Research article

A novel in-silico approach to design a multiepitope peptide as a vaccine candidate for *Aeromonas hydrophila*

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ABSTRACT

Introduction: Aeromonas hydrophila is a Gram-negative bacterium with a rod-shaped structure and a member of the *Aeromonas* genus. It is commonly found in aquatic environments such as freshwater, estuaries, and sewage. Known as an opportunistic pathogen, *A. hydrophila* can infect both aquatic animals and humans. Understanding this bacteria's virulence factors, pathogenic mechanisms, and epidemiology is crucial due to its clinical significance and potential impact on public health, to develop effective disease management and prevention strategies. *A. hydrophila* has developed resistance to numerous antibiotics, making it challenging to combat, so vaccination can be a hopeful strategy for targeting this bacterium. Despite the development of multiple vaccine candidates for this bacterium, no commercially available vaccine has demonstrated high effectiveness. This study aimed to design a vaccine candidate that has the potential to effectively combat *A. hydrophila* infections, by using an informatics server.
Methods: Servers and bioinformatics tools were used to find and evaluate vaccine candidates. The bacterial genome was extracted and open reading frames were identified. The toxicity, allergenicity, immunogenicity, and homology of ORFs were investigated and non-toxic, non-allergenic with highly immunogenic were selected as a candidate for vaccine design. Next, epitopes were predicted and combined with adjuvants, linkers, and his-tag to create the vaccine candidate. Afterward, a thorough assessment of the vaccine was carried out.
Results: After the investigations, an extracellular protein with access number WP_045528985.1 was selected as a vaccine candidate. Combining a total of 15 epitopes for B cells and T cells, the vaccine candidate was completed. The analysis showed that the structure of the vaccine is non-toxic, non-allergenic, and has a favorable immunogenicity score of 0.8573. Additionally, the designed vaccine passed all virtual tests, including analysis of physical and chemical characteristics, and valuations of secondary and tertiary structure.
Conclusion: According to the results, this multiepitope peptide can be used as a promising vaccine candidate which warrants further development. Furthermore, further investigation is required to examine the functional characteristics, in vitro and in vivo experiments, potential applications, and animal model studies to confirm the safety, effectiveness, and long-term impacts of the vaccine formulation.

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1. Introduction

Aeromonas hydrophila is a gram-negative, coccobacillus, motile, oxidase-positive, facultative, and anaerobic rod that is found in aquatic environments. *A.hydrophila* is the most common pathogen, infecting fish, amphibians, reptiles and humans [1]. It can cause multiple kinds of diseases such as diarrhea, hemorrhagic septicemia, tonsillitis, swelling of tissue, bacteremia, necrosis, asymptomatic septicemia, enterocolitis, ulceration, arthritis, peritoneal peritonitis, and meningitis in human [2,3]. Bacterial diseases are the primary factors contributing to fish mortality in aquaculture, in particular, *A.hydrophila* is the primary reason for disease outbreaks [4]. Also, the transmission of *A. hydrophila* infections from diseased fish to humans through the consumption of contaminated water and raw or undercooked seafood has been reported [5]. The mortality rate caused by this bacteria is often high; therefore, it is essential to implement effective control measures for both prevention and treatment [6].

Diseases caused by bacteria are controlled using antibiotics. Currently, the widespread use of various types of antibiotics is the main obstacle to treating disease caused by this bacteria [7]. Over time, *A.hydrophila* has demonstrated the ability to develop resistance to multiple classes of antibiotics, limiting the options available for successful treatment [8]. This resistance can arise through various mechanisms, including the acquisition of resistance genes, the production of enzymes that inactivate antibiotics, and alterations in the bacterial cell structure that prevent antibiotics from effectively targeting the pathogen [9]. Vaccination is seen as an alternative approach to safeguarding patients against diseases. *A.hydrophila*'s vaccines aim to stimulate the immune system to produce specific antibodies and immune cells that can recognize and neutralize the bacterium, protecting against infection. These vaccines may target key antigens or proteins present on the bacterium's surface to induce a protective immune response [10].

The extracellular and outer membrane protein of *A.hydrophila* plays a significant role in the pathogenicity of the bacterium by attacking the host tissues, causing the spread of tissue damage and creating a focus of infection, also enabling *A. hydrophila* to persist in wide habitats and great adaptability to environmental changes [1]. Since these proteins are recognized as foreign agents by the host's defense system, they act as defensive antigens and could function as an attractive vaccine candidate [7]. Several vaccines are developed for *A. hydrophila* but no commercial vaccine is available for uses, due to the heterologous adaptive immunity of this bacterium. Therefore, it is crucial to accurately identify immunologically safe antigens for creating a successful vaccine against *A. hydrophila*, which are capable of triggering an immune response within the host cell, which poses a significant challenge for the global scientific community [11].

By utilizing computational tools and bioinformatics, researchers can accelerate the vaccine development process and enhance the effectiveness of vaccine candidates against *A. hydrophila* infections. Multiepitope-based vaccines are protein structures that are engineered to replicate multiple characteristic epitope regions of a pathogen, triggering the host's immune system in a coordinated manner [12]. This study focus on suitable antigens based on the extracellular and outer membrane protein to formulate a potential multi-epitope peptide vaccine candidate against *A. hydrophila*, also effectiveness of this vaccine was tested and confirmed using various bioinformatics tools.

2. Materials and method

The utilization of various methods is essential for developing a multi-epitope vaccine. The process was illustrated step by step in detail in Fig. 1.
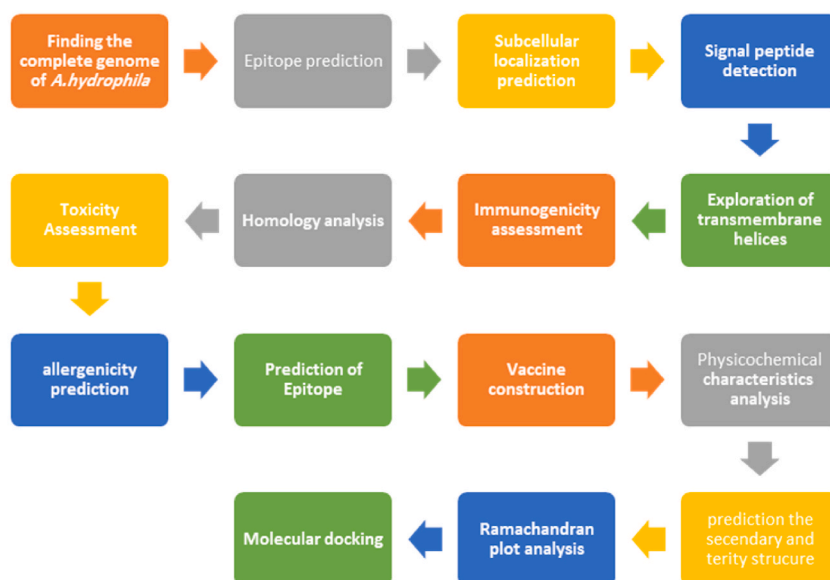

Fig. 1. Diagram depicting the methodology employed in this study.

