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The Recombinant Production of Novel Bovine Lactoferricin Engineered Peptide Using Molecular Dynamics Simulation

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ABSTRACT

Antimicrobial peptides (AMPs) are native and safe short peptides that are considered one of the best alternatives to antibiotics. Although numerous studies have been conducted on AMPs, their mode of action has not yet been fully understood. Computational peptide engineering can provide valuable insight into the stability and potency of AMPs against targets. In the present study, we performed a molecular dynamics simulation to understand the mode of action of Bovine Lfcin and to improve the interactions between Bovine Lfcin (wild and mutant types) and DNA (an important intracellular target), DNAK (an important protein in pathogenicity, mostly in gram-negative bacteria), and LysM (an important surface protein in gram-positive bacteria). The nucleotide sequence of Lfcin peptide was synthesized and cloned into pET22b (+). Induction of gene expression was performed using 1mM IPTG and recombinant peptide was purified using a His-tag marker. The antimicrobial peptide activity was tested on *Escherichia coli O157* and *Bacillus subtil*is, Gram- negative and positive bacteria,respectively, using disk diffusion methods. Our results showed that all the changes in the Bovine Lfcin wild type observed in this study improved the peptide-DNA, DNAK, and LysM interactions. Based on the results, removing the PHE25 residue from the wild- type Bovine Lfcin peptide had more significant effects on complex formation with DNA, DNAK, and LysM. The recombinant production of an Lfcin peptide with a molecular weight of ~5 KDa was confirmed by SDS PAGE. The performance of Lfcin peptide on pathogenic bacteria was strong, and it had the strongest effect at concentrations of 6 and 8 mg/ml for Gram- positive and Gram-negative bacteria, respectively.

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1. Introduction

Lactoferrin is one of the most important milk proteins, and the peptides produced from it are among the strongest AMPs (1). The cationic lactoferrin molecule interacts with a part of lipopolysaccharide (LPS) called lipid A through the positively charged cluster in the N-terminal region of the N-lobe, as part of the outer membrane of Gram-negative bacteria (2). This interaction causes damage in the cell membrane, resulting in increased permeability and the release of LPS. Experiments on human lactoferrin show that the N-terminal part of lactoferrin (lactoferrin regions 28-34) plays an essential role in binding of lactoferrin to LPS (28-30) (3). Studies show that bovine lactoferrin causes damage to the outer membrane of Gram-negative bacteria by binding with LPS (4).

Investigation of membrane dialysis shows that when metal-saturated or unsaturated lactoferrin is separated from bacterial cells, LPS is also separated from the bacterial outer membrane (2). The interaction of hydrophobic and basic amino acids with the inner core of LPS (especially tryptophan and arginine) result in the antimicrobial activity of lactoferrin (5). This interaction disrupts the structure of the outer membrane and facilitates the binding of tryptophan to lipid A (6). Studies showed that lactoferrin exhibits broad antimicrobial activity against bacterial, viral, and fungal pathogens (7,8).

The resistance of pathogenic bacteria to common antimicrobial agents has become a serious threat to human public health (9). This is mainly due to the overuse of antimicrobial agents and overtreatment with medicine (10). Antimicrobial peptides (AMPs) are an innate defense against natural microbial attacks, produced by the organism's immune system (11). AMPs have several biological functions, including antibacterial, antiviral, antiparasitic, antifungal (8). In addition, the multifunctional mechanisms of AMPs reduce the potential to develop resistance against bacteria (12). The physicochemical properties of AMPs such as: amphipathicity, peptide amino acid structure and amino acid sequence, electrical charge, and oligomerization are highly effective in the peptide action mechanism (13).

