

## In Silico Identification Of Cryptic Antimicrobial Peptides From The Ichthyophthirius Multifiliis Proteome As Templates For Next-Generation Therapeutics

**Asif Ali Khan**

Department of Zoology, Kohat University of Science and Technology, Kohat-26000, Pakistan Email: ak6910359@gmail.com

**Shehzad Zareen** (Corresponding Author)

Department of Zoology, Kohat University of Science and Technology, Kohat-26000, Pakistan Email: shehzadzareen1@kust.edu.pk

**Muhammad Arsalan** (Corresponding Author)

Department of Zoology, Kohat University of science and technology, Kohat-26000, Pakistan Email: arsalankohat70@gmail.com

**Muhammad Ali Khan**

Department of Microbiology, Kohat University of Science and Technology, Kohat-26000, Pakistan Email: ali.micro051@gmail.com

### ABSTRACT

The rapid emergence of antimicrobial resistance has intensified the demand for novel therapeutic agents. Cryptic antimicrobial peptides (AMPs), embedded within larger protein sequences, represent an underexplored reservoir of bioactive molecules with potential for next-generation antimicrobial development. Protozoan parasites, which inhabit microbe-rich host environments, are particularly promising yet neglected sources of such peptides. In this study, we performed a comprehensive in silico analysis of the *Ichthyophthirius multifiliis* proteome to uncover cryptic AMP candidates. A total of 16,761 protein sequences were analyzed using a Python/Biopython workflow integrating sequence preprocessing, physicochemical evaluation, and deep learning-based AMP prediction. In silico fragmentation produced 197,916 peptide fragments, of which 10,949 were initially predicted as high-confidence AMPs. Sequential filtering based on net positive charge, hydrophobicity, and predicted structural stability reduced this pool to 1,574 biologically plausible peptides. From these, seven unique top candidates were selected for detailed structural analysis. Secondary structure predictions and helical wheel visualization revealed amphipathic  $\alpha$ -helical organization, with clear segregation of hydrophobic and positively charged residues, consistent with classical membrane-active AMP mechanisms. Collectively, this study reveals a previously hidden repertoire of cryptic AMPs in *I. multifiliis*, providing a valuable computational framework for the rational design of next-generation synthetic AMPs for aquaculture and antimicrobial therapeutics.

**Keywords:** Ichthyophthirius Multifiliis, Cryptic Antimicrobial Peptides, Deep Learning, Proteome-Wide, Ectoparasite.

## Introduction

Antimicrobial peptides (AMPs) are short bioactive molecules ubiquitously present across almost all living organisms (Mohaideen et al., 2023). Acting as essential effectors of innate immunity, AMPs typically exert their antimicrobial activity by disrupting microbial cell membranes (Duarte-Mata & Salinas-Carmona, 2023). Their rapid action, broad-spectrum activity, and reduced propensity for resistance have positioned AMPs as promising alternatives to conventional antibiotics (Erdem Büyükkiraz & Kesmen, 2022). These properties have spurred growing interest in the AMP industry, with efforts to develop novel therapeutics, food preservatives, and aquaculture solutions, reflecting their translational potential beyond basic research (Baindara & Mandal, 2022).

Despite this promise, conventional AMP research has largely focused on well-characterized families naturally expressed in organisms, leaving much of the proteome unexplored. A substantial hidden reservoir exists in the form of cryptic antimicrobial peptides (Bosso et al., 2025). These are short sequences embedded within larger precursor proteins that are not present as free peptides under physiological conditions. Upon release or synthetic production, many cryptic AMPs display potent antimicrobial activity. Importantly, they often lack sequence similarity to established AMP families, making them invisible to traditional discovery methods (Raja et al., 2025). Such cryptic sequences offer a rich source for next-generation synthetic AMP design, enabling tailored optimization of activity, stability, and selectivity (Gawde et al., 2023).