2.1. Finding the complete genome of A.hydrophila and identification of open reading frames

The study starts with the genome analysis of *A.hydrophila* (NCBI Reference Sequence: NZ_CP065651.1, GenBank: CP065651.1). The complete genome sequence was achieved from the NCBI database (www.ncbi.nlm.nih.gov/) in FASTA format. Then open reading frames (ORFs) were predicted by the ORF finder server (www.ncbi.nlm.nih.gov/orffinder), using default parameters. ORF is a portion of a DNA sequence that does not include a stop codon [13].

2.2. Prediction of subcellular localization

The extracellular and outer membrane proteins of *A.hydrophila* are essential for the pathogenicity of the bacterium. These proteins can be recognized by the host's immune system as foreign agents, making them promising antigens. Protein localization prediction was carried out using the Psort server (www.psort.org/psortb) with the default settings [14].

2.3. Signal peptide detection

Then signal peptide sequences were identified by the SignalP server (www.services.healthtech.dtu.dk/services/SignalP-6.0/) with default parameters. Signal peptides serve as codes for cellular transport and are later removed during the post-translational modification process, so it's better to find and delete them [15].

2.4. Exploration of transmembrane helices

Some ORFs have structural sequences that integrate into the plasma membrane and remain inaccessible to the immune system, which must be excluded. As a result, an assessment of intramembrane domains was carried out. TMHMM Server v. 2. (www.services.healthtech.dtu.dk/services/TMHMM-2.0/) was used with default parameters to analyze the transmembrane helices [16].

2.5. Immunogenicity assessment

Immunogenicity refers to the capacity of a foreign substance, for example an antigen, to stimulate an immune response within the host cell. For predicting the immunogenicity of ORF the VaxiJen Server (www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html)with default parameters was used. The VaxiJen server utilizes an innovative alignment-independent approach. This method transforms protein sequences into standardized vectors that represent key amino acid characteristics through auto-covariance (ACC). The server's accuracy ranges from 70 % to 89 %, depending on the target organisms, which include bacterial, viral, and tumor protein datasets. A default threshold of 0.4 was applied [17].

2.6. Homology analysis

It is crucial to prevent any similarity between the chosen antigen sequence and the proteins of host cells, to ensure accurate identification by the immune system. BLAST server (www.blast.ncbi.nlm.nih.gov/Blast.cgi) was used with default parameters for this comprehensive evaluation of heterogeneity [18].

2.7. Toxicity assessment

Assessing toxicity is a crucial factor when contemplating both the short and long-term use of a potential vaccine component. In this study, the Toxinpred server (www.crdd.osdd.net/raghava/toxinpred/) with the default settings was used for identifying non-toxin epitopes [19].

2.8. Assessment of allergenicity

It is crucial to remove allergic epitopes to ensure safety and effectiveness in vaccine design. Allergic epitopes are specific parts of the antigen that can trigger allergic reactions in individuals who are sensitive to them. The identification and removal of allergenic epitopes were approved by using the Allertop servers (www.ddg-pharmfac.net/AllerTOP/index.html) with the default settings [20]. All parameters were set to default. At last non-allergenic epitopes were selected for further analysis.

2.9. Population coverage calculation

The overall population coverage was assessed for the chosen T lymphocyte epitopes in the vaccine construct, along with their associated MHCI and MHCII alleles, using the Population Coverage tool available in the IEDB database (http://tools.iedb.org/population/). This value is influenced by the range of MHC alleles recognized by the epitopes in the construct, which varies due to the diverse distribution of MHC alleles across different geographical regions and ethnicities worldwide [21].

2.10. Prediction of epitope

Epitopes are distinct portions of an antigen that are identified by the immune system, primarily through interaction with B cell, or T cell receptors and antibodies. The IEDB server (www.iedb.org/) with default parameters was used to analytically predict epitopes [22]. All HLA alleles were considered and analyzed. Epitopes were sorted based on their antigenicity and immunogenicity score, then underwent evaluation for toxicity and immunogenicity. Only epitopes that were found to be immunogenic, non-toxic, and non-allergenic were chosen for further analysis.

2.11. Vaccine construction

The process of constructing the vaccine needs the assembly of appropriate his-tag, adjuvant, and linker components. Subsequently, its characteristics, such as toxicity, allergenicity, and antigenicity was assessed using a previous server.

The flexible linker which use for connecting the epitopes: AAY and GGGGS. The incorporation of flexible linkers such as AAY and GGGGS in vaccine design serves multiple key functions in connecting epitopes. These flexible linkers facilitate the appropriate spatial arrangement of the epitopes, enhancing the overall conformational freedom of the protein. This increased flexibility aids the immune system in recognizing and responding to the epitopes more effectively. Linkers like GGGGS, which are rich in glycine, offer structural stability while avoiding steric hindrance. As a result, the epitopes can maintain their functional integrity and fold correctly. By utilizing flexible linkers, we reduce steric interference between neighboring epitopes, allowing for optimal presentation to immune cells.

His-tag: used to improve peptide expression.

Adjuvant: The study employed IL-12 cytokine as an effective adjuvant for acellular vaccines. IL-12 promotes differentiation into Th1 and acts as a TLR receptor agonist, boosting the immunogenicity of the contraceptive vaccine. The IL-12 sequence was obtained from the NCBI protein database with accession ID AAB37425.2 [21].

2.12. Physical and chemical characteristics

Evaluation of the chemical and physical properties of the designed vaccine is crucial. This evaluation includes factors such as half-life, molecular weight, aliphatic index, instability index, gravy, and theoretical PI. The most important feature is the stability of the vaccine structure, which should have a score below 40, which increases the chances of attaining the best outcomes, also assessing their longevity and functionality over time. Large molecules (with a minimum molecular weight of 100) are better antigens than small molecules. The isoelectric point (pI) typically varies between 4 and 12, indicating highly acidic to alkaline values. The aliphatic index provides information on protein thermal stability and should be high. The GRAVY value signifies the hydrophobic or hydrophilic characteristics. The physicochemical properties of the vaccine construct were analyzed using the ProtParam server (www.web.expasy.org/protparam/) [23].

2.13. Structural characteristics

A high concentration of alpha helices in a protein sequence can impede vaccine development by reducing its immunogenicity. To predict the two-dimensional (2D) structural modeling of the target construct, we utilized the PSIPRED V3.3 web server (http://bioinf.cs.ucl.ac.uk/psipred/), which employs a two-feed-forward neural network approach along with the query amino acid residues [24]. Additionally, we used the Prabi server (www.prabi.ibcp.fr/htm/site/web/app.php) for further analysis of the secondary structure of the vaccine sequence [25]. For structural analysis evaluations, we turned to the Robetta server (https://robetta.bakerlab.org/), which parsed the vaccine sequence into potential domains and employed either a de novo strategy or a comparative modeling technique to generate a 3D structure, utilizing suitable templates for comparison modeling [26]. To determine the tertiary structure of the potential vaccine, we accessed the Phyre server (www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) [27]. Furthermore, we evaluated the quality and structure of the 3D vaccine construct using the MolProbity server (http://molprobity.biochem.duke.edu/index.php) and the Ramachandran chart [28]. The ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) was employed to compute an overall quality score by analyzing the atomic coordinates of the model, with the ProSA-web z-score graphically representing the z-scores of experimentally obtained structures submitted to the PDB [29]. Lastly, we assessed nonbonded atom-atom interactions in the 3D structure of the vaccine using the ERRAT server (https://saves.mbi.ucla.edu/) [21].

