

# In-Silico Screening of Antimicrobial Peptides (AMPs) from *Halomonas* sp. to Combat Vibriosis in Shrimp Aquaculture

Nisrina Fitri Nurjannah<sup>1</sup>, Irfan Anwar Fauzan<sup>1</sup>, Ditta Putri Kumalasari<sup>1</sup>

<sup>1</sup>Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang, Indonesia.

Corresponding Author: Nisrina Fitri Nurjannah

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## ABSTRACT

Vibriosis caused by *Vibrio parahaemolyticus* remains one of the major challenges in shrimp aquaculture. There is an urgent need for alternative antimicrobial strategies to reduce dependence on conventional antibiotics. Antimicrobial peptides (AMPs) are antimicrobial compounds derived from natural sources which are less harmful to the environment. In this study, we employed an in-silico screening approach to identify novel antimicrobial peptides (AMPs) derived from the halophilic bacterium *Halomonas* sp., that are abundant in shrimp farming systems. Through a comprehensive computational pipeline, two AMP sequences were predicted with antibacterial activity against multiple pathogens, including *V. parahaemolyticus*. Further analysis showed short-peptide regions with high antimicrobial probability within the whole peptides. Among these, the sequence WRARGNVAQVIKLRR showed strong similarity to phage holin family proteins which are often studied as antibacterial agents. Physicochemical analyses of the sequence indicated favourable properties, including net positive charge (net charge = +5), good stability ( $\Pi = 32.69$ ), and balanced GRAVY score (-0.660). Although these findings are promising, further experimental validation is required to confirm the antimicrobial

efficacy and environmental stability of these peptides under aquaculture conditions. The discovery of such marine-derived AMPs may offer an environmentally friendly and effective alternative for disease management in shrimp aquaculture.

**Keywords:** antimicrobial peptides, *Halomonas* sp., in-silico screening, shrimp aquaculture, vibriosis

## INTRODUCTION

Shrimp aquaculture is one of the fastest-growing sectors in global food production, with demand continuing to rise each year (1). However, this growth is accompanied by serious challenges, particularly the prevalence of vibriosis, a bacterial disease caused by several species of *Vibrio* such as *Vibrio parahaemolyticus*. Vibriosis can result in lethargy and gastrointestinal disorders, and in more severe cases, lead to Acute Hepatopancreatic Necrosis Disease (AHPND), which significantly impacts shrimp survival and farm productivity (2). With the increasing restrictions on antibiotic use in aquaculture due to concerns over environmental residues and antimicrobial resistance, preventive strategies have become a central focus in recent years (3). In developing antimicrobial strategies suitable for aquaculture, compounds derived from natural sources are often favored because they are less likely to harm the surrounding

environment. Antimicrobial peptides (AMPs) represent one such class of compounds that have been extensively studied for their potent antimicrobial properties. AMPs are naturally secreted as part of the innate immune defense across a wide range of organisms, from bacteria and phytoplankton to invertebrates and mammals (4). One of the earliest AMP applications was the commercial development of bacteriocins, such as nisin, isolated from *Lactococcus lactis* and widely used as a food preservative due to its effectiveness against Gram-positive bacteria (5).

A major challenge in applying AMPs to shrimp aquaculture is their reduced stability under saline conditions. This issue is particularly relevant in euryhaline or brackish water environments, which are typical in shrimp farming (6). Marine microorganisms may offer a solution, as they have evolved proteins that remain functional in high-salinity settings. This characteristic are hypothesized to also extend to their secreted AMPs (7). In aquaculture, several AMPs have already been evaluated. For instance, pleurocidin, derived from the skin mucus of the winter flounder (*Pleuronectes americanus*), exhibits broad-spectrum antimicrobial activity (8).

Although AMPs can be derived from many marine organisms, microorganisms remain the most sustainable and scalable source. Their ability to be cultivated under controlled conditions allows for consistent and large-scale production, which is essential for long-term application (9). Therefore, bioprospecting for AMPs in marine microorganisms holds great potential. The genus *Halomonas*, for example, is known for its halophilic nature and has been isolated from various saline environments, including hypersaline lakes and marine ecosystems. Some strains have been used as probiotics, and recent studies have pointed to their potential as AMP producers (10,11).