Characteristics such as hydrophobicity and selective destruction of the cell membrane play a vital role in the performance of AMPs (14,9). Thus, the distribution of electric charge on the peptide and the presence of polar phospholipid heads are the most important factors in peptide-membrane binding (13). For example, hydrophobic peptides are able to recognize anionic lipids on the outer surface of the bacterial membrane, while in eukaryotic cells, these lipids are located inside the cytoplasmic membrane (15,16). However, in order to induce bacterial death through pore formation, three main processes must occur binding of AMPs to the bacterial membrane, (2) their accumulation inside the membrane, and (3) peptide insertion and membrane permeability (17). The binding of AMPs to negatively charged bacterial membrane does not involve a specific receptor (18). Therefore, peptide binding occurs up to a certain molecular concentration, after which they penetrate the cell through several mechanisms (18). However, peptides may interact specifically with one of the membrane components, such as lantibiotics, which bind to lipid II and prevent cell wall production (19).

There are various methods, such as microscopic fluorescence polarization, model membrane studies, fluorescent dyes, ion channel formation and molecular dynamics, to investigate the mechanism of AMPs (20, 21). Among these, the molecular dynamics method is one of the most effective and least costly methods in the process of investigating the effect of AMPs (22). The basic problem in understanding the behavior and mechanism of action of these peptides is the lack of information in molecular and atomic dimensions. Normally, tracking the behavior of these peptides in living cells with molecular resolution is very difficult and expensive (23). The number of peptides that can be generated is high, and the vast majority of experimental methods mentioned above are static and lack the ability to interpret the dynamic behavior of these macromolecules (22). Recombinant peptides with high capability and even specificity can be created through understanding their interaction mechanisms. Therefore, this study aimed to apply molecular dynamics simulation for engineering broad -spectrum antimicrobial peptides. Also, the corresponding sequence was converted into recombinant antimicrobial peptide in *E. coli*, and its effect was investigated on Gram-positive and Gram -negative bacteria.

2. Materials and Methods

2.1. Molecular Dynamic Simulation

2.1.1. Structure preparation

Three-dimensional B-forms of DNA, DNAK and Bovine Lfcin peptide were obtained from Protein Data Bank with accession numbers 1BNA, 1DKX and 1LFC,

respectively. Due to the lack of a three-dimensional structure of the LysM protein in the Protein Data Bank, the LysM structure was modeled in Modeller 9.2 software (24).

The quality of the LysM model was investigated using the PROCHECK server (http://servicesn.mbi.ucla.edu/PROCHECK/) (25).

2.2. Molecular Dynamics Simulations

The dynamic behavior of the complexes, including DNA-Lfcin, DNAK-Lfcin, and LysM-Lfcin were studied using GROMACS 2018 package with periodic boundary conditions in all directions (26). The CHARMM27 force field was used for DNA-Lfcin and Gromos54a7 was used for DNAK-Lfcinand LysM-Lfcin (27). A Simple Point Charge (SPC) water model was used to solvate the system in the cubic water box (28). To neutralize the overall charge of the systems, Na and Cl ions were added by substituting water molecules. Energy minimization was performed using the steepest descent algorithm. Subsequently, the system was equilibrated under an NVT ensemble at 300 K during 400 ps , followed by an NPT equilibration run at 1 bar pressure and 300 K temperature for 1000ps.The Nosé-Hoover algorithm with a time constant of 0.1 ps and the Parrinello-Rahman algorithm with a time constant of 0.5 ps were used for temperature and pressure coupling, respectively (29).The LINCS algorithm was used to constrain all bonds. For long-range electrostatic interactions, the Particle Mesh Ewald (PME) algorithm was applied. The nonbonded cutoff of 12 Å was also used. MD production runs were carried out for 50,000 ps under the NPT ensemble at 1 bar and 310 K. The dynamics and stability of each peptide and BDNA, including root mean square deviation (RMSD), root-mean-square-fluctuations (RMSF) hydrogen bonds, were analyzed using GROMACS built-in tools.

G-MMPBSA program was used to estimate binding free energy estimation. The average binding energy and its standard deviation were calculated using the MmPbSaStat.py python script (http://rashmikumari.github.io/g_mmpbsa/). To estimate the contribution of each residue to the total binding free energy, the MmPbSaDecomp.py python script was used (30).

