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Original Article

Antimicrobial Peptide Design, Molecular Docking and ADMET Studies Against the Methicillin-Resistant *Staphylococcus aureus* and Carbapenem-resistant and Carbapenemase-producing *Pseudomonas aeruginosa*

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HIGHLIGHTS

- Two cationic antimicrobial peptides (AMPs) were designed.
- Molecular docking of the AMPs revealed better antimicrobial activity than the reference.
- The novel AMPs had net positive charge and optimal hydrophobic amino acids.

ABSTRACT

Carbapenem-resistant and carbapenemase-producing *Pseudomonas aeruginosa* (CRPA) and methicillin-resistant *Staphylococcus aureus* (MRSA) are two pathogens that are resistant to currently available antimicrobials. As an alternative to effective medication molecules, antimicrobial peptides (AMPs) have the potential to cure superbug-caused infections effectively. Two new AMPs (ama1 and ama2) were designed utilizing a knowledge-based technique with optimal parameters. First, the PEP-FOLD 3.5 server made a *de novo* prediction of the AMPs' three-dimensional (3D) structure, which was validated using PROCHECK of SAVES v6.0 by checking the amino acid locations in the Ramachandra plot. Then, protein-peptide docking simulations of the predicted AMPs and reference AMP (Aurein 1.2) for positive control were performed using the HPEPDOCK docking web server, followed by the computation of the AMPs' physicochemical parameters and toxicity profile using the ProtParam and vNN-ADMET web servers, respectively. The sequences for ama1 and ama2 were AWGKIKALR and IKWLRLAKP, respectively. Docking analysis revealed that the antibacterial activity of ama1 and ama2 was superior to that of Aurein 1.2 against CRPA-resistant enzyme (6ew3), respectively. However, ama1, ama2, and Aurein 1.2 inhibited the activity of MRSA-resistant protein (4c12). Both the physicochemical qualities and the toxicity profiles were advantageous. Therefore, the *in-silico*-derived AMPs could serve as a pharmaceutical candidate for developing multidrug-resistant bacteria-effective antimicrobials.

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Introduction

The resistance of bacteria to modern medicines is one of

the primary challenges in addressing infectious diseases. Multidrug-resistant (MDR) infections, both Gram-negative and Gram-positive bacteria, threaten the efficacy of medical care when there is no precise treatment. Infections caused by carbapenem-resistant and carbapenemase-producing *Pseudomonas aeruginosa* (CRPA) and methicillin-resistant *Staphylococcus aureus* (MRSA) are becoming increasingly difficult to treat (Geitani et al., 2019). MRSA has inherent resistance to the majority of ß-lactam antibiotics since the mecA gene produces the penicillin-binding protein PBP2a with decreased affinity for these medicines (Temime et al., 2003).

Due to the rapid increase in antimicrobial resistance against conventional antimicrobials, the emergence of multidrug-resistant bacteria, and the sluggish development of novel antimicrobial classes, there is an urgent need to discover alternative classes of medicinal chemicals in this regard (Sarkar et al., 2021). Antimicrobial peptides, commonly known as AMPs, are a diverse class of naturally occurring compounds serving as the first line of natural defense in many living organisms. They possess enormous medical potential for eradicating dangerous germs (Dailami et al., 2016; Aliyu et al., 2021).

AMPs can combat infectious pathogens without developing resistance or slowly developing resistance. They've existed for millions of years (Yu et al., 2018). Several amphibian-derived AMPs with significant antibacterial activity against bacteria, yeast, protozoa, fungus, and viruses and some potential for cancer therapy have been found (Rabanal and Cajal, 2016). Some of these amphibian-derived AMPs are buforin II, regularin 3, dermaseptin, magainin, and japonicin 2. (Conlon and Mechkarska, 2014; Patocka et al., 2019; Aliyu et al., 2021).

The limited availability of natural AMPs, the prohibitive cost of synthesizing longer amino acid sequences, challenges with natural AMP folding, the short half-lives of natural AMPs as a result of protease degradation, systemic toxicity, and delivery challenges all limit the therapeutic potential of natural AMPs (Sarkar et al., 2021). Urgently required are short-cationic AMPs that are protease-tolerant and less toxic to humans to overcome these limitations and enable the therapeutic application of AMPs.

