**Malaysian Applied Biology (2024) 53(3): 137-146**
https://doi.org/10.55230/mabjournal.v53i3.2942

## *Research*

# *In Silico* Elucidation of Protein-Protein Interaction Network in Fish Pathogen *Flavobacterium columnare*

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**ABSTRACT**

*Flavobacterium columnare* is a virulent intracellular bacterial pathogen that causes an infection known as columnaris in many species of fish. Some economically important fish species are strongly affected by columnaris, leading to a high mortality rate and significant economic losses. Previous in silico studies have provided various biological insights into *F. columnare*, including its interaction with MHC class I alleles and the epitopic region within outer membrane proteins. However, the protein-protein interaction networks underlying the growth, defense, and pathogenesis of *F. columnare* remain largely unknown. This study was conducted to identify the protein-protein interaction (PPI) networks and hub proteins of *F. columnare* that can be used as drug or vaccine targets. A total of 500 protein sequences were retrieved from UniprotKB in FASTA format and analyzed using VaxiJen, PSORTb, STRING, Cytoscape, and BLASTp programs. The results demonstrated that 60% of F. columnare proteins were predicted as antigenic proteins, most of which were associated with catalytic activity and metabolic processes, identified as cytoplasmic proteins. Ten hub proteins with the highest number of functional interactions were identified, which were also antigenic and non-host homologous. In conclusion, *F. columnare* hub proteins represent potential therapeutic targets in drug and vaccine development against columnaris infection.

**Key words:** *Flavobacterium columnare*, columnaris, in silico, hub proteins, therapeutic targets

***Article History***
Accepted: 29 May 2024
First version online: 30 September 2024

***Cite This Article:***
Nematiasgarabad, P., Hashim, N.A.N. & Yahya, M.F.Z.R. 2024. *In silico* elucidation of protein-protein interaction network in fish pathogen *Flavobacterium columnare*. Malaysian Applied Biology, 53(3): 137-146. https://doi.org/10.55230/mabjournal.v53i3.2942



## INTRODUCTION

*Flavobacterium columnare* is a Gram-negative bacterium known for causing columnaris disease in freshwater fish, especially Siluriformes (Catfish), representing a significant threat to both wild populations and aquaculture settings (Heckman *et al*., 2023). The pathogenesis of *F. columnare* involves various mechanisms that contribute to its virulence and the development of columnaris disease. *F. columnare* first establishes infection by adhering to the mucosal surfaces of fish (Hassanien *et al*., 2023). The bacterium utilizes structures such as pili and outer membrane proteins to adhere to host tissues, particularly the skin and gills. This adhesion is a crucial step in the initiation of infection, allowing the bacterium to colonize and evade the host's defense mechanisms. The ability of *F. columnare* to form biofilms enhances its pathogenicity (De Silva & Heo, 2023). Biofilms that are heterogenous and surrounded by extracellular matrix protect against host immune responses and antimicrobial treatments (Yaacob *et al*., 2021; Kamaruzzaman *et al*., 2022; Johari *et al*., 2023), allowing the bacterium to persist in the aquatic environment and on the surfaces of fish tissues.

Protein-protein Interaction (PPI) networks have emerged as powerful tools for understanding the molecular mechanisms underlying fish disease pathogenesis. These networks provide insights into the complex web of

interactions between various proteins, shedding light on the intricate processes that contribute to the development and progression of fish diseases. PPI networks are constructed based on experimental data and computational predictions, showcasing the interactions between proteins within a biological system (Wang *et al*., 2022). In the context of fish diseases, these networks offer a holistic view of how different proteins collaborate or interfere with each other, influencing cellular processes critical for maintaining health. For instance, in zebrafish, the PPI network has been used to elucidate the mechanisms underlying disease pathogenesis and understand how alterations in protein interactions contribute to the manifestation of specific diseases (Zainal-Abidin *et al*., 2022).

PPI networks serve as a foundational framework for studying fish pathogens. By mapping the interactions between proteins, researchers gain insights into the complex molecular machinery that governs pathogenicity (Balint & Brito, 2023). Understanding these networks is essential for unraveling the key players involved in the infection process. Numerous studies have applied PPI network analysis to specific fish pathogens. For instance, in *Litopenaeus vannamei*, the PPI network has been constructed to understand the interactions among proteins, providing insights into the molecular basis of diseases affecting shrimp aquaculture (Rosilan *et al*., 2023). These networks provide valuable insights into pathogen biology, identification of virulence factors, and potential targets for therapeutic interventions (Waiho *et al*., 2021). Previous *in silico* studies have provided varieties of biological information about *F. columnare* including interaction with MHC class I alleles and epitopic region within the outer membrane proteins (Mahendran *et al*., 2016, Bhattacharya *et al*., 2019). However, PPI networks underlying the growth, pathogenesis, and defense of *F. columnare* remain largely unknown.