To explore new AMP candidates, it is essential to draw from broader protein sources. Advances in computational tools enables wide screening of potential AMPs

from protein databases (12). This study aims to screen protein datasets from the *Halomonas* genus, which are available in major biological databases, using advanced AMP prediction tools. The resulting candidates will be evaluated for their physicochemical characteristics and their antibacterial potential against several pathogens including *V. parahaemolyticus*. Through this approach, we aim to identify promising novel peptides that could lead to the development of effective antimicrobial agents for future use in shrimp aquaculture.

## MATERIALS & METHODS

### Proteome retrieval and AMP prediction

Complete proteomes of *Halomonas* spp. were retrieved from the UniProtKB database (<https://www.uniprot.org>) using the organism name “Halomonas” as the query. Protein sequences were downloaded in FASTA format (13). A custom Python script using Biopython SeqIO to remove sequences containing nonstandard amino acids. Subsequently, a length-based filtering was done and only sequences with 10-100 amino acids were retained (14). The filtered protein sequences were submitted to AMPScanner v2.0

(<https://www.dveltri.com/ascan/v2/ascan.html>) to identify putative antimicrobial peptides. Peptide segments with prediction annotated as AMP by the algorithm were retained for further evaluation (15).

### Antimicrobial activity prediction against aquatic pathogens

The candidate AMPs were analyzed for their antibacterial activity using the linear AMP activity prediction tool available at the DBAASP v3 platform (<https://dbaasp.org/tools>). From the tools menu, Antibacterial peptide prediction was done and the Strain-specific antibacterial prediction based on ML approaches and data on peptide sequences and bacterial genomes analysis menu was chosen. Prediction was performed against multiple pathogens available on the platform, which are *Escherichia coli* ATCC 25922,

*Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhimurium* ATCC 14028, *Acinetobacter baumannii* ATCC 19606, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, and *Bacillus subtilis* ACTT 6633 (16). Additionally, the genome of *Vibrio parahaemolyticus* RMID 2210633, that was obtained from GenBank, was also uploaded to be tested against the AMP candidates. Peptides showing the widest array of predicted activity were selected for further analysis.

### Cross-validation and AMP Region Prediction

The selected peptides were validated using AI4AMP (<https://axp.iis.sinica.edu.tw/AI4AMP/>). An AMP region prediction would be done automatically on AI4AMP to obtain shorter region with high AMP potential (17).

### Sequence similarity analysis

Sequences predicted as antimicrobial peptides were subjected to similarity search using BLASTp against the NCBI non-redundant (nr) protein database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (18). The search was performed using the BLASTp web interface with an E-value cut-off of  $1e^{-5}$ . Low-complexity filtering was enabled, and the maximum number of target sequences was set to 100. Functional annotations of the top hits were manually examined to identify known homologs, conserved domains, or hypothetical protein annotations. Sequences with high similarity

to known AMPs or with no known function were retained for further prioritization.

### Physicochemical characterization

Physicochemical properties of the peptides were calculated using the ProtParam tool on the ExPASy server (<https://web.expasy.org/protparam/>) (19). The following parameters were recorded: molecular weight (Da), net charge, theoretical isoelectric point (pI), instability index, aliphatic index, and grand average of hydropathicity (GRAVY) (20).

## RESULT

### Retrieval of *Halomonas* sp. Proteomes

A total of 35,152 protein sequences were retrieved from the UniProtKB database under the organism *Halomonas* sp. (organism\_id:1486246). After the removal of sequences containing non-standard amino acids and length filtering sequences 228 sequences were retained and used for AMP screening.

### Identification of putative antimicrobial peptides

AMPScanner v2.0 was used to evaluate the curated peptide dataset. Out of the 228 sequences analyzed, 42 were predicted as antimicrobial peptide. These candidates were prioritized for further functional prediction.

### Predicted activity against pathogens

The selected AMP candidates were assessed using the DBAASP platform. Among them, two peptides showed predicted antibacterial activity against all of the reference pathogen strains and the uploaded *Vibrio parahaemolyticus* RIMD 2210633 (Table 1).

**Table 1** Candidate AMP sequences from *Halomonas* sp. proteomes predicted by AMPScanner v.2 and DBAASP

No.	Sequence ID	Sequence
1	tr A0A2E2MXL1 A0A2E2MXL1 HALSX	MTTSTLVTLCCALLICTRLLTFRRRGARYRLGYSCTAWLLIAGTGTVAIRILTQSGYPAGWGIALVMAVLAWLVWRARGNVAQVIKLRRL
2	tr A0A2E2N1A8 A0A2E2N1A8 HALSX	MPSSQTRWRQRLALATAGLAALLLFTPLAWLIHHRDWGIVLMALVPLAVWLTLRLWRRLERWALQG

### Cross-validation of antimicrobial potential

The AMP predictions were further validated using AI4AMP. AI4AMP generated several short-peptide regions as potential novel AMP sequences from the full-length peptide sequence (Table 2). From the generated regions, AI4AMP predicted region 76-90

(WRARGNVAQVIKLR) from sequence 1 and region 51-66 (LTLRLWRRLERWALQG) from sequence 2 to be novel AMPs with high probability scores. The full-length peptides, as well as the short-peptide regions were then curated for the following analysis.

