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Research article

Subtractive genomics study for the identification of therapeutic targets against *Cronobacter sakazakii*: A threat to infants

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ABSTRACT

Cronobacter sakazakii is an opportunistic pathogen that has been associated with severe infection in neonates such as necrotizing enterocolitis (NEC), neonatal meningitis, and bacteremia. This pathogen can survive in a relatively dry environment, especially in powdered infant formula (PIF). Unfortunately, conventional drugs that were once effective against *C. sakazakii* are gradually losing their efficacy due to rising antibiotic resistance. In this study, a subtractive genomic approach was followed in order to identify potential therapeutic targets in the pathogen. The whole proteome of the pathogen was filtered through a step-by-step process, which involved removing paralogous proteins, human homologs, sequences that are less essential for survival, proteins with shared metabolic pathways, and proteins that are located in cells other than the cytoplasmic membrane. As a result, nine novel drug targets were identified. Further, the analysis also unveiled that the FDA-approved drug Terbinafine can be repurposed against the Glutathione/L-cysteine transport system ATP-binding/permease protein CydC of *C. sakazakii*. Moreover, molecular docking and dynamics studies of Terbinafine and CydC suggested that this drug can be used to treat *C. sakazakii* infection in neonates. However, for clinical purposes further *in vitro* and *in vivo* studies are necessary.

1. Introduction

C. sakazakii is a gram-negative non-spore-forming facultative anaerobic pathogenic bacterium belonging to the family Enterobacteriaceae [1] and is typically found on plants and other organic materials in the environment [2]. It can survive in an extremely dry environment and dehydrated products such as powdered milk, protein shakes, and infant formula [3,4]. *C. sakazakii* is acid tolerant so it exhibits significant resistance to low pH levels. It can also form biofilms which help them become resistant to antibiotics [5]. Although gastric juice in adults typically has a pH between 2 and 3, premature newborns may not have fully developed stomach acid,

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which increases the risk of complications [6].

Epidemiological studies have shown that this type of infection has been associated with contaminated powdered infant formula (PIF), however, infants who breastfed hardly get Cronobacter infections [7]. The presence of the bacteria in powdered baby food causes infection in newborns due to their underdeveloped immune systems [8]. Besides, Cronobacter-mediated neonatal systemic infection and meningitis can result in mortality rates as high as 42 % [9]. Powdered infant formula (PIF) is an alternative to breast milk which is a worldwide essential source of nutrition for infants in the early years of life [10,11]. Conventionally, powdered infant formula (PIF) is made by combining particular amounts of proteins, fats, carbohydrates, vitamins, and minerals. So, there is a significant chance that PIF will contain harmful microorganisms that could infect babies and result in life-threatening conditions [12]. However, dehumidified formulas are a breeding ground for the growth of *Cronobacter* species, especially for *C. sakazakii*. The presence of these pathogens in infant formula is a significant public health concern due to its virulence factors, which can cause fatal infections like enterocolitis and meningitis [6,13]. These microorganisms can affect the central nervous system in humans, and individuals who manage to survive the infection often suffer from severe neurological damage like hydrocephalus, quadriplegia, and developmental disabilities [14–16]. Although the exact source and transmission of *C. sakazakii* infections in newborns are not yet fully understood, some researchers suggested that powdered infant food is the primary cause of neonatal meningitis [17].

Most of the disease-causing pathogens have developed resistance to multiple drugs [18]. Nonetheless, antibiotic resistance in bacteria that cause foodborne diseases is a serious problem, leading to high rates of death and morbidity as well as large socioeconomic consequences [19]. The use of antibiotic therapy is the recommended approach for treating *C. sakazakii* infections in newborns [20]. Even as *C. sakazakii* is usually susceptible to widely used antibiotics, reports of resistance to older antibiotics such as streptomycin,