Advances in computational biology have opened avenues for systematic discovery of cryptic AMPs at the proteome scale. Machine learning and deep learning techniques can predict antimicrobial activity based on physicochemical and structural features rather than sequence homology alone. This enables high-throughput screening of complete proteomes, uncovering antimicrobial sequences that remain hidden to conventional approaches (Li et al., 2022). However, such computational strategies have been applied to only a limited number of organisms.

Protozoan parasites inhabit complex ecological niches, interacting with host tissues, immune defenses, and dense microbial communities (Shukla et al., 2024). These selective pressures may favor the evolution of bioactive peptide sequences that enhance parasite survival or modulate microbial interactions. Despite their potential, the antimicrobial capacity encoded within protozoan proteomes remains poorly understood.

Ichthyophthirius multifiliis, the causative agent of white spot disease in freshwater fish, is a ciliate protozoan of global aquaculture significance (von Gersdorff Jorgensen & Puspasari, 2022; Maezono et al., 2025). The parasite colonizes skin and gills, organs continuously exposed to dense microbial populations, suggesting that its proteome may encode cryptic AMPs. While its life cycle and pathogenicity have been extensively studied, the presence of cryptic antimicrobial peptides has not been systematically explored (von Gersdorff Jorgensen & Puspasari, 2022).

In this study, we performed a comprehensive in silico analysis to identify cryptic

AMPs in the *I. multifiliis* proteome. Our multistep computational pipeline integrated proteome fragmentation, sequence and physicochemical filtering, deep learning-based AMP prediction, and structural evaluation. This work represents the first systematic identification of cryptic AMPs in *I. multifiliis* and provides a reproducible framework for uncovering hidden antimicrobial sequences in protozoan parasites. Moreover, the identified peptides serve as novel templates for next-generation synthetic AMP development, with potential applications in aquaculture and broader antimicrobial industries.

## Materials and Methods

### Protein Dataset Collection

The complete proteome of *Ichthyophthirius multifiliis* was retrieved from the NCBI protein database in FASTA format. The file comprises all available annotated protein sequences for the organism at the time of download (accessed on 28-12-2025). All analyses were performed using Python 3.12 in the Google Colab environment to enable efficient processing of large datasets and ensure full reproducibility of the computational workflow.

### Protein Length and Biological Filtering

Protein sequences were parsed using Biopython's SeqIO module. Sequences shorter than 100 amino acids were excluded, as such proteins are less likely to contain stable internal regions suitable for cryptic peptide generation. Proteins containing extensive low-complexity or repetitive regions were identified and removed using a Shannon entropy-based sequence complexity filter implemented in Python. The isoelectric point (pI) of each protein was calculated using Biopython's ProtParam module, and only proteins with  $pI \geq 5.5$  were retained to favor neutral to basic sequences with higher potential to generate cationic antimicrobial peptide fragments.

### In Silico Protein Fragmentation

Filtered proteins were fragmented using a sliding window (central 50% of sequence) to avoid terminal regions. Fragments of 20 and 30 amino acids (step size: 10 aa) were generated, typical lengths for antimicrobial peptides. Fragments required:  $\geq 2$  positively charged residues (lysine/arginine), 4-8 hydrophobic residues, and no single-residue dominance ( $>80\%$ ). These criteria enrich for cationic, amphipathic peptides likely to interact with microbial membranes.

### Antimicrobial Peptide Prediction

Peptide fragments were analyzed for antimicrobial potential using AMP Scanner v2, a deep learning-based tool (Veltri, Kamath, & Shehu, 2018). To manage the large dataset, the sequences were divided into multiple batches and processed separately. The prediction outputs from all batches were consolidated using Python 3 with the Pandas library. Fragments with prediction scores of 0.75 or higher were retained as high-confidence antimicrobial peptide candidates. The AMP Scanner v2 tool was accessed on 13 January 2026 via <https://github.com/dan-veltri/amp-scanner-v2>.