The present work was performed to identify the PPI networks and hub proteins in *F. columnare.* Understanding PPI networks is pivotal in predicting protein functions and drug efficacy, as interactions between proteins often govern cellular processes. *F. columnare*, a bacterial pathogen, poses a significant threat to aquatic organisms, necessitating targeted interventions. This research underscores the importance of leveraging molecular interactions to develop effective strategies against pathogens, potentially leading to advancements in drug or vaccine development targeting *F. columnare* infections.

## MATERIALS AND METHODS

### Preparation of protein dataset

A total of 500 non-redundant protein sequences of *F. columnare* were randomly retrieved in FASTA format from the UniProtKB/TrEMBL database This number of protein sequences is sufficient to generate useful information about protein-protein interaction networks (Isa *et al*., 2022).

### Protein antigenicity analysis

The VaxiJen 2.0 software (Doytchinova & Flower 2007) was utilized in this study to predict the antigenicity of *F. columnare* proteins. The retrieved protein sequences were used as queries in this software. The threshold value was 0.4. Any query with a threshold above 0.4 was recorded as an antigen, and any query with a threshold below 0.4 was recorded as a non-antigen (Zulkiply *et al*., 2022). Subsequently, functional classification of recognized antigenic proteins based on molecular function and biological process was conducted by the SwissProt/TrEMBL database (Bairoch & Apweiler, 2000).

### Subcellular localization analysis

The PSORTb V3.0.3 (Yu *et al*., 2010) software was used in this study for the prediction of the subcellular localization of proteomes, whether in the cytoplasm, cytoplasmic membrane, periplasm, outer membrane, or extracellular space. The retrieved protein sequences were used as queries in the mentioned software, and information about their subcellular localization was downloaded into a separate file.

### Protein-protein interaction network analysis

The STRING 12.0 database (Szklarczyk *et al*., 2003) was used to predict the protein-protein interaction network among the selected *F. columnare* proteins. The retrieved protein sequences were utilized as queries in the STRING for obtaining information such as interaction network image, biological process, molecular function, and KEGG pathway (Yahya *et al*., 2017). The confidence limit for the analysis of protein interaction networks was set to a high confidence level (0.7). Prediction of protein-protein interactions was based on neighborhood, fusion-fission events, occurrence, co-expression, text mining, and data imported from public databases of physical interactions.

### Functional enrichment analysis

Functional enrichment analysis was performed using the STRING 12.0 database. Significant

biological processes and molecular functions were identified when $p<0.05$.

### Identification of the hub protein

The protein information obtained from the STRING 12.0 database was further analyzed using Cytoscape version 3.7.0. The software was used to analyze i) different levels of centrality and degree over the interactions, and ii) the cascade of interactions with the highest total degree score, to identify the proteins with the highest functional interaction which are also known as hub proteins (Wu *et al*., 2022).

### Identification of non-host homologous proteins

BLASTp was utilized for the detection of non-host homologous proteins. The parameters of the search against catfish (Siluriformes) proteome were set to *E*>1e-06 and sequence similarity < 30% (Liu *et al*., 2016).

## RESULTS

### Antigenicity and functional categories

A total of 298 proteins (60%) of *F. columnare* were predicted to be antigenic proteins while 202 proteins (40%) were predicted to be non-antigenic (Figure 1A). Most of the *F. columnare* proteins were associated with catalytic activity (57%) (Figure 1B), metabolic process (41%) (Figure 1C), and cytoplasm (73%) (Figure 1D).