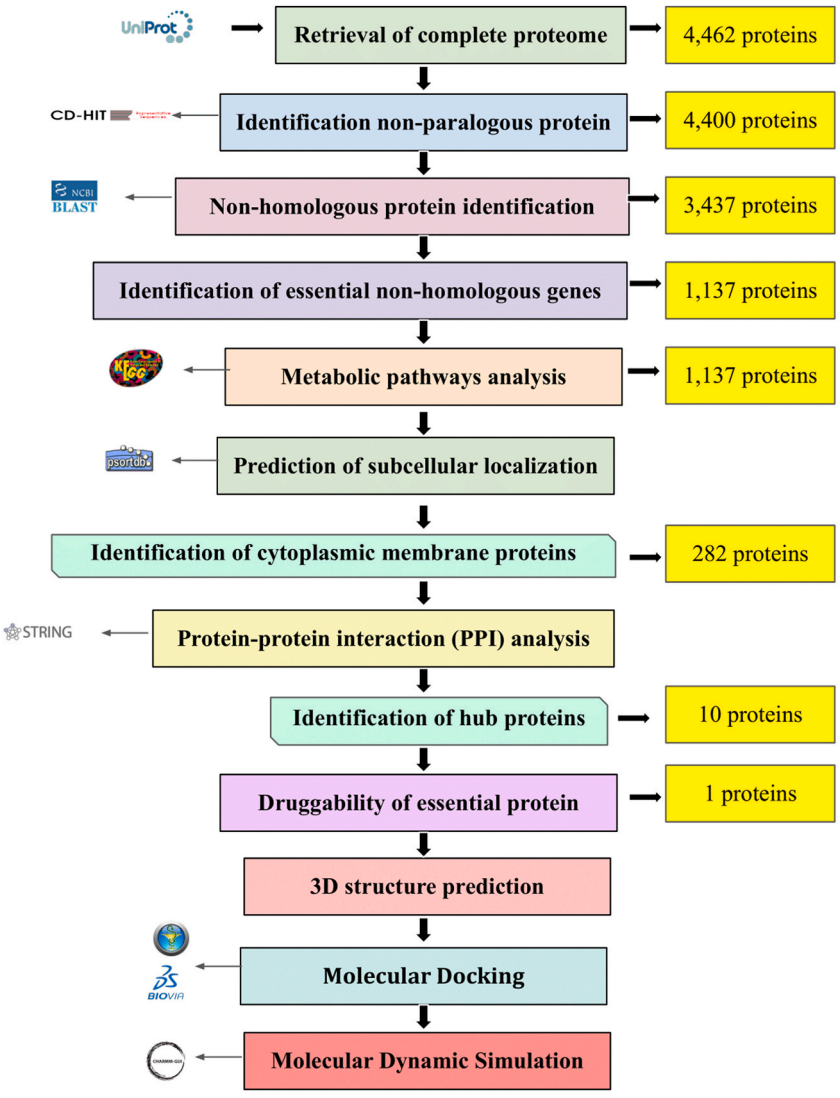

Fig. 1. Workflow of the study. The study has been carried out in 8 sequential steps.

tetracycline, cephalothin, and gentamicin have emerged [21]. However, the infection caused by this pathogen, particularly those resulting in septicemia and meningitis, require effective antibiotic treatments [22].

The conventional approach to developing drugs requires extensive time and financial investment in research and development [23]. Nowadays, the process of developing new drugs is becoming dependent on the integration of computational biology and bioinformatics in various fields [24]. Bioinformatics-based analysis often utilizes diverse approaches to identify potential drug or vaccine candidates, design structure-based drug molecules, assess the effectiveness of repurposed drugs, analyze host-pathogen interactions, evaluate the drug potential of newly proposed therapeutic agents, facilitate genome-based comparative studies to identify host-specific drug targets and more. These approaches have minimized the need for traditional laboratory-based experimental studies [25,26]. The entire host and proteome of the bacteria are currently the focus of the subtractive genomics approach and the objective is to identify proteins with unique metabolic pathways in the pathogenic genome that have specific therapeutic effects by excluding any host proteins that are homologs to human [27].

In this study, the entire proteome of *C. sakazakii* was analyzed through a subtractive genomics approach to discover a potential therapeutic target to counteract the harmful effects of the pathogenic organism on infants. The goal here was to identify several compounds with favorable properties that could potentially be optimized through further experimental studies. It is also important to keep in consideration that the approach to developing new drugs is challenging and intricate. Even promising outcomes from *in silico* research such as this one should be supported through experimental validation by subsequent studies.

2. Methods

To identify novel drug targets against the pathogenic bacterium *C. sakazakii*, a subtractive genomic approach was utilized. The process is outlined in Fig. 1, which provides a brief overview of the workflow.

2.1. Retrieval of complete proteome

The whole proteome of the target organism *C. sakazakii* 701 (proteome ID: UP000009355) was retrieved from the UniprotKB (https://www.uniprot.org/help/uniprotkb) server in FASTA (Canonical) format [28].

2.2. Non-paralogous protein identification

The entire proteome was run through the CD-HIT (http://weizhong-cluster.ucsd.edu/cdhit-web-server) suite server [29]. This server removed the paralogous sequences of *C. sakazakii* 701 (proteome ID: UP000009355). Prior to the execution of the search, the cutoff value was fixed to 0.6 which indicates sequences with 60 % identity marking redundant sequences. The paralogous proteins were then removed and non-paralogous ones were selected for further analysis.