## Physicochemical Property Filtering

High-confidence peptides were further evaluated for physicochemical properties using Biopython's ProtParam at pH 7. Peptides with a net charge of +2 or higher were retained to ensure sufficient electrostatic interaction with negatively charged microbial membranes. Fragments containing four to eight hydrophobic residues were selected to support amphipathic structure formation, while peptides with an instability index below 40 were kept to favor predicted structural stability. These criteria were applied to enrich for sequences with balanced charge, hydrophobicity, and stability, consistent with potential antimicrobial activity.

## Secondary Structure Propensity and Candidate Selection

The  $\alpha$ -helical propensity of filtered peptides was assessed using the Chou–Fasman method. Peptides were ranked according to net charge, hydrophobicity, instability index, and sequence diversity, with higher-ranking peptides considered more favorable for antimicrobial activity. Redundant sequences with near-identical properties were removed to ensure uniqueness. From this curated set, a representative subset of peptides was selected for structural visualization, prioritizing those with balanced physicochemical properties and predicted  $\alpha$ -helical potential.

## Helical Wheel Visualization

Selected peptide candidates were analyzed for  $\alpha$ -helical organization using NetWheels (<http://lbqp.unb.br/NetWheels>) on 15 January 2026. The tool generates helical wheel projections, allowing visualization of the spatial arrangement of hydrophobic and positively charged residues along the helix (Mol, Castro, & Fontes, 2018). This analysis was performed using default settings to assess amphipathic organization, which is important for potential antimicrobial activity.

## Data Handling and Visualization

Peptide sequences were stored in FASTA format, and numerical data were stored in CSV format. Data processing, filtering, and ranking were performed using Pandas. Graphical visualizations, including histograms and scatterplots of key physicochemical parameters and prediction scores, were generated using Matplotlib to support data quality assessment and candidate selection. Summary tables were prepared to document dataset progression and peptide characteristics.

## Results

The complete proteome of *Ichthyophthirius multifiliis* contained 16,761 protein sequences. Length filtering retained 15,527 proteins of  $\geq 100$  amino acids. Subsequent removal of low-complexity sequences and exclusion of proteins with isoelectric points ( $pI < 5.5$ ) resulted in 13,300 proteins suitable for cryptic antimicrobial peptide mining.

In silico fragmentation of the central 50% of these proteins, using fragment lengths of 20–30 amino acids and a step size of 10, generated 197,916 peptide fragments, which constituted the input dataset for antimicrobial peptide prediction. Summary of proteome filtering and fragment generation is listed in table 1.

**Table 1.** Summary of proteome filtering and fragment generation. This table shows the stepwise reduction of the *I. multifiliis* proteome from 16,761 proteins to 197,916 peptide fragments.

| Step | Processing Stage        | Criteria applied                                                              | Sequences retained        |
|------|-------------------------|-------------------------------------------------------------------------------|---------------------------|
| 1    | Raw proteome            | Complete <i>I. multifiliis</i> proteome from NCBI                             | 16,761 proteins           |
| 2    | Length filtering        | Proteins $\geq 100$ amino acids                                               | 15,527 proteins           |
| 3    | Biological filtering    | Removal of low-complexity sequences and exclusion of proteins with $pI < 5.5$ | 13,300 proteins           |
| 4    | In silico fragmentation | Central 50% of proteins: fragment lengths 20-30aa; step size 10               | 197,916 peptide fragments |

All 197,916 peptide fragments were analyzed using AMPScanner v2. Application of a probability threshold of  $\geq 0.75$  identified 10,949 high-confidence antimicrobial peptide candidates. The distribution of AMP prediction probabilities demonstrated clear enrichment of peptides within the high-confidence range following threshold application as shown in figure 1.