According to Vishnepolsky et al. (2019), who employed bioinformatics tools to develop AMPs, the most successful method is simple, fast, reliable, and inexpensive. Ferrando and Solomon (2021) cited *de novo* peptide/protein design as a potent instrument for studying biological mechanisms in the context of synthetic proteins. In this instance, the peptide or protein of interest is produced from a completely non-biological scaffold utilizing chemical principles to induce mutations that predict how the protein will fold (Regan et al., 1988). Using computer models based on peptide qualities, such as hydrophobicity, hydrophilicity, and amphiphilicity, we can better comprehend the potency of AMPs (Pirtskhalava et al., 2021).

Three antimicrobial peptides containing the α-helix structure, GH8 (GLLWHLLHNH2), GH12 (GLLWHLLHHLLH-NH2), and GH16 (GLLWHLLHHLLHH-NH2), were developed using an *in silico* model by Wang et al. (2017), and these AMPs are short cationic sequences and contain high content of hydrophobic amino acids. Similarly, the current study intends to produce short antimicrobial peptides to safely and effectively treat multidrug-resistant MRSA and CRPA using computational approaches.

Materials and Methods

De novo design of AMPs and validation

As a promising technique to obtain the most probable parameters for the *de novo* design of improved AMPs, database filtering technology (DFT) has been proposed (Wang et al., 2016), in which peptide activity, peptide length, amino acid frequency, charge, hydrophobicity, structure profile, and motif have used as filters (Mishra and Wang, 2012; Wang et al., 2022).

This knowledge-based approach was used to design two new AMPs using optimum parameters of net charge between +1 and +4, high AMP amino acid frequency of L, F, I, G, S, and K, and very notably the presence of hydrophobic amino acid residues in the range of 40-60%. The generated sequences were modeled using PEP-FOLD 3.5 (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/), which is an advanced and fast server for *de novo* prediction of three-dimensional (3D) peptide structures from FASTA format of the peptide [amino acid sequences] (Hashemi et al., 2021). It accommodates a vast range of peptide sizes ranging from 5 to 50 amino acids (Lamiable et al., 2016) and utilizes a hidden Markov model (HMM) to make the predictions. The method is based on structural alphabet (SA) letters to describe the conformations of four consecutive amino acid residues and couples the predicted series of SA letters to a greedy algorithm and a coarse-grained force field. The 3D of the best-ranked predicted models was retrieved for validation.

The 3D structures were validated using PROCHECK of SAVES v6.0 (https://saves.mbi.ucla.edu/) by examining the amino acid positions in the Ramachandra plot. The antimicrobial activity of these AMPs was ascertained by iAMP-2L (http://www.jci-bioinfo.cn/iAMP-2L) and the collection of antimicrobial peptides $CAMP_{R3}$ (http://www.camp.bicnirrh.res.in/).

Retriever of resistant genes and reference AMP from protein data bank (PDB)

Research Collaboratory for Structural Bioinformatics (RCSB PDB), a database for the three-dimensional structural data of large biological molecules, such as proteins and nucleic acids (https://www.rcsb.org/), was used.

UDP-N-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase (*murE*) of MRSA was retrieved from PDB with identification number 4C12. The *mbl*VIM-2 protein of CRPA (6EW3) was also retrieved from PDB. The *murE* increases the transcription of *pbpB* and *mecA*, which are the structural genes of penicillin-binding protein 2 (PBP2) and PBP2A that are known to play critical roles in the mechanism of staphylococcal methicillin resistance. Therefore, any agent that could inhibit the action of *murE* of MRSA could serve as a lead molecule for an anti-MRSA drug candidate. Moreover, Verona Integron-encoded Metallo-β-lactamase-2 (*mbl*VIM-2) producing carbapenem-resistant *Pseudomonas aeruginosa* is a widespread Metallo-β-lactamase with a broad substrate spectrum and hence could serve as an interesting drug target for the treatment of β-lactam-resistant infections.

Aurein 1.2 is a 13 amino acid residue (GLFDIIKKIAESF) AMP isolated from Southern bell frog *Litoria aurea* and *Litoria raniformis* with 53 % hydrophobic residues. Its structure was downloaded from PDB and used as a reference (positive control).

