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Ph.D. Dissertation

Unveiling the Potent Antimicrobial Activity and Mechanism of Action of Novel Peptide YS12 from *Bacillus velezensis* against Drug-Resistant Strains: A Promising Strategy for Combating Antibiotic Resistance

Graduate School of Chosun University

College of Pharmacy

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*Bacillus velezensis*에서 분리한 신규 펩타이드 YS12의 항미생물 활성 기전 연구 및 항생제 저항성 병원 미생물 제어 전략

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Advisor: Prof. Jin Cheol Yoo

This dissertation is submitted to the Graduate School of Chosun University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Graduate School of Chosun University

Department of Pharmacy

Suzia Aktar Suchi

This is to certify that the Ph.D. dissertation of Suzia Aktar Suchi has successfully met the dissertation requirements of Chosun University

Chairman : Prof. Cho, Seung Sik, Mokpo Natl. University

Member : Prof. Lee, Hyo Jeong, Gwangju University

Member : Prof. Choi Eun Joo, Chosun University

Member : Prof. Yang, Young-Mo, Chosun University

Member : Prof. Yoo, Jin Cheol, Chosun University

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Graduate School of Chosun University

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Abbreviations

AMP: Antimicrobial peptide

APD: Antimicrobial Peptide Database

HDP: Host defense peptide

LAB: Lactic Acid Bacteria

AU: Arbitrary Unit

AUC: Area Under the Curve

BLAST: Basic Local Alignment Search Tool

CFU: Colony Forming Unit

MALDI-TOF: Matrix-Assisted Laser Desorption - Ionization-

Time of Flight MDR: Multi-Drug Resistance

MH: Mueller-Hinton MHA: Mueller Hinton Agar

MHB: Mueller Hinton Broth

MIC: Minimum Inhibitory Concentration

MRS: Media-De Man-Rogosa-Sharpe

MRSA: Methicillin-resistant Staphylococcus aureus

FICI: Fractional Inhibitory Concentration Index

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

LUVs: large unilamellar vesicles PG: Phosphoglycerol

PE: Phosphoethanolamine PC: Phosphatidylcholine

NPN: 1-N- phenyl naphthylamine

DISC-3: 3,3-dipropylthiadicarbocyanine iodide

PI: Propidium Iodide

ONPG: Ortho-nitrophenyl-galactose

CLSM: Confocal Laser Microscopy

Abstract

Unveiling the Potent Antimicrobial Activity and Mechanism of Action of Novel Peptide YS12 from *Bacillus velezensis* against Drug-Resistant Strains: A Promising Strategy for Combating Antibiotic Resistance

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Nowadays, the abuse of antibiotics has led to the rise of multi-drug-resistant bacteria. Antimicrobial peptides (AMPs) have attracted considerable attention as possible alternatives to traditional antibiotics. In this work, we aimed to evaluate the antimicrobial, anti-biofilm activity with the mechanism of action of an antimicrobial peptide designed as YS12 derived from *Bacillusvelezensis* CBSYS12. The strain CBSYS12 was isolated from Korean food kimchi and purified, followed by ultrafiltration and sequential chromatographic methodology. Hereafter, Tricine-SDS PAGE revealed a single protein band of around 3.3kDa that further confirmed insitu inhibitoryactivity of the gel. A similar molecular weight (~3348.4 Da) protein also appeared in MALDI- TOF confirming the purity and homogeneity of the peptide YS12.

AMPs are considered potential substitutes for traditional antibiotics due to their broad antimicrobial spectrum against multidrug-resistant (MDR) pathogenic bacteria and the inability of microorganisms to easily develop resistance to them. Intriguingly, our data revealed a strong antimicrobial activity with a minimum inhibitory concentration (MIC) value ranging from 6-12 μg/ml for different bacteria h as *E. coli*, *P. aeruginosa*, MRSA 4-5, VRE 82, and *M. smegmatis*. Peptide YS12 provides a synergistic action with commercial antibiotics and thus creates a more effective approach to the treatment of multi-drug resistant bacteria. In addition, the anti-biofilm assay demonstrated that peptide YS12 was able to inhibit biofilm formation around 80% for both bacterial strains *E. coli* and *P. aeruginosa* at 80 μg/ml. Notably, YS12 exhibited a greater biofilm eradication activity than commercial antibiotics. Cytotoxicity towards mouse macrophage Raw 264.7 of peptide YS12 was measured. However, peptide YS12 causes a low level of hemolysis of red blood cells compared with the standard well-known antimicrobial compound melittin. In terms of mechanism of action, most AMPs do not target specific molecular receptors of pathogens, which is different from the mechanisms of traditional antibiotics. The results from fluorescent measurement revealed that peptide YS12 bound with LPS through electrostatic interaction. The principal mechanism of AMPs is that can act directly, interacting with cytoplasmicmembranes and intracellular molecules.

We carried out a membrane depolarization assay, N- phenyl-1-naphthyl amine (NPN) assay (which measures the permeability of the outer membrane),and propidium iodide assay (inner membrane permeability) to elucidate the possible mechanisms underlying the peptide's mode of action using different fluorescent dyes.

Peptide YS12 exhibited direct antimicrobial activity by damaging the integrity of the bacterial membrane. Finally, confocal microscopy confirmed that the peptide carried out an intracellular targeting mechanism by cell penetration. In summary, our study proposed that the antimicrobial peptide YS12 may be a promising therapeutic agent against antibiotic-resistant bacteria.

Keywords: Antimicrobial peptides (AMPs), Multidrug-resistant bacteria, Time-killing kinetics,Anti-biofilm activity, Fractional Inhibitory concentration Index (FICI); Mechanism of action, Fluorescence spectroscopy.

국문 초록

*Bacillus velezensis*에서 분리한 신규 펩타이드 YS12의 항미생물 활성 기전 연구 및 항생제 저항성 병원 미생물 제어 전략

수치 수지아 아카타

지도교수 : 유진철

조선대학교 약학대학 약학과

항생제의 남용으로 다제 내성 균주가 증가하는 가운데, 항균 펩타이드(AMPs)는 전통적인 항생제 대안으로 큰 주목을 받고 있다. 본 연구에서는, 한국의 전통식품인 김치에서 분리한 *Bacillus velezensis* CBSYS12 균주로부터 유래된 항균 펩타이드 YS12의 항균성, 항바이오필름 작용 및 작용 메커니즘을 규명하였다. CBSYS12 균주는 김치로부터 분리 후, 최적배지를 결정하여 대량배양 하였고, 배양액으로부터 ultrafiltration 및 sequential chromatographic 기법을 사용하여 정제하였다. Tricine-SDS PAGE를 통해 대략 3.3kDa의 단일

단백질 밴드를 확인하였고, 또한 같은 위치에서 anti-biofilm을 확인할 수 있었다. 비슷한 분자량(~3348.4 Da)의 단백질이 MALDI-TOF에서도 나타나, 펩타이드 YS12의 순도와 균일성을 확인하였다.

AMPs는 다제 내성 감염성 균에 대한 넓은 항균 스펙트럼과 미생물이 쉽게 내성을 개발하기 어렵다는 특성 때문에, 전통적인 항생제의 잠재적인 대체재로 간주된다. 본 연구의 결과는 *E. coli, P. aeruginosa*, MRSA 4-5, VRE 82, 그리고 *M. smegmatis*와 같은 그램 양성 및 그램 음성 균에 대해 6-12 μg/ml 범위의 최소 억제 농도(MIC) 값을 가진 강력한 항균 활성을 보여주었다. 펩타이드 YS12는 상용 항생제와 시너지 효과를 보여주어, 다제 내성 균에 대한 효과적인 치료 방법을 제시하였다. 더불어, 항 바이오필름 검사에서는 펩타이드 YS12가 80 μg/ml에서 *E. coli*와 *P. aeruginosa*의 바이오필름 형성을 약 80% 억제하였다. 특히, YS12는 상용 항생제보다 더 뛰어난 바이오필름 제거 활성을 보였다. 또한, 펩타이드 YS12의 마우스 대식세포인 Raw 264.7에 대한 세포독성도 측정하였다. 그러나, 펩타이드 YS12는 잘 알려진 항균 화합물인 멜리틴에 비해 적은 수준의 적혈구 용해작용을 보였다.

작용 메커니즘 측면에서, 대부분의 AMPs는 병원체의 특정 분자 수용체를 대상으로 하지 않는 것으

CHAPTER ONE

INTRODUCTION

1.1. Antibiotic development and resistance

One of the greatest medical achievements of the 20th century was the discovery of antibiotics [1].Salvarsan was one of the first drugs to successfully treat syphilis without causing harm to the patients. However, the development of antibiotics did not begin until 1928, when Alexander Fleming accidentally discovered penicillin. This led to the "golden age" of antibiotic research, which lasted between the 1950s and 1960s [2]. Between 1930 and 1962, more than 20 new typesof antibiotics were developed. But as new resistant bacteria have emerged, the pharmaceutical industry has found it more difficult than ever to find novel compounds with antimicrobial properties [3].

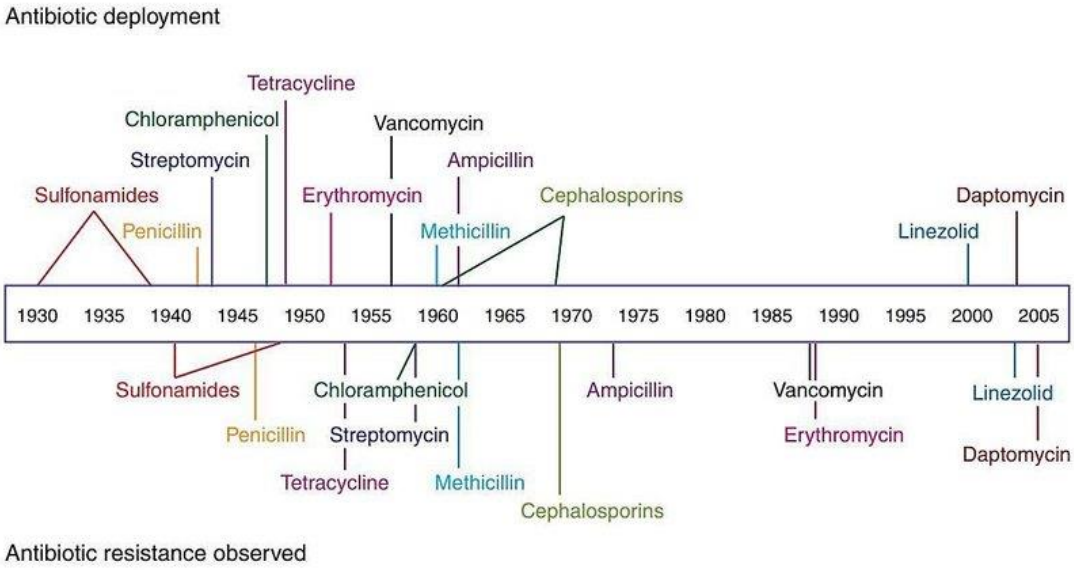

Figure 1.1. Antibiotic development and resistance

(Clatworthy, A.E. et al. 2007)

Overuse of antibiotics has promoted the growth and spread of bacteria that are resistant to medicines [4]. The extensive (over- and incorrect-) usage of antibiotics over the last few decades has encouraged the development and spread of resistant microorganisms. Due to the continued lack of research and commercial acceptance of effective novel antibacterial medications, clinically relevant bacteria's sensitivity to commonly used antibiotics continues to be a worldwide health issue. The rising occurrence of antibiotic resistance in bacteria is a major healthcare concern globally since drug-resistant infections increase morbidity, mortality, and overall healthcare expenditures in both human and veterinary medicine [5]. Antimicrobial-resistant diseases result in about 700,000 fatalities per year, and if this trend persists, 10 million deaths will be predicted by 2050. Antimicrobial resistance in bacteria is difficult to measure, but it is predicted to cost the European Union's economy roughly EUR 1.5 billion annually and the US healthcare system between $21 and $34 billion [6]. Antibiotic resistance is expected to result in higher morbidity and mortality rates, as well as enormous increases in healthcare costs and other indirecteconomic effects on the global economy. The history of antibiotic resistance vs antibiotic discovery is depicted in Figure 1.1, with a focus on how quickly bacterial resistance develops. Antibiotics act on different mechanisms on bacterial cell membranes presented in Figure 1.2

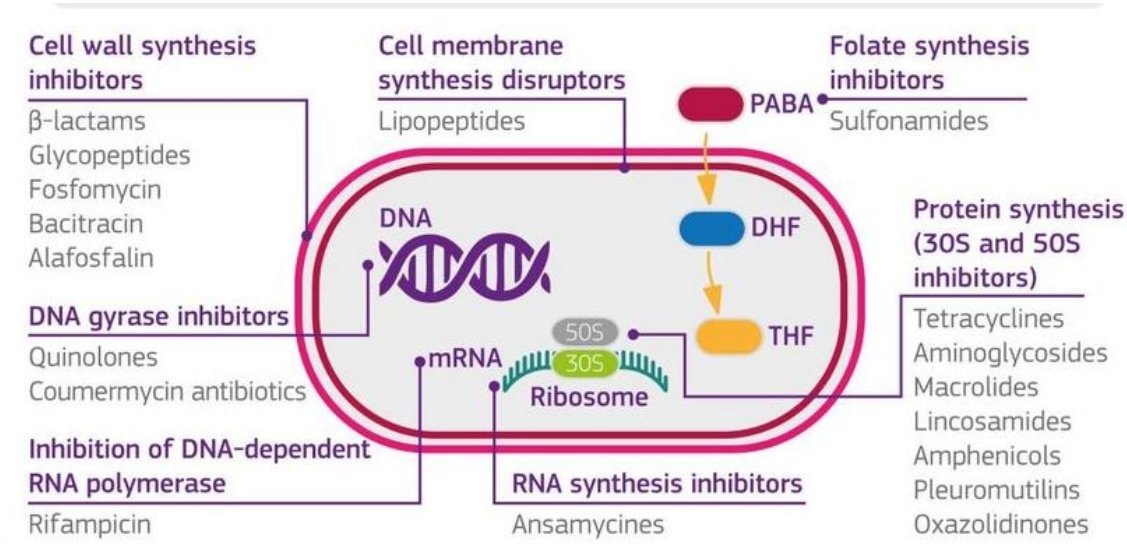

Figure 1.2. Antibiotic mechanism pathway (Sanseverino, I. et al. 2018).

1.2. Alternatives to traditional antibiotics

The rapid growth of antibiotic resistance has attracted researchers to discover safe, efficient, therapeutic, and alternative strategies to treat complicated drug-resistant infections [7]. The pharmaceutical industry has consistently accomplished this need by efficiently developing new antibiotics along with modifications to older antibiotics. Thesesuccessful attempts led to the broad range of antibiotic drug classes that are currently on the market,including beta-lactams (penicillin, carbapenems, and cephalosporins), glycopeptides, macrolides, ketolides, aminoglycosides, fluoroquinolones, and oxazolidinones. Numerous investigations on non-antibiotic approaches to destroying harmful microbes, such as the use of bacteriophages, synthetic antimicrobial peptides, and naturally occurring bacteriocins, are being prompted by the phenomenon of antibiotic resistance. The search for new secondary antimicrobial compounds from bacteria has become more intense due to the abundance of uncharacterized biosynthetic gene clusters (BGCs) and an increasing level of interest in natural product development. The effectiveness and affordability of vaccines as a technique for preventing bacterial infectious illnesses have made them prevalent in earlier times. Even when new technologies and vaccinationpractices emerge, there are still many obstacles to overcome, including novel and volatile infections, complicated

illnesses (such as polymicrobial diseases), and hard-to-reach population.

1.3. Bioactive peptide

Bioactive peptides are distinct protein fragments that have been found to have a significant impact on functions in the body and conditions that could potentially affect health [8]. Peptidomics is the meticulous study of all the peptides in a biological sample using quantitative and qualitative methods. With the aid of current separation, analysis, and computing technology, an emerging field has emerged from proteomics. Bioactive peptides are proteins that the cell synthesizes as propeptides, then cleaves and transforms into usable products [9]. Bioactive peptides play crucial functions as signaling agents in disease and physiological activities.

1.4. Increasing Interest in New Bioactive Antimicrobial Peptides

Compared to times before the COVID-19 pandemic, the search for antibacterial bioactive substances assumed higher importance. While their variety offers a range of opportunities that are still largely unexplored, new biomolecules could play a significant role in the fight against infectious diseases. Antimicrobial peptides are one class of biomolecules that are attracting significant scientific attention (AMPs) [10,11]. AMPs are a novel class of therapeutic drugs aimed at solving the underlying issue of pathogen invasion, possessing a variety of characteristics that includes tiny size, quick

activity, and minimal risk of the pathogenic targets developing resistance [12,13]. They are ancestral molecules in evolution, having initially appeared in living things more than 2.6 billion years ago [14]. AMPs played a crucial part in the multicellular organisms' success in evolution. Such compounds are still effective weapons for organism defense against bacteria, fungi, viruses, and protozoa [15,16,17].

1.5. Antimicrobial peptide

However, the rapid rise of resistance poses a more significant tissue for life-threatening viral infections [18]. Small biological molecules known as antimicrobial peptides (10 kDa) are produced by prokaryotic microbes and complex eukaryotes (e.g., humans, animals, plants, bacteria, and fungi) as a part of innate immunity [19, 20]. Although anionic peptides have also been described, most AMPs are cationic. The microbial membrane is primarily the target of cationic AMPs, whereas anionic AMPs typically have intracellular targets (such as ribosomes) [21]. Compared to traditional antibiotics, AMPs are now under intensive investigation as promising antimicrobial agents for combating drug-resistant pathogens.

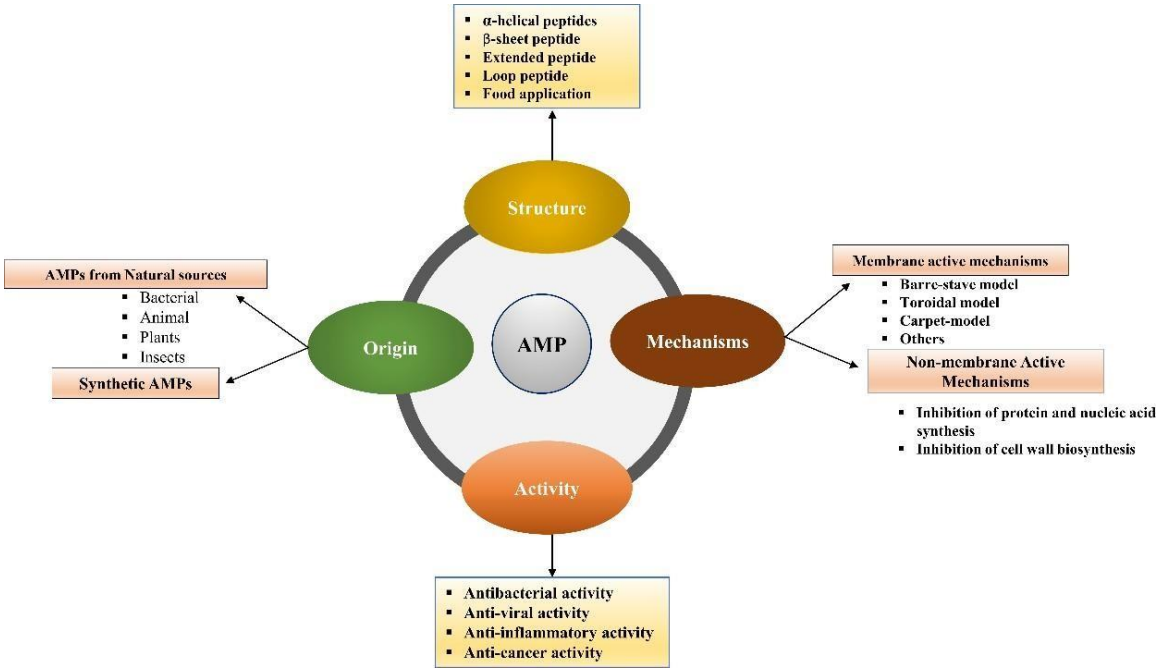

Figure 1.3. Overview of antimicrobial peptides (AMPs)

1.6. Bacteriocins

Bacteriocins are small, thermally stable peptides produced by bacteria that are used by certain microorganisms to compete with other members of their species or with bacteria from different genera (broad-spectrum). They are generated by bacteria and some types of organisms with the aim of preventing the growth of bacterial strains that are similar to or closely related to them. These substances are capable of killing harmful and degrading microorganisms, which indicates their biotechnological potential.