2.14. Protein-protein interactions (PPI)

Utilizing the STRING protein interaction database at (https://string-db.org)/, we examined protein-protein interactions (PPI) to identify potential metabolic functional connections among antigenic proteins and other proteins. The STRING database is designed to offer a thorough analysis and integration of both direct (physical) and indirect (functional) protein interactions. Initially, the antigenic protein was entered into the STRING database, with *Aeromonas hydrophila* serving as the reference strain for the PPI analysis [30].

2.15. In Silico Immune Simulation

The immunological response to the developed vaccine was simulated using C-IMMSIM v10.1 (https://kraken.iac.rm.cnr.it/CIMMSIM). This tool was employed to conduct in silico immunological simulations to evaluate the immunogenic potential of the

final vaccine. The C-ImmSim simulator incorporates machine learning methods and a specific scoring matrix (PSSM). Most current vaccines recommend administering 2–3 doses within a four-week period. Therefore, in this study's immune simulation, we provided three doses of 1000 vaccine units, configured all parameters to default, and scheduled the three injections at time steps 1, 84, and 168, respectively [31].

2.16. Molecular docking

Molecular docking experiments were carried out to study how the designed vaccine (ligand) interacts with MHCI (PDB ID: 5YXU) and MHCII (PDB ID: 4MD4) receptors. To investigate these interactions Hdock server (www.hdock.phys.hust.edu.cn/) was used with default parameters. 5YXU was selected as a model for MHC I because its structure has been well-characterized, allowing for effective binding and presentation of endogenous antigens to CD8$^+$ T cells. This interaction is crucial for eliciting a cytotoxic T-cell response, which is vital for targeting infected or malignant cells. Similarly, 4MD4 was chosen for MHC II due to its established role in presenting exogenous antigens to CD4$^+$ T cells. This interaction is vital for activating helper T cells, which in turn stimulate B cells and enhance the overall immune response [32].

3. Result

3.1. Discovery of effective antigen

A. hydrophila has a single chromosome with a length of 4.7 Mb. The genome profile of this bacterium is detailed in Table 1.

By using the ORF finder and Psortb server, 229 and 71 ORFs were predicted as extracellular and outer-membrane proteins, respectively. The SignalP server discovered 97 ORFs among extracellular proteins and 58 ORFs among the outer-membrane proteins, which contains signal peptides. After being identified, the signal peptides were eliminated from the protein sequences. Out of the total 229 extracellular proteins and 71 outer-membrane proteins, 6 and 7 proteins were found to have intramembrane domains, respectively, by utilizing the TMHMM Server. Due to being inaccessible to the immune system, these proteins were deleted.

The threshold of the Vaxijen server for immunogenicity of ORFs is 0.4. Through analysis of this server, 16 ORFs among 223 extracellular proteins and 3 ORFs among 64 outer-membrane proteins was non-antigen which was deleted. Among 268 ORFs, those with high immunogenicity were selected for further analysis. Utilizing the Blast server, 6 proteins were identified to have significant differences from the host, indicating they could be suitable candidates for additional research. Assessing toxicity with the Toxinpred server identified 3 non-toxin proteins for further examination in the next phase. After allergenicity assessment with the Allertop servers, 1 protein showed potential for investigation and progression in the next research phase.

Due to the result, fimbrial protein (protein 18) was selected as a vaccine candidate, with the accession number WP_045528985.1. According to the NCBI database (www.blast.ncbi.nlm.nih.gov/Blast.cgi), this protein is expressed in several pathogenic strains, including PartN-Ahydrophila-RM8376, FDAARGOS_916, MAH-4, M894, FUJ01265, FUJ01809, MMO-14, MMO-36, MMO-41, MMO-44, and M894. These strains are among the most common and are known for their pathogenicity. Allergenicity, immunogenicity, toxicity and homology analysis of this protein were investigated with Allertop, Vaxijen, toxinPred and blast servers, respectively. The immunogenicity score of 0.8573 indicates the optimal immunogenicity of the designed vaccine (the threshold score is 0.4), also no toxic area was seen in the vaccine formula. The protein sequence and analysis are shown in Tables 2 and 3.

3.2. Population coverage calculation

We assessed the global population coverage of the 13 epitopes using the IEDB Population Coverage tool. The vaccine is expected to cover approximately 92.1 % (average hit (average number of epitope hits/HLA combinations recognized by the population): 2.13, pc90 (minimum number of epitope hits/HLA combinations recognized by 90 % of the population): 1.09) of the global population.

Table 1
Genome profile of *A.hydrophila.*

Aeromonas hydrophila FDAARGOS_916		
NCBI RefSeq		NZ_CP065651.1
GenBank		CP065651.1
Genome size(bp)		4733702
GC content (%)		61.5
CDSs	(total)	4236
	(with protein)	4203
	(without protein)	33
Genes (total)		4396
Genes (coding)		4203
Pseudo Genes (total)		33

Table 2

Selected protein for epitope prediction.

	Localization	Sequence	Toxicity	Antigenicity	Allergenicity
Protein 18	Extracellular	APRAGDDLELLGSTVNLHGRIIDQP CVIAPESLDQAVDMGGVDVRELYA NGTGAQVPFVIRLTNCKPGIFRMAK VTFTGSKDAQLSGGLAFSAGSAKGA GIRLYDVTQSPLMLGTPSRGYLLGGT TENELQFSARVEGHPDALAAKNIQPG DYTAVANFIVAYE	non toxin	0.8267	NON-ALLERGEN

Table 3

Homology of Selected protein.

3 top matched protein	Max score	Total score	Query score	E value	Pre. ident	Accession
fimbrial protein [Aeromonas hydrophila]	335	335	100 %	2e-115	100 %	WP_045528985.1
fimbrial protein [Aeromonas hydrophila]	333	333	100 %	1e-114	99.39 %	WP_282466816.1
TPA type 1 fimbrial protein [Aeromonas hydrophila]	328	328	100 %	1e-112	98.17 %	HAT6344355.1

3.3. Epitopes selection

Using the IEDB server, 2 epitopes binding to B cells, 8 epitopes binding to MHCI, and 5 epitopes binding to MHCII were found. Allergenicity, immunogenicity, and toxicity of each epitope were analyzed with Allertop, Vaxijen and toxinPred servers and represented in Table 4.

3.4. Vaccine construction

Adjuvant, linker, and his-tag components were added to complete the vaccine's sequence. The toxicity evaluation of the vaccine's sequence by the ToxinPred server did not detect any 10-mer toxin peptide sequences. The Allertop server analysis of the vaccine sequence suggested that it is non-allergenic. Also utilizing the Vaxinjen server, the antigenicity of the vaccine surrenders a favorable antigenicity score of 0.8573. The final sequence is shown in Table 5.

3.5. Physical and chemical characteristics

Physicochemical measurements provide useful data on the protein's size, stability, and hydrophilicity, offering insights into its potential, as outlined in Table 6. The results were extracted from the ProtParam server.

3.6. Structural characteristic

Secondary structure of the candidate vaccine predicted by the Prabi server (Table 7 and Fig. 2). Also, structural modeling was successfully generated using the PSIPRED V3.3 web server. The analysis provided detailed predictions of secondary structure elements, including alpha helices and beta sheets, based on the input amino acid sequence. The resulting 2D model highlighted the distribution and arrangement of these structural features, offering insights into the potential folding and stability of the construct. using the two-

Table 4

Types of the selected epitopes and toxicity, antigenicity, and allergenicity analysis.