2.3. Production of Lfcin peptide

2.3.1. Gene Synthesis and Production of Recombinant Protein

The pel B signal sequence was used at the beginning of the structure for periplasmic protein production (31). The Lfcin peptide was codon-optimized for *E. coli* expression and synthesized in a pGH vector by the GenRay Biotechnology (Shanghai, China).

The target genes in pGH vector were sub-cloned and then cloned into the pET22b (+) expression vector between Xba I and Hind III restriction sites. *E. coli* BL 21DE3 cells containing recombinant pET22b (+) were cultured in LB broth medium overnight at 37°C with shaking at 180 rpm. Next, 200 ml of overnight culture was inoculated in 2L of LB broth containing 100 µg/ml ampicillin. The culture was grown at 37°C until the optical density (OD) at 600 nm was reached. For induction of expression, isopropyl β-D- thiogalactoside (IPTG) was added to the culture to a final concentration of 1.5 mM and the culture was incubated at 37°C for 5 hours. To collect the induced cells, the cells were centrifuged at 12,000 × g for 20 minutes at 4 °C, and the supernatant was discarded. The supernatant was first analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Protein Extraction and Sepharose Chromatography

To extract the proteins, osmotic shock methods were applied (32). Briefly, the 25 ml of hypertonic buffer (Tris-Hcl 30mM, EDTA 1 mM and Saccharose 20%, pH=8) was added to the induced cells and the solution was incubated on ice for 30 minutes. The solution was centrifuged at 8000 g for 20 minutes , and the supernatant was collected. The pellet was suspended in 25 ml of hypotonic 5 mM MgSo4, incubated for 30 minutes on ice, and centrifuged at 8000 g for 20 minutes. The supernatants from the hypertonic and hypotonic solutions were mixed and dialyzed in lysis solution (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH: 8.0) for 16 hours. To condense the proteins, the solution was filtered using Amicon Ultra-0.5 mL (Merck, USA). Q-sepharose chromatography was used to extract the target protein. Briefly, the Q-Sepharose column was first washed by lysis solution three times, and 5 ml of the supernatant containing soluble recombinant protein was filtered three times at 4°C. Next, the lysis solution was washed with six concentrations of NaCl concentration (0.1, 0.2, 0.3, 1, 2, 3 Molar) in the Q-Sepharose column. Then, the solution was collected for the SDS-PAGE analysis. To extract the target protein, the best concentration of NaCl was used for the entire amount of the soluble recombinant proteins based on SDS-PAGE.

Finally, Bradford PROTEIN ASSAY was used to determine the protein concentration (33).

2.4. Investigation of Lfcin Peptide Effect on Gram-Positive and Gram-Negative Bacteria

One of the simplest tests to evaluate the antimicrobial activity of a peptide is the disk diffusion method (34). Five different disks were coated with five different concentrations of the antimicrobial peptide and placed on culture media containing Gram-positive *Bacillus subtilis* and Gram-negative *Escherichia coli* O157. Gentamycin was used as a control.

3. Results

3.1. Molecular Dynamics Simulation

After homology modeling, the structure of the LysM protein was examined for overall quality. The Ramachandran plot for LysM protein showed that 93.3% of the residues were situated within the most favored region, while the remaining residues were found within the additional allowed region (Figure 1).

3.2. Dynamics Behavior and Interaction Between DNA, DNAK, Lysm and Bovine Lfcin

RMSD measures how much the protein structure deviates from the the initial structure over the course of the simulation. The RMSD patterns of Bovine Lfcin in interaction with DNA, DNAK and LysM did not deviate drastically over 50,000 psin all simulations (Figure 2). The dynamic behavior of individual amino acid residues in Lfcin was analyzed using the RMSF value, which was defined by peak elevation in all simulations. Rational residue fluctuation behavior was observed for Lfcin.