1.7. Structure of Antimicrobial Peptides

Antimicrobial peptides are small molecules (12–50 amino acids) consisting of a positive charge andan amphipathic structure. AMPs can be categorized into four primary groups according to their secondary structures [22].The structure of the -helical AMPs, which include cecropins, magainins, and LL-37, is distinguished by an incredibly high tendency for α -helix formation. Human α - and β -defensins, plectasin, and protegrins are slightly rigid AMPs with two to four disulfide bridges stabilizing them. It is anticipated that understanding the mode of action used by AMPswill be significantly aided by knowledge of their structures, which will also accelerate the developmentof novel AMPs that are more appropriate for pharmaceutical applications.

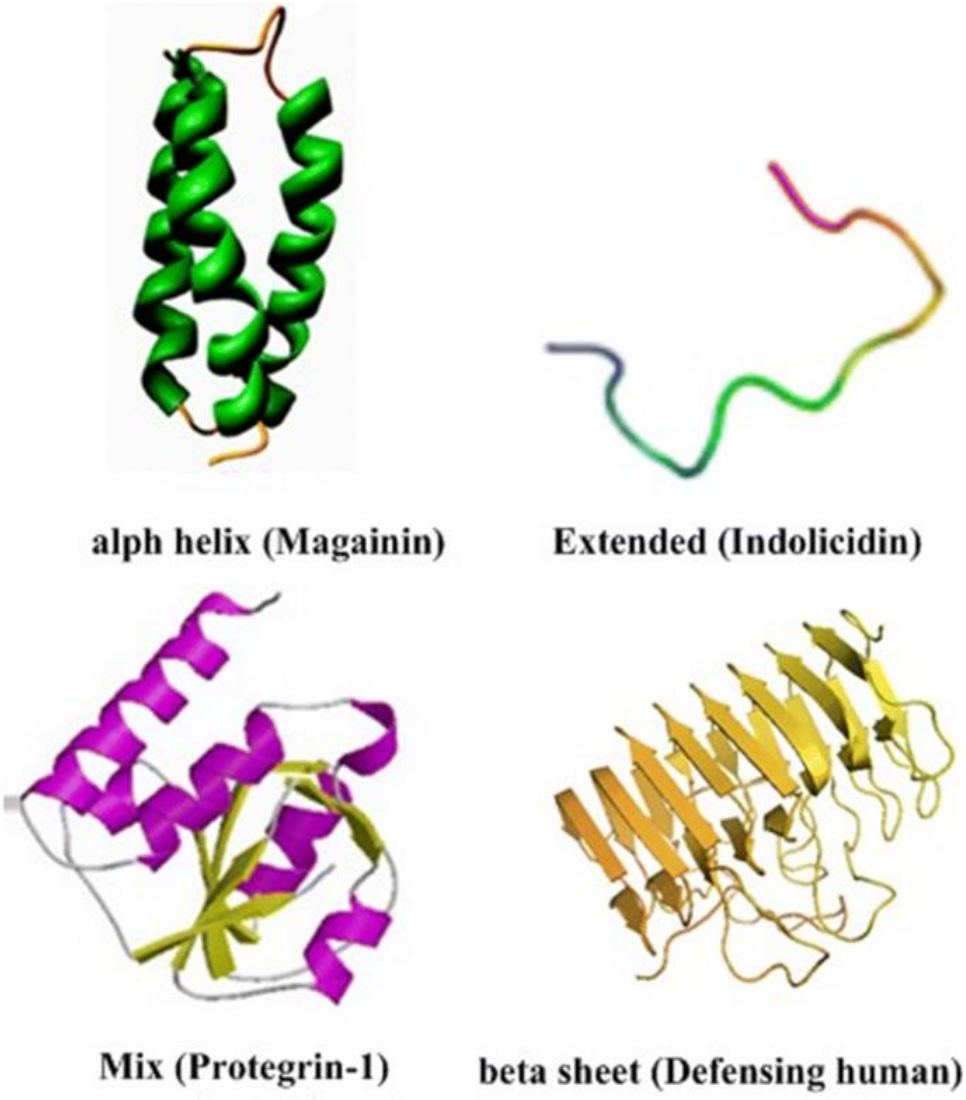

Figure 1.4. Examples of three-dimensional conformations of antimicrobial peptide (Seyfi, R. et al. 2020)

1.8. Production and Processing of Bioactive Peptides Derived from Food Protein

1.8.1. Enzymatic Hydrolysis

The commonly used methods for producing bioactive peptides are fermentation and enzyme hydrolysis of protein from food [23]. In this procedure, the protein material is hydrolyzed by enzymes at a specific pH and temperature [24,25]. Enzymatic hydrolysis is strongly suggested for producing bioactive peptides than microbial fermentation owing to a rapid reaction time, predictability, and ease of scalability. No known proteolytic enzymes produce particular bioactive peptides in food, are typically produced when subtilisin is hydrolyzed. However, to achieve a high degreeof hydrolysis, the enzyme-to-substrate ratio must be taken into consideration.

1.8.2. Microbial Fermentation

This involves growing specific microbes on protein substrates to hydrolyze the proteins using their enzymes. The bacterial growth secretes its proteolytic enzymes into the protein substrate to facilitate the biopeptides release from parent proteins. The selected strain, protein type, and the period of fermentation all affect how much proteinis hydrolyzed. Since various microbes have different proteolytic systems, protein hydrolysates may have varying functional properties depending on the culture [26].

Additionally, peptides can be synthesized upon confirmation of the composition of a bioactive peptide. Three methods are widely used at present.

i. Synthesizing compounds

ii. Enzymatic synthesis and

iii. Recombinant technology with DNA

1.9. Fermented foods

Fermented foods have unique functional qualities which provide some health advantages to consumers due to the presence of beneficial microorganisms with probiotic, antimicrobial, antioxidant, and peptide-producing characteristics. Some health advantages of globally consumed fermented foods include nutrition synthesis, diabetes prevention, cancer prevention, cardiovascular disease prevention, gastrointestinal disorders prevention, and allergy reaction prevention.

1.10. Fermented foods in Korea

Throughout the year, both Koreans and many other people throughout the world consume a wide variety of fermented foods and beverages, which constitute a crucial component of Korean cuisine.These fermented food items are appreciated by people worldwide and have gained popularity in Western nations. In Korea, the major fermented food items can be categorized into three groups.

Category	Name	Preparation process	Photo	Medicinal value
First category	Chongkukjang	Short fermentation of cabbage with an additional ingredient		Rich in vitamins, minerals, antimicrobial, antioxidant etc.
	Doenjang	Fermentation of fried soybean seeds using naturally existing microorganisms		Anti-cancer, anti-oxidative activity
	Gangjang	Fermented soybean is used to make soybean sauce.		Antioxidant potential
	Gochujang	Red chili powder paste that has been fermented		Anti-oxidant.
Second category	Fish and shellfish products	Fermented fresh fish and shellfish		An essential source of vitamins, protein, and minerals.
Third category	kimchi	Short fermentation of napa cabbage with other components		Antibacterial, anti-oxidant, anti-obesity, immune potential, source of functional foods

1.11. Kimchi fermentation and its microorganisms

According to the level of acidity, the fermenting process for kimchi typically involves four stages: the initial stage (acidity of 0.2), the immature stage (acidity of 0.2–0.4), the optimal ripening stage(acidity of 0.4–0.9), and lastly the overripening or rancid stage (acidity of >0.9). As the kimchi ferments, the LAB profile changes with various factors, including pH and acidity.

1.12. Mode of action of AMPs

Although several hypotheses have been suggested to evaluate the molecular mechanisms caused by AMPs, it still needs to be explained. Different Bacillus species produce bacteriocins or BLSs with various mechanisms. The majority of bacteriocins are hydrophobic or amphiphilic cationic peptides [27]. Four primary steps are involved in killing bacteria; forming membranes, membrane binding, aggregation within the membrane, and channel development [28, 29]. Channel formation causes leakage of intracellular cell contents, which ultimately results in cell death. AMPs must pass through the negatively charged lipopolysaccharide (LPS)-containing the outer cell wall of Gram-negative bacteria or the acidic polysaccharide-containing outer cell wall of Gram-positive bacteria. [30].

Lipopeptides can immediately attach to the microbial membrane and modify the local lipid organizational linkages on negatively charged fatty acids [31]. The AMPs accumulate initial electrostatic and hydrophobic interaction with the microbial membrane. To explain how AMPs work on bacterial membranes, several models (Carpet model, toroidal pore and barrel-stave model) have been employed. Models can be classified into two different types: transmembrane pore models and non-pore models. The toroidal pore and barrel-stave pore models are additional subcategories of transmembrane pore models.

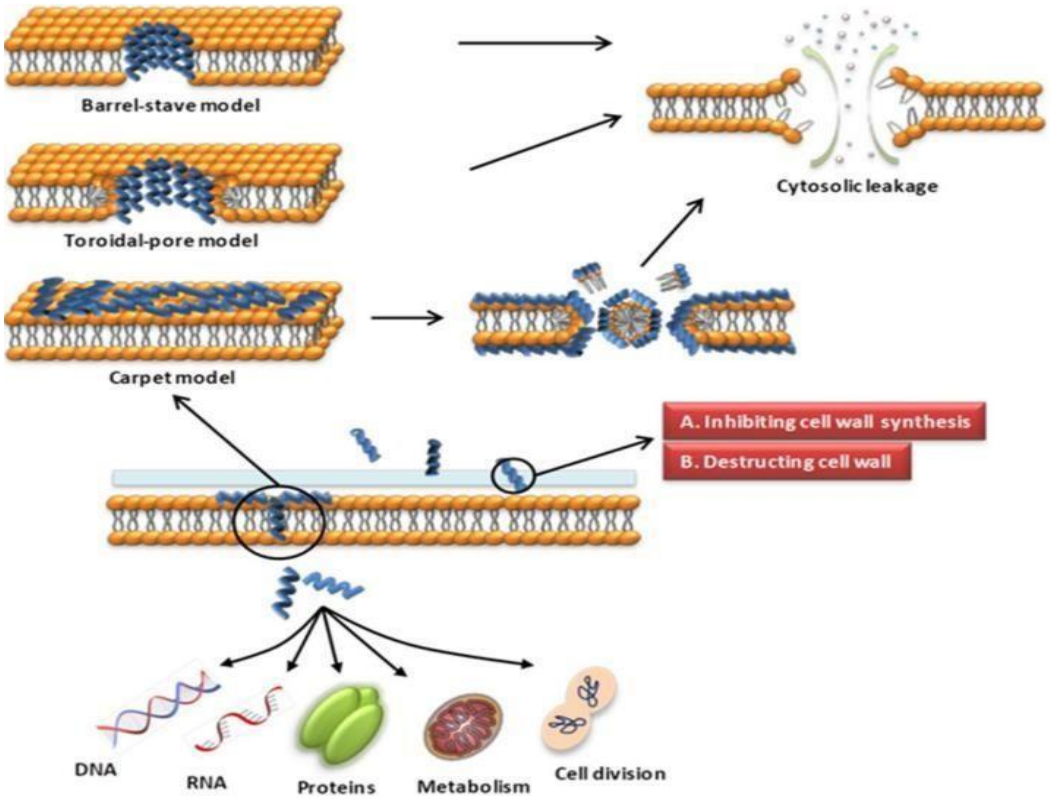

Figure 1.5. Schematic presentation of the antibacterial mechanisms of AMPs

(Xin Li. et al. 2022)

1.13. Resistance to AMPs by Gram-positive and Gram-negative bacteria

In Gram-positive bacteria, low G+C concentration and resistance modules frequently facilitate processes that lead to antimicrobial resistance [32]. Multiple mechanisms exist for gram-negative bacteria to impede natural AMPs. Active AMPs can be degraded into inactive forms by bacterial proteases that are released at the outside membrane of Gram-negative bacteria. Secondly, AMPs canbe resistant to certain types of polysaccharides that exist in bacterial cell envelopes, including capsule polysaccharides, exopolysaccharides that form biofilms, and the polysaccharide of lipopolysaccharide. These polysaccharides bind with AMPs and inhibit AMPs from penetrating the bacterial membrane [33]. Thirdly, lipopolysaccharides, the exterior membrane of Gram-negative bacteria, play a significant role in the resistance to AMPs through altering. Finally, another method of resistance involves pumping AMPs into or out of cells via members of the transporter and resistance nodulation-division (RND) efflux pump.

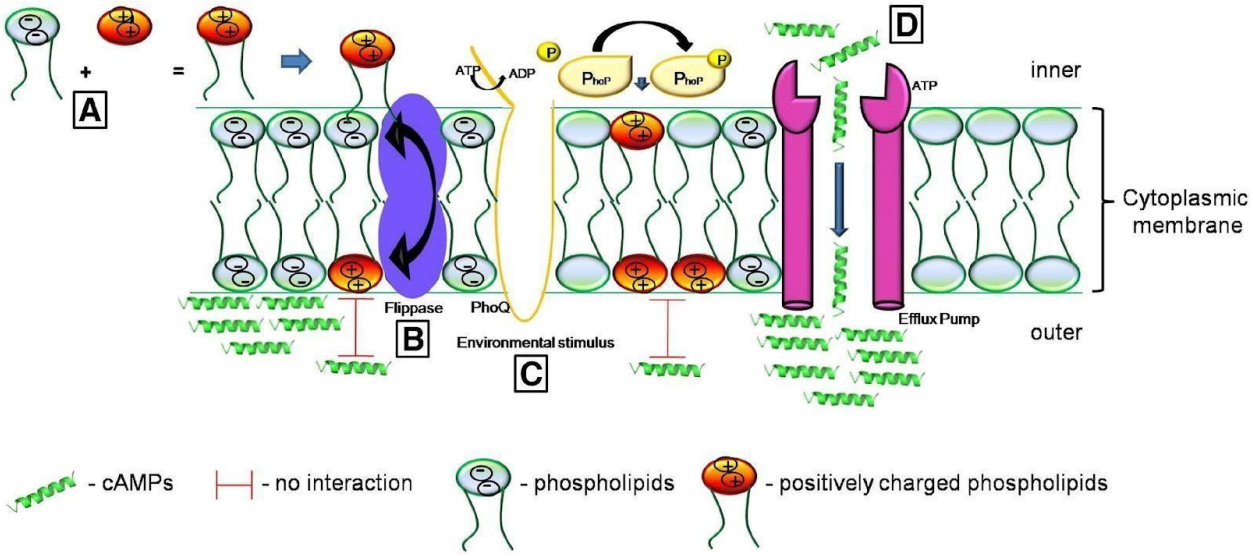

Figure 1.6. Membrane alterations that influence AMPs bacterial resistance

(S Maria- N, et al, 2015)

1.14. Challenges

Although the benefits of using AMPs are well-established due to their antibacterial properties, onlya small number of them are undergoing clinical trials and research**.** Despite the early promise of AMPs as alternatives to antibiotics, many of them are not suitable for therapeutic applications dueto their high manufacturing cost and ease of degradability. There were initially two methods for extracting antimicrobial peptides: chemical production or separation from the host organism [34]. After separating these protective molecules from host tissues through a number of fractionation and purification procedures, microbiological tests were conducted to ascertain their bactericidal capabilities. Using this method, several cationic peptides from various species have been detected. However, this AMP isolation method is laborious, necessitates large volumes of the parent material, often produces low yields, and needs to be revised for clinical studies [35].

On the other hand, chemical peptide synthesis utilizing the current solid-phase techniques is more rapid, simpler to automate, and requires fewer purification steps. However, the high expense and difficulty of integrating extensive post-translational enhancements limits chemical AMP production. The heterologous expression of eukaryotic AMPs by microbes, such as lactic acid bacteria, may also be a cost-effective technique for medicinal and practical studies [36]. The production of AMPs by genetically modified microorganisms is remarkable due to the low costs, quick growth on affordable substrates, predictable laboratory settings, relatively well-characterized genetic histories, and the availability of a wide range of vectors and host strains. Despite the possibility of isolating or creating AMPs using chemical synthesis or microbial cell factories, the cost of producing sufficient numbers of AMPs for therapeutic application exceeds the cost of antibiotics already in use. Another major obstacle to AMPs' development as therapeutics is their sensitivity to salt [37]. However, recent research employs a more comprehensive strategy to design AMPs that overcome many existing challenges.

1.15. Strategies to improve AMPs

Directly extracted AMPs from natural sources must be adequately diverse for therapeutic use. Nevertheless, they have the potential to improve their efficacy, safety, and stability by undergoing a variety of tactics, such as current developments in recombinant technologies, appropriate fusion protein partners, and other molecular engineering strategies for enhanced AMP synthesis.

PURPOSE AND SUMMARY OF THIS THESIS

It has been proposed that the therapeutic use of AMPs could provide an alternative strategy for controlling the spread of pathogenic microorganisms that are multidrug-resistant (MDR). This study investigates the effectiveness of naturally occurring AMPs in treating illnesses caused by various pathogenic bacteria. Our laboratory used fermented foods fromseveral regions of Korea to isolate about 200 distinct bacterial strains. Around the world, traditional household processing is still used for about 90% of naturally fermented foods and alcoholic beverages. Fermented foods contain a variety of naturally occurring microbes, both beneficial and detrimental.

- To screen microbial strains producing relatively higher bacteriocin of interest and identify the strain
- To purify and characterize with maximum purity by using several purifications steps
- To biochemically characterize the purified peptide
- To estimate the antimicrobial and anti-biofilm activities of the antimicrobial peptide againstseveral pathogenic microbes.
- Exploring the antimicrobial peptide's proposed mode of action against Drug-resistant bacteria.

CHAPTER TWO

Isolation, identification, and purification of CBSYS12

2.1. Introduction

Naturally derived or originated compounds play a major role as drugs [38]. Over 60% of authorizedmedications and therapeutic candidates either come from natural sources or are derived from them. A total of 100,000 secondary metabolites with molecular weights under 3500 Da have been identified and originated from both microorganisms and plants. Consequently, research investigating AMPs isolated from various microorganisms has dramatically increased [39]. AMPsand bacteriocins have attracted significant interest as antimicrobial agents against bacteria. AMPs are small molecular weight proteins (2-9kDa) produced by microorganisms that are considered as novel antimicrobial agents. There are a number of *Bacillus* peptides with biological activities that are currently being investigated for their potentialantibacterial properties. Fermented vegetables have long been used as a source of beneficial microorganisms. Koreans have been eating salted and fermented vegetables like kimchi for more than 3000 years. Korean traditional food kimchi is food used as a relevant resource for isolating microorganisms such as *Bacillus* and lactobacillus that can produce promising antimicrobial agents and other valuable compounds [40].

Importantly, kimchi is rich in nutritional fiber, vitamins, minerals, and other essential components. Kimchi has been shown in numerous earlier research to have anticancer, antimicrobial, antioxidant, anti-atherosclerotic, and anti-obesity properties. Bacteriocins have garnered a lot of attention as antimicrobial agents. However, researchers found that the AMPs produced by microbes *Bacillus* are capable ofproducing a variety of active proteins that can act broadly against different pathogenic bacteria and their collaborators [41]. The genus *Bacillus* bacteria can produce a wide range of peptides and bacteriocins [42]. The production and characterization of AMP by *Bacillus* strain obtained from fermented food kimchi have been explored in this study. The nutritional and optimum conditions have a great influence on the growth of antimicrobial cells [43]. An experimental strategy called Response Surface Methodology (RSM) is frequently used for optimizing the culture media of antimicrobial agents [44]**.** We effectively utilized RSM to evaluate the potential effects of different nutritional media.

2.2. Methods and Materials

2.2.1. Materials and bacterial strains

The strain CBSYS12 was mainly derived from fermented Korean food kimchi. The columns Sephadex G-50 and DEAE Sephadex A-50 column were purchased from Pharmacia (Uppsala, Sweden). Commercial media (MRS) De Man, Rogosa and Sharpe, and Muller-Hinton (MH) were acquired from Becton-Dickinson (Spark, USA).

2.2.2. In vitro screening, isolation, and identification of the antimicrobial peptide-producingstrain

The bacterial strain CBSYS12, which is able to generate an AMP, was derived from kimchi. The isolation process was conducted by combining 1 gram of raw kimchi with NaCl (0.85%) while incubating at 37 °C for 24 h, according to our previous article [45]. The mixture was carefully incubated before being sequentially adjusted up to 10^{-7} in (MH) broth. The sample was streaked onto the surface of MHB plates to check the proper CFU, and the diluted sample was maintained and kept at -70 °C. Strain CBSYS12 identification was mainly established based on morphologic characteristics. For further identification of strain, the phylogenetic tree wasconducted by analyzing the 16S ribosomal RNA (rRNA) gene sequences [46].