	Epitope	Score of Vaxijen server	Result of Toxinpred server	Result of Allertop server
B cell	GVDVRELYANGTGAQV	1.794	Non-Toxin	NON-ALLERGEN
	HPDALAAKNIQPG	0.9628	Non-Toxin	NON-ALLERGEN
MHC 1	AQVPFVIRL	1.0034	Non-Toxin	NON-ALLERGEN
	AVDMGGVDVR	2.181	Non-Toxin	NON-ALLERGEN
	GGVDVRELY	1.8563	Non-Toxin	NON-ALLERGEN
	GTTENELQF	1.5995	Non-Toxin	NON-ALLERGEN
	AVANFIVAY	0.5864	Non-Toxin	NON-ALLERGEN
	EGHPDALAAK	1.3645	Non-Toxin	NON-ALLERGEN
	MGGVDVRELY	1.4995	Non-Toxin	NON-ALLERGEN
	YTAVANFIV	0.5233	Non-Toxin	NON-ALLERGEN
MHC 2	GVDVRELYANGTGAQ	1.7982	Non-Toxin	NON-ALLERGEN
	GLAFSAGSAKGAGIR	1.5889	Non-Toxin	NON-ALLERGEN
	AFSAGSAKGAGIR	1.7512	Non-Toxin	NON-ALLERGEN
	ENELQFSARVEGHPD	0.9933	Non-Toxin	NON-ALLERGEN
	GAGIRLYDVTQSPLM	0.7766	Non-Toxin	NON-ALLERGEN

Table 5

Vaccine construct.

Vaccine's sequence	Score of Vaxijen server	Result of Allertop server	Result of Toxinpred server
AQVPFVIRLAAYGTTENELQFAAYAVDMGGVDVRAAYGG VDVRELYAAYAVANFIVAYAAYEGHPDALAAKAAYMGGV DVRELYAAYYTAVANFIVGPGPGGVDVRELYANGTGAQGP GPGGLAFSAGSAKGAGIRGPGPGAFSAGSAKGAGIRGPGPGE NELQFSARVEGHPDGPGPGGAGIRLYDVTQSPLMGPGPGHPD ALAAKNIQPGGPGPGGVDVRELYANGTGAQVEAAAKMAMW ELEKDVYVVEVDWRPDAPGETVTLTCDSPEEDDIHWTSDQRR GVIGSGKTLTITVREFLDAGQYTCHRGGETLSHSHLLLHKKEN GIWSTEILKNFKNKTFLKCEAPHHHHHH	0.8573	NON-ALLERGEN	Non-Toxin

Table 6

Physicochemistry properties of the vaccine candidate.

| Physicochemical Properties	
Number of amino acid	368
Molecular weight	38439.79
Theoretical pI	5.42
Total number of atoms	5315
Extinction coefficients	Ext. coefficient 47455
in units of M − 1 cm-1, at 280 nm measured in water	Abs 0.1 % (=1 g/l) 1.235, assuming all pairs of Cys residues form cystines
	Ext. coefficient 47330
	Abs 0.1 % (=1 g/l) 1.231, assuming all Cys residues are reduced
Estimated half-life	4.4 h (mammalian reticulocytes, in vitro).
	>20 h (yeast, in vivo).
	>10 h (*Escherichia coli*, in vivo).
Instability index	23.95
	This classifies the protein as stable.
Aliphatic index	71.96
Grand average of hydropathicity	−0.273

Table 7

The characteristics of the secondary structure prediction.

Substructure	percentage
Alpha helix (Hh)	86 is 24.23 %
310 helix (Gg)	0 is 0.00 %
Pi helix (Ii)	0 is 0.00 %
Beta bridge (Bb)	0 is 0.00 %
Extended strand (Ee)	76 is 21.41 %
Beta turn (Tt)	0 is 0.00 %
Bend region (Ss)	0 is 0.00 %
Random coil (Cc)	193 is 54.37 %
Ambiguous states (?)	0 is 0.00 %
Other states	0 is 0.00 %

feed-forward neural network approach enhanced the accuracy of the predictions, reinforcing the model's reliability for further functional and structural analyses (Fig. 3) According to this server, 71(21 %) amino acids in the entire vaccine formed extended Beta strands,88 (24 %) amino acids were found in alpha helixes, and 196 (55 %) amino acids formed coils. The Phyre2 Server was used to predict the third structure (Fig. 4). Also, the Robetta server was utilized to perform structural analysis evaluations. The most optimal model, along with the error estimate in Angstroms, is visually depicted in Fig. 5. The PDB file of the predicted tertiary structure was analyzed using the MolProbity server, to evaluate the quality and structure of the three-dimensional structure of the vaccine construct. Analyzing the result, it was noted that 95.8 % of the amino acids resides within the favored region of the Ramachandran chart, signifying a correct conformational alignment. Also, 100 % of the amino acids were within the allowed region, indicating admissible deviations from ideal conformations (Fig. 6). These results offer crucial insights into the quality and dependability of the predicted tertiary structure of the vaccine construct. The final selected 3D model had the ERRAT value of 95 % (Fig. 7). The ProSA-web analysis yielded a negative Z-score of −5.11, suggesting that the 3D protein model is highly consistent (Fig. 8).

3.7. Protein-protein interactions

Using the STRING protein interaction database, we performed an extensive analysis of protein-protein interactions (PPIs) to investigate potential metabolic functional connections between fimbrial proteins and other related proteins. The STRING database
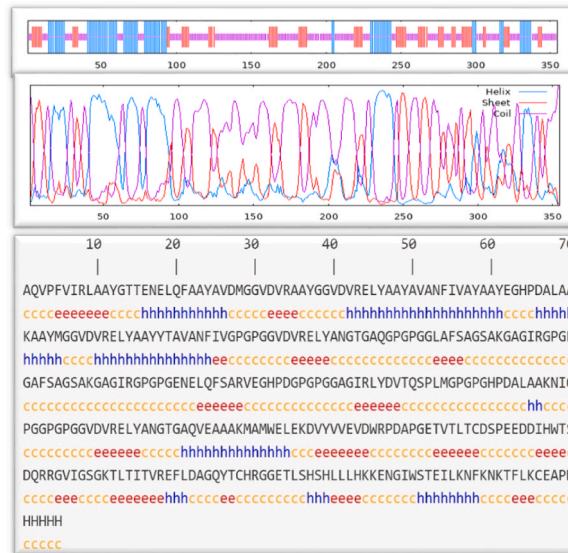
Fig. 2. The Prabi server was utilized for Secondary structure prediction of the candidate vaccine. The amount of alpha helixes is optimally observed.

provides a comprehensive network of interactions that reveal direct (physical) and indirect (functional) relationships between proteins. Our analysis highlighted a significant number of interactions, indicating a robust network of metabolic pathways involving antigenic proteins. In particular, we observed that several proteins interact with key metabolic enzymes, suggesting a potential role in metabolic regulation and bacterial pathogenicity. This extensive interaction network underscores the significance of fimbrial protein as a candidate for vaccine design. The communication network and related proteins are shown in Fig. 9 and Table 8.