The radius of gyration (Rg),which shows the compactness of the protein,was plotted for alpha-carbon atoms vs time at 310 K interaction with DNA, DNAK and LysM (Figure 2). The Rg plot showed that Lfcin did not exhibit major changes in compactness during these interactions.

3.3. Number of Hydrogen Bonds

The number of hydrogen bonds between Lfcin and DNA showed significant variation during the simulation (Figure 3A). The average number of hydrogen bonds between Lfcin and DNA over the 50 ns simulation was 5.76±0.5 ($P<0.05$). A representative snapshot of the Lfcin-DNA interaction is illustrated in Figure 3B. In this frame, it can be observed that Arg and Glu have hydrogen bonding with DNA-backbone phosphate groups. The average number of hydrogen bonds between Lfcin and DNAK during the 50,000 ps simulation was 9.72±0.3 ($P<0.05$). The most important region of DNAK for interaction with targets is the aD-aE region. The results showed that Lfcin, on average, formed only two hydrogen bonds with this site (Figure 3C). The results indicated that the interaction between Lfcin and LysM was weak, with only two hydrogen bonds observed on average (Figure 2D).

3.4. Binding Free Energy Estimate

The binding free energy was calculated by the MM/PBSA method. The results revealed that the free binding energy between Lfcin and DNA and DNAK was -342±9.21 and -426±8.24 kJ/mol, respectively ($P<0.05$). The free energy values for the Lfcin-DNA system were decomposed into residue contributions using the MmPbSaDecomp.py python script. The results suggested that positively charged residues are more relevant for binding (Figures 4A and B). On the other hand, the last residue of Lfcin-DNAK (PHE25) had a detrimental effect on interacting with DNA (Figure 4B). Complex formation and stability between LysM and Lfcin were highly correlated with electrostatic interaction (Figure 4C). The binding energy contribution per amino-acid residue indicates that ARG, LYS, TRP and LEU residues in the bovine Lfcin peptide play a major role in interaction with LysM over 50,000 ps simulation. Overall, complex stability in this system was mainly due to electrostatic interaction.

3.5. Peptide Engineering

Given the strong interaction between DNA, DNAK and LysM with Bovine Lfcin, engineering of the Lfcin peptide was carried out. The last amino acid in the Lfcin (PHE25) had highest inhibitory effect on interaction with the DNA and DNAK. PHE25 was substituted with ALA amino acid. The results of the binding energy contribution per amino acid indicate that substitution of PHE with ALA at the last position of the peptide in all targets did not affect complex formation, and the change could not improve the free binding energy (Figure 5).

3.6. Production of Recombinant Lfcin

The production of Lfcin antimicrobial peptide was confirmed by SDS PAGE (Figure 6). Protein purification was successfully performed using osmotic shock in the Q-Sepharose column (Figure 6). Although our findings confirmed protein expression, no recombinant proteins were observed in the LB medium, indicating that almost all Lfcin AMPs were expressed either inside the bacterial cell or moved into the periplasmic area. According to the Bradford analysis, the concentration of the recombinant proteins extracted was 10.78 mg/ml for Lfcin antimicrobial peptide ($P<0.05$).

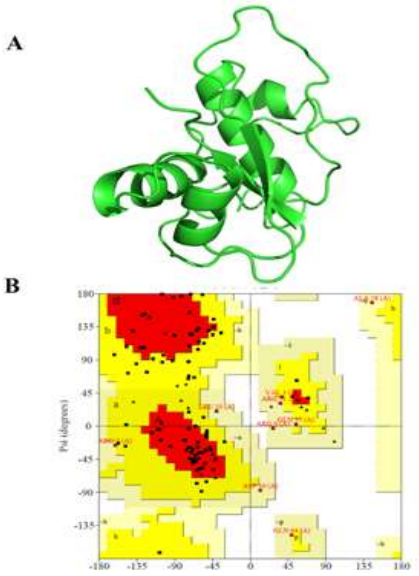

Figure 1. The structure of LysM protein and their Ramachandran plot. Each black point in the Ramachandran plot refers to one amino acid residue of protein.