The 16s rRNA sequence was submitted to the Gen bank, and an isolated strain has been preserved at Korean TypeCulture Collection (KTCC).

2.2.3. Effects of Nutrition Media

Optimization of the nutrient medium for strain CBSYS12 was carried out utilizing media supplementedwith various percentages (0.25%, 0.50%, 0.75%, 1%, 1.25%, 1.50%, 1.75%, and 2%) of carbon sources such as maltose, glucose, sucrose, starch, mannitol, lactose) and nitrogen sources, including tryptone, yeast extract, peptone, oatmeal, soymeal, and beef extract. Furthermore, different metal ions origins such as $MgCl_2$, NaCl, $CaCl_2$, KH_2PO_4, $ZnSO_4$, $FeSO_4$, Na_2HPO_4, and KCl in variousproportions (0.005%, 0.0075%, 0.01%, 0.0125%, and 0.015%) were also used for optimizing the culture media. As a control medium, commercially available MH and MRS broth media were employed.

2.2.4. Statistical bioprocess design and optimization of culture media through RSM

The peptide YS12 derived from the Bacillus strain exhibited significant antimicrobial activity when peptone, maltose, and NaCl were used as nitrogen, carbon, and metal ion source, respectively in the production medium. The optimization of culture media was assessed to determine the optimum levels of significant variables. The Box-Behnken experimental design was adopted to measure the maximum peptide production using design-Expert Software [47]. In this model, the most significant variables, namely peptone (A), maltose (B), and NaCl (C) were included. A partial factorial model was used to conduct a total of seventeen exploratory runs. The expected bacteriocin activity (AU/mL) is provided by the software. The statistical impacts of each important variable on bacteriocin activity were identified.

2.2.5. Production and purification of YS12

The production procedure was incorporated by inoculating the strain CBSYS12 in 250-ml flasks filled with 50 ml of MRS broth under continuous agitation on a shaker at 37 °C (150rpm). The seed culture was then transferred aerobically in a 2-L baffled flask including 300 ml of optimum medium analyzed by RSM, kept at 37 °C, and agitated at 150 rpm. After being cultivated for 40 h, the fermented broth was centrifugated at 10,000 rpm for 30 minutes.

Ammonium sulfate (20-60% saturation) was included to the supernatant and was left at 4 ℃ overnight. The next day, the pellets were retrieved and dissolved in Tris-HCl (pH 7) buffer. In this step, the precipitate was fractionated through ultrafiltration membranes (Billerica, Massachusetts, USA) using a molecular mass cut-off of 30 and 10 kDa (Millipore Corp), respectively. Furthermore, peptide components (MM <10kDa) thus obtained were purified by operating Sephadex G-50 column (1.5×65cm) pre-equilibrated with 10mmol/L pH 7 buffer. Fractions having antimicrobial activity wereconcentrated, followed by lyophilization, and finally purified utilizing the DEAE Sephadex A-50 column (1.2×35 cm). Collected samples were extracted applying chloroform precipitation. The antimicrobial activity of the samples was checked against *Mycobacterium smegmatis* .

2.2.6. Protein estimation and analysis of molecular weight

The Bradford method was executed to measure the peptide concentration, whereas bovine serum albumin (Bio-Rad) was used as a standard [48]. To reveal, the homogeneity with apparent molecular mass, the antimicrobial peptide YS12 was exposed to Tricine SDS-PAGE) [49]. The gel was stained with Coomassie Brilliant Blue R-250 and subsequently distained with a (1:8:1, v/v/v) solution of methanol, distilled water, and glacial acetic acid. For in situ evaluation, the gel was successively cleaned with 50 mmol/L Tris-HCL pH (7.0) buffer consisting of Triton X-100 2.5% (v/v). The processed gel was then

repeatedly washed and placed in a petri dish with agar (0.6 %) over Muller-Hinton broth (DIFCO, San, CA, USA), containing indicator strain *Mycobacterium smegmatis* (about 10^8 CFU), and evaluated the inhibition after overnight incubation .

2.2.7. MALDI-TOF/MS analysis

The molecular mass of peptide YS12 was determined utilizing MALDI-TOF/MS analysis by Ultra- flex III (Burker, Bremen, Germany) [50]. The tests were carried out on mass spectra ranging from500 to 40,000 m/z.

2.2.8. Stability of YS12 against pH, temperature, and protease enzymes

To evaluate the thermal stability of peptide YS12, the supernatants were treated at 20, 30, 40, 50,60, 70, 90, and 100 ℃, kept for 30 min, and 121 ℃/105 kPa for 15 min (autoclave condition) before testing the remaining activity [51]. Additionally, the peptide sample was exposed to different pH values (4-12) and assessed for antimicrobial activity using the disc diffusion method. The control reaction was carried out without peptides to determine residual activity.

Additionally, the influential role of different protease enzymes on the activity of peptide YS12 wasalso tested. YS12 was incubated with trypsin, proteinase, and chymotrypsin (final concentration 1mg/ml) prepared in 10mml/L Tris-HCl (pH 7.0) buffer, incubated for 2, and 4h and residual activity was determined.

2.3. Result and Discussion

2.3.1. Isolation and identification of antimicrobial peptide-producing strain

In recent years, the isolation of novel antimicrobial peptides from microorganisms has increased dramatically. This part of our study explained the isolation and identification of the strainCBSYS12. The 16SrRNA-gene sequence indicated that the isolated strain showed a maximum homology of 99.9% with the Bacillus strain.Nowadays, 16SrRNA is the most extensively used method for bacterial species identification dueto its substantially higher percentage accuracy [52]. Based on the 16S rRNA gene sequencing, the isolated strain was identified as *Bacillus velezensis* CR-502CBSYS12 and was deposited at the Gen-Bank under the accession number AY603658. In Fig. 2.1, the neighbor-joining phylogenetic tree is presented. Therefore, many bacterial strains producing AMP were isolated from kimchi found in numerous studies [53,54].

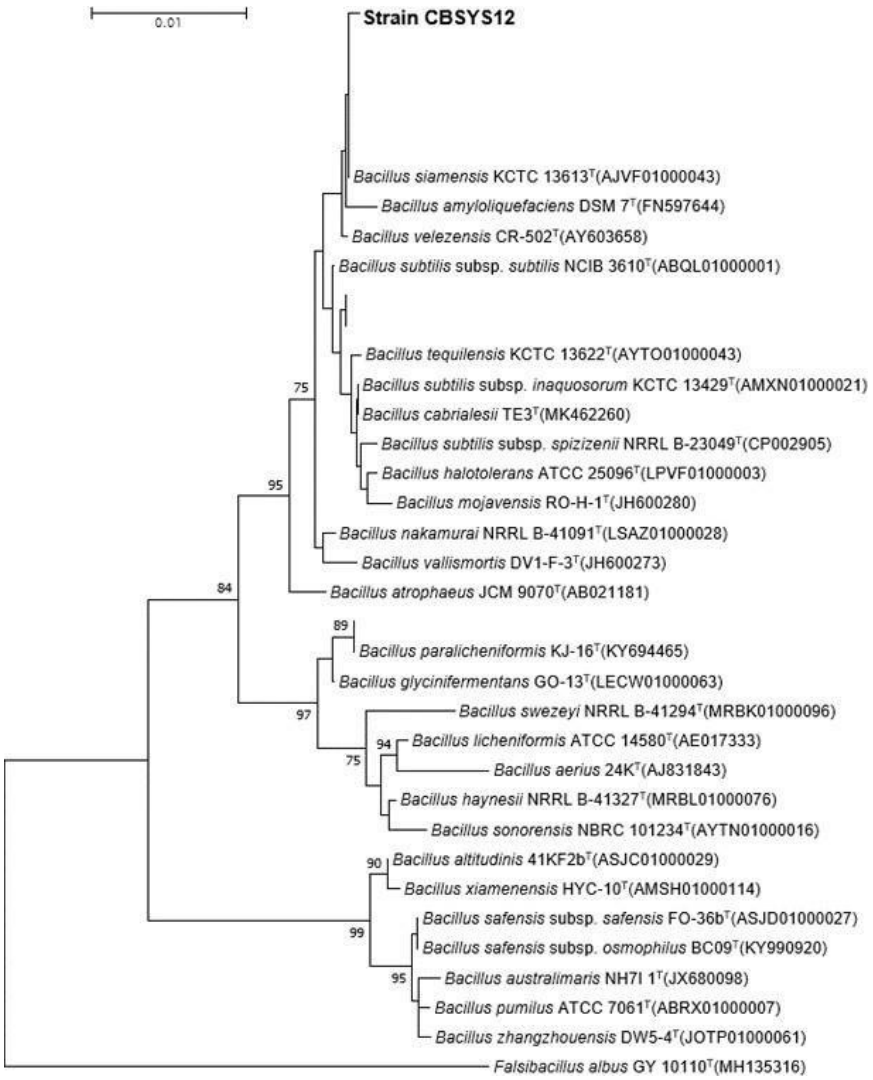

Figure 2.1. The phylogenetic tree is based on the entire 16S rRNA gene sequence, illustrating therelationships between strain CBSYS12 and some closely related *Bacillus* taxa. Reference sequences were obtained from Gen-Bank using the accession inparentheses.

2.3.2. Optimization and production of antimicrobial peptide

It has been envisaged that culture media, including carbon, nitrogen, and metal sources, have enormous potential for promoting microbial growth and peptide production [55]. The optimum culture medium for producing antimicrobial compounds was emphasized. The strain was grown inmedia containing carbon, nitrogen, and metal ion sources at 37º C at 150 rpm. In the caseof carbon sources (0.25%-2%), maltose (1.25%) caused the maximum production for peptideYS12.Peptone (1%) stimulated the highest output under the impact of different nitrogen sources (0.25%–2%), and NaCl (0.01%) seemed to be the best among all metal sources (0.005%-0.015%). The composition of growth media has a significant influence on the production of antimicrobial substances [56]. Finally, maximum production was obtained in optimized media (1.25% maltose,1% peptone, and 0.01% NaCl) analyzed by RSM based on our previous report [57]. A significant increase in the production of the peptide YS12 was detected at 24 to 48 h (Fig. 2.2 a). The peptide production was increased to the highest at 40 h, showing 17 mm of the clear zoneof inhibition after 12 h of growth (Fig. 2.2 b).

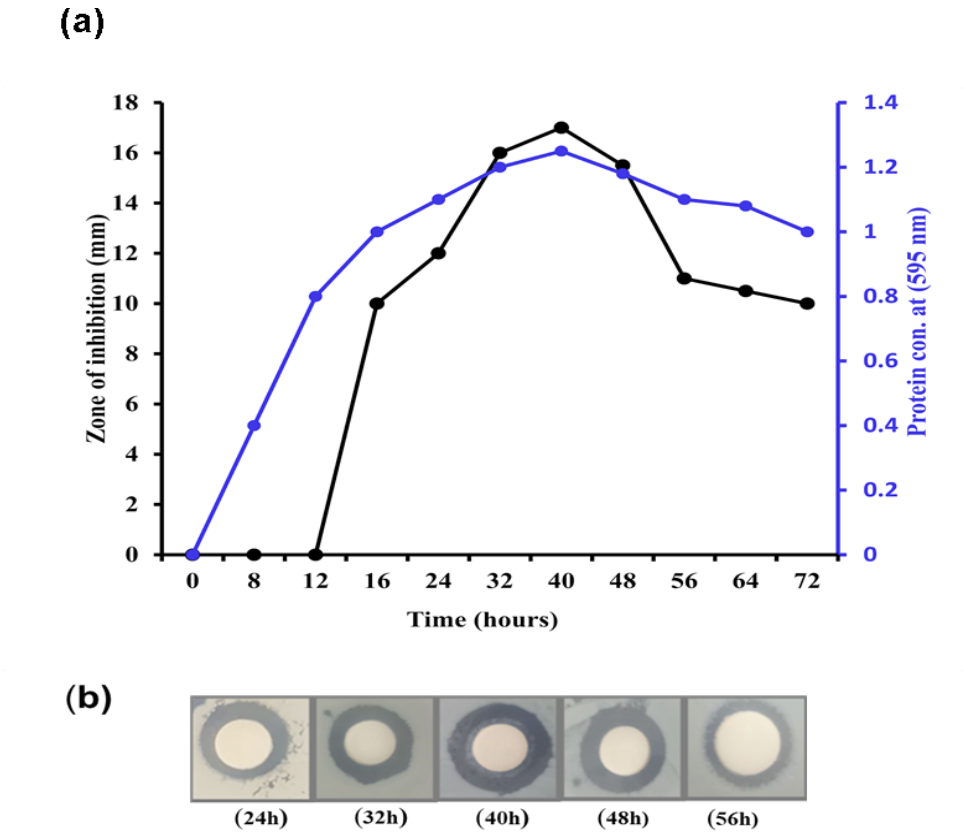

Figure 2.2. (a) The zone of inhibition and protein concentration for the indicator strain is presented at each 8-hour interval. Cultivation took in 250ml flasks containing the medium with 150 rpm on a shaking incubator at 37 °C, (b) At 40 hours, the maximum activity is observed against *Mycobacterium smegmatis* ATCC 9341.

2.3.3. Design of experiments and Box-Behnken analysis using RSM

RSM was applied to assess the influence of three significant variables: (A) maltose, (B) peptone (C) NaCl, yielding a set of experiments (Table 1.1).

Table 2.1. Numerous independent variable levels (-1, 0, +1) were used for optimizing the culturemedia.

Factor	Name	Units	High level (+1)	Centre point 0	Low level (-1)
A	Maltose	g/L	20	15	10
B	Peptone	g/L	15	10	5
C	NaCl	g/L	0.15	0.1	0.05

The equivalent experiment was carried out in four duplicates, and our actual value was obtained close to the predicted value provided by the model analysis suggesting the efficacy of the experimental and response surface methodologies in the optimization of the culture media. The findings (Table 1.2) showed that the three compounds maltose (A), peptone (B), and NaCl had higher values as a result of the degree of independent amount choice and were further examined at three distinct levels of highest experimental value (12,505 AU/mL) and the lowest value (9,174 AU/mL) were assessed using RSM in production media comprising maltose, peptone, and NaCl respectively.

Table 2. Experimental design of the actual and predicted level of variables in the Box-Behnken experiment.

	Maltose (g/L)	Peptone (g/L)	NaCl (g/L)	Bacteriocin	Activity (AU/ml)
Run order	**(A)**	**(B)**	**(C)**	**Actual value***	**Predicted value**
1	20	10	0.15	11478	11478
2	10	10	0.05	10428	10428
3	15	5	0.1	11471	11471
4	20	10	0.15	12505	12505
5	15	15	0.1	9842	9842
6	15	10	0.1	9174	9174
7	20	5	0.15	12438	12438
8	10	15	0.1	9998	9998
9	10	10	0.05	9674	9674
10	15	5	0.1	10124	10124
11	20	10	0.15	11027	11027
12	15	10	0.1	12348	12348
13	10	15	0.1	11426	11426
14	10	15	0.05	10245	10245
15	15	5	0.1	9876	9876
16	15	10	0.15	9654	9654
17	20	10	0.05	12409	12409

* From the mean of the triplicate experiment, actual bacteriocin activity was determined. To find the optimum media, the two levels of each variable were applied.

Bacteriocin activity was assessed using an ANOVA test. Following an ANOVA analysis of the results, we noted that three linear coefficients (A, B, and C) and three interaction coefficients (AB,AC, and BC) in the bacteriocin activity were determined to be statistically significant. The highestpeak for bacteriocin activity was observed at 0.1 g/L NaCl, 12.5 g/L maltose, and 10 g/L peptone(Fig. 2.3). Several regression analyses were applied to investigate data to produce the final equation to represent bacteriocin activity. Thus, the experiment design using RSM had potential use for the optimization of the fermentation medium [58].

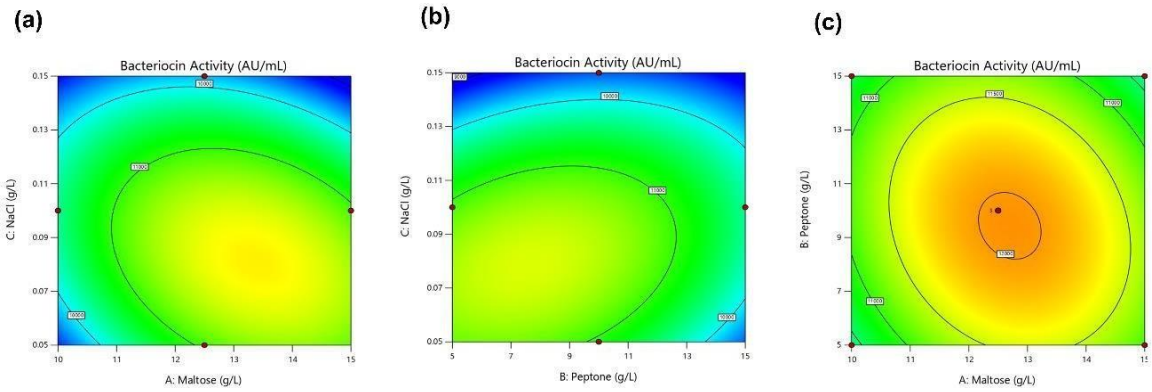

Figure 2.3. (a) The interaction of maltose (g/L) with peptone (g/L) (b) The interaction of peptone (g/L) with NaCl (g/L) (c) The interaction of maltose (g/L) and NaCl(g/L) was adjusted for bacteriocin activity.

2.3.4. Purification and molecular weight identification of YS12

The highest production of YS12 was accomplished in the MPN (1.25% maltose, 1% peptone, 0.01% NaCl) media at 150 rpm at 37 ºC. YS12 was purified from culture-free supernatants (20- 40% ammonium saturation), followed by ultrafiltration and sequential column chromatography. Toget low molecular weight peptides from a crude sample, ultrafiltration is an efficient purification technique [59]. Thus, the peptide sample was separated through 30kDa and 10kDa filtration membranes. Purification and fractions (MW<10kDa) of the peptide YS12 were then employed forhomogeneity by column chromatography procedure using Sephadex G-50 column (Fig. 2.4 a) andDEAE Sephadex A-50 column (Fig. 2.4 b).

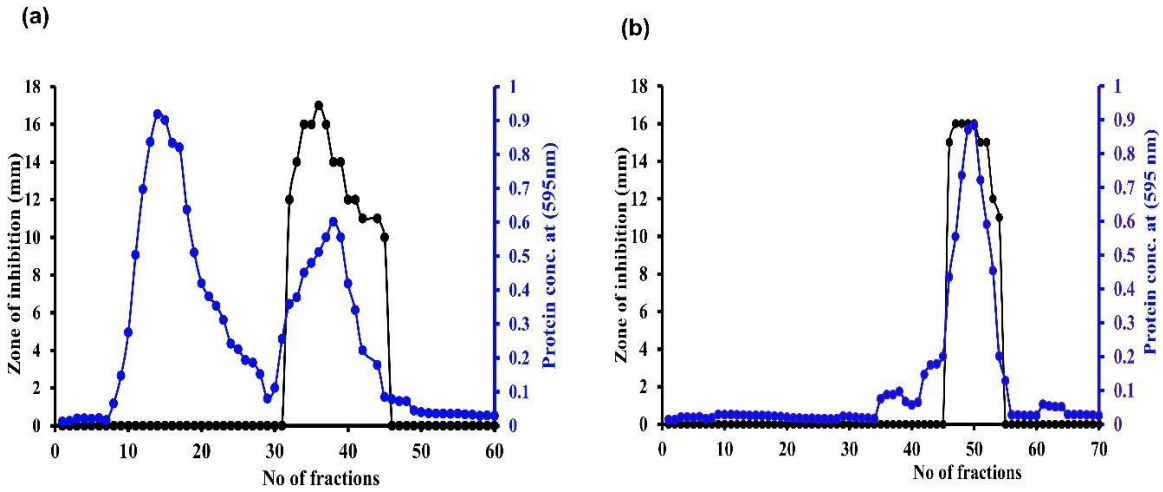

Figure 2.4. The elution profile of peptide YS12 (a) Sephadex G-50 column (2.5×85 cm) and (b) DEAE Sephadex A-50 (1.5×37 cm) column.