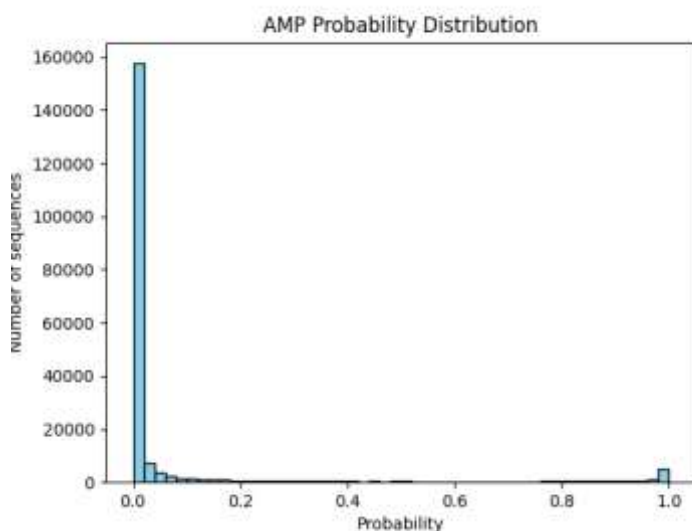


**Figure 1.** Distribution of AMP prediction probabilities for *I. multifiliis* peptide fragments. The bar graph shows the frequency of peptide fragments across prediction probability scores assigned by AMP Scanner v2, highlighting the enrichment of high-confidence candidates ( $\geq 0.75$ ).

Physicochemical properties, including net charge at pH 7 and hydrophobic residue count, were calculated for all high-confidence antimicrobial peptides. Filtering based on cationicity (net charge  $\geq +2$ ) and amphipathicity (4–8 hydrophobic residues)

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reduced the dataset to 6,241 peptides. Further refinement using the instability index eliminated peptides predicted to be structurally unstable, yielding 1,574 biologically plausible antimicrobial peptides. Distribution of net charge, Hydrophobicity, and instability index of AMPs is shown in figure 2-4 respectively. Summary statistics of physicochemical properties of AMPs is listed in table 2.

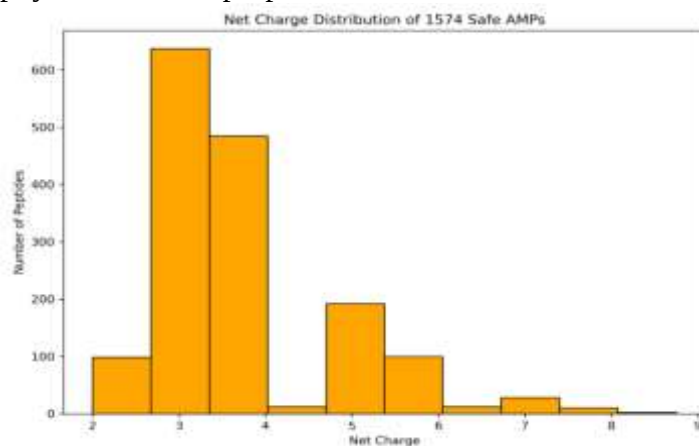


**Figure 2.** Distribution of net charge among high-confidence AMP candidates. The bar graph illustrates the number of peptides at each net charge value, emphasizing the prevalence of positively charged peptides suitable for interacting with microbial membranes.