3.8. In Silico Immune Simulation

The immune response generated by the C-ImmSim immune simulator in reaction to a pathogen closely mirrored the actual immune response (Fig. 10). In Fig. 10A, it is evident that antibody levels (IgM, IgG1, IgG2) were significantly higher during secondary and tertiary responses, which correlated with a decrease in antigen concentrations. Long-lasting B cell isotypes were also observed, indicating the development of memory B cells and their ability to switch, as illustrated in Fig. 10B. Likewise, memory development was confirmed in both T-helper and cytotoxic T-cell populations, playing a vital role in enhancing the immune response, as shown in Fig. 10C. Fig. 10D highlighted a marked increase in macrophage activity and interactions, along with a substantial expansion of dendritic cells.

3.9. Molecular docking

To investigate the molecular docking interaction, the HDOCK server was utilized, providing insights into the binding affinity and structural compatibility between the vaccine construct and specific receptor models. The obtained docking scores indicate the strength of interaction, with a score of −257.58 for the vaccine construct with 5YXU, which serves as a model for MHC I, and −262.89 for 4MD4, representing MHC II (Fig. 11). These scores suggest that the vaccine construct exhibits a strong affinity for both MHC I and MHC II, which is crucial for eliciting an effective immune response. The negative values of the docking scores imply that the interactions are energetically favorable, indicating a stable binding conformation.

4. Discussion

Aeromonas hydrophila consists of various strains or genotypes, with many demonstrating high pathogenicity in the host. For many years, this bacterium has been recognized as a primary cause of vertebrate and invertebrate diseases [1]. The widespread occurrence of *A.hydrophila* in water environments indicates constant and unavoidable contact with fish, which may play a role in their ability to cause infections opportunistically. *A. hydrophila* has been associated with global fish deaths in the last decade, leading to significant financial losses [33]. In aquaculture, bacterial diseases are typically managed with antibiotics. However, the indiscriminate use of antibiotics can lead to development of antibiotic-resistant patterns, rise of residual effects, disrupt the gut microbiota, and pose potential risks to human health [34,35]. Cases of *A. hydrophila* infections have been documented in humans due to the consumption of contaminated water and undercooked seafood from infected fish. These challenges are increasingly difficult to control and eliminate [5]. *A. hydrophila* is resistant to a variety of antibiotics including ampicillin, erythromycin, rifampicin, ciprofloxacin, sulfadiazine, kanamycin, and piperacillin. Therefore, innovative approaches are essential for managing bacterial infections in the aquaculture sector
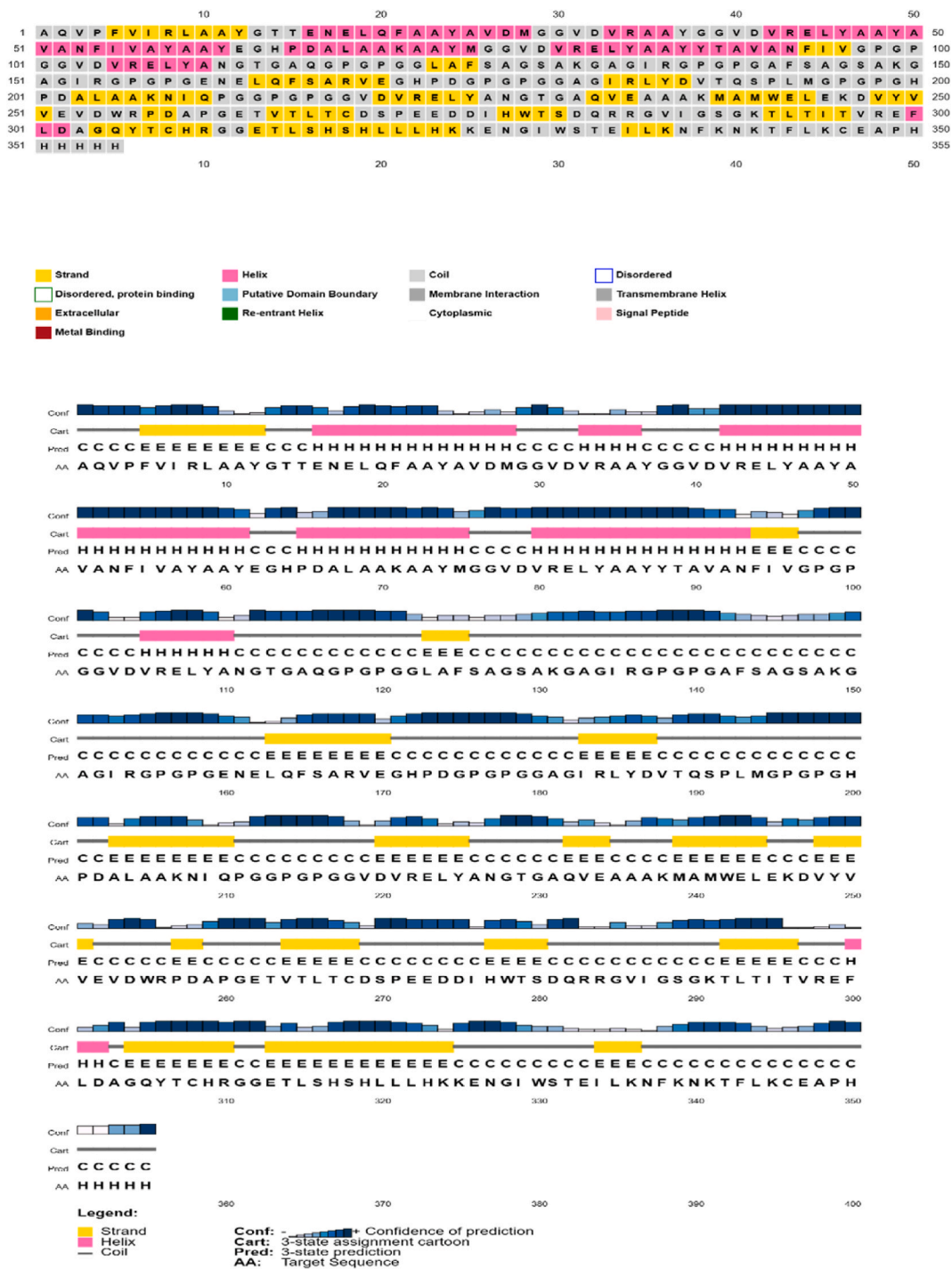
Fig. 3. Secondary structure of the candidate vaccine predicted by PSIPRED V3.3 web server.

to ensure sustainability and minimize economic losses due to the diseases like those caused by *A. hydrophila* [36]. Vaccination is a key strategy in this regard, helping to decrease the reliance on antibiotics.