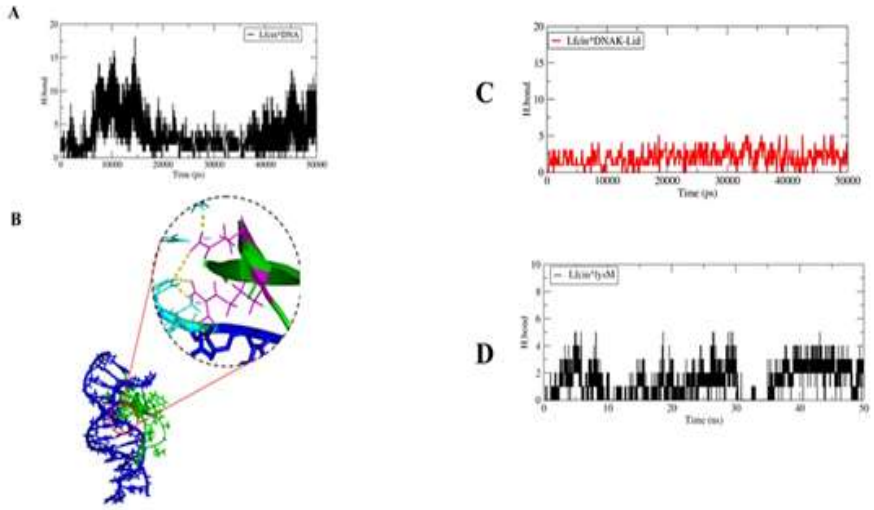

Figure 2. RMSD, RMSF and Radius Gyration analysis for Bovine Lfcin during interaction with DNA, DNAK, LysM.

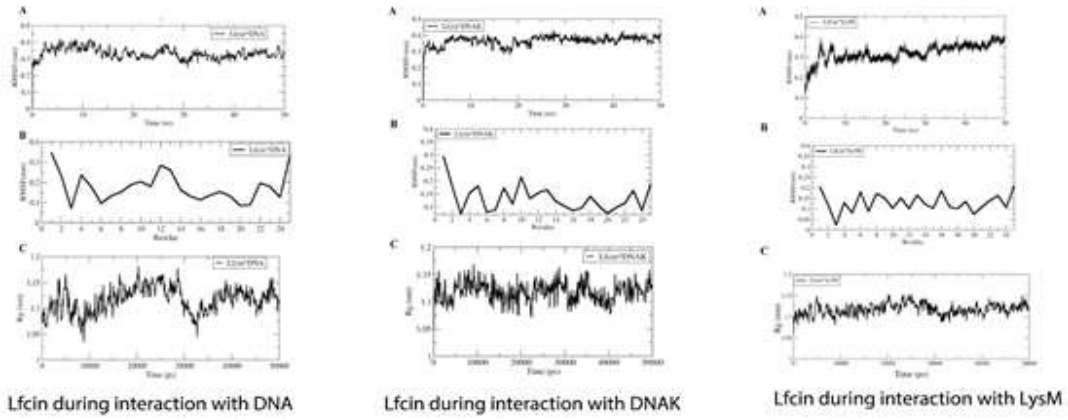

Figure 3. Hydrogen bonding between DNA, DNAK and LysM and Lfcin. (A) number of hydrogen bonds, (B): Snapshot at t = 45 ns, indicating hydrogen bonds (yellow dashed), (C) Number of hydrogen bonding between DNAK active site (aD-aE) and Lfcin.