Molecular docking studies of the AMPs and the receptors

Molecular docking is a computer-aided prediction of the bound geometry between a drug molecule (ligand) and the protein (receptor) using computer algorithms. The molecular flexibility and conformational changes upon binding are treated differently in rigid-body and flexible docking algorithms. In this study, because peptides are highly flexible molecules that adopt different functional conformations, docking servers that use flexible algorithms were used.

HPEPDOCK (http://huanglab.phys.hust.edu.cn/hpepdock/) is a blind peptide-protein docking server by fast modelling of peptide conformations and global sampling of binding orientations and was used to carry out the peptide-protein docking studies as reported by Zhou *et al*. (2018). The downloaded crystal structures of the resistant genes (receptors) of *murE* MRSA (PDB ID: 4C12) chain A and *Pseudomonas aeruginosa mbl*VIM-2 (PDB ID: 6EW3) were uploaded on Chimera standalone software (http://www.rbvi.ucsf.edu/chimeram) and prepared by adding hydrogen, charges and energy minimization using the software algorithms as described by Pettersen *et al*. (2004). The two novel designed peptides and Aurein 1.2 were also prepared using UCSF Chimera. Both the prepared receptors and the peptide molecules were uploaded onto the HEPEPDOCK server for protein-peptide docking to get the docking scores, best pose and conformation of the peptide in the binding pocket of the receptor.

In silico physicochemical properties and ADMET studies

ProtParam (https://web.expasy.org/protparam/) is a tool that allows the computation of various physical and chemical parameters for a given protein or peptide stored in Swiss-Prot (https://www.sib.swiss/swiss-prot) or for a user-entered protein sequence to compute physicochemical parameters such as molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY) as described by Gasteiger *et al*. (2005). To get the physicochemical properties of the designed peptides, their amino acids' sequence one-letter codes were pasted on the ProtParam input field, and the physicochemical parameters were computed.

The vNN-ADMET webserver is a publicly available online platform to predict absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties and to build new models based on the variable near neighbor (vNN) methodology reported by Schyman *et al*. (2017). The PDB format of the peptides was submitted to the vNN-ADMET server (https://vnnadmet.bhsai.org/vnnadmet/admetpredinput.xhtml), and the predicted ADMET properties were recorded.

Results and Discussion

AMPs design and validation

Knowledge-based database filtering technology (DFT) *de novo* approach was used to design two novel AMPs with improved antimicrobial activity and with secondary structural conformation folding as alpha helixes (Fig. 1). The generated short cationic sequences and their molecular weights were AWGKIKALR (1042.29 Da) and IKWLRLAKP (1124.44 Da), which we named ama1 and ama2, respectively. They have a hydrophobic amino acid composition of 55.6 %. More so, the 3D alpha-helical structures of ama1 and ama2 were validated by the Ramachandra plot that showed 100 % of the amino acid residues in the most favored region of alpha-helical structures, i.e., the fourth quadrant (Fig. 1).

AMP activity predicting server, iAMP-2L that uses a fuzzy K-nearest neighbor algorithm, and conserved sequence signatures captured as patterns, and Hidden Markov Models (HMMs) in $CAMP_{R3}$ confirmed that ama1 and ama2 might have antibacterial activity.

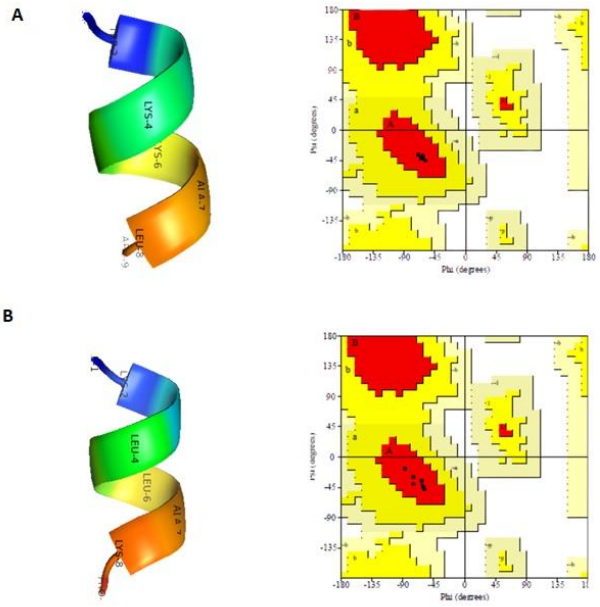

Figure 1: Predicted structure of the AMPs along with the Ramachandran plot. (A) ama1, (B) ama2. Both ama1 and ama2 are α-helical in 3D and further confirmed by the concentration of amino acid in the most favoured region of the α-helical structure of the Ramachandra plot.