The extracellular proteins of *A. hydrophila* have significant potential as vaccine candidates because they can trigger robust protective immunity in human. These proteins consist of various compounds related to virulence, digestion, and host-cell attachment, which are believed to play a vital role in the bacterium's widespread presence, high adaptability, and pathogenicity [37]. After predicting out membrane and extracellular ORFs, we have identified a potential vaccine candidate for *A. hydrophila* accessible in the GenBank database, the accession number is WP_045528985.1. This accession number belongs to the fimbrial protein. Fimbrial proteins
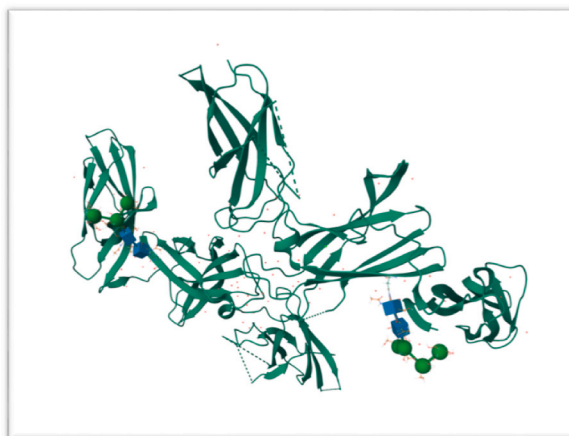
Fig. 4. Tertiary structure prediction of constructed vaccine, predicted by the Phyre2 Server.
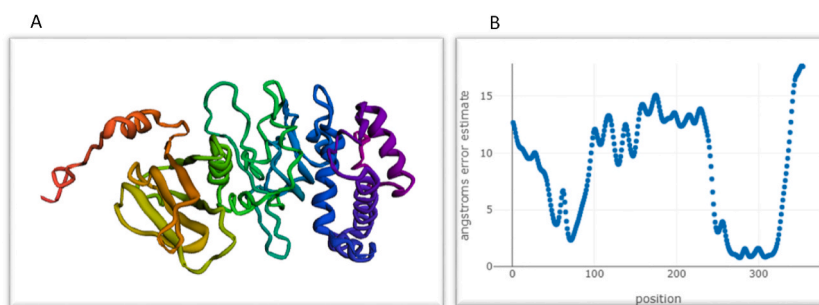
Fig. 5. The Robetta server was utilized to predict 3D view of the obtained best mode of the vaccine(A) and Angstroms error estimate(B).
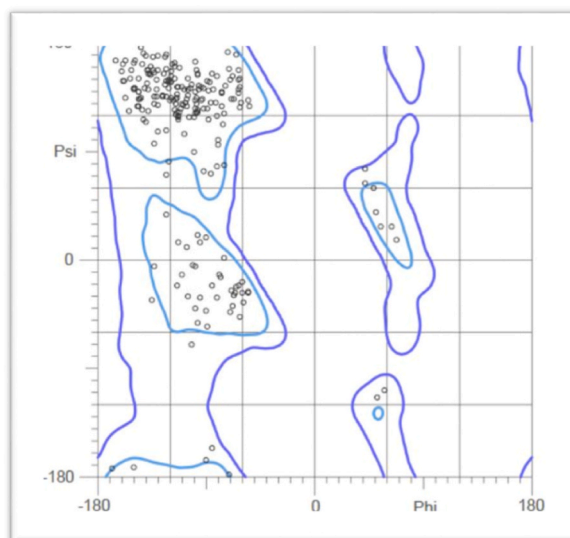
Fig. 6. Ramachandran plot analysis. It was observed that 95.8 % of the amino acids fell within the favored area, indicating proper conformational alignment. Additionally, 100 % of the amino acids were located within the allowed region, suggesting that any deviations from ideal conformations are acceptable.
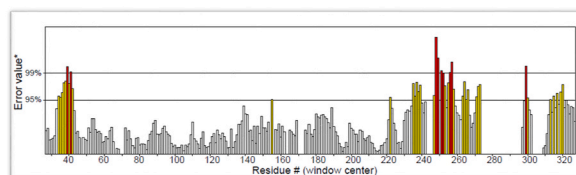
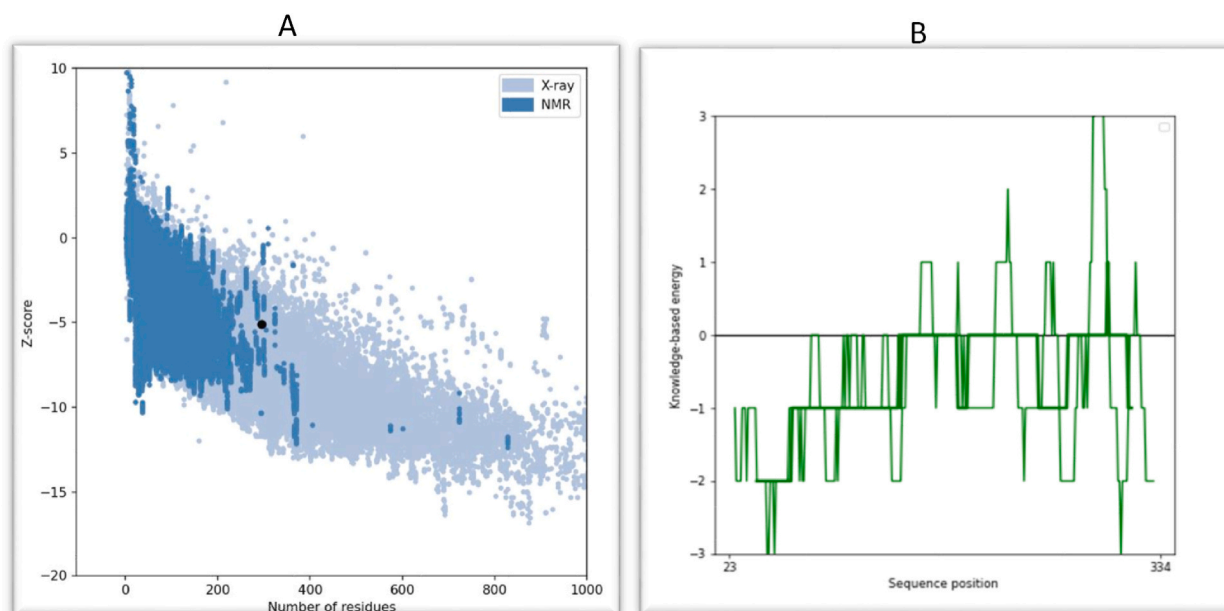**Fig. 7.** The final selected 3D model had the ERRAT value of 95 %.

Fig. 8. A: The ProSA-web analysis yielded a negative Z-score of −5.11. B: ProSA graphical plot (local model quality.

in *A.hydrophila* play a crucial role in the adhesion of the bacterium to host cells in the gastrointestinal tract, skin, and other tissues, allowing it to colonize and establish infection [38]. Moreover, these proteins are also important for bacterial biofilm formation, this matrix is composed of proteins and polysaccharides produced by bacteria. It allows them to adhere to surfaces and protects them from the host immune system and antibiotics. Also, Biofilm formation allows *A. hydrophila* to persist in the environment and cause chronic infections in humans and animals [39]. The fimbrial protein is expressed in several pathogenic strains of *A. hydrophila*. These strains are among the most common and are well-documented for their pathogenicity, and its presence across these strains underscores its potential as a vaccine target, as it may elicit a robust immune response that could provide protection against multiple pathogenic variants of *A. hydrophila*. Through comprehensive computational evaluations, our study unveiled that this specific protein lacks any toxin regions. Also, the protein exhibited a high antigenicity score, suggesting its capacity to elicit an immune response without presenting any potential harm to the host. In this research, we utilized immunoinformatic and a reverse vaccination strategy to create an epitope-based vaccine directed toward the protein WP_045528985.1. The vaccine construct also addressed population coverage for both MHCI and MHCII alleles. Even if a person has one of the alleles to the vaccine that recognizes the epitope will respond to the vaccine as there are more than a thousand human MHC alleles in the world. Therefore, the IEDB population coverage tool was utilized to estimate this coverage, revealing that the vaccine covers nearly 92.1 % of the population.