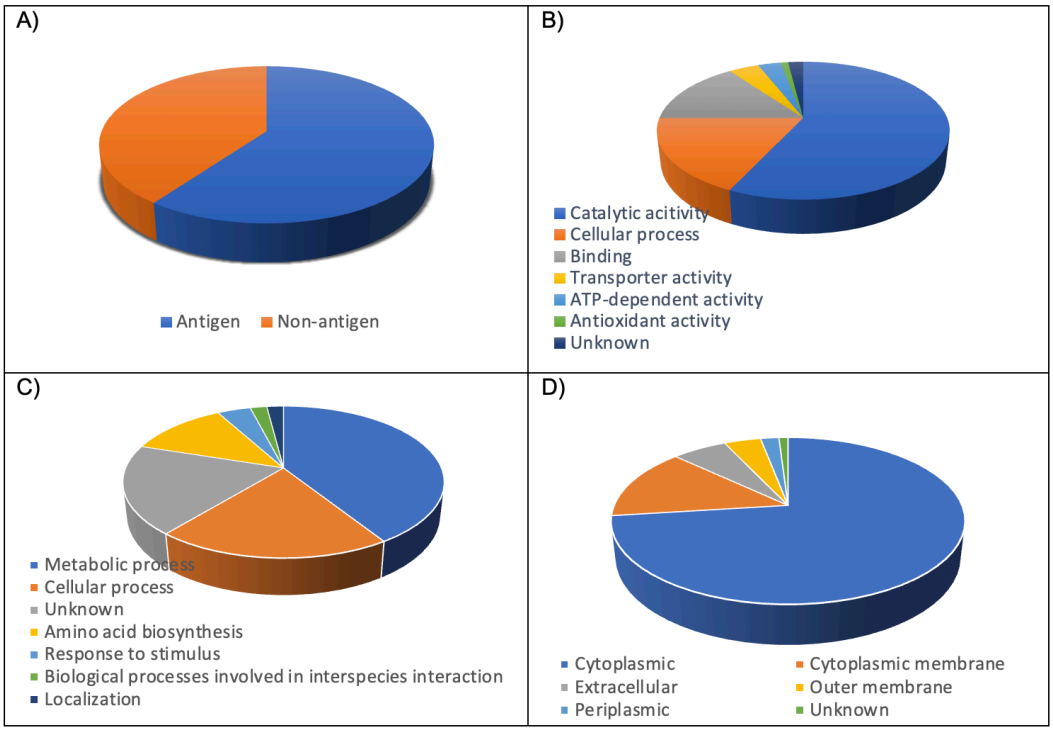


**Fig. 1.** Distribution of retrieved *F. columnare* proteins. A) Antigenic and non-antigenic proteins; B) Functional classification based on molecular function; C) Functional classification based on biological process. D) Functional classification based on subcellular localization.

### Protein-protein interaction network

Figure 2 denotes the STRING analysis to visualize the PPI networks in *F. columnare*. The network nodes and edges represented the *F. columnare* proteins and the predicted functional associations, respectively. A total of 6543 functional interactions were produced among the retrieved *F. columnare* proteins.

### Functional enrichment

Table 1 represents functional enrichment data. All biological processes, molecular functions, cellular components, and pathways from the network enrichment analysis were significant ($p<0.05$). In terms of biological processes, proteins involved in stress response, cellular metabolism, DNA repair, and biosynthesis of amino acids exhibited particularly low *p*, indicating their critical involvement in *F. columnare*. Molecular functions such as GTPase activity, catalytic activity, and binding were significantly

enriched, suggesting their crucial roles in cellular processes. Furthermore, the KEGG pathway analysis highlighted several pathways with notable enrichment, including biosynthesis of amino acids, nucleotide excision repair, and metabolic pathways, indicating their involvement in the underlying biological processes. These findings provide valuable insights into the molecular mechanisms underlying the studied condition and suggest potential targets for further experimental validation and therapeutic interventions.