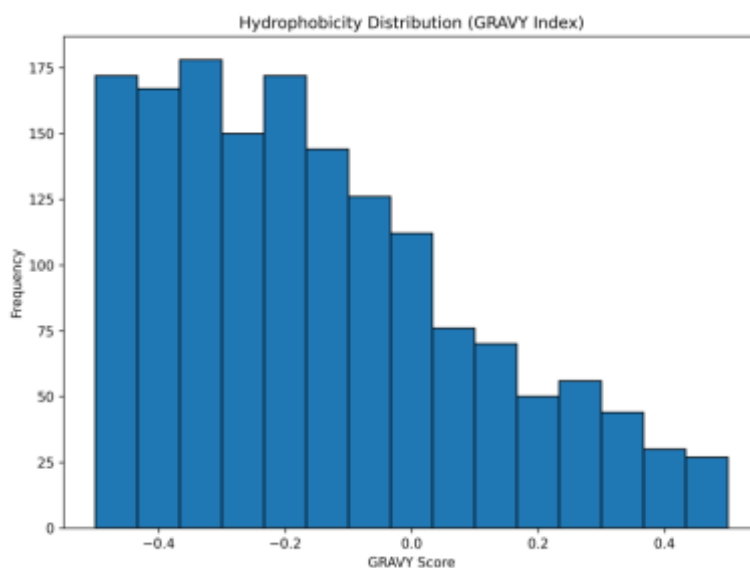


**Figure 3.** Distribution of hydrophobic residues in filtered AMP candidates. This bar graph shows the frequency of peptides with different numbers of hydrophobic residues, supporting amphipathic  $\alpha$ -helix formation.

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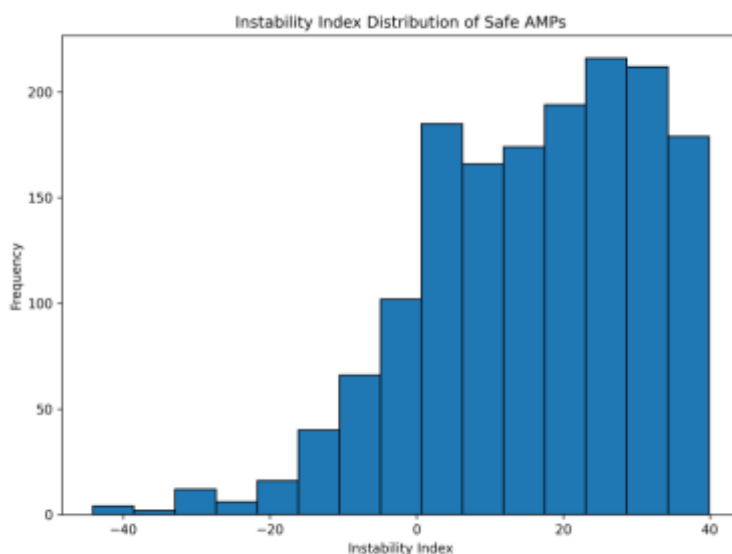
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**Figure 4.** Distribution of instability indices for filtered AMP candidates. The histogram displays predicted structural stability of peptides, with lower instability indices indicating sequences likely to persist long enough to exert antimicrobial activity.

**Table 2.** Summary statistics of physicochemical properties of the final filtered AMP dataset. This table summarizes the effect of successive filtering steps, from AMP prediction to redundancy removal, highlighting the refinement process that produced 7 high-confidence AMP candidates.

| Step | Filtering stage                | Criteria applied                                                   | Peptides retained |
|------|--------------------------------|--------------------------------------------------------------------|-------------------|
| 1    | AMP prediction                 | Prediction using AMPScanner v2                                     | 197,916           |
| 2    | High-confidence selection      | Prediction probability $\geq 0.75$                                 | 10,949            |
| 3    | Physicochemical filtering      | Net charge $\geq +2$ ;<br>Hydrophobic residues 4-8                 | 6,241             |
| 4    | Stability filtering            | Instability index $< 40$                                           | 1,574             |
| 5    | Redundancy removal and ranking | Removal of overlapping fragments: selection of top unique peptides | 7                 |

Secondary structure prediction of the 1,574 filtered peptides revealed a strong predominance of  $\alpha$ -helical conformations, with fewer peptides predicted as  $\beta$ -sheet or random coil structures as shown in figure 5 (Supplementary File S3).