2.3. Non-homologous protein identification

To avoid unwanted interactions between potential drugs and human proteins, non-paralogous proteins were aligned (https://blast.ncbi.nlm.nih.gov/) against the *Homo sapiens* proteome using the BLASTp algorithm. Non-homologous proteins were removed from the analysis based on their e-value <10–4, query coverage ≤70 %, and identity ≤30 %, as they were considered homologous to humans [30].

2.4. Essential non-homologous gene identification

Essential proteins of *C. sakazakii* were identified using the Database of Essential Genes (DEG) (http://origin.tubic.org/). Proteins with expectation value $\leq 10^{-100}$, identity ≥25 %, and bit score ≥100 were regarded as essential proteins [31].

2.5. Analysis of metabolic pathways

Each of the essential proteins was run through KAAS (KEGG Automatic Annotation Server) (https://www.genome.jp/kaas-bin/kaas_main) for metabolic pathway analysis. KAAS uses BLAST searches against the KEGG GENES database to annotate the functional properties of genes. For each protein, KO (KEGG Orthology) designations and automatically generated KEGG pathways were unveiled. Proteins with KOs that corresponded to both the pathogen and the host were discarded [32].

2.6. Prediction of subcellular location

Identification of proteins as potential drug or vaccine targets requires an understanding of protein subcellular localization. Membrane proteins may be thought of as both prospective vaccination candidates and therapeutic targets while cytoplasmic proteins can be thought of as drug targets only. PSORTb web server (https://www.psort.org/psortb/) was employed to identify possible locations of the shortlisted proteins. Proteins that localized to the cytoplasmic membrane were then considered as potential drug targets [33].

2.7. Protein-protein interaction analysis

The STRING Database (https://www.string-db.org/) was used for the construction of the protein-protein interactions (PPI) network [34]. The PPI network was then visualized using Cytoscape software (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC403769/). Cytoscape is enabled to implement different plugins/apps to execute different analyses on the STRING-generated PPI. The interactions between the proteins include both direct (physical) and indirect (functional) associations. From the PPI network, the top 10 proteins with the highest number of interactions (degree parameter) were selected as the 'hub' proteins. The interactions were calculated using the CytoNCA plugin in Cytoscape [NEW CITATION: https://pubmed.ncbi.nlm.nih.gov/25451770/]. A threshold value of 50 was used in terms of degree centrality for selecting the hub proteins [35]. Additionally, we have calculated the betweenness centrality and closeness centrality of the hub proteins.

2.8. Druggability of essential protein

The hub proteins were queried in the DrugBank database (https://go.drugbank.com/) as drug targets using BLASTp. Proteins were considered potential druggable targets if their sequences had a high degree of similarity (E value- $<10^{-100}$, bit score>100) to those in the DrugBank database. Those that had no hits were regarded as novel targets [36].

2.9. Homology modeling, structure prediction, and validation

The 3D structures of the selected proteins were modeled using the HHpred interactive server (https://toolkit.tuebingen.mpg.de/tools/hhpred) [37]. Using the PROCHECK in the SAVES 6.0 web server (https://saves.mbi.ucla.edu/), the quality of the protein structures was evaluated. A Ramachandran plot was generated for each of the proteins [38].

2.10. Molecular docking

The chemical structure of Terbinafine was obtained from DrugBank. It was docked against the target protein (Glutathione/L-cysteine transport system ATP-binding/permease protein CydC) using AutoDock vina within the PyRx software (https://pyrx.sourceforge.io/) [39]. Following successful molecular docking, the protein-ligand interactions were visualized using BIOVIA Discovery Studio Visualizer.

2.11. Molecular dynamic simulation

The ligand-free apo-CydC ABC transmembrane type-1 domain and the ligand bound CydC CydC ABC transmembrane type-1 domain were embedded in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayer using Chemistry at Harvard Macromolecular Mechanics (CHARMM)-Graphical user interface (GUI) (https://www.charmm-gui.org/) [40]. The bilayer system was energetically minimized using CHARMM36 m force field [41]. Waterbox with 11.0 nm length were created on the bilayer surfaces with the TIP3 water model. K^+ and Cl^- ions were used to neutralize the systems. The simulation was conducted using the GROningen MAchine for Chemical Simulations (GROMACS) (version 2020.6) for 100 ns.

The results of the simulations were analyzed via the Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), Radius of gyration (Rg), and Solvent Accessible Surface Area (SASA). The plots for each of these studies were produced using the ggplot2 package (https://ggplot2.tidyverse.org/) in R Studio (https://posit.co/).

3. Results

In this study, new potential therapeutics were explored for *C. sakazakii* by analyzing its entire set of proteins using a subtractive genomic method. To carry out this investigation, several bioinformatics tools were being used. Table 1 provides a summary of the outcomes of the study.