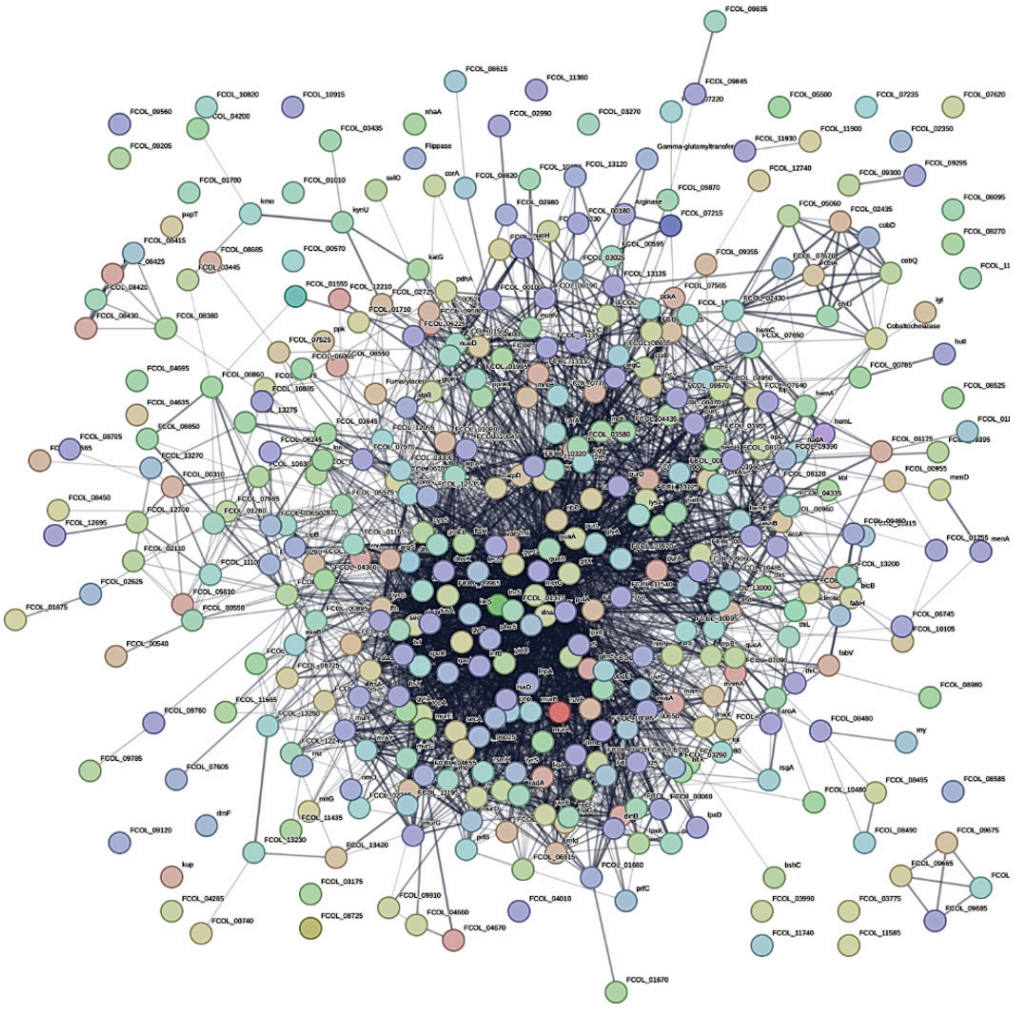

**Fig. 2.** PPI network visualization and their predicted interaction by STRING 12.0 database.

## Hub proteins

Figure 3 represents the PPI network degree of the top 10 expressed proteins in *F. columnare*. Network degree represents the total number of functional linkages of a protein, indicating the essentiality and centrality of the proteins within the PPI network. The identified hub proteins include bifunctional malic enzyme, 3,4-dihydroxy-2-butanone 4-phosphate synthase, serine hydroxymethyltransferase, chaperone protein DnaK, phosphoribosylformylglycinamidine synthase, phenylalanine--tRNA ligase beta subunit, inosine-5'-monophosphate dehydrogenase, metG (Methionine--tRNA ligase), guaA (GMP synthase [glutamine-hydrolyzing]), and polA (DNA polymerase I).

## Non-host homologous hub-proteins

BLASTp was used to identify sequence similarity between selected hub proteins and the host (Siluriformes). Table 2 shows the BLASTp results for the hub proteins. It was found that all the hub proteins showed no sequence similarity with Siluriformes, making them non-host homologous.

**Table 1.** Functional enriched biological process, molecular function, and KEGG pathway

| Biological process | *p* |
|---|---|
| Response to stress | 0.00013 |
| Cellular metabolic process | 0.0004 |
| DNA repair | 0.0035 |
| Metabolic process | 0.0078 |
| Cellular amino acid biosynthetic process | 0.0183 |
| Vitamin metabolic process | 0.023 |
| Regulation of cell shape | 0.0388 |
| **Molecular function** | ***p*** |
| GTPase activity | 0.0011 |
| Catalytic activity | 0.0019 |
| Binding | 0.0032 |
| Pyrophosphatase activity | 0.0054 |
| Anion binding | 0.0054 |
| Hydrolase activity | 0.0088 |
| Lyase activity | 0.0088 |
| Transferase activity | 0.0112 |
| Ribonucleotide binding | 0.015 |
| **KEGG pathways** | ***p*** |
| Biosynthesis of amino acids | 0.00041 |
| Nucleotide excision repair | 0.004 |
| Metabolic pathways | 0.0091 |
| Pyruvate metabolism | 0.0122 |
| RNA degradation | 0.0147 |
| Methane metabolism | 0.015 |
| Mismatch repair | 0.0436 |
| Vancomycin resistance | 0.0436 |