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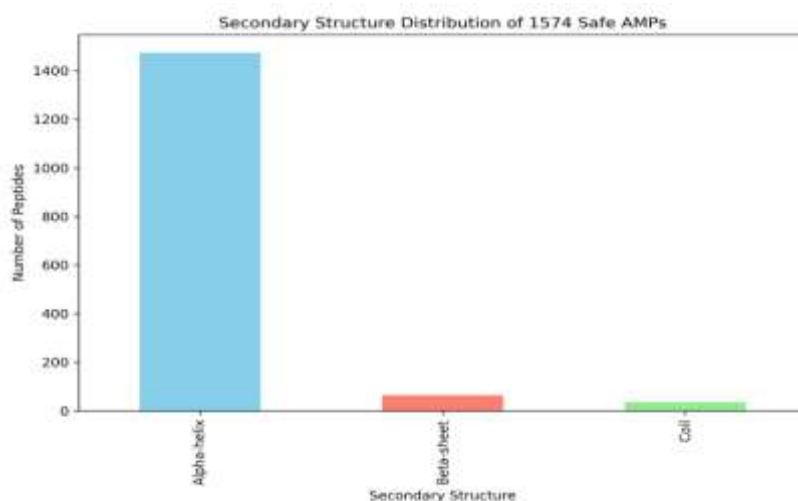
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**Figure 5.** Secondary structure distribution of filtered AMP candidates. The bar graph depicts the predicted proportions of  $\alpha$ -helix,  $\beta$ -sheet, and random coil conformations among the peptides, highlighting the predominance of  $\alpha$ -helices.

From the final set of 1,574 peptides, candidates were ranked based on net charge, instability index, hydrophobic residue content, and peptide length. Redundant sequences arising from overlapping fragments were removed, resulting in the selection of 7 unique antimicrobial peptide candidates for downstream structural analysis as given in table 3. These peptides represent the highest-confidence AMP candidates identified in this study.

**Table 3.** Properties of the 7 unique high-confidence AMPs. The table lists the sequence, net charge, hydrophobicity, instability index and sequence length of the top peptides, illustrating their physicochemical features relevant to antimicrobial activity.

| PeptideID            | Sequence                      | NetCharge | Hydrophobicity | Instability Index | Sequence length |
|----------------------|-------------------------------|-----------|----------------|-------------------|-----------------|
| EGR31116.1_frag51_70 | KIIIIITRKIRIIKKKQKKT          | 8.753089  | -0.26          | 8.585             | 20              |
| EGR30421.1_frag1_30  | PSINSSCKISKCNRIKKKKIFLIGKCSFH | 8.008997  | -0.40333       | 32.77667          | 30              |

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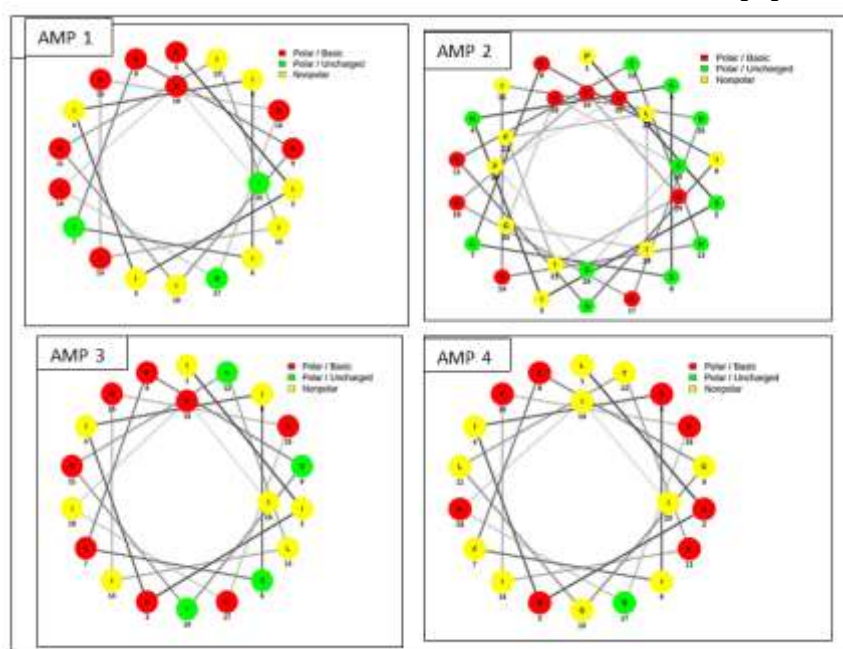
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|                                                     |                           |          |        |        |    |
|-----------------------------------------------------|---------------------------|----------|--------|--------|----|
| <b>EGR32782.1</b><br><b>_frag61_80</b>              | IIKIIQKKQTRNLIRK<br>KIKI  | 7.754088 | -0.415 | 6.265  | 20 |
| <b>EGR32462.1</b><br><b>_frag31_50</b>              | LRKIRIFKGGLYKIK<br>KQKII  | 7.753089 | -0.255 | 8.38   | 20 |
| <b>EGR30216.1</b><br><b>_frag291_31</b><br><b>0</b> | KVKSIIQKIKIKQVKF<br>FKKSV | 7.75211  | -0.405 | -14.07 | 20 |
| <b>EGR27267.1</b><br><b>_frag111_13</b><br><b>0</b> | IGSINRKKVKKIYIKK<br>KIYI  | 7.751101 | -0.395 | -8.92  | 20 |
| <b>EGR30165.1</b><br><b>_frag21_40</b>              | LFKKVKSTDISLRKK<br>KKAVV  | 6.754219 | -0.415 | 5.93   | 20 |