Table 1
Stepwise selection of *C. sakazakii* proteins through subtractive genome analysis.

Serial No	Steps and Description	*Number of proteins*
1.	*C. sakazakii* proteome	4462
2.	Non-paralogous sequences in the *C. sakazakii* proteome	4400
3.	No. of proteins non-homologous to H*omo sapiens* using BLASTp (E value $\leq 10^4$ were excluded)	3437
4.	Essential proteins in DEG ($E \leq 10^{-100}$, bit score >100)	1137
5.	Essential proteins involved in metabolic pathways unique to *C. sakazakii* (KAAS at KEGG)	1137
6.	Cytoplasmic membrane proteins identified by PSORTb web servers	282
7.	Protein-protein interaction (PPI) analysis using STRING Database	10
8.	Drug target analysis using Drugbank Database	1

3.1. Non-paralogous protein identification

The proteome of *C. sakazakii* (UniProtKB proteome ID: UP000009355) was composed of 4462 proteins which were subjected to the CD-HIT server. Proteins with more than 60 % sequence identity were removed from the dataset [58,59]. After the exclusion of paralogous and duplicate sequences, 4400 proteins were left.

3.2. Non-homologous protein identification

4400 non-paralogous proteins were screened through BlastP analysis against the *Homo sapiens* proteome and 963 proteins were discovered to be homologous to humans. To prevent probable complications regarding cytotoxicity and drug cross-reactivity, these homologous proteins were omitted from consideration as potential drug candidates for treatment. The remaining 3437 proteins were