The antimicrobial peptide purification was done using column chromatography reported inprevious studies [60]. The purification of YS12 exhibited a very effective step at column chromatography from where the antimicrobial activity eluted free of mostly undesirable proteins. The purification process of strain CBSYS12, which resulted in a total activity of 31,000 AU witha 30.17-fold and a final recovery of 7.73%, was summarized in Table 1.3.

Table 3 Purification steps of YS12

Purification steps	Vol	Total protein	Total activity	Specific activity	Purification
	(mL)	(mg)	(AU)	(AU/mg)	Fold
Cell-free supernatant	1000	425.36	401,000	942.73	1
Ammonium sulfate	48	73.21	179,000	2445.02	2.59
Sephadex G-50	12	7.84	87,000	11,096.93	11.77
DEAE-Sephadex A-50	3.5	1.09	31,000	28,440.366	30.17

Several conventional purification methods used to recover the antimicrobial peptide have been widely used in other reports, including ultrafiltration, ammonium sulfate precipitation, and chromatographic steps [61]. The Tricine SDS-PAGE profile displayed a single protein band of peptide YS12 (~3.3kDa) presented in Fig. 2.5a). In addition, in situ analysis, a portion of the gel exhibited antimicrobial activity showing zone (Fig. 2.5b) against the reference bacteria *Mycobacterium smegmatis.*

2.3.5. Identification of antimicrobial compound by MALDI-TOF MS

YS12 was further subjected to MALDI-TOF analysis to measure the accurate molecular mass. MALDI-TOF was employed to assess the molecular mass of YS12 in the 500 Da ~40 kDa acquisition mass range. Analysis of the MALDI-TOF results revealed the molecular weight of YS12 was ~3348.42 Da derived from a single prominent peak (n+1) (Fig. 2.5c). The molecular weight of YS12 is comparatively lower than other antimicrobial peptides produced from *Bacillus velezensis* [62]. However, we could not determine the peptide sequence probably due to the complex cyclic backbone structure /and or modified sidechains.

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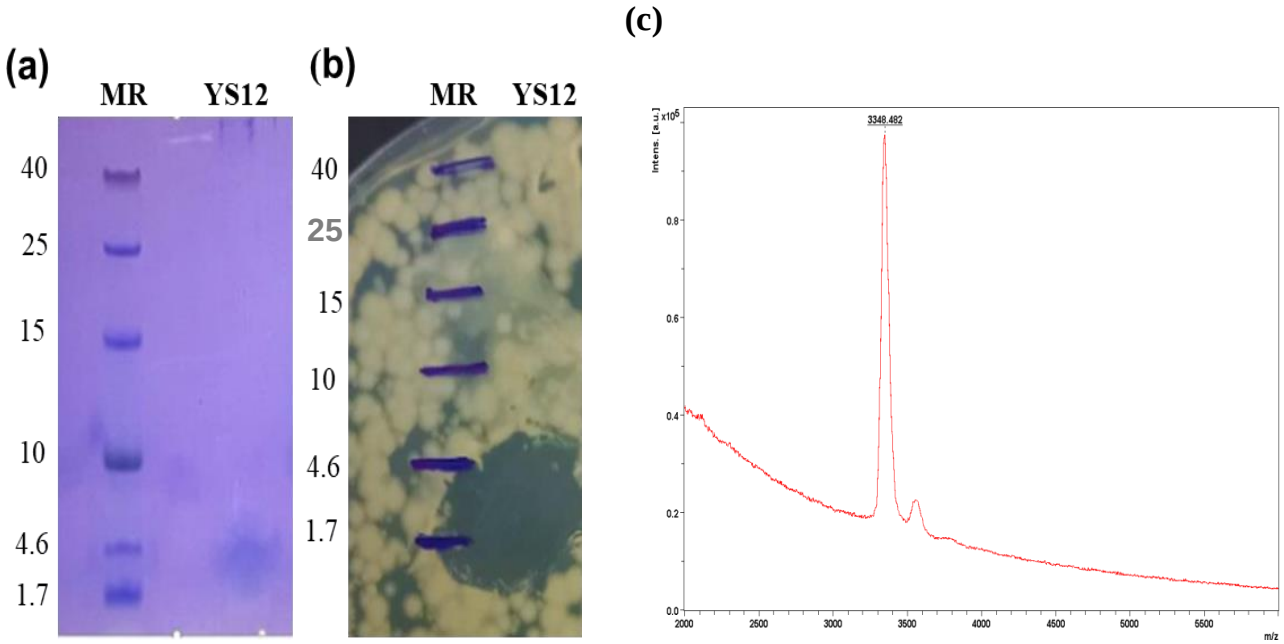


Figure 2.5. SDS-PAGE and in situ analysis of the purified peptide from *Bacillus velezensis* CBSYS12 strain (a) Tricine SDS-PAGE: Lane 1; protein molecular weight markers (MR) , Lane 2 purified peptide YS12 (b) In Situ analysis (bioassay) . Lane 1; protein molecular weight markers (MR)Lane 2 purified peptide YS12 (b) MALDI-TOF Analysis revealed the peptide YS12's molecular weight as 3348.4 Da.

2.3.6. Stability of YS12

Mounting evidence suggests that the antimicrobial activities of AMP are significantly attenuated by specific physical parameters, such as pH and temperature [63]. The stability of YS12 against pH was shown over a range of pH 4.0-10.0 using 1M NaOH or HCl, shown in Fig. 2.6a. Althoughpeptide YS12 was resistant to different pH values between 6.0 and 10.0, its maximum activity was particularly observed around pH 7.0-8.0. After autoclaving (105kPa for 15 min), YS12 completely lost its promising activity. Furthermore, our analysis indicated the effects of temperatures on the stability of YS12 as represented in Fig. 2.6b. The peptide YS12 was stable around 20-60 ℃ the newly identified strain exerted high thermos- stability and a broad range of pH stability [64, 65].

Intriguingly, we found that antimicrobial compounds remained unchanged over diverse proteolytic enzymes (including proteinase K^+, trypsin, and chymotrypsin,) compared to non–treatment control. Comparable findings have displayed that AMPs derived from the microbialstrain are stable against pH and temperature [66]. Taken together, our findings revealed that peptide YS12 had a high stability against a broad range of pH, temperatures, and different protease enzymes.

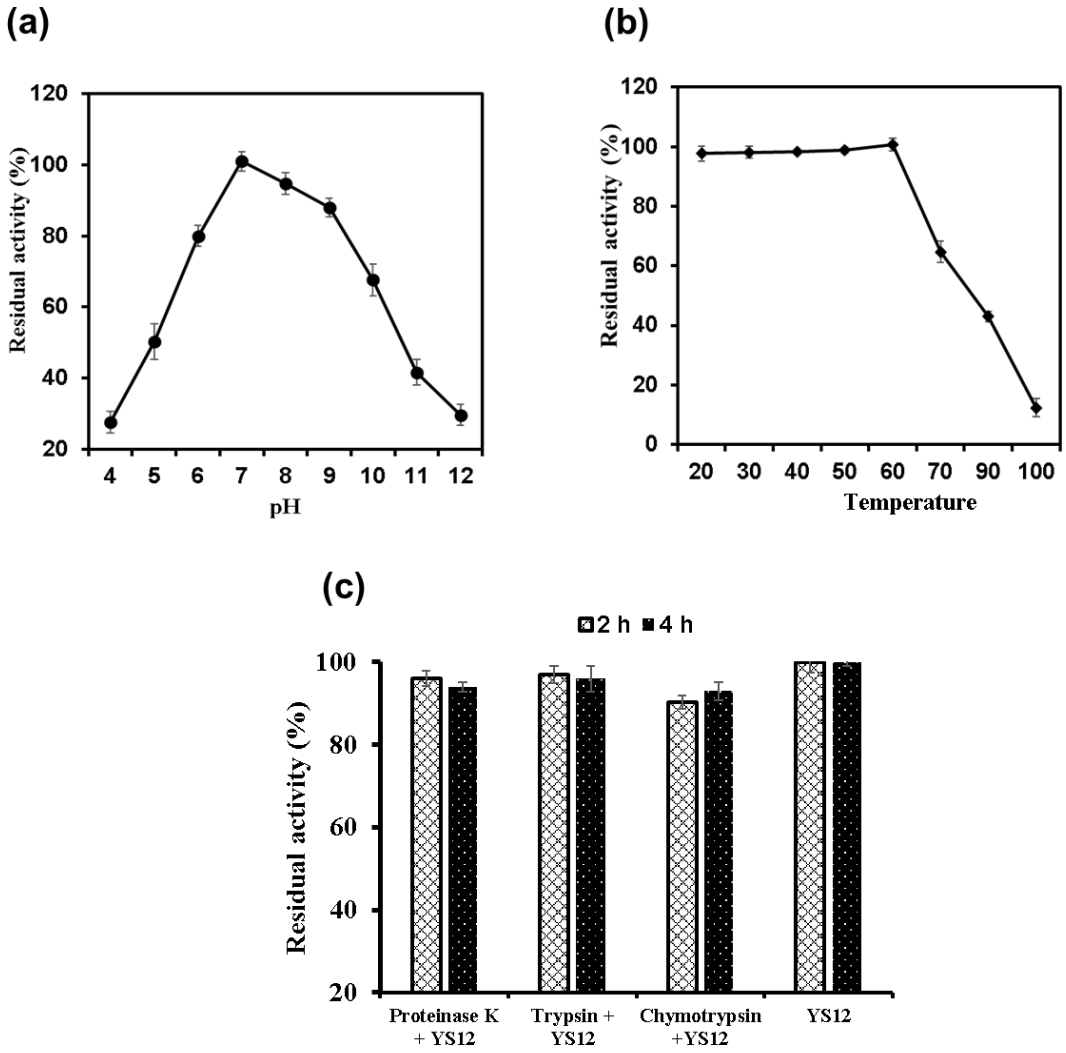


Figure 2.6. (a) Effects of temperature on YS12 stability (b) Effects of pH on YS12 stability (C)Effect of protease enzymes on YS12 stability

2.4. Conclusion

In conclusion, to develop novel antimicrobial agents, we identified and purified the peptide YS12 from *Bacillus velezensis* CBSYS12 derived from kimchi. Further studies regarding antimicrobial activity and mechanism of actionare being carried out.

CHAPTER THREE

Antimicrobial activity and mechanism of action of peptide YS12

3.1. Introduction

AMPs are considered potential substitutes for traditional antibiotics due to their broad antimicrobial spectrum against multidrug-resistant (MDR) pathogenic bacteria and the inability of microorganisms to easily develop resistance to them [67,68]. Biofilm development by pathogenic bacteria is an essential clinical issue regarding economic impairments, morbidity, and mortality that has gained considerable attention in current history. Biofilms are typically a group of colonies in which bacteria permanently enveloped in a self-produced matrix of polysaccharides, DNA, and protein [69].

Biofilm protects bacteria from the host immune system and enables them to survive in hostile environmental conditions [70]. Some multi-drug-resistant strains are prone to biofilm formation, making it more difficult to treat for conventional antibiotics that can enhance antibiotic resistance [71]. As a result, biofilms increase bacterial survival and multiplication in a range of infectious diseases by reducing bacterial susceptibility to antimicrobial drugs and immune responses [72]. An unconventional approach to overcome this problem is to use anti-biofilm agents. that prevent or eradicate biofilm formation.

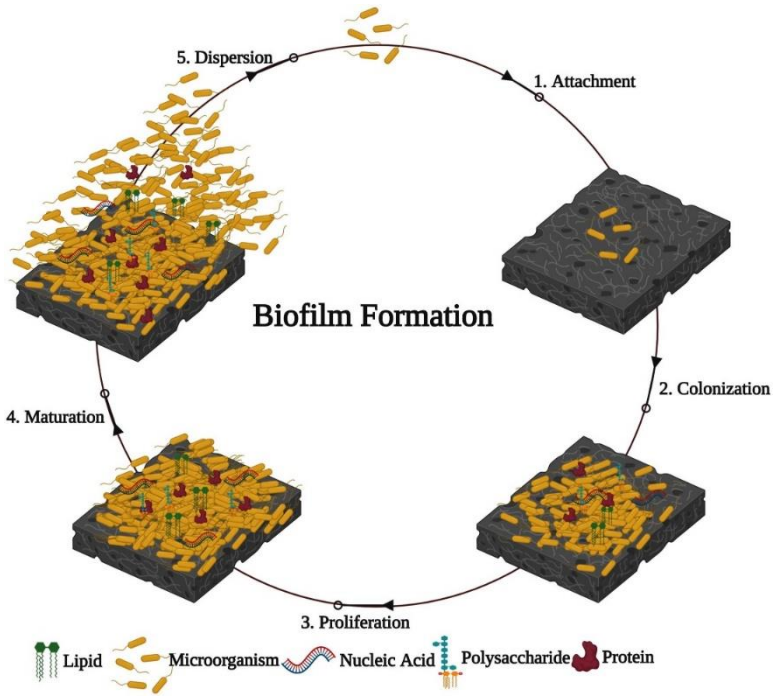


Figure 3.1. Biofilm development cycle (Srinivasan, R. et al. 2021)

Antimicrobial peptides (AMPs) have different multi-cellular function including direct antimicrobial activity as well as anti-biofilm, anti-inflammation, immunomodulation, cytokine release, chemoattraction, and histamine release from mast cells.

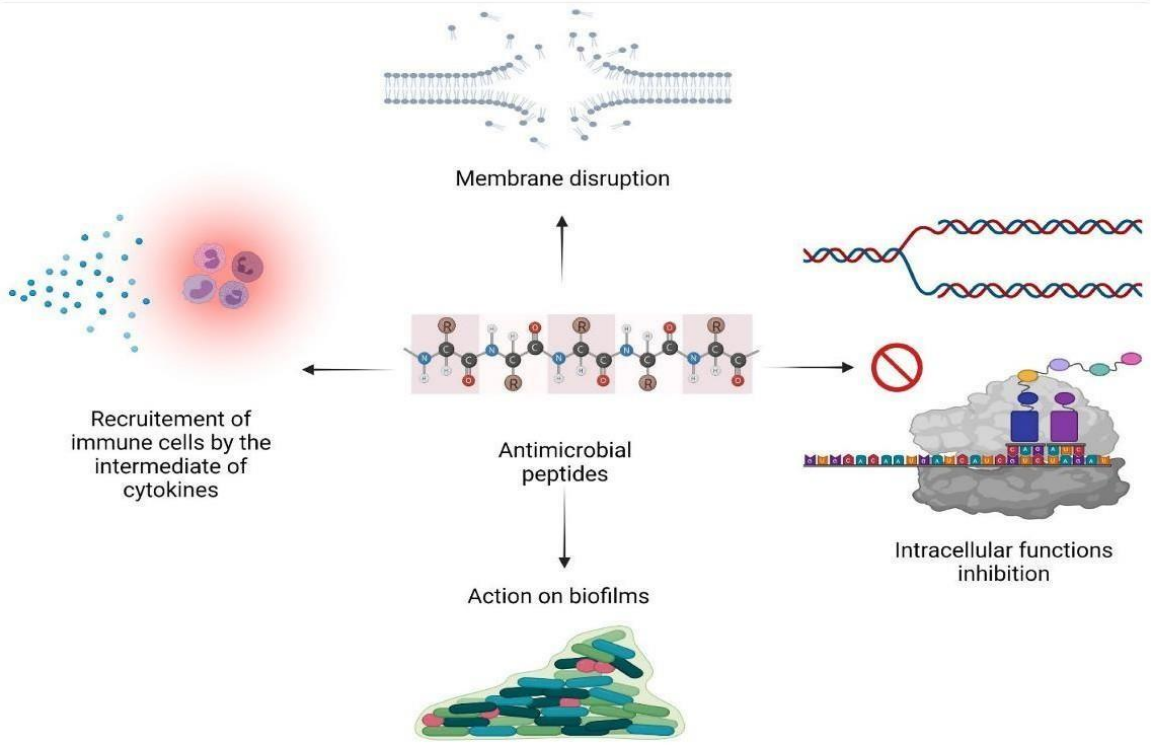


Figure 3.2. The multi-functional activity of antimicrobial peptide (AMP)

(Rima, M. et al. 2021)

Our research has found that AMPs displayed remarkable anti-biofilm activity by reducing bacterialbiofilm formation. Therefore, the antimicrobial peptide was able to inhibit bacterial biofilm formation compared with traditional antibiotics. Unlike conventional antibiotics, unlike the actions of conventional antibiotics, the majority of AMPs do not specifically target pathogen molecular receptors. The principal mechanism of AMPs is that AMPs can act and interact with cytoplasmic membranes and intracellular molecules [73].

Most AMPs exhibit amphipathic structures that reflect the presence of hydrophobic amino acids and have a cationic charge. The positively charged residues of AMPs facilitate an electrostaticinteraction with negatively charged phospholipid head groups that are prevalent in bacteria's cytoplasmic membrane. Some AMPs exert antimicrobial activity on pathogenic bacteria by inhibiting DNA and protein synthesis [74].

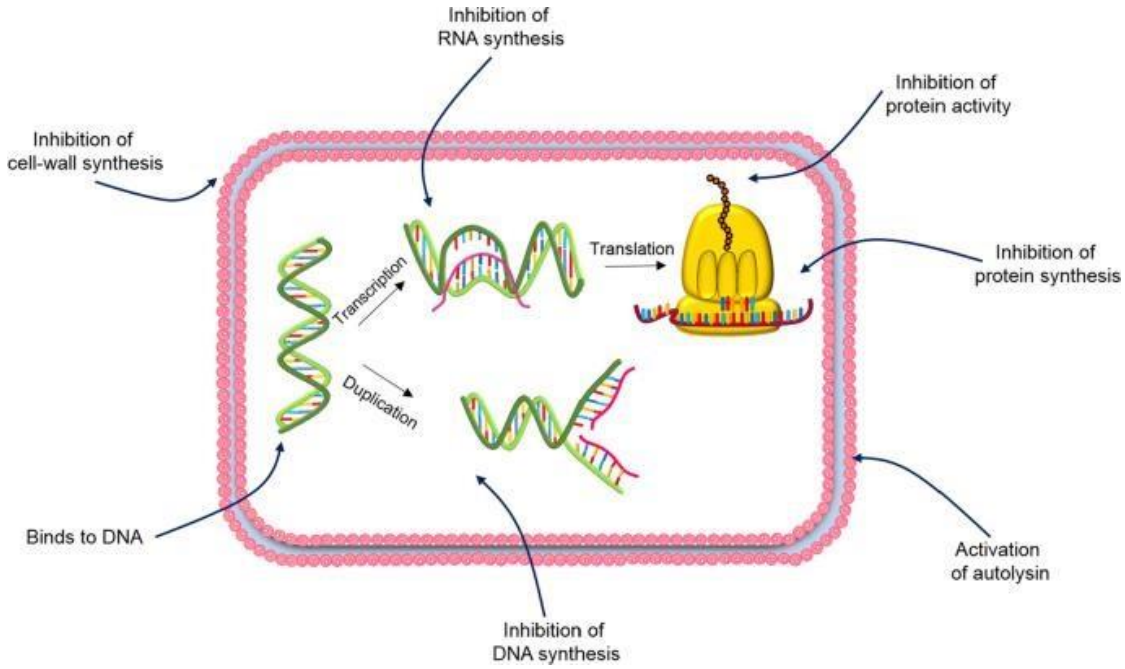


Figure 3.3. Nonmembrane targeting action of AMPs

(Parchebafi, A. et al. 2022)

Gram-negative bacterial membrane typically consists of lipopolysaccharide that act as a permeability barrier, making the development of antibiotics challenging. Some AMPs can kill the bacteria without causing membrane permeabilization. These AMPs exert antimicrobial activity on pathogenic bacteria by inhibiting DNA and protein synthesis [75]. Through this process, AMPs disrupt the integrity of the microbial cell membrane and cell wall indicating to cell death.