Resistant genes and Aurein 1.2 AMP from protein data bank (PDB)

The resistant gene, *murE*, controls the expression of penicillin-binding protein (pbpB) and mecA (pbp2 & pbp2A), which are the structural genes of penicillin-binding protein 2 (pbp2), and pbp2A responsible for the action of methicillin-resistant *Staphylococcus aureus* with PDB ID 4c12, which was retrieved from PDB with the high-resolution value of 1.80 Å. It comprises homodimers, chain A and chain B, and each chain contains 501 amino acids sequence length. The active binding site of the receptor and the amino acid residues for the hydrogen and other bonding that would bring about the inhibition of the resistance gene were SER 30, ASN 151, ARG 187, ARG 383, GLU 460, and others that were not as important as these ones. They were retrieved from PDBSum (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html)

Another resistant gene retrieved from the PDB was the crystal structure of the Metallo-beta-lactamase VIM-2 (*mbl*VIM-2) with PDB ID 6ew3 with a structure resolution of 2.14 Å. The protein is a hydrolase from *Pseudomonas aeruginosa* that is responsible for destroying the beta-lactam ring of the carbapenem. It has 229 amino acid residues, and ASP 118, CYS 198, HIS 240, and others compose the receptor's active site. Any drug that forms hydrogen bonds with these active residues will inhibit the carbapenemase action, thereby presenting the susceptible organism to carbapenem antibiotics.

Aurein 1.2 is an AMP from a frog, *Litoria aurea* , with a sequence length of l2 amino acids and was extracted from PDB (with PDB ID of 1vm5) to serve as a positive control and a reference to the *in silico* antibacterial activity studies of the AMPs.

Molecular docking studies of AMPs against the resistant genes (receptors)

The docking results to determine the extent of inhibition of the MRSA-resistant (4c12) and *mbl*VIM-2 of *Pseudomonas aeruginosa* by AMPs (ama1 and ama2) and the reference AMP (Aurein 1.2) using HPEPDOCK webserver are presented in Fig. 2. From the HPEPDOCK results, ama2 was most effective against both 4c12 and 6ew3 resistant genes. This was closely followed by the action of ama1 against both 4c12 and 6ew3 receptors, while the reference AMP, Aurein 1.2, was not as effective as ema2 and ama1.

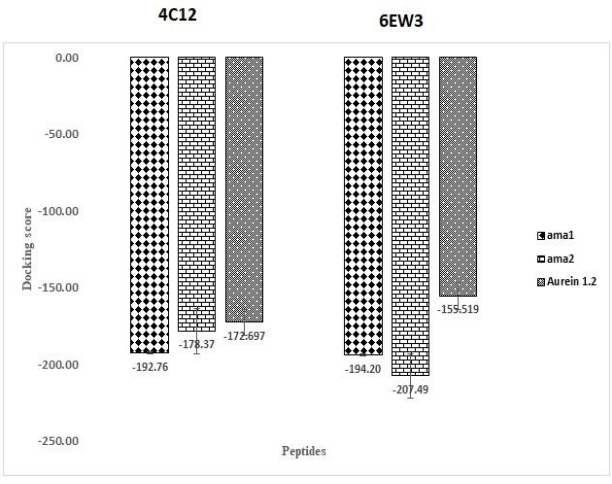

Figure 2: Docking scores of AMPs against resistant receptors. Inhibition profile of the resistance genes by the designed AMPs: ama1 and ama2, and reference AMP: Aurein1.2. The designed peptides showed better inhibition activity numerically than the reference AMP. However, it is statistically not significant at a p-value of 0.1952.