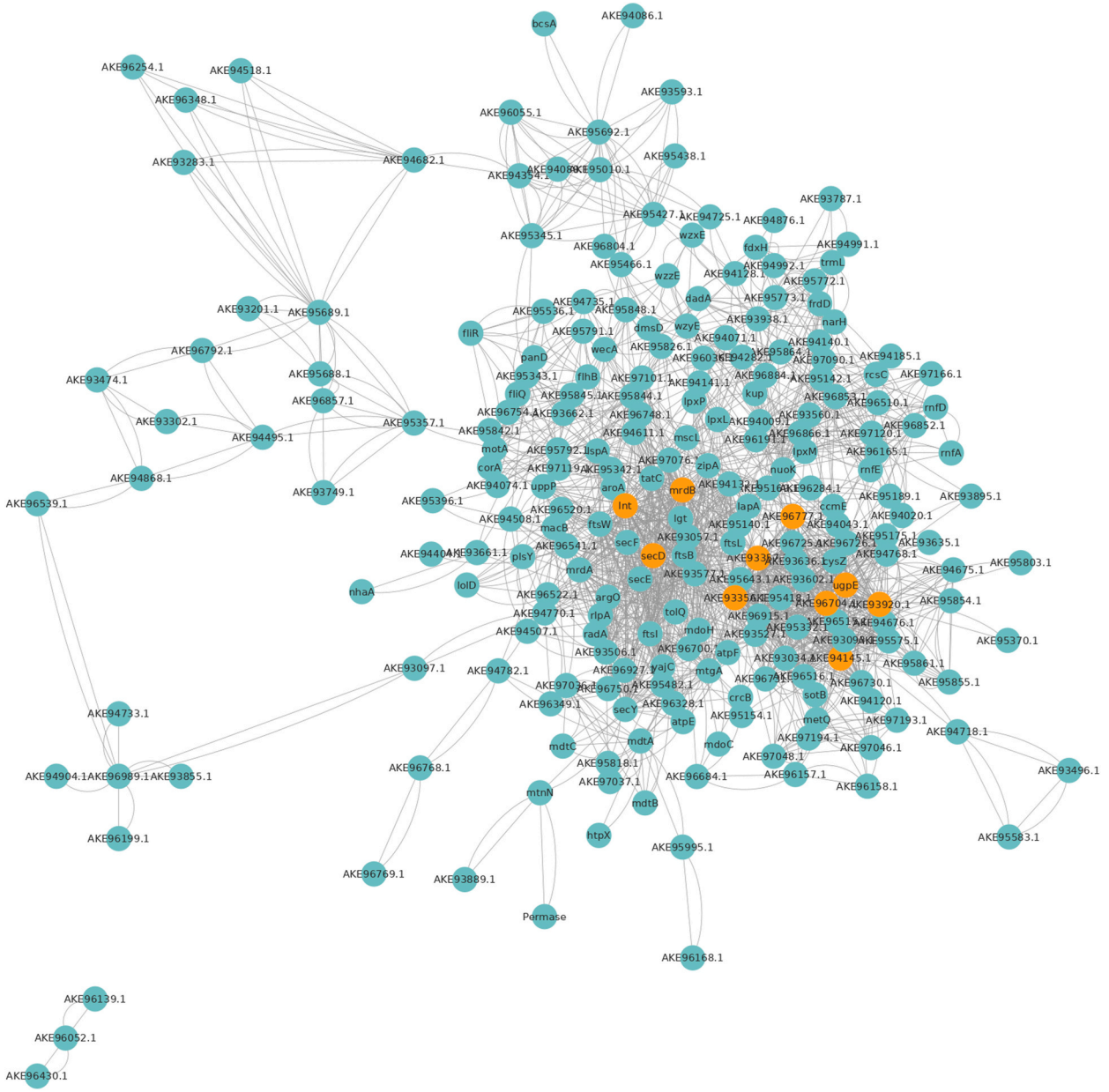

Fig. 2. Protein-protein interactions network of PPI network of pathogen-specific cytoplasmic membrane proteins. The network contains 236 nodes (proteins) and 1988 edges (interactions). Hub proteins of the network (n = 10) have been highlighted in orange. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

identified as non-homologous proteins.

3.3. Essential non-homologous gene identification

The identification of essential genes is crucial for developing novel drugs or vaccines that can inhibit microbial activity. As part of this study, a similarity search was conducted against the essential proteins of bacteria in the DEG database using a specific set of parameters (E-value 0.0001 and bit score >100), leading to the discovery of 1137 proteins that were deemed essential.

3.4. Analysis of metabolic pathways

The KAAS server was used to analyze 1137 essential proteins of *C. sakazakii* in order to find out their association with specific metabolic pathways. None of these proteins were found to be part of any pathways shared with humans. This eliminated any possibility for cross-reactivity with human-specific pathways. Therefore, these essential proteins can be exploited as potential therapeutic targets in the future.

3.5. Prediction of subcellular localization

During drug discovery, it is crucial to know how proteins are localized within cells. Modern bioinformatics tools can predict subcellular localization which reduces time, resources, and labor used for the selection of the most effective therapeutic agents. Here, the PSORTb server was used to find out the subcellular localization. Among all the proteins, 282 proteins were identified as cytoplasmic membrane proteins. These were further subjected to drug target analysis.

3.6. Protein-protein interaction (PPI) analysis

Identifying protein-protein interactions (PPI) can enhance the understanding of infection mechanisms and facilitate the development of new drugs and treatment approaches. In this study, the protein-protein interactions (PPI) network of 282 cytoplasmic membrane proteins was constructed using theSTRING Database and then visualized using Cytoscape software (Fig. 2). Furthermore, 10 hub proteins were selected among those 282 cytoplasmic membrane proteins which can be considered as potential drug targets (Table 2, Fig. 2). Supplementary Fig. 1 contains the network of only the hub proteins. The result of the betweenness centrality and closeness centrality analysis of the hub proteins can be found in **Supplementary File 1**.

3.7. Druggability of essential protein

The DrugBank was used to analyze the 10 hub proteins. It revealed that only one protein named ATP-binding protein CydC (Accession number- AKE96704.1, UniProt ID- A7MES4) had a corresponding FDA-approved drug called Terbinafine. The remaining hub proteins did not match any drugs in the DrugBank database, suggesting that they can be considered as potential novel therapeutic targets.

3.8. Homology modeling, structure prediction, and validation

The 3D structure of ATP-binding protein CydC was generated using the HHpred interactive server which performs pairwise comparison of profile hidden Markov models (HMMs). The structural quality of the protein was assessed and the Ramachandran plot of the model revealed that 93.4 % of the residues were in the most favored regions, 5.6 % were in the additional allowed regions, 0.6 % were in the generously allowed regions, and 0.4 % were in disallowed regions (Supplementary Fig. 2). According to ERRAT, the overall quality factor of the protein was 78.8732.

Table 2
List of hub proteins and their degree of protein-protein interactions.

Serial No.	Accession Number	Uniprot ID	Protein name	Number of interactions	Druggability of the protein
1	AKE93356.1	A7MG47	lipopolysaccharide ABC transporter permease LptG	88.0	Novel target
2	AKE93357.1	A7MG48	lipopolysaccharide ABC transporter permease LptF	76.0	Novel target
3	WP_007848231.1	A7MK36	Peptidoglycan glycosyltransferase MrdB	62.0	Novel target
4	WP_007848259.1	A7MQS9	Apolipoprotein N-acyltransferase	60.0	Novel target
5	AKE93920.1	A7MMV6	ribose ABC transporter permease	58.0	Novel target
6	WP_012126359.1	A7MGF9	sn-glycerol-3-phosphate transport system permease protein UgpE	56.0	Novel target
7	AKE96704.1	A7MES4	ATP-binding protein CydC	54.0	Terbinafine drug target
8	MDK1145284	A7MLX6	Protein translocase subunit SecD	50.0	Novel target
9	AKE94145.1	A7MGF8	glycerol-3-phosphate transporter permease	50.0	Novel target
10	AKE96777.1	A7MEY8	glutamine transport system permease protein GlnP	50.0	Novel target