Helical wheel projections were generated for the 7 unique peptides using NetWheels to visualize amphipathic  $\alpha$ -helical organization (figure 6) . All peptides exhibited clear spatial segregation of hydrophobic residues in the helix and positively charged residues , consistent with membrane-active antimicrobial peptides.



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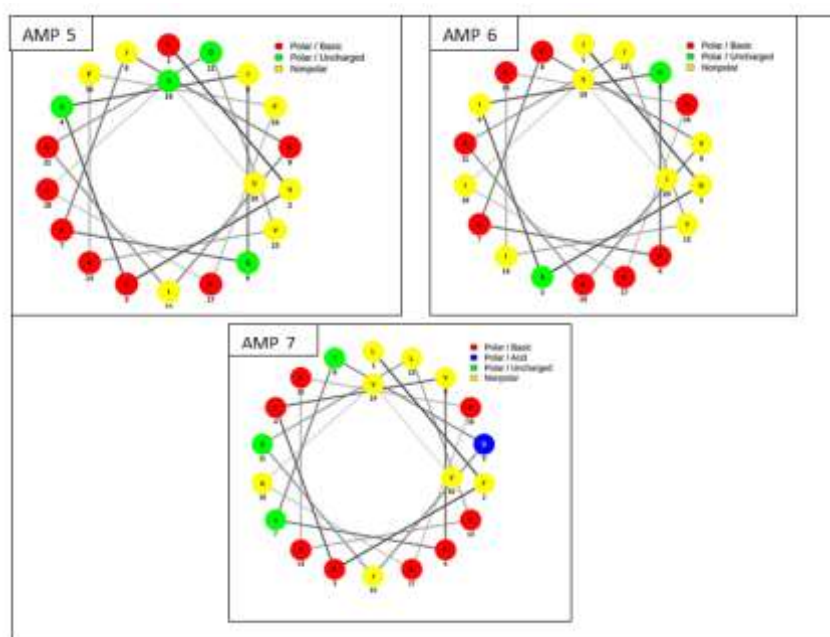
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**Figure 6.** Helical wheel projections of the 7 unique AMP candidates. The projections visualize the amphipathic  $\alpha$ -helical organization, showing hydrophobic residues on one face and positively charged residues on the opposite face, consistent with membrane-targeting potential.

## Discussion

Parasites such as *Ichthyophthirius multifiliis* inhabit environments with intense microbial exposure, particularly on fish skin and gills where dense and diverse bacterial communities persist (von Gersdorff Jorgensen & Puspasari, 2022). Continuous contact with these microbial populations likely exerts strong selective pressure for intrinsic antimicrobial defenses, suggesting that parasite-derived peptides may encode bioactive motifs optimized for microbial targeting.