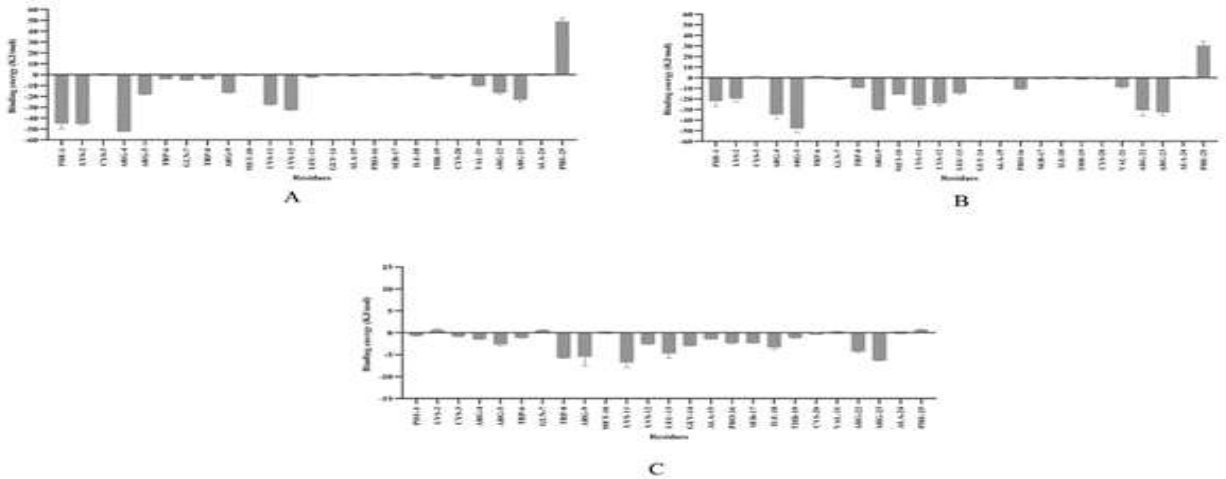

Figure 4. Contribution binding free energies of amino-acid residues in Lfcin*DNA (A), Lfcin*DNAK (B) and Lfcin*LysM (C) interaction.

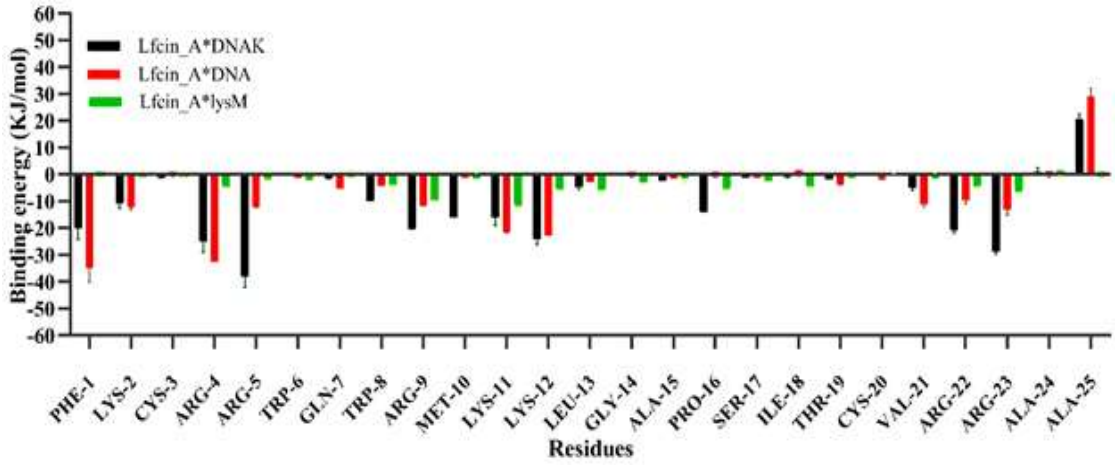

Figure 5. Contribution binding free energies of amino-acid residues Lfcin_A and DNA, DNAK and LysM.