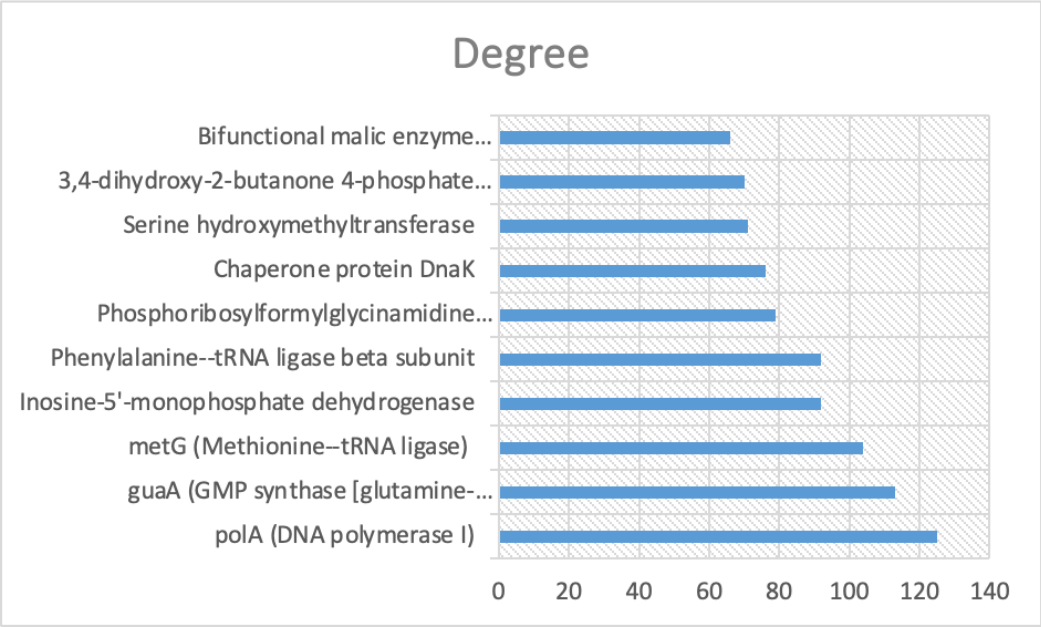


**Fig. 3.** PPI network degree of the top 10 *F. columnare* proteins (hub proteins).

## DISCUSSION

Over the past years, proteome analysis of pathogenic microorganisms has garnered significant interest. This technique has been employed to identify potential vaccine or drug targets that are absent in the host itself. It has proven successful in developing efficient therapeutic strategies that do not have any negative impact on the host. The present study identified some potential proteins from the entire proteome of *F. columnare* that can be used as vaccines or drug targets in the future against columnaris infection. The *in silico* approach taken in the present study has also been utilized in other computational works (Othman & Yahya, 2019; Zulkiply *et al*., 2022).

**Table 2.** The top 10 hub proteins in *F. columnare*

| Accession | Hub proteins | Biological process | Degree | Sequence similarity | Remarks |
|---|---|---|---|---|---|
| G8X579_FLACA | DNA polymerase I | DNA replication | 125 | | |
| G8X4L0_FLACA | GMP synthase [glutamine-hydrolyzing] | GMP biosynthesis | 113 | | |
| G8X8N3_FLACA | MetG (Methionine—tRNA ligase) | Protein biosynthesis | 104 | | |
| G8XAV8_FLACA | Inosine-5'-monophosphate dehydrogenase | GMP biosynthesis | 92 | | |
| G8XAA6_FLACA | Phenylalanine—tRNA ligase beta subunit | Protein biosynthesis | 92 | *E*>1e-06 | Non-host homologous |
| G8X4T3_FLACA | Phosphoribosylformylglycinamidine synthase | Purine biosynthesis | 79 | Similarity < 30% | |
| G8X623_FLACA | Chaperone protein DnaK | DNA replication | 76 | | |
| G8XAA9_FLACA | Serine hydroxymethyltransferase | Amino-acid biosynthesis | 71 | | |
| G8X4F1_FLACA | 3,4-dihydroxy-2-butanone 4-phosphate synthase | Riboflavin biosynthesis | 70 | | |
| G8X5M7_FLACA | Bifunctional malic enzyme oxidoreductase/ phosphotransacetylase | Malate metabolic process | 66 | | |