**Table 2 Predicted short-peptide AMP sequences of *Halomonas sp.* from AI4AMP**

No	Peptide	Score	AMP Prediction
1	MXL1 1 49	0.38960344	No
2	MXL1 26 74	0.18319726	No
3	MXL1 51 90	0.00028428435	No
4	MXL1 76 90	0.9328973	Yes
5	N1A8 1 49	6,03E-01	No
6	N1A8 26 66	3,01E+01	No
7	N1A8 51 66	0.925617	Yes

### Homology to known AMPs and hypothetical proteins

BLASTp analysis revealed that sequence 1 shared highest similarity with phage holin family protein from unclassified *Halomonas* (Sequence ID: WP\_222568385.1). Homology analysis of region 76-90 of sequence 1 also shows the same result. In contrast, AMP sequence 2 were annotated as hypothetical proteins from *Halomonas sp.* (Sequence ID: MAY71362.1), suggesting they may represent previously uncharacterized AMPs. The same results were obtained from analysis of region 51-66 of sequence 2. From this results, only the shorter peptide would be kept for further analysis.

### Physicochemical Properties of Candidate Peptides

The physicochemical characteristics of the two candidate peptides were analyzed using the ExPASy ProtParam tool, and the results

are summarized in Table 3. Both peptides display basic isoelectric points (pI), with sequence 1 having a higher theoretical pI than sequence 2 which indicated stronger cationic nature. Sequence 1 also exhibited a higher net charge (+5) compared to Peptide 2 (+3), suggesting a potentially stronger electrostatic interaction with negatively charged bacterial membranes (21). In terms of stability, the instability index (II) classified sequence 1 as stable (II = 32.69), whereas Peptide 2 was predicted to be unstable (II = 118.79). The aliphatic index of sequence 2 was higher than that of sequence 1 which indicates greater thermal stability (22). However, this was accompanied by a lower net charge and higher instability. The hydropathicity score (GRAVY) showed that both peptides are moderately hydrophilic, with sequence 1 showing slightly more hydrophilicity which may suggests enhanced solubility and reduced host toxicity (23).

**Table 3 Physicochemical properties of candidate short-peptide AMPs analyzed using ExPASy ProtParam**

Property	Sequence 1	Sequence 2
Number of amino acids	15	16
Molecular weight	1823.18 Da	2067.47 Da
Theoretical pI	12.48	12.00
Net charge	+5	+3
Instability index (II)	32.69	118.79
Aliphatic index	104.00	128.12
GRAVY (hydropathicity)	-0.660	-0.444

## DISCUSSION

The present study demonstrates the utility of in-silico bioprospecting strategies to identify novel antimicrobial peptides (AMPs) from proteomes of marine microorganisms. Specifically the genus *Halomonas*, as a promising source for combating aquaculture-related pathogens such as *Vibrio parahaemolyticus* (10,11). This approach responds to a critical need in shrimp aquaculture, where the overuse of antibiotics has raised environmental and regulatory concerns (24). The synthesis of novel AMP may be the solution to solve our dependence toward antibiotics. Especially, the use of novel AMPs from marine microorganism may increase its efficacy in brackish aquaculture setting. Marine-derived peptides are more likely to retain their activity in the brackish or saline waters (25). Thus, sourcing from marine-adapted organisms offers a biologically logical and ecologically compatible strategy.

Marine ecosystem has such a wide array of genetic resources and it would be nearly impossible to isolate every proteins from every organisms and test it in in-vitro settings. The use of bioinformatics helps us to focus on certain potential candidates organism and test large datasets of proteomes compiled on online database (11,26). In this case, *Halomonas* sp. was chosen due to its prevalence in brackish water including in shrimp farms. Moreover, several studies showed their ability to act as probiotics in a biofloc system in shrimp aquaculture and reduce the amount of pathogens in pond (10,27). Selecting a certain peptide produced by *Halomonas* sp. will open up the possibilities of synthesis of a standalone AMPs that can be easily produced and packed to be directly used in place of antibiotics.