3.9. Molecular docking analysis

Utilizing PyRx software, a molecular docking analysis was carried out between the FDA-approved drug Terbinafine and the predicted structure of the ATP-binding protein CydC. The molecular interactions between the ligand Terbinafine and the ATP-binding protein CydC indicate a significant binding energy value of −7.3 kcal/mol. According to docking analysis, only one carbon-hydrogen bond was formed between Arg 96 and the naphthalene ring structure of Terbinafine. Five alkyl bonds were observed between Leu 103, Leu 119, Leu 120, Leu 123, and Val 127 amino acids of the protein and terbinafine. One pi-sigma bond was also seen between Val 124, and the naphthalene ring structure of the Terbinafine (Fig. 3). Fig. 4 highlights the hydrophobic surface around the binding pocket of Terbinafine and CydC. The degree of hydrophobicity is indicated by color range according to Fig. 4.

3.10. Molecular dynamic simulation

RMSD has depicted the distinctness between the backbones of the receptor and ligand-bound receptor from its initial 3D structure conformation (0 ns) to its final structural conformation (100 ns). The RMSD for apo-CydC ABC transmembrane type-1 domain receptor was 0.6 nm throughout the simulation. Whereas, the RMSD value of the Terbinafine bound receptor gradually increased and finally showed a value around 0.9 nm (Fig. 5a). RMSF values demonstrated the regional flexibility of the receptors. The highest peaks of the mobilities were different for apo-receptor and ligand-receptor complexes (Fig. 5b). The Rg is a measure to determine the degree of compactness. The Rg values were significantly distinct through the simulation. The ligand-receptor complex had lower Rg values than only the receptor (Fig. 5c). SASA predicted the hydrophobic core of the proteins. Ligand-receptor complexes were lower SASA than the apo-CydC ABC transmembrane type-1 domain (Fig. 5d).

4. Discussion

C. sakazakii is a gastrointestinal pathogen that poses a significant risk to infants worldwide [42]. Powdered infant formulas (PIF) have been identified as the primary source of transmission of *C. sakazakii* which has a much higher potential to cause infection compared to other *Enterobacter* spp. [43]. Therefore, it is important to monitor commercial PIF for the presence of *C. sakazakii* and discover potential therapeutic drugs for treatment. Recent advancements in the field of bioinformatics and computational biology have led to the discovery of various techniques for drug discovery. This has resulted in the reduction of time and costs associated with the trial-and-error process of developing new drugs [44].

This study implemented a subtractive genomics approach to predict potential therapeutic targets for the clinically important *C. sakazakii*. The methodology followed a systematic process, and with each subsequent step, the outcomes became increasingly precise and restrictive. From the whole set of proteins in *C. sakazakii*, non-redundant and essential proteins were selected since they were important for bacterial survival [45], and make them desirable targets for the development of vaccines and antibacterial therapeutics [46]. Targeting homologous genes to humans could be problematic as they can interfere with human metabolism and lead to fatal consequences [47]. To minimize such risk of cross-reactivity and adverse effects, targeting non-homologous proteins is the best

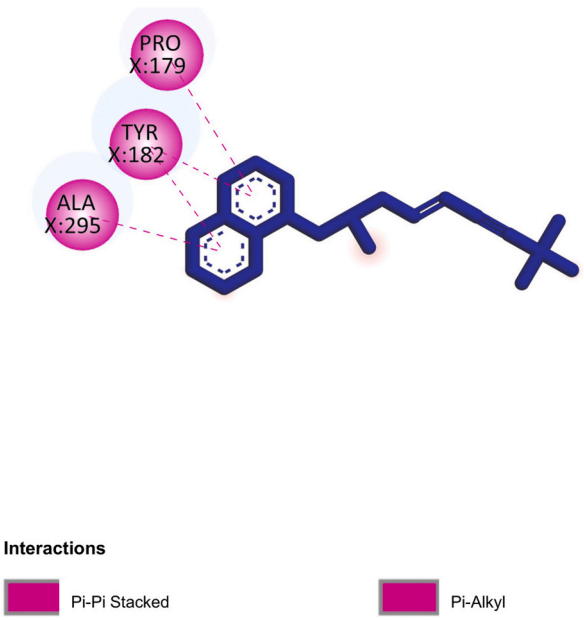

Fig. 3. Molecular Docking between the CydC and Terbinafine. CydC interacts with Terbinafine through Arg 96, Leu103, Leu 119, Leu 120, Leu 123, Val 124, Val 127.