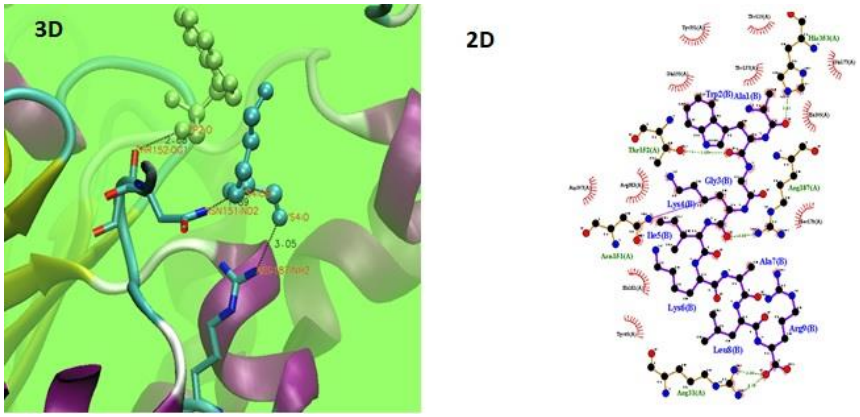

Figure 3: 3D and 2D representation of ama1 against 4c12 showing interactions with different amino acid residues of the receptors. In both 3D and 2D, Thr2 of the ama1 had H-bond with Thr152 of the 4c12. More so, Lys4 of ama1 formed H-bonds with Asn151 and Arg187 of the 4c12.

The hydrogen bonds between ama1 and ama2 with the active amino acid residues of the 4c12 receptor in 3D and 2D are presented in Fig. 3 and 4, respectively. The ama1 had hydrogen bonds with 4c12 through some active amino acid residues: ARG 31, ASN 151, THR 152, and ARG 187, and many other non-bonded interactions, which made it exhibit high inhibitory actions against the MRSA. The action of ama2 against the 4c12 receptor was demonstrated by the presence of hydrogen bonds between this peptide and HIS 205, ASN 407, and GLU 460.

The presence of hydrogen bonds between the peptides and the active amino acid residues indicated that the AMPs were in the right active pocket of the receptors and also inhibited the proper amino acids.

From HPEPDOCK results, the inhibitory activities of these AMPs witnessed from *in-silico* studies showed that the reference AMP, Aurein 1.2, is not as effective as the designed AMPs. Accordingly, ama2 and ama1 were able to inhibit the β lactamase and carbapenemase enzymes and could serve as lead molecule for the treatment of multiple antibiotic-resistant organisms. Using an *in-silico* approach, Bakare *et al.* (2020) reported the inhibition of pneumonia pathogens by an experimentally validated AMP. Moreover, Liscano *et al.* (2020) expressed that through molecular docking, two amphibian AMPs, caerin 1.6 and caerin 1.10 could inhibit the ACE2 receptor of the SARS-CoV2. In addition, Zouhir *et al.* (2021) docked the experimentally validated AMPs against MRSA receptors (4c12) and found that three AMPs gave satisfactory inhibitory interactions and could serve as lead peptides in the search for drugs against MRSA.

Physicochemical parameters of the designed AMPs and their ADMET

The Isoelectric point (pI) is the *p*H at which the net charge on the peptide will be zero. For the ama1 and ama2, both pIs were calculated to be 11.17 (basic), and the predicted half-lives of the AMPs were predicted to be greater than 10 hours. The net charges on both AMPs were +3.

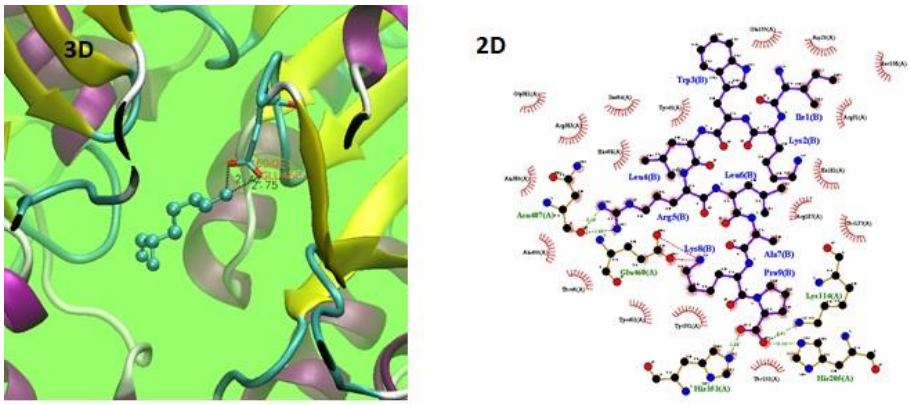

Figure 4: 3D and 2D representation of ama2 against 4c12 showing interactions with different amino acid residues of the receptors. Though the 2D representation showed many H-bonds, the 3D only showed two between Lys8 of ama2 and Glu460 of the 4c12.

