for the treatment of skin lesions in diseased fish.

Aquaculture needs successful preventive measures and effective treatments to reduce the prevalence of infectious diseases caused by various bacteria. This is especially true for sturgeon aquaculture because sturgeon farms are expensive to run, and it takes a long time before sturgeons' spawn (Knapp et al. 2006). Indeed, bacterial pathogens are the main reason for the deaths of these fish (Nurzhanova et al. 2021; Bakiyev et al. 2022; Bakiyev et al. 2023); however, due to the appearance of antibiotic-resistant and multidrug-resistant pathogens, the research into alternative and adjunctive therapeutic modalities is needed.

To our knowledge, the current study is the first to report an endolysin efficacy tests regarding wound healing in *A. baerii* fish naturally affected by aeromonosis. Although the use of a protein might be problematic for the control over bacterial pathogens in aquaculture, endolysins can be delivered by food grade organisms such as *Lactococcus lactis* or *Saccharomyces cerevisiae* (Gaeng et al. 2000; Khatibi et al. 2014). Further analysis of appropriate delivery systems and the nature of their specificity will facilitate their use as novel agents for control over bacterial pathogens in aquaculture.

Author contributions AB, designing, conceptualizing and supervision, formal analysis and writing the original draft; SB, data collection, data analysis and writing, review, and editing; IS, data collection, data analysis, and writing, review and editing. All authors read and approved the final manuscript.

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