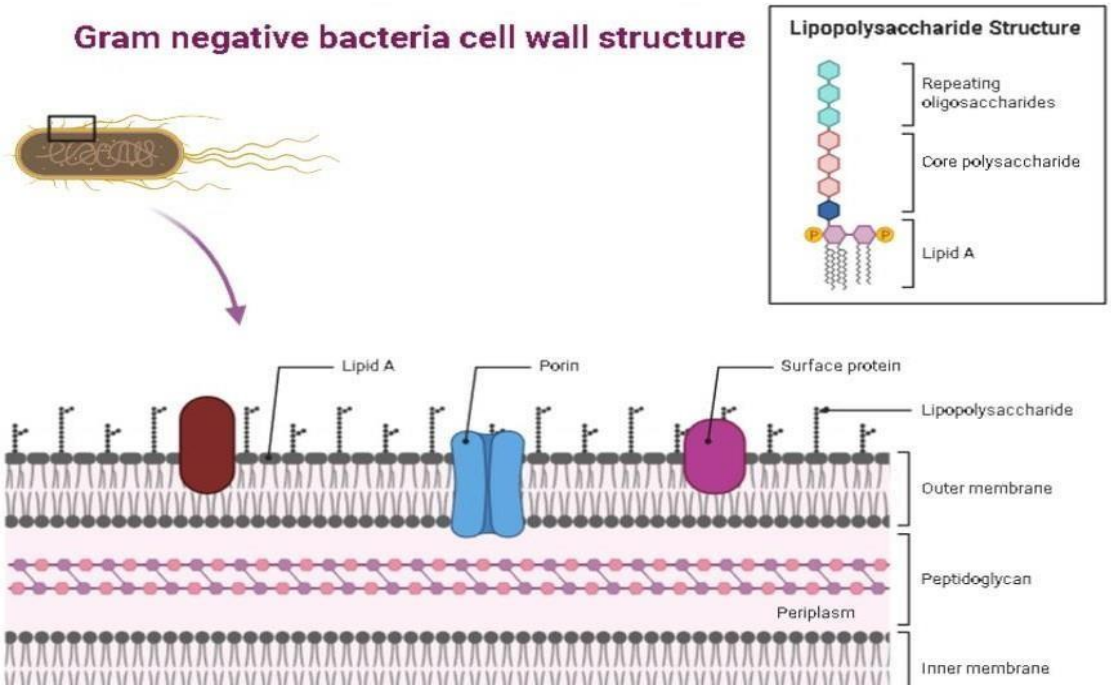


Figure 3.4. Gram-negative bacteria structure

In a previous study, we isolated bacterial strain from the Korean traditional food kimchi. In the current investigation, we evaluate the antimicrobial and anti-biofilm activity of the peptideYS12 derived from *Bacillus velezensis* CBSYS12 against multi-drug resistant strains. We also explored the possible mechanisms of low molecular weight peptide YS12 against drug-resistant strains.

3.2. Method and materials

3.2.1. Antimicrobial activity assay in vitro

To determine antimicrobial activity, the disk diffusion method was checked consistent with the previous research [76]. A disk of filter paper (8 mm, Toyo Roshi Kaisha, Japan) containing peptides was placed and overlaid with indicator bacteria on MH agar plates at 37 °C. Afterwards 12 h incubation, the diameter of the inhibitory zone of each disk was measured. MIC was determined through a micro-dilution assay with different pathogenic multi-drug-resistant bacteria[77]. For this test, agar solutions containing a specific number (1.5×10^{8}CFU/ml) were quickly transferred to agar plates covering various concentrations of peptide YS12 and two commercial antibiotics, such as Vancomycin and Bacitracin. MIC was foundto be the lowest concentration of antibiotics with no discernible growth after incubation compared to the control plate. The following several microorganisms were used as test organisms:

Gram- Negative bacteria

Pseudomonas aeruginosa KCTC 1637, *Escherichia coli* KCTC 1923, *Salmonella Typhimurium* KCTC 1925, *Alcaligenes faecalis* ATCC 1004.

Gram-positive bacteria

Enterococcus faecalis ATCC 29,212, *Bacillus subtilis* ATCC 6633, Methicillin-resistant *Staphylococcus aureus* 4–5, *Staphylococcus aureus* KCTC 1928, *Micrococcus luteus* ATCC 9341, *Mycobacterium smegmatis* ATCC 9341, VRE 4, VRE 82, VRE 89

3.2.2. Time-killing kinetics of YS12

Time-killing assay of peptide YS12 was determined against *Pseudomonas aeruginosa* (KCTC 1637), VRE82, Methicillin-resistant *Staphylococcus aureus* 4–5, and *Escherichia coli* (KCTC 1923) at different MIC concentrations using the viable plate counting method [78]. The growth control study was conducted with an untreated peptide sample. Briefly, the bacteria were grown overnight and then diluted in fresh Mueller–Hinton broth to a viable cell density of 1.5×10^8 CFU/ml. Various concentrations (2 × MIC, 3× MIC, 4 × MIC) of peptide YS12 were added to the diluted bacterial suspension and maintained at 37 °C and 150 rpm in a rotating incubator. Then, the sample was withdrawn from the central unit at different time intervals throughout the observation period. The samples were plated onto Muller-Hinton agar plates and left in the incubator for 12–16 h. Finally, CFU/ml was analyzed throughout all visible colonies.

3.2.3. Synergistic effect of peptide and different antibiotics

The synergistic effects of peptide YS12 with other conventional antibiotics were investigated using the two-dimensional checkerboard method [79]. In a nutshell, peptide YS12 was added withserially diluted antibiotics into the 96-well plates, respectively. Then bacteria (approximately 106 microorganisms) were added to each plate, and growth-control wells were filled with only the medium. OD_{600} was measured by a spectrophotometer after 18h of incubation. The fractional inhibitory concentration (FIC) index was obtained using the equation.

FIC index = FICA + FICB

[(MIC of drug A in combination /MIC of drug A alone)+ (MIC of drug B in combination /MIC of drug B alone) FIC index was represented as follows: FICi≤0.5 indicated synergy, 0.5<FICi≤1 denoted partialsynergism.

3.2.4. Time-dependent killing of the synergistic group

Combination therapy against the Gram-negative bacterium *E. coli* was used to analyze the synergistic impact of peptide YS12 with commercial antibiotic melittin [80]. Peptide YS12 at the concentration of 2×MIC was mixed with the same concentration of melittin. At different time intervals (30, 60, 90, 120,150, and 180) minutes, the mixture was collected and put onto MHB agar plates. The bactericidal effect was determined by colony-forming units (CFUs). The experiment was also carried out without using antibiotics as the control.

3.2.5. Stability assay

The salt sensitivity assay of the peptide was measured to determine antimicrobial activity under different physiological salts [81]. In Mueller Hinton Broth media, *P. aeruginosa* KCTC 1637, and *S. aureus* KCTC 1928 bacterial strains were grown and diluted to 2×10^5 CFU/mL. The bacterial cells incubated with peptide supplemented with different physiological salts such as NaCl (100 mM, 150 mM), $MgCl_2$ (0.5 mM, 1 mM), and $FeCl_3$ (2 µM, 4 µM). To determine MIC,the subsequent processes were carried out according to the method previously explained [13].

3.2.6. Biofilm formation inhibition

The anti-biofilm effect of the peptide was determined using the (TCP) tissue culture plate method with minor alterations [82]. The biofilm inhibition abilities of the peptide YS12 and well-known antibiotics (oxacillin and ampicillin) were assessed following the micro-broth dilution method. To conduct this experiment, different drug-resistant strains were grown at 37 °C and, after overnightincubation, diluted in an MHB medium supplemented with 0.2% glucose. The diluted bacterial suspension (*E. coli* and *P. aeruginosa*), peptide, and antibiotics with concentrations 5 to 80 µg/ml were placed on 96-well TCPS and kept for incubation. Positive control was carried out in wells depleted with antibiotics. After proper incubation, the culture supernatants were removed, and adherent cells were attached by adding methanol (100%) solution for 15 min. The biofilm was further stained with a (0.1%) solution of crystal violet (Sigma, Aldrich, Munich, Germany). Furthermore, each well-containing solution was repeatedly cleansed with DW and put for drying. Finally, 100% ethanol was added to each test well, and the absorbance value at 620 nm was determined using ELISHA.

3.2.7. Effects of YS12 on biofilm eradication

To evaluate the eradication activity of the peptide YS12, *E. coli* biofilm supplemented with Mueller–Hinton Broth (MHB) medium was first developed in 96-well plates at 37 ℃ for 24 h [83]. After the non-adherent cells were removed, the biofilm was treated with various doses (10–160 μg/ml) of peptide YS12, ampicillin, and oxacillin. Planktonic cells were removed after a 24h incubation period, and the biofilms were retained with 100% methanol before being dyed by 0.1% CV. ELISHA reader was utilized to assess the absorbance at 620 nm.

3.2.8. In vitro Cytotoxicity assay of peptide YS12

The cytotoxicity of peptide YS12 was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide (MTT) reagent towards Raw 264.7 cells [84]. In brief, Raw 264.7 cells were plated in a 96-well plate, which was then left to incubate at 37 ℃ with 5% CO2 overnight. Cells were then incubated with increasing concentrations (5-120µg/ml) of peptide for 24 h at 37° C under a 5% CO2 atmosphere. MTT was added to the well the following day at a concentration of 0.5 mg/ml. The absorbance was measured at 570 nm. At least two replicates were given to each measurement.

3.2.9. Hemolytic activity of peptide YS12

The hemolytic activity of peptide YS12 using fresh red sheep blood was measured as previouslyreported with slight alteration [85]. In a nutshell, fresh red sheep blood cells were washed several times using PBS, and the supernatants were removed by centrifuging at 1000×g for five minutes. The peptide YS12 at a wide range of concentrations (5-150µg/ml) was added. Melittin was used as a control. 4% RBC was put into separate well and put at 37°C. At 1000×g, the plate was centrifugated again at 4° C, and supernatants were placed on a fresh 96-well plate. The optical density (OD) at 414 nm was measured. The absorbance of PBS-treated cells indicated zero hemolysis, while 0.1% Triton X-100 indicated complete hemolysis.

3.3. The potential antimicrobial mechanisms study of peptide YS12

3.3.1. Preparation and aggregation of liposomes

large unilamellar vesicles (LUVs) were developed using the freeze-thaw technique [86]. The liposome mixture was prepared by dissolving PE: PG (7:3, w/w), PG, and PC in chloroform. The dispersed liposomes were then removed with argon gas. The lipid film was dried, and then the solution was vortexed in PBS with a pH of 7.2 until a milky appearance developed in the suspension. LUVs were created utilizing nine freeze-thaw cycles under a water bath and liquid nitrogen. A polycarbonate membrane with a thickness of 0.2 m was used to extrude the suspensions.The peptide was added at concentrations of 5, 10, 20, and 40 µg/mL to 400 µM LUVs composed of PE: PG, PG, and PC. Using a microplate reader, the enhanced absorbance was determined at 405 nm.

3.3.2. Effect of Lipopolysaccharides (LPS)

The biological action of AMPs is dependent on their binding to the cell membrane [87]. The peptide YS12's antimicrobial activity against *Escherichia coli* KCTC 1923 in the presence of LPS was identified. *E. coli* cells (approximately 6.0×10^{7}CFU/ml) were grown and diluted with tryptic soy broth. The cell suspension was mixed with lipopolysaccharides from *E. coli* O111: B at aconcentration of 5, 10, 40, or 80µg/ml. Peptide YS12 was added to a final concentration (50µg/mL)against *E. coli.* Ampicillin at 10 µg/mL and Polymyxin at 5 µg/mL were used to serve as controls. The suspensions were

placed at 37 °C for 60 min while being stirredat 200 rpm. By spreading plating on tryptic soy agar, Stayers cells were measured.

3.3.3. LPS binding assay using Dansyl-polymyxin B

Danyl-polymyxin B displacement experiment was also carried out to establish the antimicrobial peptide's binding affinity for LPS [88]. LPS from *E. coli* and Danyl-polymyxin B were treated with a pH 7.2 HEPES buffer (1ml; 5mM)) to achieve optimal fluorescence. Followingthat, fluorescence was assessed prior to the addition of peptide YS12 ranging from (2-40 µg/ml) to the cuvette, and a spectrometer was used to measure the decrease in fluorescence. Excitation and emission wavelengths of the fluorescence were detected at 340 nm and 485 nm, respectively.

3.3.4. Neutralization of LPS by peptide

A chromogenic limulus amebocyte lysate assay was used to measure the LPS neutralization [89]. Various doses of the peptide (5 -40µg/ml) were incubated with a constant concentration of LPS (from *E. coli*) (1 ng/ml) at 37° C in the wells of a pyrogenic sterile microtiter plate. The solutions were then mixed and treated at 37° C for 10 minutes with equivalent amounts of Limulusamebocyte lysate reagent. Following the addition of a chromogenic substrate solution, a yellow color emerged. After adding acetic acid (25%) to terminate the reaction, the absorbance was recorded at 580 nm.

3.3.5. Outer membrane permeability assay

The outer membrane permeability of the peptide was analyzed on gram-negative bacteria by using 1-N- phenyl naphthylamine (NPN) uptake assay as previously described [90]. Specifically, *E. coli* (KCTC 1923) and *P. aeruginosa* (KCTC 1637) bacterial cells were cultivated in MHB media at 37° C and suspended to an OD_{600} in 5mM HEPES buffer containing 5mM glucose (pH 7.4). Next, the cell suspensions were placed in 96 black plates to which NPN (final concentration 10μM) mixed in ethanol (95%) was mixed. Subsequently different concentrations of peptide YS12 (1×MIC, 2×MIC, and 4×MIC) put to each plate. Fluorescence was recorded (excitation λ= 350nm, emission λ = 420nm) for 25 min at 5-min intervals using a fluorescence spectrophotometer. Usingthe following equation, the results were converted to percent NPN uptake:

$$\text{NPN uptake (\%)} = (F_{obs} - F_0)/(F_{100} - F_0) \times 100$$

Where Fobs signifies the detected fluorescence at a particular peptide concentration, F_{obs} denotesthe original fluorescence of NPN with *E. coli* or *P. aeruginosa* cells in the absence of peptide.

3.3.6. Cytoplasmic membrane depolarization

The cytoplasmic membrane depolarization activity of the peptide using fluorescent dye 3,3-dipropylthiadicarbocyanine iodide (DiSC3-5) [91]. Briefly, *E. coli* and *S. aureus* bacteria were grown to the mid-log phase at 37º C and washed with 5 mM HEPES buffer (pH 7.2) including 20 mM glucose. With the same buffer, the bacterial cells were resuspended at 600 nm and placed on 96 well plates. Then the diSC3-5 dye (final concentration 1 μ M) was added to each well. The peptide at 1× MIC, 2× MIC, and 4× MIC was added to the mixture. After the peptide addition, the fluorescence was observed at excitation and emission wavelengths of 622 nm and 670nm, respectively.

3.3.7. Inner membrane permeability activity

Membrane permeabilization by the peptide was performed by membrane-sensitive dye propidiumiodide (PI) [92]. Briefly stated, *E. coli* and *S. aureus* bacteria were grown in MHB medium to themid-log phase and were further diluted in sodium phosphate buffer (10 mM) to an OD_{600} value of 0.25. Then, on a dark 96-well plate, each bacterium was combined with PI (final concentration, 20μM). After mixing, peptide YS12 at various concentrations (1×,2×, and 4×MIC) was added to each well. A fluorescence spectrophotometer was employed to assess the absorbance at 580 nm and 620 nm for the excitation and emission, respectively, and the percentage (%) of fluorescence intensity obtained from peptides was represented relative to the control.

Ortho-nitrophenyl-galactose (ONPG) was used to test the inner membrane permeabilization of the peptide against *E. coli* bacteria [93]. Bacterial cells were cultured, collected, and resuspendedin PBS (pH 7.2) to a final OD_{420} of 1.2. Finally, the peptide (1×, 2×, 4 ×MIC) and ONPG (final concentration 1.5 mM) were placed on 96 well plates. After that, a versa-microplate reader was used to measure the absorbance at 420 nm.

3.3.8. Calcein dye leakage assay

Calcein-loaded liposomes were prepared to imitate bacterial membranes [94]. The liposomes were prepared according to the method described in the earlier report. Peptide YS12 at 1× MIC, 2× MIC, and 4× MIC was loaded into liposomes (1:1, v:v). Following the addition of YS12, the fluorescence intensity (excitation 490 nm, emission 530 nm) was recorded at 0, 3, 5, 10, 15, and 20 min. The calcein release rate was calculated in accordance with the following equation.

The extent of calcein output was calculated as,

$$(Ft - F_0) / (F_{max} - F_0),$$

where Ft represents the fluorescence of a peptide liposome/calcein solution at a certain time, and F_0 and F_{max} indicate the initial fluorescence. where Ft is the fluorescence of a peptide liposome/calcein solution at the time.

3.3.9. Confocal Microscopy

E. coli and *P. aeruginosa* bacteria were grown In LB broth to the mid-logarithmic phase. The cell suspensions were centrifuged and washed three times with a 10 mM PBS buffer (pH 7.5). Afterward, the cells were treated with fluorescein isothiocyanate (FITC) labeled peptide YS12 (2×MIC) at 37° C. After an hour of incubation, the bacterial cells were centrifuged, pipetted into PBS buffer (pH 7.5), and then allowed to stand on a glass slide. Cell images with FITC-labeled peptides were observed using a laser confocal microscope (LSM 510, Carl Zeiss, Germany) [95]3.4.0.

3.4.0. Result and discussion

3.4.1. Zone of inhibition assay

One of the most commonly used methods for evaluating the peptide's antimicrobial activity is the agar diffusion assay, also known as the inhibition zone assay.

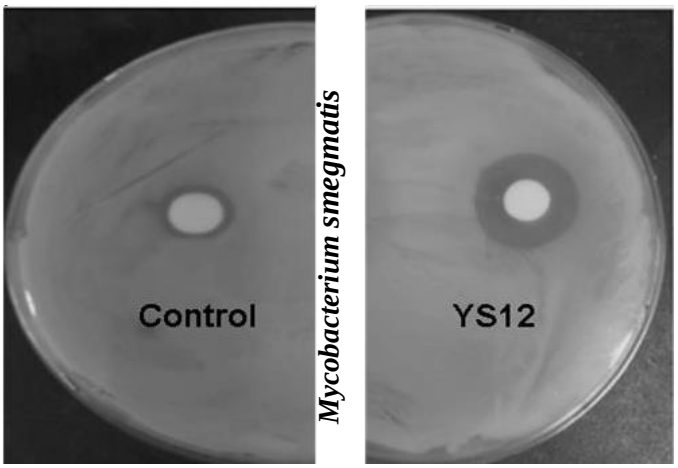


Figure 3.5. Antimicrobial activity of peptide YS12 was observed by measuring the zone of inhibition against *Mycobacterium smegmatis* ATCC 9341

3.4.2. Antimicrobial activity of the peptide YS12

The antimicrobial effects of peptide YS12 against different bacteria interms of MIC were determined compared with already recognized antibiotics Vancomycin and Bacitracin. The results presented in Table 1.4 revealed that the peptide YS12 showed a stronger antimicrobial activity against different MDR strains. Bacterial strains, *Pseudomonas aeruginosa* KCTC 1637, Vancomycin-resistant *Enterococci* 82, and *Mycobacterium smegmatis* ATCC 9341 were susceptible to 12µg/ml for peptide YS12. This concentration acts as a minimum inhibitory concentration for the following strains. With the lowest MIC value of 6 µg/ml, *Escherichia coli* ATCC 1923 was confirmed to be the most sensitive strain. A lower MIC indicates potent antimicrobial efficacy. Intriguingly, the resultant effect of peptide YS12 against pathogens such as Methicillin-resistant *Staphylococcus aureus* 4-5 (12 µg/ml) and *Micrococcus luteus* ATCC9341 (24 µg/ml) was similar to that of vancomycin and significantly less potent than that of bacitracin against similar bacteria.

Interestingly, traditional antibiotics showed less antimicrobial activity for most of the test organisms. The antimicrobial actions of YS12 against *Salmonella typhimurium* KCTC 1925 and *Alcaligenes faecalis* ATCC 1004 were relatively weaker than the other strains. Importantly, CBSYS12 exerted promising antimicrobial activity on drug-resistant strains of bacteria compared to the other AMP isolated from the *Bacillus* strain [96, 89]. Collectively, our result indicated that the antimicrobial peptide was considered an effective antimicrobial agent against the standard strains. The result is similar to other reports that AMPs exhibit potent antimicrobial activity [97,98].