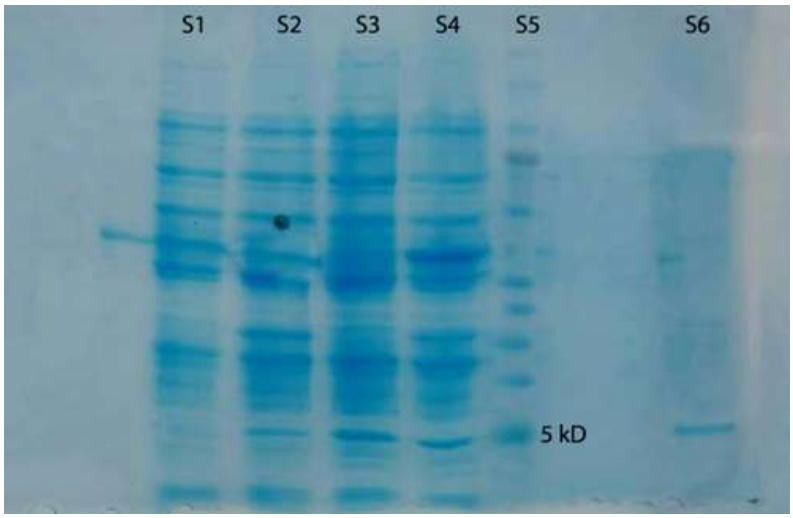

Figure 6. The confirmed Lfcin antimicrobial peptide expression in pET 22b (+). (S1) The SDS PAGE of Lfcin protein in E. coli BL21 (DE3) in zero time. (S2-S4) The SDS PAGE of Lfcin protein in E. coli BL21 (DE3) in 1, 2 and 3 hours after inducing by IP.

3.7. Cytotoxicity of Lfcin for Positive and Negative Bacteria

The effect of the peptide on Gram-positive and Gram-negative bacteria was evaluated using the disk diffusion method (DDM).This analysis showed that at a concentration of 6 mg/ml, this peptide can inhibit the growth of *E. coli* O157 and 8 mg/ml for *Bacillus subtilis* ($P<0.05$).The diameter of the disk radius of a clear zone on media plate indicated the inhibiting effect of Lfcin on bacteria growth (Figure 7).

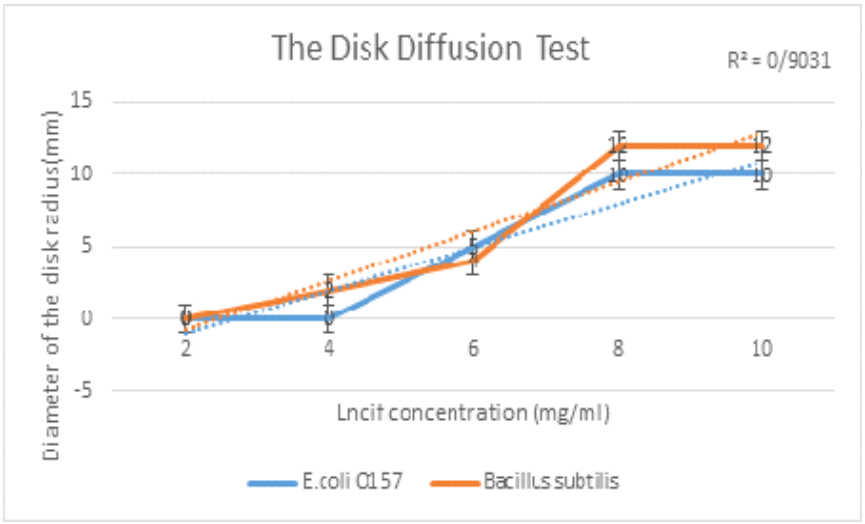

Figure 7. Comparison of recombinant peptide effect to inhibit the growth of E. coli O157 and B. subtilis with disk diffusion method.