In the present study, the majority of *F. columnare* proteins (60%) were predicted to be antigenic proteins (Figure 1). This indicates that they are molecules recognized by the host immune system to induce an immune response (Doytchinova *et al*., 2007). *F. columnare* proteins conferring immunity can serve as excellent vaccine targets and are preferable for vaccine candidates in future studies. In this analysis, *F. columnare* proteins underwent classification based on their physicochemical attributes, utilizing an alignment-independent method (Salod & Mahomed, 2022). This approach transforms protein sequences into uniform, equal-length vectors through auto-cross covariance. The employed reverse vaccinology strategy in this study excels over traditional vaccinology due to its rapid and efficient nature. It also ensures safety, as there is no need to cultivate the pathogen in a laboratory. Additionally, it facilitates the identification of all potential vaccine candidates, including those not expressed *in vitro*.

Prediction of subcellular localization is essential for studying bacteria, and diseases, as well as protein function and genome annotation. Research on protein subcellular localization is valuable for identifying clues between bacteria and host cells, which can aid in the design of targeted drugs or vaccines. The present study demonstrated that the majority of *F. columnare* proteins (70%) are associated with the cytoplasm (Figure 1). These cytoplasmic proteins can be considered potential drug targets (Hafsa *et al*., 2022) because are readily accessible to small-molecule drugs, facilitating drug binding and interaction. Meanwhile, cytoplasmic proteins can also induce good antibody responses and are potential vaccine targets (Guo *et al*., 2004).

The STRING 12.0 database collects and integrates PPI information from diverse sources, including scientific literature, databases of experiments, annotated complexes/pathways, and computational predictions. This comprehensive approach ensures a global network of direct and indirect interactions. In the present study, enriched molecular functions, biological processes, and pathways were successfully identified based on the constructed PPI networks (Figure 2, Table 1). The obtained information allows us to understand the function of a protein and the molecular mechanisms governing the pathogenesis and survival of *F. columnare* (Yahya *et al*., 2017; Zulkiply *et al*., 2022). The vancomycin resistance pathway was identified in the PPI networks herein. This finding corroborates Sarker *et al*. (2019) who reported the resistance of Flavobacterium sp. to several antibiotics including vancomycin. The emergence of vancomycin resistance in infectious agents has been linked to an increased risk of microbial pathogenesis and the development of diseases in fish (Osman *et al*., 2016). Additionally, the present study unveiled the presence of the pyruvate metabolism pathway in the PPI networks of *F. columnare,* shedding light on its role in sustaining a balance between energy production and defense against the host immune response (Echlin *et al*., (2020). The mismatch repair pathway was also identified in the PPI networks of *F. columnare*. In 2014, Martina *et al*., (2014) revealed that the mismatch repair contributed to hypermutation in *Burkholderia cepacia* which is prevalent in cystic fibrosis chronic respiratory infection. To our knowledge, the present study represents the pioneering effort to report the PPI networks in *F. columnare*.

In the present study, 10 cytoplasmic proteins of *F. columnare* were predicted as hub proteins, as they showed a degree level of more than 60 and therefore have the greatest number of interactions with other proteins (Figure 3). Major molecular mechanisms are often controlled by many protein components that are modulated by PPI networks, which include both direct and indirect contacts that

take place between two or more proteins and cause the creation of certain biochemical events. Several independent studies have demonstrated that hub proteins are more likely to be essential to cell function than non-hub proteins (Prava & Pan, 2022). Thus, essentiality has a direct relation with hub proteins. This claim has also been supported by Kim *et al*. (2020), who, based on the PPI networks produced using five public datasets, namely Uetz, Ito, DIP, SGD, and BioGRID, suggest that hub proteins are most likely to be essential compared to non-hub proteins.