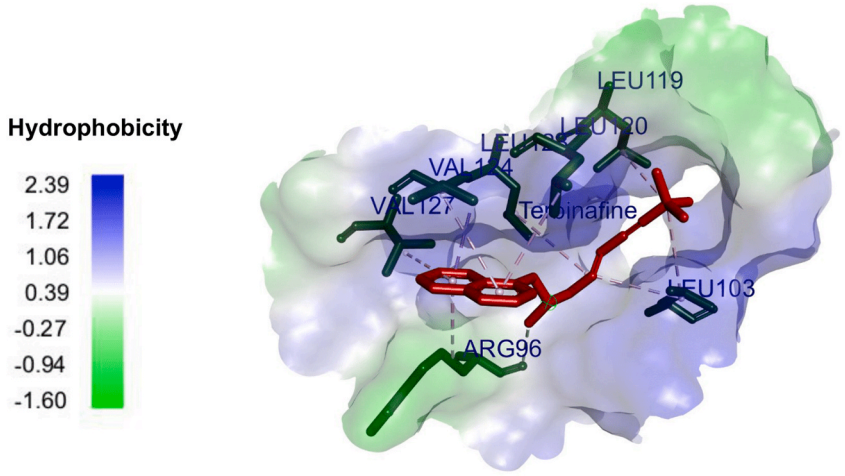

Fig. 4. Hydrophobic surface around the Terbinafine binding site in CydC. The binding pocket is mostly hydrophobic in nature.