The findings of our investigation offer important information on allergenicity, toxicity, antigenicity, secondary structure, physicochemical features, Ramachandran plot analysis, and docking of the constructed vaccine. The toxicity evaluation showed that the vaccine construct does not contain any 10-mer toxin peptide sequences, so it can be administered without posing any toxic risks to the recipient. The analysis of antigenicity showed a promising antigenicity score of 0.8573. This score indicates that the vaccine construct has the potential to effectively trigger an immune response, which is crucial for its effectiveness as a preventive measure against the target. Also, the vaccine construct is assessed for allergenicity, revealing that the vaccine construct is non-allergenic. It implies that the vaccine may be well-tolerated by people with various sensitivities or allergies. This is encouraging as it lowers the risk of triggering adverse allergic responses upon administration.

The physical and chemical characteristics of the vaccine design offer valuable information about its attributes. The vaccine construct is expected to remain stable in typical physiological conditions, due to its stability index of 23.95. Additionally, the estimated half-lives in various biological systems provide insights into the potential duration of effectiveness and breakdown patterns of the
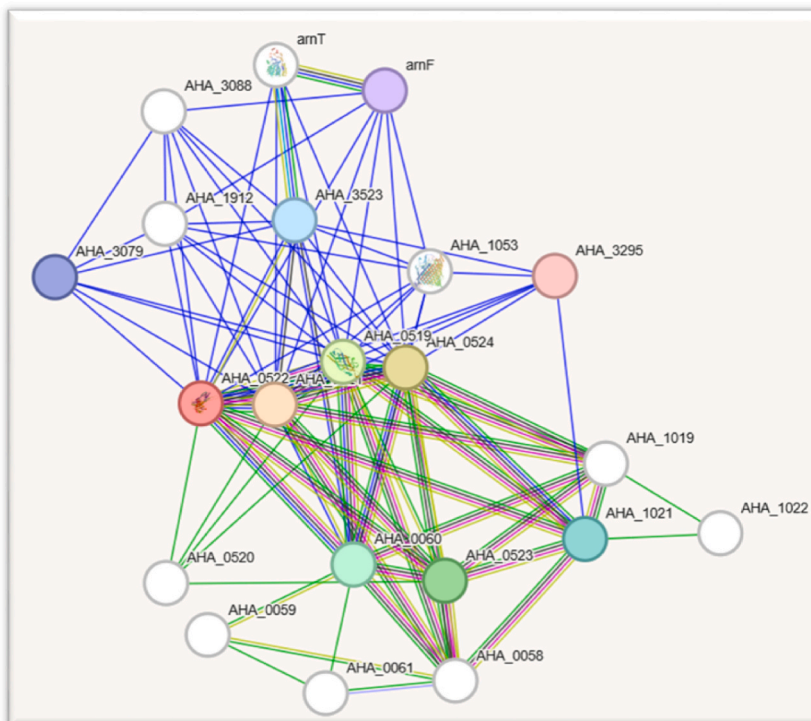
Fig. 9. Using the STRING protein interaction database, we conducted an analysis of protein-protein interactions (PPIs). Our findings revealed a substantial number of interactions, suggesting a strong network of metabolic pathways involving antigenic proteins.

Table 8
Protein-protein interactions (PPIs) by the STRING protein interaction database.

proteins	Identifier
Inner membrane protein YeeA	AHA_3079
Conserved hypothetical protein; Belongs to the ArnF family.	AHA_0986, arnF
Fimbrial protein; (GB: AAV78666.1)	AHA_0523
Fimbrial chaperone protein; match to protein family HMM PF02753.	AHA_0522
Outer membrane usher protein; match to protein family HMM PF00577.	AHA_0521
match to protein family HMM PF01569.	AHA_3523
Conserved hypothetical protein; match to protein family HMM PF06476.	AHA_3295
Conserved hypothetical protein.	AHA_0060
Conserved hypothetical protein.	AHA_1021
match to protein family HMM PF00419.	AHA_0524
match to protein family HMM PF00419.	AHA_0519

vaccine. It has a molecular weight of 38439.79 and an aliphatic index of 71.96, suggesting that it is a hydrophobic protein. The analysis of the secondary structure showed that the vaccine construct is mainly composed of alpha helices (24.23 %), which is beneficial for the overall stability and functionality of the protein. The analysis of the Ramachandran plot showed that a substantial percentage (95.8 %) of the vaccine construct's amino acids were located within the favored region, suggesting a correct conformational arrangement. In Silico immunological simulations showed that the designed vaccine has a high potential to stimulate the host's immune system and produce antibodies. According to the results, B and T memory cells create long-term immunity, and this vaccine also showed a noticeable increase in macrophage activity and interaction. The docking analysis offered information on how the vaccine design interacts with the MHCI and MHCII receptors. The top cluster models showed robust interactions, as shown by the weighted scores and the significant number of interacting amino acids. These results imply that the vaccine construct can effectively bind to the target receptors, which could help trigger an immune response.