4. Discussion

Antibiotic resistance (AMR) is a pervasive global issue, leading to 700.000 fatalities annually worldwide (35). Scientists believe that antibiotic resistance could lead to the death of 10 million people by 2050 (36). A comprehensive review of studies shows that AMPs exhibit broad-spectrum activity and strong effects against both Gram-positive and Gram-negative bacteria (37-39). Today, the mechanism of action of AMP can be predicted using molecular dynamics (MD) simulations (40, 41). MDS studies on AMPs have led to several important products (42-44), and this technique has proven useful in discoveries of new paths (22).

In this study, BDNA, DNAK and LysM were selected as targets of Gram-negative and Gram-positive bacteria for engineering antimicrobial peptides. B-DNA is the most important and abundant pattern of DNA in nature that is found in both Gram-negative and Gram-positive bacteria (45). DNAK is a heat shock protein located in membrane of Gram negative bacteria that plays a major role in pathogenic bacteria (46). This protein is a highly important target in AMPs design. LysM is a surface protein in Gram-positive bacteria and is important to keep bacteria alive. Studies have shown that LysM blocking can destroy bacteria (47). The results of MDS showed that mutant Lcf successfully interacts with DNA, DNA and LysM that confirms Lcf can be classified in broad spectrum AMPs against gram-negative and positive bacteria. Rizzetto et al (2023) reported similar results about the effect of antimicrobial peptides against gram-positive and negative bacteria (48). None of the AMPs in this study were able to destroy gram-positive and gram-negative bacteria in a native state. However, reports show that various mammalian lactoferrins are highly suitable candidates as antimicrobial peptides (49).

AMPs typically consist of 10-50 amino acids, with smaller AMPs being more effective (50). The molecular weight of Lcf is about 5 KDa, including residues from restriction enzyme and pET vectors. It is expected that removing excess amino acids will enhnce peptide performance.

The antimicrobial potency of Lcf on *E. coli* and *B. subtilis* was assessed in this study. The results showed that Lcf was more effective against against Gram-negative than Gram-positive bacteria. Therefore, the amphipathicity of the peptide is a more effective parameter (51), along with hydrophobicity and hydrophilicity (52). Chen et al (2020) investigated the effect of Chol-37(F34-R) peptide on *Staphylococcus aureus* (Gram-positive) and *Salmonella typhimurium* (Gram-negative) (51). They showed that Chol-37 peptide has a more significant effect on Gram-positive bacteria. The effect of their peptide on *S. aureus* was 64 times stronger than on *S. typhimurium*, while the strength of Lcf peptide was only 1.3 times stronger. These comparisons showed that Lcf peptide has a similar effect on Gram-positive and Gram-negative bacteria and confirmed that Lcf is a broad-spectrum AMP. The main purpose of this study was to investigate the interaction of Bovine Lfcin as one of the derived peptides of bovine lactoferrin with DNA, DNAK, and LysM protein as the main targets of AMPs and antibiotics in Gram-negative and Grampositive bacteria. To achieve this objective, we performed molecular dynamics simulations. The results indicated that Lfcin showed weak interaction with these targets.

Base on our findings, hydrogen plays a crucial role in the formation and stabilization of the Lfcin and targets interaction. To improve the effectiveness of this peptide, we opted to change some residues in the Lfcin. According to our studies, removal of the PHE25 in the last position of the Lfcin could cause an increase in the antimicrobial effect of the peptide. Our findings in the present study provided valuable information regarding the significance of residues in AMPs and could be of considerable value in the field of peptide engineering to achieve stronger and higher spectrum AMPs with intracellular activity.

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Authors' Contribution

Study concept and design: M.AZ, A.A.
Acquisition of data: A. M, M. N.
Analysis and interpretation of data: M. AZ, A. M, M. N.
Drafting of the manuscript: M. AZ, A. A.
Critical revision of the manuscript for important intellectual content: M. N, A. M.
Statistical analysis: A. A, M. AZ.

Administrative, technical, and material support: M. AZ, A. M, M. N, A. A.

Ethics

The authors of this study hereby declare that all the ethical standards were followed in the preparation of the submitted article.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data generated and/or analyzed during the current study are available from the corresponding author upon request.

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