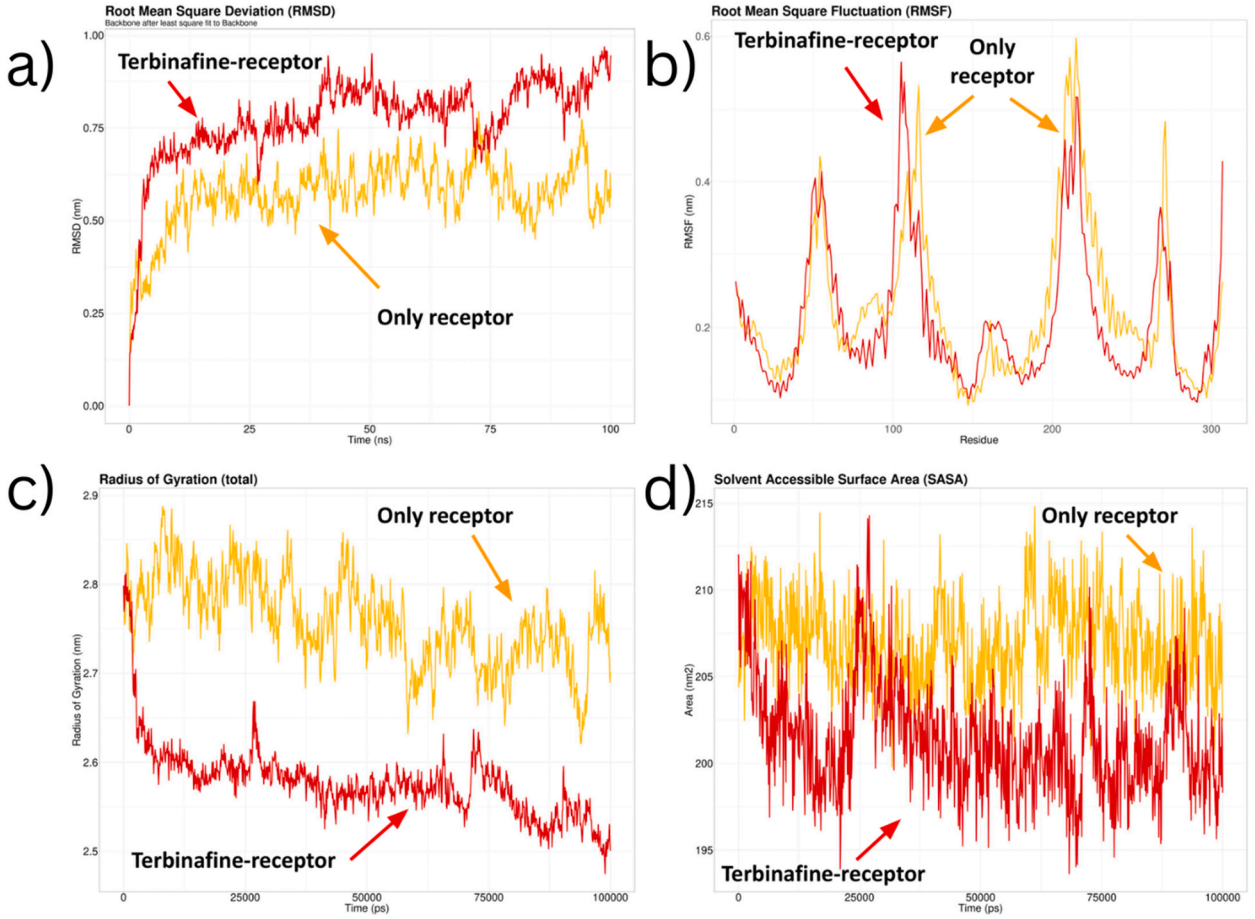

Fig. 5. Results of Molecular Dynamics Simulation a) Root Mean Square Deviation (RMSD) b) Root Mean Square Fluctuation (RMSF) c) Radius of gyration (Rg), and d) Solvent Accessible Surface Area (SASA) of CydC ABC transmembrane type-1 domain (Yellow) or Only Receptor and Terbinafine bound CydC ABC transmembrane type-1 domain (Red) or Terbinafine-Receptor. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

approach for developing new drugs and inhibitors [48,49]. In this study, no essential proteins were found to be associated with any pathways that are common to humans. This eliminated the likelihood of cross-reactivity. Subsequently, the subcellular localization of the proteins was revealed. Only cytoplasmic membrane proteins were kept as they are more accessible for therapeutic compounds to interact with compared to proteins located inside the cell [50].

Protein-protein interactions (PPIs) are important for various biological processes. Hub proteins are proteins in a protein-protein interaction network that have a significantly higher number of interactions (or degrees) compared to other proteins in the system [51]. Thus, information about hub proteins can provide valuable insights for selecting or prioritizing targets during the drug development process [52]. From the PPI network, hub proteins were selected and checked for their druggability. As a result, an FDA-approved drug, Terbinafine, was predicted to be effective against the ATP-binding protein CydC (AKE96704.1).

HHpred, an interactive web server, was implemented to model the 3D structure of the ATP-binding protein CydC, which greatly aided in the study of protein functions, dynamics, ligand interactions, and other protein components [53]. Analysis of the Ramachandran plot showed that most residues were localized in the favored and acceptable regions, whereas very few residues were found in the disallowed regions [54]. The ERRAT quality factor and z-score indicated that the ATP-binding protein CydC had a high-quality structure. Molecular docking was utilized to identify the compounds that exhibited the strongest residue interaction with the target protein [55].

In this study, CydC protein and Terbinafine drug showed effective interaction with a high binding energy (−7.3 kcal/mol) which indicated that Terbinafine can potentially inhibit the protein's activity. 100 ns long molecular dynamics simulation of the CydC ABC transmembrane type-1 domain and Terbinafine-CydC ABC transmembrane type-1 domain complex revealed that Terbinafine interrupted the structural conformation of the receptor in dynamic simulations. The protein (in the absence of ligand) exhibited distinct variations in solvent accessibility and compactness right from the onset of the simulation. Furthermore, the mobility patterns were altered as a result of the binding of Terbinafine. The drug formed a stable bond that changed the 3D structure of the protein, which may have changed its function, as seen by the stable RMSD value, increased compactness, and decreased SASA value. Plausibly, in the presence of Terbinafine, the transmembrane domain of CydC could not function properly. Terbinafine might hinder Glutathione/L-cysteine transportation and it can be a potential drug to repurpose against *C. sakazaki*. Previous studies have demonstrated that Terbinafine has broad-spectrum antimicrobial activities against different bacteria [56,57]. However, further molecular studies using *in vitro* and *in vivo* models are required to establish prospective therapeutics against the pathogen.

5. Conclusion

The identification of potential therapeutic targets for various pathogens has been considerably enhanced by the use of genomic and proteomic analysis. A subtractive genomic approach was utilized in this study, which resulted in the identification of non-homologous essential druggable proteins in *C. sakazakii*. This approach enabled the identification of possible therapeutic targets that are unique to this particular pathogen. It might be useful for developing effective therapeutics against *C. sakazakii* related infections.

Data availability

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Ishtiaque Ahammad: Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Anika Bushra Lamisa:** Writing – original draft, Visualization, Data curation. **Sadia Sharmin:** Writing – original draft, Formal analysis. **Arittra Bhattacharjee:** Writing – review & editing, Methodology, Formal analysis. **Zeshan Mahmud Chowdhury:** Writing – original draft, Visualization, Formal analysis, Data curation. **Tanvir Ahamed:** Validation. **Mohammad Uzzal Hossain:** Formal analysis. **Keshob Chandra Das:** Writing – review & editing. **Md Salimullah:** Writing – review & editing. **Chaman Ara Keya:** Writing – review & editing, Supervision, Conceptualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30332.

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