In recent years, several studies have explored vaccine candidates against *A. hydrophila*, primarily focusing on traditional methods such as inactivated whole-cell vaccines or subunit vaccines derived from specific proteins. For instance, previous research has demonstrated the efficacy of using heat-killed or formalin-inactivated bacterial extracts, which, while effective in some cases, they have limitations related to strain variability and often require multiple doses for adequate immunity. Our vaccine candidate achieved an immunogenicity score of 0.8267 and an ability index of 23.95 indicating a favorable stability profile compared to existing vaccines,
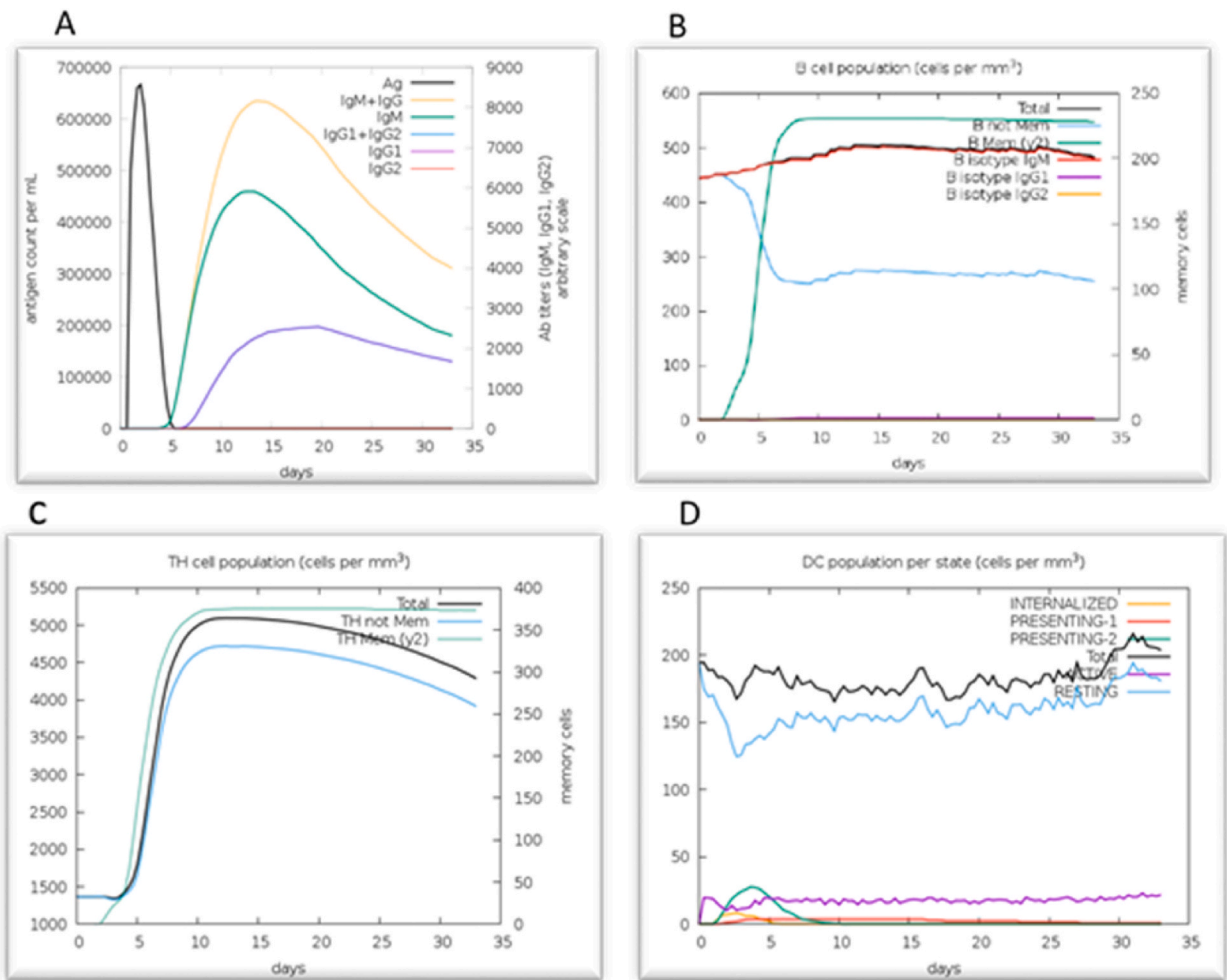
Fig. 10. In Silico Immune Simulation against the mRNA vaccine retrieved from the C-ImmSim server. Antibody levels production after antigen injection(A). The B cell population after three injections (B). The B Cell Population per state(C). Macrophage Population per state (E).
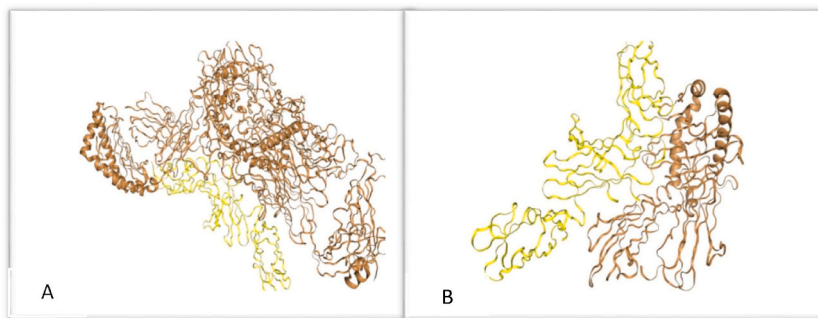
Fig. 11. Molecullar docking, A: 5YXU binding to MHCI, B: 4MD4 binding to MHCII.

many of which have reported lower stability indices. some traditional vaccines have reported immunogenicity scores below 0.7, indicating a less robust potential for eliciting an immune response. By achieving a higher stability index and immunogenicity score, our vaccine demonstrates a promising advantage in terms of ease of storage and transport, and capacity to stimulate a strong immune reaction which is essential for widespread use. while some previous studies have identified specific proteins associated with pathogenicity, they have not explored the potential of fimbrial proteins as vaccine candidates. Adhesion caused by fimbrial proteins is crucial for colonization and infection, as it allows *A. hydrophila* to establish itself in both vertebrate and invertebrate hosts. Many

traditional vaccines have struggled with strain variability, leading to reduced efficacy against diverse isolates, but Fimbrial proteins, being conserved among multiple strains, enhance the likelihood that a vaccine targeting these proteins will confer broad protection. Studies have shown that fimbrial proteins can elicit strong immune responses, often resulting in the production of both humoral and cell-mediated immunity. This dual response is crucial for effective protection against bacterial infections.

These results highlight the significance of this protein as a promising subject for further research. Also, enhance our understanding of the vaccine design's potential and suitability for development. Exploring its functional characteristics, immunogenicity, and possible uses could provide valuable information on its significance in the field and contribute to progress in areas like vaccine development, immunotherapy, or diagnostics. In our study, we employed various bioinformatics tools to predict epitope immunogenicity, protein stability, and other relevant characteristics of our vaccine candidate. While these tools provide valuable insights, it is essential to acknowledge their inherent limitations. For instance, Factors such as the dynamic nature of protein folding, post-translational modifications, and the influence of the immune system on epitope recognition can significantly impact the accuracy of these predictions. Additionally, the reliance on available databases may introduce biases, as the quality and comprehensiveness of the data can vary. Therefore, while the results obtained from these bioinformatics analyses are informative, they should be interpreted with caution, and further experimental validation is necessary to confirm the predicted outcomes and ensure the robustness of our vaccine candidate.

This designed multi-epitope vaccine can be delivered to the target organism by liposome. Vaccine delivery through liposomes represents a promising approach to increase its efficacy and stability. Liposomes are spherical vesicles composed of two phospholipid layers that can enclose hydrophilic and hydrophobic components and enable the effective delivery of vaccine antigens. This method not only protects the encapsulated epitopes from degradation but also facilitates their uptake by antigen-presenting cells, thereby improving the immune response. In addition, liposomes can be engineered to contain adjuvants, which can further enhance immunogenicity and promote a stronger and longer-lasting immune response. Their biocompatibility and ability to target specific tissues make liposomes an attractive option for vaccine delivery.

5. Conclusions

Considering the great economic losses caused by *A.hydrophila* to the aquaculture industry, and causing variable disease in human as well as its resistance to all kinds of antibiotics, it is necessary to have a commercial vaccine for this bacteria. Vaccine development plays a vital role in the prevention and control of infectious diseases. In recent years, in silico approaches have emerged as powerful tools to optimize vaccine design, maximize immunogenicity, and minimize potential adverse effects. In this study, we successfully identified a promising candidate protein with accession ID WP_045528985.1 using bioinformatics tools and servers. This vaccine is based on identifying non-toxic, immunogenic, and antigenic epitopes derived from WP_045528985.1 protein. The findings from this study present a promising strategy for designing vaccines against *A.hydrophila* diseases. The utilization of computational analyses and epitope-based approaches enable the generation of highly targeted and effective preventive measures.

CRediT authorship contribution statement

Mahdieh SobhZahedi: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mohammad Hossein YektaKooshali:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability

All relevant data are within the paper and its Supporting Information files.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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