After identifying the hub proteins, to avoid cross-reactivity or side effects of future drugs designed with the mentioned hub proteins as their targets, Blastp against Siluriformes (Catfish) was performed to exclude any hub protein that is homologous with the host. All 10 selected hub proteins showed no significant sequence similarity to Siluriformes proteins and were considered non-homologous, making them excellent target points for drug design (Table 2). A similar approach was undertaken in another study by Zulkiply *et al*. (2022) to identify the best antigenic hub proteins in *Staphylococcus aureus*, where Blastp was employed to identify sequence similarity of bacterial proteins against the *Homo sapiens* proteome. In another research on the identification of potential vaccine targets using a reverse vaccinology approach, Blastp was used to identify non-host homologous essential proteins of *Corynebacterium pseudotuberculosis* against their hosts, namely *Homo sapiens* and *Ovis aries* (Abd Rashid *et al*., 2022).

The selected hub proteins identified herein have previously been proposed as ideal drug targets. DNA polymerase I is an enzyme that synthesizes DNA. This protein plays an essential role in genome duplication and is critical for protecting the cell against the effects of DNA damage. Therefore, it has been suggested in previous studies that this protein can act as an effective drug target (Lange *et al*., 2021). During oxidative stress, methionine-tRNA ligase (MetG) gets phosphorylated, resulting in the promiscuity of this enzyme, where it aminoacylated methionine to various non-Met tRNAs. This substitution of amino acids in proteins with methionine helps relieve oxidative stress in the cell. Torrie *et al*. (2020) reported that MetG has been chemically validated as a drug target in the kinetoplastid parasite. Inosine-5'-monophosphate dehydrogenase is a metabolic enzyme that catalyzes an important step in guanine nucleotide biosynthesis and is therefore at the center of cell growth and proliferation. This enzyme has been exploited as a potential target for the development of immunosuppressive, anticancer, and antiviral agents, as well as a promising antiprotozoan drug target (Fotie, 2018). The bacterial chaperone protein DnaK is an enzyme that pairs cycles of ATP binding, hydrolysis, and ADP release with an N-terminal ATP-hydrolysing domain to cycles of sequestration and release of unfolded proteins with a C-terminal substrate binding domain (Hosfelt *et al*., 2022). Gestwicki (2022) reported that the molecular chaperone DnaK is a potential drug target for treating bacterial infections. Moreover, in the pathogen *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), DnaK, and its cofactors are suggested as potential antimycobacterial targets (Hosfelt *et al*., 2022).

Bioinformatics studies of PPI networks in *F. columnare* pathogens face several limitations that hinder comprehensive understanding and necessitate further research directions. Firstly, the availability and quality of experimental data for constructing PPI networks pose a challenge. Experimental methods to study PPIs, such as yeast two-hybrid assays or affinity purification coupled with mass spectrometry, have limitations in terms of coverage, specificity, and false positive rates (Mendez-Rios & Uetz 2010). Thus, bioinformatics approaches heavily rely on integrating heterogeneous data sources, which may introduce biases or inaccuracies (Bulgakov & Tsitsiashvili 2013). Another limitation pertains to the identification and characterization of hub proteins, which are critical nodes in PPI networks. While network topology analysis identifies highly connected proteins, experimental validation of their biological relevance and functional significance is often lacking. Future research directions should address these limitations by integrating multi-omics data to improve network reconstruction accuracy and prioritize experimentally validated interactions (Wani & Kaza 2019). To validate hub proteins, targeted biochemical assays like co-immunoprecipitation coupled with mass spectrometry or bacterial two-hybrid systems can confirm protein interactions and assess their functional consequences (Mendez-Rios & Uetz 2010).

## CONCLUSION

In conclusion, information about the PPI network and hub proteins is useful for identifying potential drug or vaccine targets. A total of 10 *F. columnare* hub proteins were selected as potential drug or vaccine targets because they fulfilled common criteria, including antigenicity, essentiality, and non-host homology. The findings in this study can potentially serve as a basis for future studies on the pathogenesis of *F. columnare* and the development of novel drugs and vaccines against it. Vaccines developed using this method are specific to *F. columnare*; therefore, one of the greatest advantages is that the occurrence of resistance and toxicity for the host can be minimized. Experimental validation for

the selected hub proteins deserves further attention.

**ACKNOWLEDGEMENTS**
This research was funded by the Pembiayaan Yuran Penerbitan Artikel (PYPA), Universiti Teknologi MARA.

**ETHICAL STATEMENT**
Not applicable

**CONFLICT OF INTEREST**
The authors declare no conflict of interest.

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