Table 1. ADMET properties of ama1, ama2 and Aurein 1.2

Query	Liver Toxicity		Metabolism Cyp Inhibitors for						Membrane Transporters			Others			
	DILI	Cyto-toxicity	HLM	1A2	3A4	2D6	2C9	2C19	BBB	P-gp Inhibitor	P-gp Substrate	hERG Blocker	MMP	AMES	MRTD (mg/day)
ama1	-	-	-	-	-	-	-	-	+	-	+	-	-	-	4566
ama2	-	-	-	-	+	-	-	-	-	-	-	-	-	-	33034
Aurein 1.2	-	-	+	-	-	-	-	-	-	-	-	-	-	-	965

The cytotoxicity, mutagenicity, cardiotoxicity, drug-drug interactions, microsomal stability, and the likelihood of causing the drug-induced liver injury of the AMPs were assessed using vNN-ADMET, and it was found that while reference AMP, Aurein 1.2 passed all the 15 parameters, ama1 and ama2 passed 11 out of 15 *in silico* toxicity tests (Table 1).

Similar to the reference AMP, Aurein 1.2, ama1, and ama2 were not likely to cause any liver toxicity, cytotoxicity, cardiotoxicity, mitochondrial toxicity, or mutagenicity. However, both ama1 and ama2 might be highly metabolized by the liver, while ama2 was predicted as likely inhibitor of one (3A4) out of five main drug metabolizing cytochrome P450 enzymes inhibitors necessary for drug metabolism. More so, ama1 might be a Pgp-substrate that would make it be transported out of the microbial cell through an efflux mechanism. Besides, ama1 could cross BBB. Importantly, the Maximum Recommended Therapeutic Dose (MRTD), an estimated upper daily dose that is safe in both ama1 and ama2, was found to be higher than the reference peptide, Aurein 1.2.

In drug development, early assessments of pharmacokinetic and toxic properties are important stepping stones to avoid costly and unnecessary failures (Schyman *et al*., 2017). Since many drug candidates that enter clinical trials usually fail due to toxicity, both ama1 and ama2 were predicted to be safe from causing toxicity. Ebbensgaard *et al.* (2015) had earlier reported that melittin was highly cytotoxic while indolicitin at 128 mg/mL was slightly cytotoxic; however, some AMPs such as cecropin B and pyrrhocoricin were not cytotoxic at the concentration of antibacterial activity. Recently, Tong *et al.* (2022) revealed that a short linear peptide, RW-BP100-4D, obtained from optimization of RW-BP100 showed a remarkable reduction in cytotoxic effect with improved antibacterial activity against a wide range of microorganisms, including MRSA.

Conclusion

Global antimicrobial resistance has necessitated the urgent discovery of AMPs that would be clinically effective against MRSA and CRPA. Using *in silico* approach, ama1 and ama2 were designed, and their functional 3D structure was modeled and validated. Through computational analysis, it was predicted that both AMPs could have potent antibacterial activity on both multidrug-resistant MRSA and CRPA without causing toxicity. In addition to their predicted strong and rapid antibacterial activity, these two cationic AMPs have low resistance potential, making them interesting alternatives to conventional antibiotics. However, ama1 and ama2 could provide future therapeutic solutions to infections caused by multidrug-resistant bacteria. Nevertheless, the results presented in this paper are *in silico* and should be further validated by experimental studies.

Ethical Statement

The authors of this article have not conducted any studies involving animals or humans.

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Competing Interests

The authors declared that there is no conflict of interest.

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Authors' Contributions

A. Aliyu and Y.K.E. Ibrahim conceived of the presented idea. Y.K.E. Ibrahim and B.A. Tytler developed the theory, and A. Aliyu performed the computations. A. Aliyu and A. Olowo-okere verified the analytical methods. Y.K.E. Ibrahim and B.A. Tytler supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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