Despite this ecological and functional relevance, antimicrobial peptides (AMPs) in protozoan parasites remain largely unexplored. Through a systematic proteome-wide in silico approach, the present study uncovers a previously hidden repertoire of cryptic AMP-like sequences within the *I. multifiliis* proteome. Beyond advancing our understanding of parasite biology, these cryptic peptides represent a valuable blueprint for the design of synthetic AMPs, offering a route to engineer molecules with enhanced potency, stability, and selectivity.

By integrating AI-based AMP prediction with physicochemical filtering, the study reduced an initial dataset of 16,761 proteins to 1,574 biologically plausible AMP candidates and ultimately to seven high-confidence, non-redundant sequences for detailed structural evaluation. This sequential refinement illustrates the power of combining machine learning with biophysical constraints to prioritize peptides not only predicted as AMPs but also amenable to synthetic optimization. Similar proteome-wide screening strategies have successfully guided synthetic AMP development in bacterial and protozoan systems, demonstrating the translational potential of this workflow.

Selection criteria focused on net positive charge, hydrophobic residue content, and predicted structural stability. A net positive charge enhances electrostatic attraction to negatively charged microbial membranes, a critical step in AMP-mediated membrane disruption (Felsztyna, Galassi, & Wilke, 2025). Amphipathic distribution of hydrophobic residues supports membrane insertion and bilayer destabilization, while structural stability suggests resilience against degradation, improving the likelihood of effective synthetic designs.

Secondary structure predictions indicated  $\alpha$ -helical conformations in most selected peptides, a motif widely leveraged in synthetic AMP engineering. Helical wheel projections revealed clear amphipathic segregation, providing mechanistic insight for rational design: hydrophobic faces for membrane insertion and cationic faces for selective microbial binding (Felsztyna, Galassi, & Wilke, 2025). By choosing seven unique peptides, the study highlights diverse sequence scaffolds suitable for synthetic optimization, avoiding redundancy while maximizing functional diversity.

The translational significance is clear: these cryptic peptides offer ready-made templates for next-generation synthetic AMPs, potentially applicable for controlling protozoan pathogens, opportunistic bacterial infections, and other aquaculture-relevant microbes. Integrating mechanistic insights at each filtering step strengthens the design pipeline, bridging computational prediction with experimental feasibility for synthetic AMP development.

Li et al. (2025) demonstrated that deep learning-driven discovery of antimicrobial peptides from protist genomes provides strong experimental validation but still limitations exist. The study relies on computational predictions, and functional activity, selectivity, and safety require experimental validation. Length-based filtering may have excluded longer precursor proteins, potentially underestimating the full repertoire. Future work should focus on peptide synthesis, in vitro antimicrobial testing, cytotoxicity assessment, and mechanistic studies to validate and refine these candidates for practical synthetic AMP applications.

## Conclusion

Using deep learning and physicochemical filtering, this study systematically uncovered cryptic antimicrobial peptides in the *Ichthyophthirius multifiliis* proteome sequences that are largely hidden and rarely observed individually. Thousands of candidate peptides were identified, with a subset displaying strong structural and functional potential. These findings reveal an untapped reservoir of bioactive molecules shaped by the parasite's microbe-rich environment and provide novel templates for next-generation synthetic AMPs, demonstrating how computational approaches can bridge fundamental parasitology with translational antimicrobial development.

## Declaration of Competing Interest

The authors confirm that they have no conflicts of interest related to this work.

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## Author Contributions

Asif Ali Khan designed and conceptualized the study, carried out all data analyses, and drafted and revised the manuscript. Shehzad Zareen provided supervision for the project and contributed to manuscript writing and revision. Muhammad Arsalan assisted in manuscript writing. Muhammad Ali Khan critically reviewed the manuscript. All authors have read and approved the final version of the manuscript.

## Data Availability

All datasets generated or analyzed during this study are available with first author .

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