Table 3.1. The antimicrobial spectrum of peptide YS12 in terms of the Minimum InhibitoryConcentration (MIC) test against diverse MDR microorganisms

Microorganisms	MIC (μg/ml)		
	YS12	**Bacitracin**	**Vancomycin**
Gram-negative bacteria			
Salmonella typhimurium KCTC 1925	>96	>96	>96
Escherichia coli KCTC 1923	6	48	>96
Pseudomonas aeruginosa KCTC 1637	12	>96	12
Alcaligenes faecalis ATCC 1004	>96	>96	>96
Gram-positive bacteria			
Staphylococcus aureus KCTC 1928	48	>96	>96
Vancomycin-resistant *Enterococci* 4	24	48	>96
Vancomycin-resistant *Enterococci* 82	12	24	>96
Vancomycin-resistant *Enterococci* 89	24	48	1.5
Enterococcus faecalis ATCC 29212	48	>96	6
Methicillin-resistant *Staphylococcus aureus* 4-5	12	24	12
Micrococccus luteus ATCC 9341	24	12	24
VRSA	>96	>96	>96
Mycobacterium smegmatis ATCC 9341	12	48	3
Bacillus subtilis ATCC 6633	48	48	3

3.4.3. Time-killing kinetics of YS12

The bactericidal action of peptide YS12 was assessed by performing a time-course antimicrobial assay followed by CFU counting. Both gram-positive and gram-negative indicator strains were exposed to diverse concentrations of peptide YS12 (2×MIC, 3×MIC, and 4×MIC). The bactericidal actions of antimicrobial peptide YS12 against *P. aeruginosa* (Fig. 3.6a), VRE 82 (Fig. 3.6b), Methicillin-resistant *Staphylococcus aureus* 4-5 (Fig. 3.6c), and *E. coli* (Fig. 3.6d) were significantly dependent on the concentration of peptide and exposure time. In this study, we foundthat peptide YS12 exhibited robust growth inhibitory activity. Intriguingly, YS12 exerted a rapid decline of approximately 5log to 2log on Methicillin-resistant *Staphylococcus aureus* 4-5 and *E. coli* at a low MIC (2×MIC) within 8h. However, peptide YS12 caused a reduction of CFU counts on all bacterial strains at 4×MIC value. The regrowth pattern was observed on *P. aeruginosa* and VRE 82 after 8h exposure to peptide YS12 at a lower concentration.

AMPs also resulted in significant bactericidal effects found in an earlier report [99, 92]. Taken together, these findings ensure that AMPs can remarkably inhibit drug-resistant and drug-susceptible bacterial growth owing to their rapid and effective bactericidal actions.

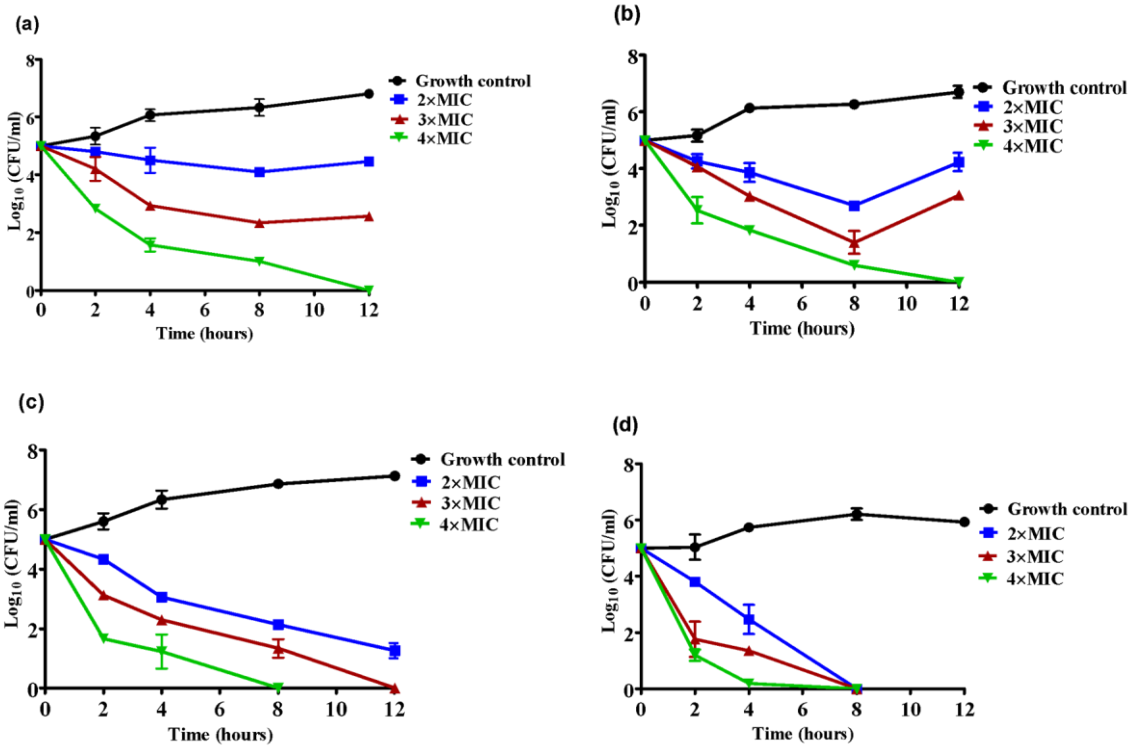


Figure 3.6. Time killing kinetics of peptide YS12 against **(a)** *P. aeruginosa* KCTC 1637 **(b)** VRE 82(c) Methicillin-resistant *S. aureus* 4-5 **(d)** *E. coli* KCTC 1923

3.4.4. Synergistic effect of peptide YS12 with conventional antibiotics

Unfortunately, the number of bacteria that exhibit resistance to antibiotics considerably increased over time [100]. Combination antibiotic therapy has promising approaches to reducing bacterial resistance and regaining the potency of antibiotics [101]. Specifically, the combination therapy utilizing AMPs with antibiotics has drawn attention as it often exhibits a synergistic bacterial effect [102]. In the above study, the synergistic antimicrobial activity of peptide YS12 with antibiotics, including oxacillin, ciprofloxacin, or erythromycin, was tested using the fractional inhibitory concentration index (FICI against *E. coli* KCTC 1923 and *S. aureus* KCTC 1928. In Table 2, the greater synergistic effect was obtained for YS12 and ciprofloxacin against *E. coli* KCTC 1923, which achieved a fractional inhibitory concentration index (FICI) of 0.15625. Similarly, the combination of peptide YS12 with oxacillin or erythromycin exhibited a synergistic effect (FICI of 0.282 to 0.501) on *E. coli* and *S. aureus*, respectively. The partial synergism (FICI >0.5) was observed when YS12 was combined with ciprofloxacin or erythromycin on *P. aeruginosa* and *S. aureus*. Peptide YS12 combined with erythromycin also showed additive or partial synergism (FICI of 0.78) against P. *aeruginosa*. The overall finding of this study illustrated that the peptide resulted in a synergistic

effect with different antibiotics.

Table 3.2. The FIC index of peptide YS12 and antibiotics by the checkerboard method

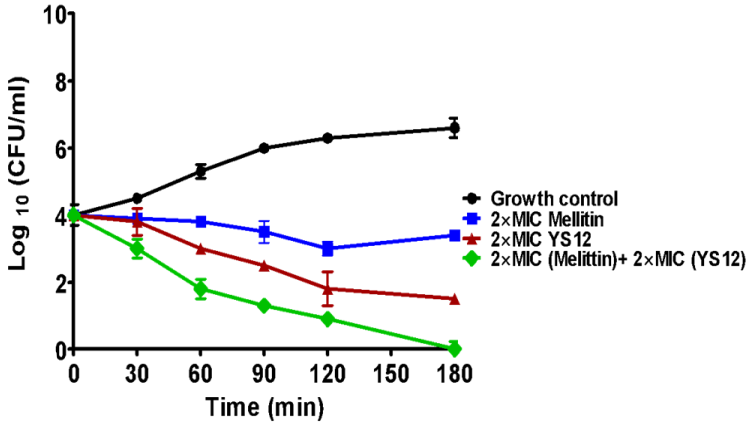
Bacterial strain	**Peptide-YS12 Oxacillin**		**Peptide-YS12 Ciprofloxacin**		**Peptide-YS12 Erythromycin**	
	ΣFIC	**Interpretation**	**ΣFIC**	**Interpretation**	**ΣFIC**	**Interpretation**
Gram-negative						
E. coli KCTC 1923	0.282	Synergy	0.1562	Synergy	0.501	Synergy
P.aeruginosa KCTC 1637	0.45	Synergy	0.65	Partial Synergism	0.78	Partial Synergism
Gram-positive						
S. aureus KCTC 1928	0.31	Synergy	0.79	Partial Synergism	0.435	Synergy

*FIC of < 0.5 was interpreted as synergy, 0.5<1.0 as an additive or partial

3.4.5. Time-dependent killing of the synergistic group

Using the killing test, we observed the synergistic effect of peptide YS12 combined with melittin. In Figures3.7a and 3.7b, the combined treatment of YS12 and melittin at 2×MIC had intense synergistic antibacterial activity compared to the use of YS12 or the antibiotic itself against microbial strains (*Escherichia coli* and *Pseudomonas aeruginosa*) respectively. Some studies also revealed the promising synergistic of AMPs [103].

(a)



(b)

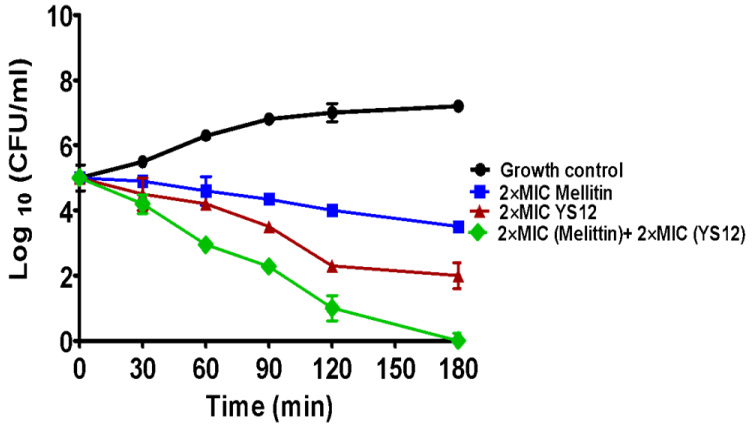


Figure 3.7. Synergistic effect of peptide YS12 with melittin against (a) *E. coli* KCTC 1923 (b) *P.aeruginosa* KCTC 1637. The activity of the combination of 2×MIC of peptide and 2×MIC melittin was compared with the result of 2×MIC of peptide, 2× MIC of melittin alone, or untreated control.

3.4.6. Stability assay

A significant limitation of natural antimicrobial peptides (AMPs) is their possible degradation by the physiological concentration of salts [102]. AMPs may exhibit a partial or even total loss ofantimicrobial activity under different salt concentrations [105]. To be effective as therapeutic agents, AMPs must maintain antimicrobial activities in salts-containing environments. Numerous studies have suggested that monovalent Na^{+}, $Mg^{2+,}$ and Fe^{3+} may reduce the antimicrobial actionof naturally occurring AMPs. Therefore, we analyzed the antimicrobial activity of peptide YS12 and melittin using different physiological salt concentrations. As shown in Table 3.3, the peptide YS12 against *P. aeruginosa* and *S. aureus* had minimal effect on Na^{+}, Mg^{2+}, and Fe^{3+} cations. The peptide YS12 maintained strong activity (MIC ~2 µg/ml) against *P. aeruginosa* in the presence of $Fe^{3+.}$ Similarly, for *S. aureus*, YS12 retained its stability (MIC ~2-4 µg/ml) at various salt concentrations. The peptide exhibited a strong tolerance to different salts, which was consistentwith the finding from various AMPs [106, 107]. These findings confirm that the peptide would bethe perfect candidate for application as a therapeutic agent.

Table 3.3. Antimicrobial activity of peptide YS12 in the presence of different physiological salts

	NaCl (mM)		MgCl2 (mM)		FeCl3 (μM)	
	100	**150**	**0.5**	**1**	**2**	**4**
***P.aeruginosa* KCTC 1637**						
Peptide YS12	2	2	4	2	2	2
Melittin	2	2	2	2	4	4
***S. aureus* ATCC 1234**						
Peptide YS12	2	2	4	2	2	2
Melittin	2	4	4	2	4	2

3.4.7. Biofilm inhibition of peptide YS12

The development of pathogenic bacterial biofilm caused an increased risk of antibiotic resistance and chronic diseases [108]. Consequently, there is an urgent need for novel AMPs that can reduce bacterial biofilm. This study investigated the promising anti-biofilm activity of peptide YS12. For this purpose, some bacterial strains were initially allowed to form biofilm. Specifically, our results revealed that the bacterial strains *E. coli* and *P. aeruginosa* formed significant amounts of biofilms (Fig. 3.8 a). We then selected these strains to check the biofilm inhibitory activity of YS12 using the crystal violet staining procedure. As shown in Fig. 3.8b and Fig. 3.8c, peptide YS12 obstructedbiofilm formation in a dose-dependent manner (5-80 µg/ml). Peptide-treated samples significantly suppressed biofilm generation compared with the untreated group that served as control. With an increased concentration of peptide samples, bacterial biofilm formation was decreased, but the effects of the targeted peptide were not the same for the selected strains.

The strains were markedly inhibited at a high concentration of AMP. In contrast, antibiotics (ampicillin or oxacillin) treated wells showed less inhibition than peptide samples. Interestingly, peptide YS12 remarkably contributed to reducing the biofilm formation of drug-resistant strains. In the case of *E. coli*, approximately 70.6% of biofilm formation was observed at 40 µg/ml, which decreased to 85.5% at 80 µg/ml. YS12 inhibited biofilm formation at a maximum concentration of 80 µg/ml for both bacterial strains. It was so clear that YS12 served as a strong biofilm inhibitor for laboratory reference bacterial strains. The promising anti-biofilm effect of AMPs against the MDR pathogenicbacterial strains was also noted in another research [109, 110]. Our findings confirmed that AMP acted as a potent biofilm inhibitor against the biofilm of drug-resistant strains.

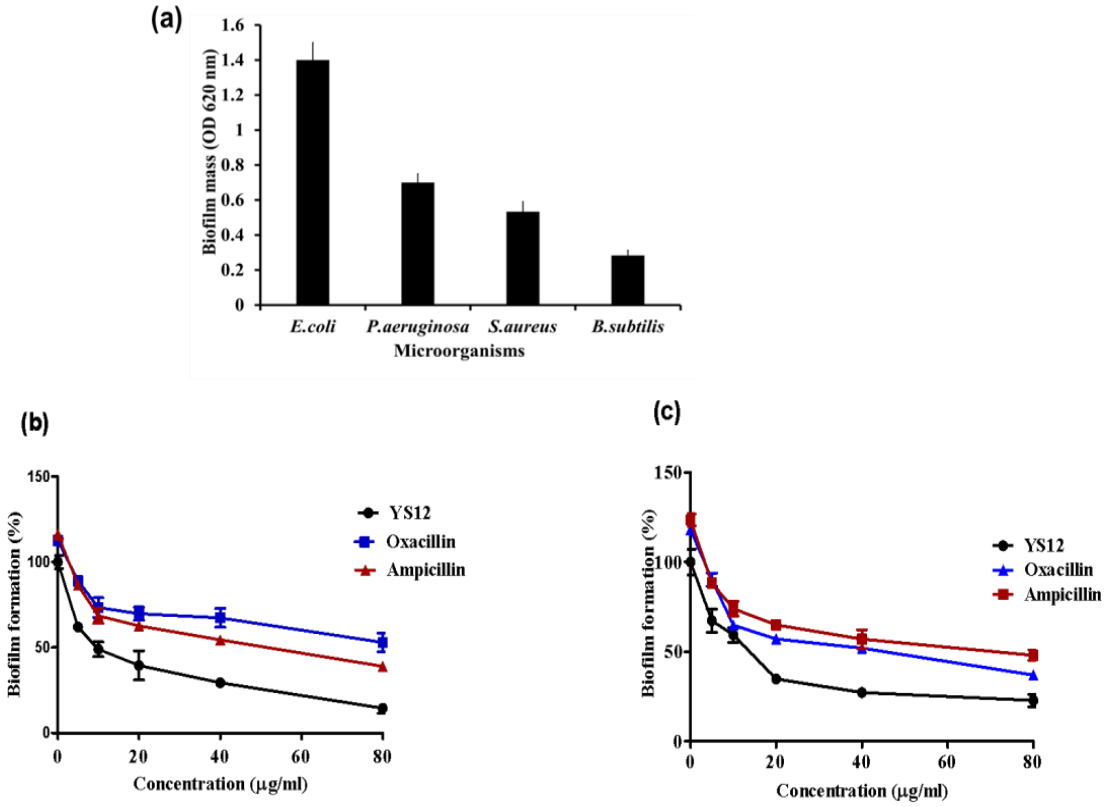
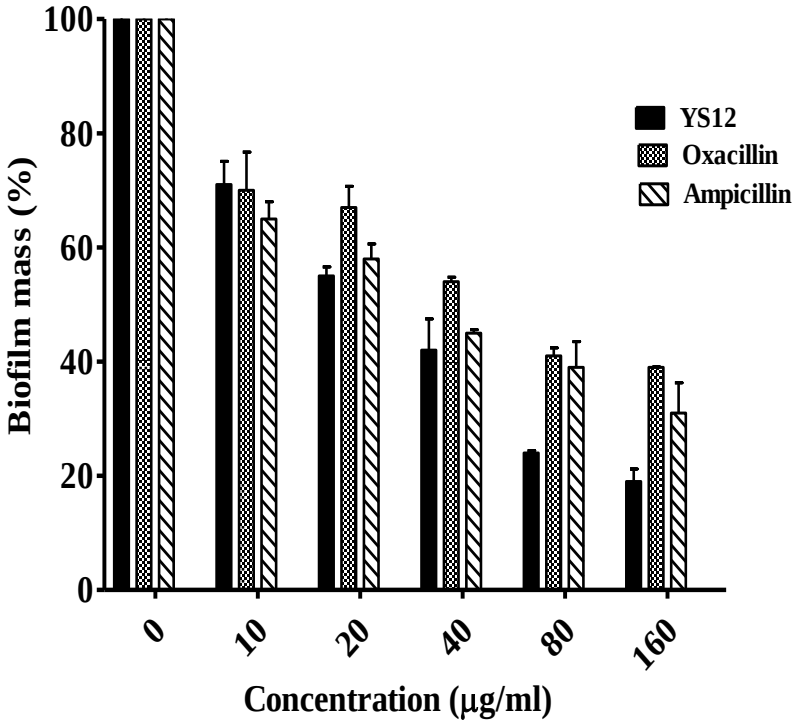


Figure 3.8. Anti-biofilm activity of peptide (a) Biofilm formation by different pathogenic bacteriain MHB + 0.2% glucose media for 24h at 37ºC. Peptide YS12, ampicillin, and oxacillin at concentrations (5-80μg/ml) were treated for (b) *E. coli* KCTC 1923 and (c) *P. aeruginosa* KCTC1637 in 96-well plates and incubated at 37 ºC.

3.4.8. Effects of the peptide on biofilm eradication

The biofilm removal activity of peptide YS12 was measured using crystal violet staining. As displayed in Fig. 3.9a, the biofilm mass was decreased from 71% to 19% when treated with peptidesamples at concentrations of 10-160 µg/ml, respectively. Importantly, our results indicated that treatment of oxacillin and ampicillin could impede the biofilm mass. In this assay, supplementation of peptide YS12 could highly eradicate the formed biofilm. In Fig. 3.9b, the peptide YS12 containing well exhibited the complete elimination of biofilm at 160 µg/ml compared to control and wells induced with antibiotics. Antimicrobial peptides are promising candidates that influence the prevention of bacterial biofilms overviewed in other reports [111,99]. However, the removal rate of biofilm mass was consistent with earlier data using other anti-biofilm agents [112, 113, 114]. Therefore, these findings revealed that AMPs obstructed biofilm formation by blocking bacterial growth via regulating bactericidal growth action orprohibiting the attachment of bacterial cells. Thus, such AMPs with anti-biofilm properties are considered potential drug candidates against pathogenic bacterial biofilm.

(a)



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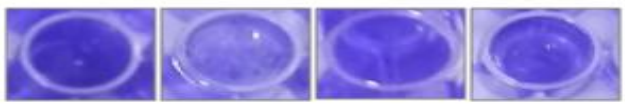

Figure 3.9. (a) Effects of YS12, ampicillin, and oxacillin on eradication of preformed E. coli biofilm (b) The formed biofilm of *E. coli* was treated with 160 μg/ml of YS12, ampicillin, and oxacillin. Crystal violet was used to stain the wells after 24. After 24 h, the retaining biofilm was ruined with CV , and the optical density was assessed at 620 nm.