In this study, using a comprehensive computational pipeline, we successfully identified 42 promising AMP candidates from AMPScanner v.2. AMPScanner v.2 employs a deep neural network (DNN) classifier to recognize putative AMP sequences (15). Comparative study of

AMPScanner v.2 showed higher specificity of the platform compared to several other AMP predictors (28). However, these putative AMP sequence may not work against the target pathogen in aquaculture. DBAASP platform enables the prediction of antimicrobial activity of tested sequence (16). While several sequences shows antibacterial activity against *Vibrio parahaemolyticus*, a broad spectrum AMPs will be more beneficial in the context of aquaculture, where several type of opportunistic pathogens exist (29). Thus, sequences with antibacterial activity against all pathogens listed on DBAASP were chosen.

While the combination of AMPScanner v.2 and DBAASP gave strong AMP candidates, it is worth noting that AMP predictions result may differ with different algorithm (28). Often, a cross-validation using other programs are needed to find sequences with strong confidence (30). In present study, the potential AMP candidates were subsequently analysed using AI4AMP. AI4AMP works by comparing tested sequences against combination of data from several known AMP databases. The platform will predict several short regions (15-20 amino acids) within the analysed sequences to find regions with high AMP probability score (17). Shorter amino acid sequence were more advantageous due to reduced synthetic cost, lower immunogenicity, and increased tissue penetration (31,32). From two candidate sequences, one short-peptide region with high AMP probability was predicted and were subjected to similarity analysis using BLASTp.

The most promising region of sequence 1 (WRARGNVAQVIKLR), shares sequence similarity with phage holin family proteins. Holins are known to form membrane pores during bacterial lysis, which is an important and common mechanism that parallels the membrane-disrupting action of many AMPs. This structural analogy strengthens the hypothesis that sequence 1 may act by compromising membrane integrity (33). In contrast, short-region of sequence 2

(LTLRLWRRLERWALQG) aligns with hypothetical proteins lacking characterized function. While this could suggest a novel AMP discovery, they also require careful consideration as the lack of annotation could reflect incomplete genomic data rather than functional uniqueness (34). Nonetheless, both sequences hold potential for future experimental validation.

Lastly, the physicochemical properties analysed via ProtParam provided important insights into AMP functionality. Sequence 1 showed a higher net positive charge, a higher pI, and was more stable based on the instability index. These factors are critical for AMP interaction with negatively charged bacterial membrane. In AMP, positively charged residues such as Arg and Lys facilitate binding to lipopolysaccharides and peptidoglycan. Sequence 1 also exhibited lower molecular weight that would increase the potency of AMP in penetrating bacterial membrane (21). Another important property of an AMP is its hydrophobicity presented as GRAVY, which represent the capability of AMP insertion into the lipid bilayer membrane of the bacteria and while maintaining its solubility. GRAVY scores close to zero are more desirable in an AMP (23). Sequence 2 exhibit slightly better GRAVY score as well higher aliphatic index, which suggest greater thermal stability (22). However, this was counterbalanced by a much higher instability index, indicating that it may degrade more rapidly in the environment.

Despite these promising findings, all predictions remain computational and must be validated in vitro and in vivo. The AMP activity prediction platforms used here rely on machine learning models trained on known peptides, which may overlook non-canonical features or underrepresented structural motifs (35). For future work, peptide synthesis and MIC testing against *V. parahaemolyticus* and other aquaculture pathogens should be prioritized. Evaluation of peptide stability in seawater and in the presence of organic matter (e.g., shrimp feed,

biofilms) would also needed to ensure its applicability in fields (36).

## CONCLUSION

This study employed a systematic in silico approach to identify novel antimicrobial peptides (AMPs) from the halophilic bacterium *Halomonas* sp. with the goal of discovering candidates suitable to fight Vibriosis shrimp aquaculture. Out of 35,152 proteins screened, 42 were predicted as potential AMPs, and two candidates demonstrated broad-spectrum predicted antibacterial activity, including against *Vibrio parahaemolyticus*. Further analysis predicted potent short-peptide AMP regions, which were then analysed for their physicochemical properties. Sequence WRARGNVAQVIKLR emerged as the most promising AMP candidate with homologous similarity to phage holin family protein. While these findings are promising, experimental validation both is essential before field application. The integration of marine-derived AMPs into aquaculture could offer an eco-friendly and effective alternative to conventional antibiotics, contributing to sustainable shrimp farming and improved disease management.

## Declaration by Authors

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