3.4.9. Cytotoxicity assay and Hemolytic Activity of peptide

It has been assumed that AMPs exhibit cell selectivity. Cytotoxicity and hemolysis are frequentlyused methods to assess the cellular toxicity of AMPs [115]. To evaluate the cell selectivity more apparently, we checked the toxicity assay of peptide YS12 on Raw 264.7 cells using the MTT reagent. According to the findings (Figure 3.10a), no cytotoxicity was observed up to 40µg/ml with Raw 264.7 macrophages. Cell viability for YS12 was 83% at 120 µg/ml. Our finding in thisstudy revealed that the peptide had great survival in Raw 264.7. Figure 3.10b displays the peptide'shemolytic action on red sheep blood. Hemolytic activities showed that peptide YS12 exhibited less than 15% hemolysis at the maximum concentration of 150 µg/ml. In contrast, melittin was used asa negative control and demonstrated 76% hemolytic activity at 100 µg/ml. The promising low cytotoxic and hemolytic effects of AMPs were also reported in other studies [116,117]. These outcomes reveal new insight that is essential and useful for evaluating the therapeutic effectivenessof AMPs for systemic applications.

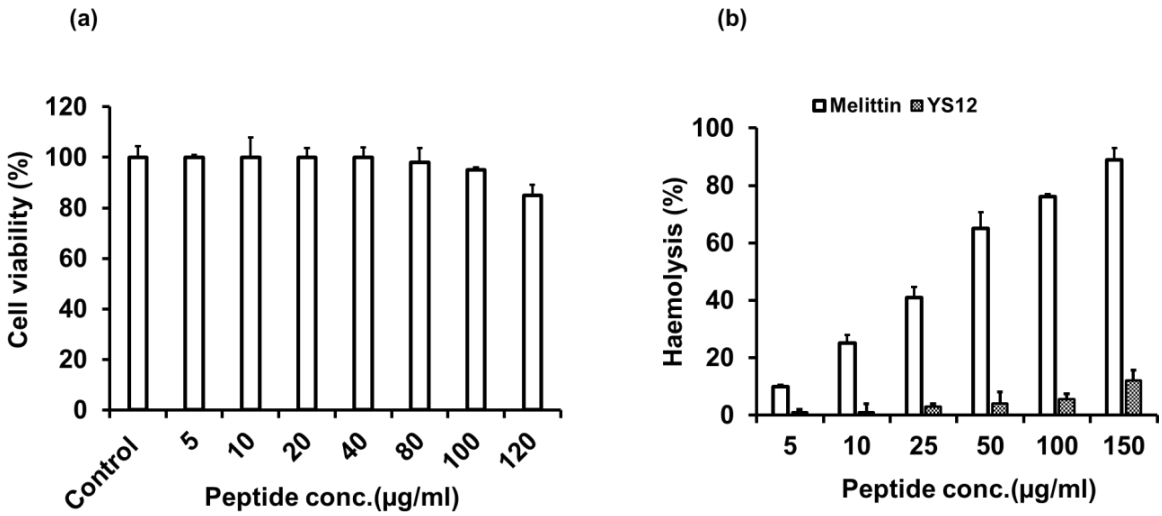

Figure 3.10. (a) Cell viability using Raw 264.7 cells was measured. Raw 264.7 cells were treated with the peptide YS12 (5-120 μg/ml) (b). The hemolytic activity of peptide YS12 and Melittin with different concentrations (5-150 μg/ml) was determined. Each bar represents the mean ± SV of three independent trials that were carried out in triplicate.

3.5. Antimicrobial mechanism studies

Although the precise AMPs mechanisms is unknown, it is predicted that it is distinctfrom that of conventional antibiotics. Fluorescence spectroscopy was used to determine how the developed antimicrobial peptideworked against different bacteria. We used different fluorescent dyes, such as NPN, and DiSC3-5dyes, to evaluate the possible mechanism of action of the peptide.

3.5.1. liposome preparation and aggregation

LUVs are widely employed to mimic bacterial membranes [118]. The lipid bilayer of the microbial membrane is composed of phospholipids containing phosphoglycerol (PG), phosphoethanolamine (PE), phosphatidylcholine (PC), and cardiolipin that have negative charges. To investigate the interaction between peptide YS12 and cell membranes, we established differenttypes of LUVs with PE: PG (7:3, w/w), PG, and PC, respectively [119]. The amount of aggregationcaused by the peptide YS12 was measured using the turbidity of liposomes. PE: PG liposome suspension turbidity increased gradually and consistently at a peptide/lipid (P/L) ratio from 0.05 to 0.025. When the peptide/liposome ratio was 0.025, the turbidity was achieved at 0.025, as shown in Figure 3.11a. With increased peptide/liposome ratios, the turbidity of PG arises slowly compared with PE: PG.

However, the peptide didn’t interrupt with phosphatidylcholine (PC) liposomes. The peptide's strongest interactions with bacterial membranes were confirmed throughthe aggregation of PE: PG and PG liposomes. The result of this study was consistent with other research that reported that AMPs had an impact on bacterial membranes [120].

3.5.2. LPS binding affinity of peptide YS12

We performed several molecular and cellular investigations to ascertain the peptide YS12's mode of action on bacteria. It is believed that most AMPs contain a positive that interacts with the negatively charged bacterial membrane through electrostatic interaction. Gram-positive and Gram-negative bacterial membranes differ significantly in their molecular components and structure. The interaction between microbial membranes and AMPs plays a critical role in the destruction of bacteria and has drawn more attention in terms of developing drugs [121, 122]. Typically, LPS acts as a barrier for gram-negative organisms. Thus, we evaluated the LPS binding affinity of peptide YS12. YS12 was able to bind with LPS by electrostatic attraction to the *E. coli* surface.

In Figure 3.11b, it was observed that LPS only slightly decreased the antibacterial activity of YS12 at lower concentrations of 5 µg/ml, but at 80 µg/ml LPS successfully neutralized the killing effects of YS12. Polymyxin B, an effective outer membrane binding agent, was employed as a positive control, and the bacteria was no longer sensitive at 80 µg/ml. The negative control of ampicillin, which works to prevent cell wall production, did not affect LPS. Based on the findings, YS12 seems to have a strong affinity for the LPS of Gram-negative bacteria.

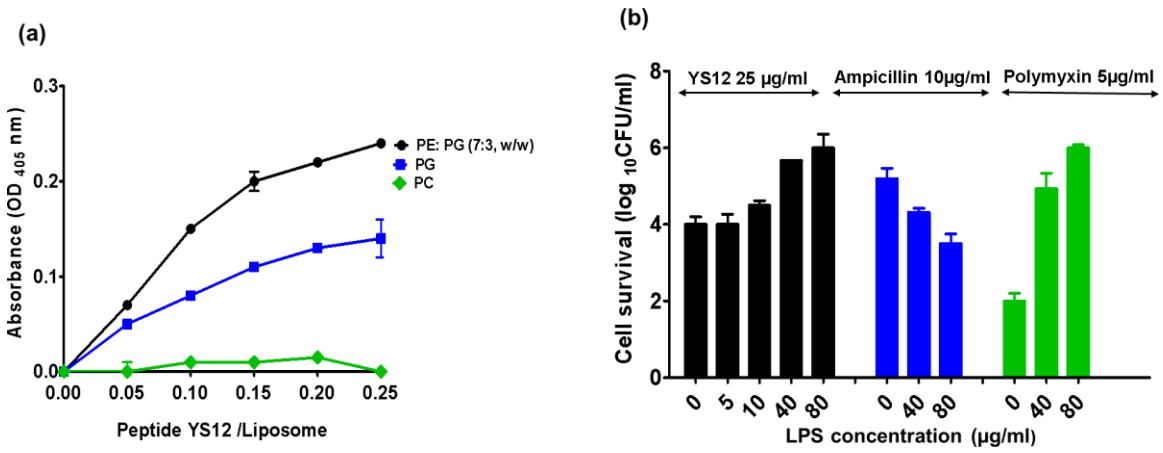

Figure 3.11. (a) Aggregation of large unilamellar vesicles (LUV). 400μM LUV with PE: PG (7:3,w/w) PG and PC were added to a solution containing different ratios of peptide, and aggregation was observed at 405 nm. (b) *E. coli* survival was checked after treatment with lipopolysaccharide (LPS) either itself or in combination with ampicillin (10 μg/ml), polymyxin (5 μg/ml), and peptide(50 μg/ml).

We also conducted the dansyl-polymyxin B displacement experiment to quantify the LPS binding.DPX works effectively as an indicator for cationic bindings on both purified LPS and whole bacterial cells [123]. Although DPX is nonfluorescent in free solution but exhibits high fluorescence when bound with LPS [122]. As seen in Figure 3.12a, peptide YS12 was bound to LPS, and it was able to replace the dansyl PMB resulting in decreasing fluorescence from 90% to 40% at 2.5 and 40μg/ml, respectively. According to the findings, the peptide YS12 exerted a potentelectrostatic interaction with LPS and then exhibited notable membrane rupture properties that enhanced its excellent antimicrobial activity.

Additionally, we tested the antimicrobial peptide's capacity to neutralize LPS using a Limulus amebocyte lysate (LAL) assay using differentconcentrations of YS12 (2.5-40 μg/ml) and 1 ng/ml LPS. In Figure 3.12b, we found that peptide YS12 neutralized LPS in a dose-dependent manner and showed 78% neutralization at 40 μg/ml. Taken together, the peptide affects LPS, which is similar inthat other AMPs act on drug-resistant strains [125].

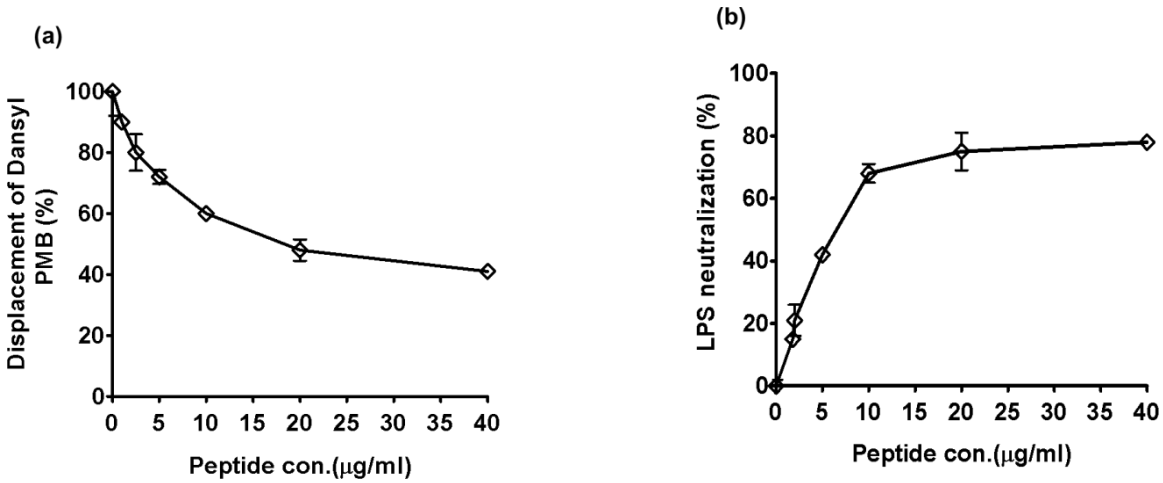

Figure 3.12. (a) Binding affinities of peptide for LPS (from *E. coli*), as analyzed using dansyl PMB (b) LPS neutralization by peptide YS12, determined using an endotoxin quantification kit. Each bar represents the mean ± SV of three independent trials that were carried out in triplicate

3.5.3. Outer membrane permeability

Numerous investigations have shown that most antimicrobial peptides act against drug-resistant bacteria via disrupting membranes [126, 127]. The outer membrane of gram-negative and gram-positive bacteria contains anionic components lipopolysaccharide (LPS) or lipoteichoic acid (LTA), which can interact with cationic amphipathic AMPs. These AMPs were proposed to act on bacteria by disrupting membranes via barrel stave, toroidal pores, or carpet-likemechanisms [128]. Thus, we investigated the mechanisms of action on pathogenic microbes in this study. We first evaluated the potentiality of the peptide to damage the outer membrane of *E. coli* and *P. aeruginosa* bacteria using the fluorescent NPN dye. When the bacterial outer membrane is disrupted, NPN reacts to show fluorescence. As shown in Fig 3.13 (a) and Fig (b), peptide YS12 caused membrane disruption to both bacteria in different concentrations. In *E. coli* bacteria, the fluorescence intensity gradually increased to 33%, 49%, and 66% within 5 minutes at 1× MIC, 2× MIC, and 4× MIC, respectively.

In contrast, the peptide increased NPN uptake by about (50% in *P. aeruginosa*) at 4 × MIC within 5 min, which was slightly lower than the *E. coli* bacteria. Subsequently, the ability to depolarize the bacterial inner membrane in the presence of peptide YS12 was confirmed using diSC3-5. DiSC3-5, a membrane potential fluorescent dye was employed to determine whether the peptide affected the bacterial membrane. Fig. 3.13 (c)and 3.13 (d) showed that the cytoplasmic membrane potential change of gram-negative bacteria *E.coli* and gram-positive bacteria *S. aureus* induced by peptide concentration ranging from 1× to 4×MIC. The peptide YS12 caused stronger depolarization of the cytoplasmic membrane at the concentration (4× MIC) of peptide, reaching approximately 165 AU and 141 AU for *E. coli* and *S.aureus,* respectively. Collectively, the results suggest that peptide affects the bacterial outer membrane which is compatible with the mode of action of different AMPs [110].

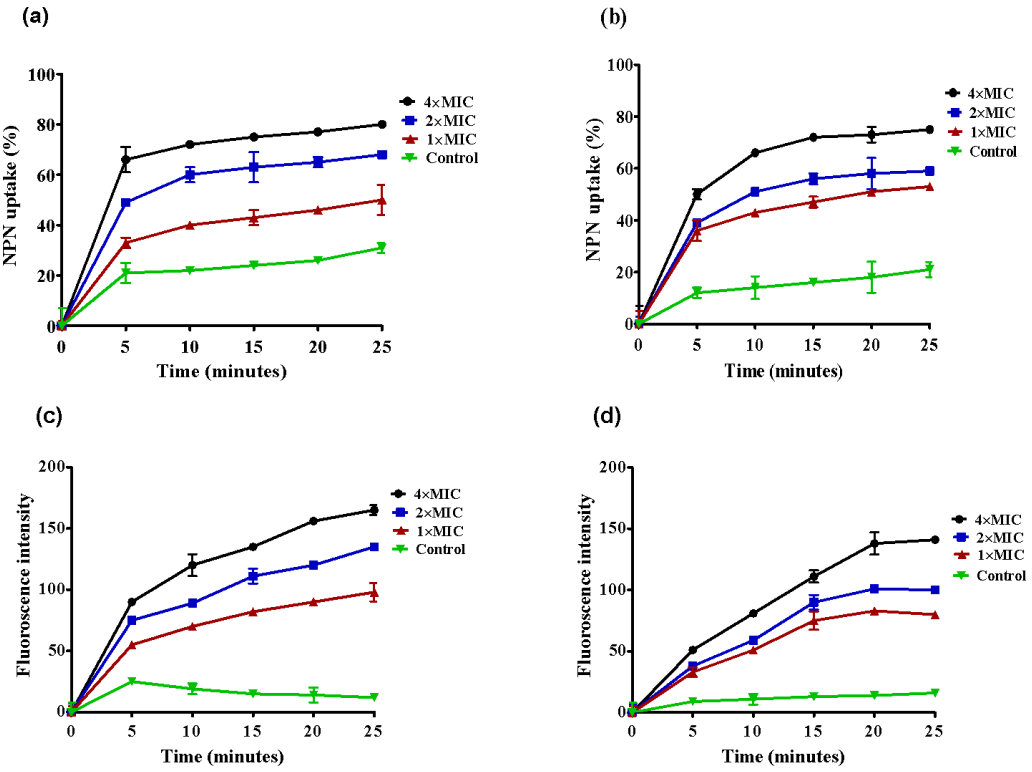

Figure 3.13. Peptide YS12 mechanism of action. (a, b) The outer membrane permeability of YS12 was determined using a fluorescent dye (NPN). (c, d) Depolarization of cytoplasmic membrane induced by YS12 determined using fluorescent dye DisC3-5. (a, c: *E. coli* KCTC 1923. b: *P. aeruginosa* KCTC 1637 d: *S. aureus* KCTC 1928).

3.5.4. Peptide YS12 damaged the membrane integrity of bacteria.

The positively charged AMPs have a variety of modes of action on bacteria, including disruption of the integrity of the cell membrane and leakage of intracellular material [129,130]. In the initial stage of the mechanism study, we determined the affinity of the peptide, which confirmed that the peptide was able to bind with LPS. In this work, we explore the effects of peptide YS12 on microbial cell membrane integrity using PI. Typically, the peptide treatment allowed PI to enter into bacteria [131]. In Figure 3.14a and Figure 3.14b, it was shown that the presence of peptide YS12 at 1×, 2×, and 4×MIC increased fluorescence intensity, respectively over time. The results proved that peptide YS12 caused maximum fluorescence of approximately 75% and 71% at 4×MIC against *E. coli* and *S. aureus*, respectively. The time and concentration-dependent permeabilization ability of antimicrobial peptides (AMPs) against drug-resistant strains has been reported [132].

Moreover, a colorimetric and spectrophotometric substrate (ONPG) is used to measure the activityof β-galactosidase in the inner membrane. To assess the peptide YS12's effects on the inner membrane of *E. coli*, ONPG was used as a-galactosidase. In Figure 3.14c, Peptide YS12 caused rapid permeabilization of the *E. coli* membrane by increasing the fluorescence in a time and concentration-dependent manner. Furthermore, by measuring the release of calcein from negatively charged liposomes over time, membrane permeabilization was discovered. Within 25 minutes Peptide YS12-caused membrane rupture by inducing approximately 83% leakage of calcein from calcein-loaded liposomes at the maximum concentration of 4×MIC (Figure 3.14d). The findings were consistent with prior work that demonstrated increased membrane permeabilization and calcium leakage after peptide treatment [133].

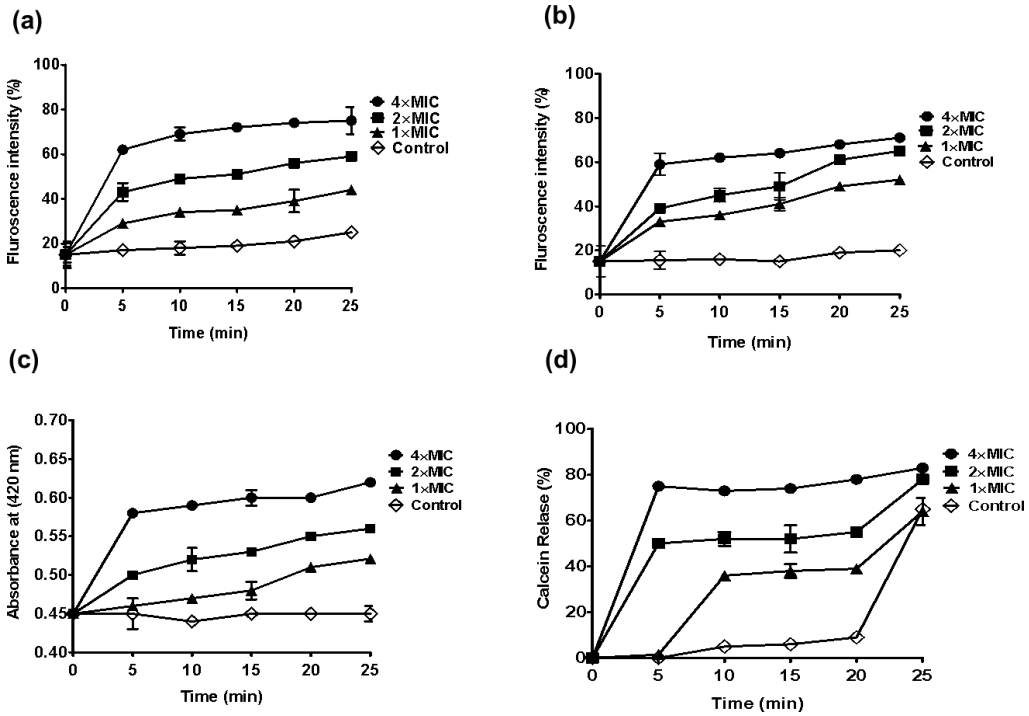

Figure 3.14. Peptide YS12 mechanism of action. PI uptake assay was used to measure the permeability of the inner membrane on (a) *E. coli* ATCC 1923 (b) *S. aureus* KCTC 1928 (c)Release of cytoplasmic B-galactose of *E. coli* ATCC 1923 treated with peptide YS12 at different concentrations (1,×2×, and 4×MIC) (d) Calcein release measured by peptide YS12 at different concentrations. At the specified time, the calcein release rate was determined. 0.1% TritonX-100 was used as a positive control.

3.5.5. Confocal microscopy

We proposed that peptides may cause bacterial membrane damage suggesting a potential AMPsmechanism of action. In this study, we used Fluorescein isothiocyanate (FITC)- labeled YS12 dyeto assess the localization of the peptide in the bacterial membrane under a confocal lasermicroscope. (FITC)-labeled YS12 corresponding to a 2× MIC value was applied to the *P. aeruginosa* and *E. coli* bacteria for 30 minutes.In Figure 3.15a and Figure 3.15b, it was observed that FITC-leveled YS12 translocated into the bacterial cell by aggregating in the cytoplasm, confirming that YS12 exhibits a targeting mechanism by cell penetration. Finally, the findings of this study could help us to understand the possible mechanism of our novel AMPs and may facilitate the development of promising antimicrobial agents.

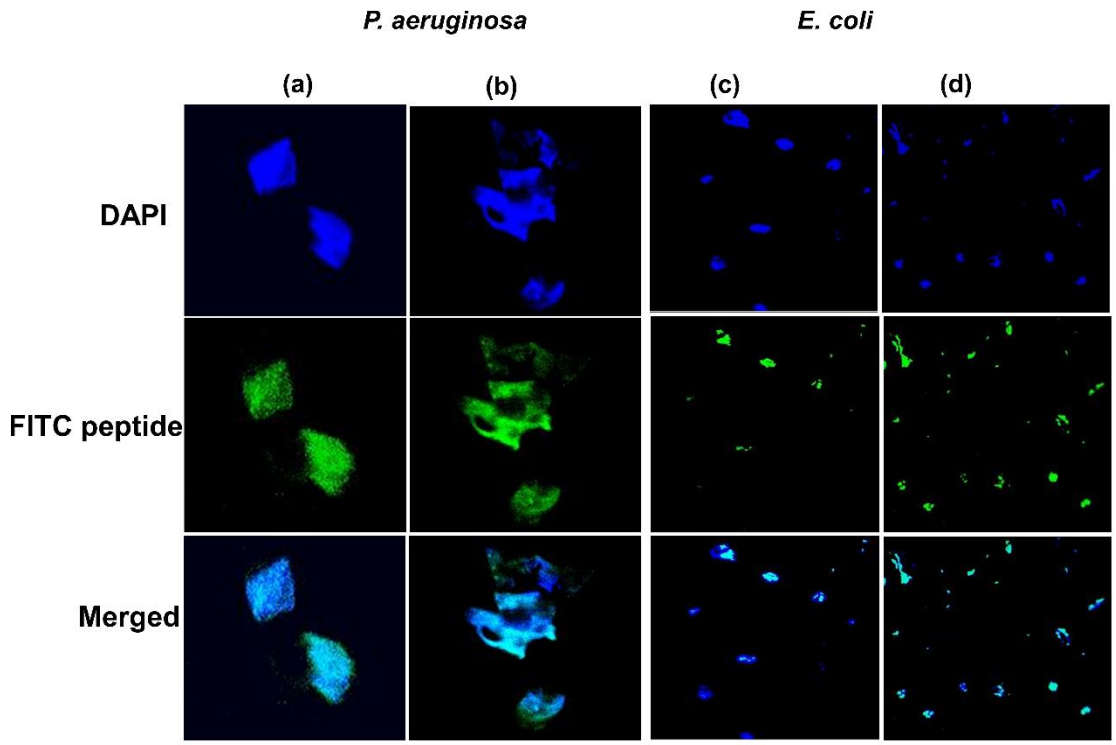

Figure 3.15. Localization of FITC-YS12 peptide on the bacteria. **(a, b)** *P. aeruginosa* **(c,d)** *E. coli*cells were incubated with peptide at theconcentration of 2 ×MIC. The bacteria were washed, fixed, and stained with DAPI (blue).

3.6. Conclusion

The present study has demonstrated the bactericidal and synergistic effect of peptide YS12 with traditional antibiotics against different drug-resistant strains. Peptide YS12 also confirmed salt resistance and cell selectivity properties. Finally, weassumed a possible mechanism of our novel peptide that may be used as a promisingantimicrobial agent for treating infectious illnesses caused by drug-resistant strains.

4. Future directions:

- Antimicrobial peptides are extensively studied for their structure, purpose, and method of action. Despite this research, there are many protected fields of study where new expertise, including newinsights into the manufacture of drugs for multiple therapeutic applications, can be acquired. Below are some of the crucial topics that need to be discussed in the near future.
- In nanobiotechnology, the self-assembly of tiny peptides into nanostructures is a developingarea of study. Core-shell nanoparticles made from amphiphilic peptides that self-assemble have potent antibacterial capabilities against a wide variety of bacteria, fungi, and yeasts. Create novelnanoparticles that include beneficial medicinal ingredients. Additional study is required.
- More research is required to create peptidomimetic antimicrobials that are non-toxic, shorter,and more effective. They should also be very stable and possess the requisite antimicrobial activity.Animal models should be utilized to study the pharmacokinetics, toxicity studies, and biological activity of antimicrobial peptides.
- To enable the development of a greater variety of antimicrobial peptides in the near future, additional study is needed to categorize or

discover potential amino acids that may play a crucial role in the antimicrobial and cytotoxic activity of anti-microbial peptides.

5. References

1. Saga, Tomoo, and Keizo Yamaguchi. "History of antimicrobial agents and resistant bacteria." Jmaj 52.2 (2009): 103-108.

2. Nassar, Nazim, et al. "The History of Antibiotics Illumes the Future of Antimicrobial Peptides Administered Through Nanosystems." Nano-Strategies for Addressing Antimicrobial Resistance: Nano-Diagnostics, Nano-Carriers, and Nano-Antimicrobials. Cham: Springer International Publishing, 2022. 1-74.

3. Coates, A. R. M., and Yanmin Hu. "Novel approaches to developing new antibiotics for bacterial infections. "British journal of pharmacology 152.8 (2007): 1147-1154.

4. Rudramurthy GR, Swamy MK, Sinniah UR, Ghasemzadeh A (2016) Nanoparticles: alternatives against drug-resistant pathogenic microbes. Molecules 21:836

5. Sang Y, Blecha F (2008) Antimicrobial peptides and bacteriocins: alternatives to traditional antibiotics. Anim Health Res Rev 9:227-35

6. Pulingam, Thiruchelvi, et al. "Antimicrobial resistance: Prevalence, economic burden, mechanisms of resistance and strategies to overcome." European Journal of Pharmaceutical Sciences 170 (2022): 106103.

7. Li, Xingshu, et al. "Supramolecular antibacterial materials for combatting antibiotic resistance."Advanced Materials 31.5 (2019): 1805092.

8. Korhonen H, Pihlanto A. Bioactive peptides: Production and functionality. International dairy journal. 2006 Sep 1;16(9):945-60.

9. Sánchez, Adrián, and Alfredo Vázquez. "Bioactive peptides: A review." Food quality and safety 1.1 (2017): 29-46.

10. Zupin, Luisa, et al. "Bioactive Antimicrobial Peptides: A New Weapon to Counteract Zoonosis." Microorganisms 10.8 (2022): 1591.

11. Drayton, Matthew, Jayachandran N. Kizhakkedathu, and Suzana K. Straus. "Towards robust delivery of antimicrobial peptides to combat bacterial resistance." Molecules 25.13 (2020): 3048.

12. Zhang, Qi-Yu, et al. "Antimicrobial peptides: mechanism of action, activity and clinical potential." Military Medical Research 8 (2021): 1-25.

13. Datta, Shreeja, and Arpita Roy. "Antimicrobial peptides as potential therapeutic agents: a review." International Journal of Peptide Research and Therapeutics 27 (2021): 555-577.

14. Petkov, Peicho, et al. "Self-association of antimicrobial peptides: A molecular dynamics simulation study on bombinin." International journal of molecular sciences 20.21 (2019): 5450.

15. Moravej, Hoda, et al. "Antimicrobial peptides: features, action, and their resistance mechanisms in bacteria." Microbial Drug Resistance 24.6 (2018): 747-767.

16. Torres, Marcelo Der Torossian, and Cesar de la Fuente-Nunez. "Reprogramming biological peptides to combat infectious diseases." Chemical Communications 55.100 (2019): 15020-15032.

17. Singh, Mangal, Sandeep Chaudhary, and Dipti Sareen. "Non-ribosomal peptide synthetases: identifying the cryptic gene clusters and decoding the natural product." Journal of biosciences 42 (2017): 175-187.

18. Albenzio, Marzia, et al. "Bioactive peptides in animal food products." Foods 6.5 (2017): 35.

19. Pihlanto, Anne. "Lactic fermentation and bioactive peptides." Lactic acid bacteria–R & D for food, health and livestock purposes 2 (2013): 310-331.

20. Pessione, Enrica, and Simona Cirrincione. "Bioactive molecules released in food by lactic acid bacteria: Encrypted peptides and biogenic amines." Frontiers in microbiology 7 (2016): 876.

21. Tamang, Jyoti P., et al. "Functional properties of microorganisms in fermented foods." Frontiers in microbiology 7 (2016): 578.

22. Patra, Jayanta Kumar, et al. "Kimchi and other widely consumed traditional fermented foods of Korea: a review." Frontiers in microbiology 7 (2016): 1493.

23. Kim, Soon Hee, et al. "Erratum: Corrigendum to “Korean diet: Characteristics and historical background”(Journal of Ethnic Foods (2016) 3 (1)(26–31)(S2352618116300099)(10.1016/j. jef. 2016.03. 002))." Journal of Ethnic Foods 3.2 (2016): 165-165.

24. Singh, Digar, Sunmin Lee, and Choong Hwan Lee. "Metabolomics for empirical delineation of the traditional Korean fermented foods and beverages." Trends in Food Science & Technology 61 (2017): 103-115.

25. Park, Kun-Young, et al. "Health benefits of kimchi (Korean fermented vegetables) as a probiotic food." Journal of medicinal food 17.1 (2014): 6-20.

26. Bahar, Ali Adem, and Dacheng Ren. "Antimicrobial peptides." Pharmaceuticals 6.12 (2013): 1543-1575.

27. Malanovic, Nermina, and Karl Lohner. "Antimicrobial peptides targeting gram-positive bacteria." Pharmaceuticals 9.3 (2016): 59.

28. Kumar, Prashant, Jayachandran N. Kizhakkedathu, and Suzana K. Straus. "Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo." Biomolecules 8.1 (2018): 4

29. Khosa, Sakshi, Marcel Lagedroste, and Sander HJ Smits. "Protein defense systems against the lantibiotic nisin: function of the immunity protein NisI and the resistance protein NSR." Frontiers in microbiology 7 (2016): 504.

30. Yount, Nannette Y., and Michael R. Yeaman. "Peptide antimicrobials: cell wall as a bacterial target." Annals of the New York Academy of sciences 1277.1 (2013): 127-138.

31. Grein, Fabian, Tanja Schneider, and Hans-Georg Sahl. "Docking on lipid II—a widespread mechanism for potent bactericidal activities of antibiotic peptides." Journal of molecular biology 431.18 (2019): 3520-3530.

32. Guilhelmelli, Fernanda, et al. "Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance." Frontiers in microbiology 4 (2013): 353.

33. Omardien, Soraya, Stanley Brul, and Sebastian AJ Zaat. "Antimicrobial activity of cationic antimicrobial peptides against gram-positives: current progress made in understanding the mode of action and the response of bacteria." Frontiers in cell and developmental biology 4 (2016): 111

34. Armengol, Eva, et al. "Efficacy of combinations of colistin with other antimicrobials involves membrane fluidity and efflux machinery." Infection and drug resistance (2019): 2031-2038.

35. Klein, Stefanie, et al. "Adaptation of Pseudomonas aeruginosa to various conditions includes tRNA-dependent formation of alanyl-phosphatidylglycerol." Molecular microbiology 71.3 (2009): 551-565.

36. van Heijenoort, Jean. "Lipid intermediates in the biosynthesis of bacterial peptidoglycan."Microbiology and Molecular Biology Reviews 71.4 (2007): 620-635.

37. Münch, Daniela, and Hans-Georg Sahl. "Structural variations of the cell wall precursor lipid II in Gram-positive bacteria—Impact on binding and efficacy of antimicrobial peptides." Biochimica et Biophysica Acta (BBA)-Biomembranes 1848.11 (2015): 3062-3071.

38. Gordon, Y. Jerold, Eric G. Romanowski, and Alison M. McDermott. "A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs." Current eye research 30.7 (2005): 505-515.

39. Rončević, Tomislav, Jasna Puizina, and Alessandro Tossi. "Antimicrobial peptides as anti- infective agents in pre-post-antibiotic era?." International journal of molecular sciences 20.22 (2019): 5713.

40. Stawikowski, Maciej, and Gregg B. Fields. "Introduction to peptide synthesis." Current protocols in protein science 69.1 (2012): 18-1.

41. Baindara, Piyush, et al. "Characterization of two antimicrobial peptides produced by a halotolerant Bacillus subtilis strain SK. DU. 4 isolated from a rhizosphere soil

42. Urdaci, M. C., and I. Pinchuk. "Antimicrobial activity of Bacillus probiotics." Bacterial spore formers–Probiotics and emerging applications. Norfolk, UK: Horizon Bioscience (2004): 171-82.

43. Sidooski T, Brandelli A, Bertoli SL, Souza CK, Carvalho LF (2019) Physical and nutritional conditions for optimized production of bacteriocins by lactic acid bacteria–A review. Crit Rev Food Sci Nutr 59:2839-49

44. Mackowiak, Philip A. "Recycling Metchnikoff: probiotics, the intestinal microbiome and the quest for long life." Frontiers in public health 1 (2013): 52.

45. Belkaid, Yasmine, and Timothy W. Hand. "Role of the microbiota in immunity and inflammation." Cell 157.1 (2014): 121-141.

46. Rössler, Dieter, et al. "Phylogenetic diversity in the genus Bacillus as seen by 16S rRNA sequencing studies." Systematic and applied microbiology 14.3 (1991): 266-269.

47. Mnif, Ines, Semia Ellouze-Chaabouni, and Dhouha Ghribi. "Optimization of inocula conditions for enhanced biosurfactant production by Bacillus subtilis SPB1, in submerged culture, using Box–Behnken design." Probiotics and antimicrobial proteins 5 (2013): 92-98.

48. Gotham, Simon M., Peter J. Fryer, and William R. Paterson. "The measurement of insoluble proteins using a modified Bradford assay." Analytical biochemistry 173.2 (1988): 353-358.

49. Schägger, Hermann. "Tricine–sds-page." Nature protocols 1.1 (2006):

50. Ayed, Hanen Ben, et al. "Identification and biochemical characteristics of lipopeptides from Bacillus mojavensis A21." Process Biochemistry 49.10 (2014): 1699-1707.

51. Gaur, Smriti, Sarita Agrahari, and Neeraj Wadhwa. "Purification of protease from Pseudomonas thermaerum GW1 isolated from poultry waste site." The Open Microbiology Journal 4 (2010): 67.

52. Clarridge III, Jill E. "Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases." Clinical microbiology reviews 17.4 (2004): 840- 862.

53. Kang, Nam Joo, et al. "New approaches towards the discovery and evaluation of bioactive peptides from natural resources." Critical Reviews in Environmental Science and Technology 50.1 (2020): 72-103.

54. Yamanaka, Hayato, et al. "Degradation of bisphenol A by Bacillus pumilus isolated from kimchi, a traditionally fermented food." Applied biochemistry and biotechnology 136 (2007): 39-

55. Wibowo, David, and Chun-Xia Zhao. "Recent achievements and perspectives for large-scale recombinant production of antimicrobial peptides." Applied microbiology and biotechnology 103 (2019): 659-671.

56. Yang, En, et al. "Influence of culture media, pH and temperature on growth and bacteriocin production of bacteriocinogenic lactic acid bacteria." AMB express 8.1 (2018): 1-14.

57. Khan, Md Maruf, et al. "Response surface optimization of culture conditions for cyclic lipopeptide MS07 from Bacillus siamensis reveals diverse insights targeting antimicrobial and antibiofilm activity." Processes 8.6 (2020): 744.

58. Singh, Vineeta, et al. "Strategies for fermentation medium optimization: an in-depth review." Frontiers in microbiology 7 (2017): 2087.

59. Zacharof, Myrto-Panagiota, et al. "Separation of lactobacilli bacteriocins from fermented broths using membranes." Process Biochemistry 48.8 (2013): 1252-1261.

60. Choi, Yun Hee, et al. "A novel thermotolerant and acidotolerant peptide produced by a Bacillus strain newly isolated from a fermented food (kimchi) shows activity against multidrug-resistant bacteria." International journal of antimicrobial agents 40.1 (2012): 80-83.

61. Pingitore, E. Vera, et al. "Different strategies for purification of antimicrobial peptides from lactic acid bacteria (LAB)." Communicating current research and educational topics and trends in applied microbiology 1 (2007): 557-568.

62. An, Junying, et al. "Purification and characterization of a novel bacteriocin CAMT2 produced by Bacillus amyloliquefaciens isolated from marine fish Epinephelus areolatus." Food Control 51 (2015): 278-282.

63. Khan, Md Maruf, et al. "Reduction of oxidative stress through activating the Nrf2 mediated HO-1 antioxidant efficacy signaling pathway by MS15, an antimicrobial peptide from Bacillus velezensis." Antioxidants 9.10 (2020): 934.

64. An, Junying, et al. "Purification and characterization of a novel bacteriocin CAMT2 produced by Bacillus amyloliquefaciens isolated from marine fish Epinephelus areolatus." Food Control 51 (2015): 278-28

65. Panthi, Sandesh, et al. "Antimicrobial Peptide from Bacillus Strain K1R Exhibits Ameliorative Potential Against Vancomycin-Resistant Enterococcus Group of Organisms." International

Journal of Peptide Research and Therapeutics 23 (2017): 419-430

66. Ayed, Hanen Ben, et al. "Isolation and biochemical characterisation of a bacteriocin-like substance produced by Bacillus amyloliquefaciens An6." Journal of global antimicrobial resistance 3.4 (2015): 255-261.

67. Kim, Soon Hee, et al. "Korean diet: characteristics and historical background." Journal of Ethnic Foods 3.1 (2016): 26-31.

68. Ferre, Rafael, et al. "Synergistic effects of the membrane actions of cecropin-melittin antimicrobial hybrid peptide BP100." Biophysical journal 96.5 (2009): 1815-1827

69. Beaudoin, Trevor, et al. "Activity of a novel antimicrobial peptide against Pseudomonas aeruginosa biofilms." Scientific reports 8.1 (2018): 14728.

70. Khosa, Sakshi, Marcel Lagedroste, and Sander HJ Smits. "Protein defense systems against the lantibiotic nisin: function of the immunity protein NisI and the resistance protein NSR." Frontiers in microbiology 7 (2016): 504.

71.Appiah, Theresa, Yaw Duah Boakye, and Christian Agyare. "Antimicrobial activities and time- kill kinetics of extracts of selected Ghanaian mushrooms." Evidence-Based Complementary and Alternative Medicine 2017 (2017).

72. Lv, Jia, et al. "Mechanism of antibacterial activity of Bacillus amyloliquefaciens C-1 lipopeptide toward anaerobic Clostridium difficile." BioMed Research International 2020 (2020).

73. Huang, En, and Ahmed E. Yousef. "The lipopeptide antibiotic paenibacterin binds to the bacterial outer membrane and exerts bactericidal activity through cytoplasmic membrane damage." Applied and environmental microbiology 80.9 (2014): 2700-2704.

74. Kalelkar, Pranav P., Milan Riddick, and Andrés J. García. "Biomaterial-based antimicrobial therapies for the treatment of bacterial infections." Nature Reviews Materials 7.1 (2022): 39-54.

75. Le, Cheng-Foh, Chee-Mun Fang, and Shamala Devi Sekaran. "Intracellular targeting mechanisms by antimicrobial peptides." *Antimicrobial agents and chemotherapy* 61, no. 4 (2017): 10-1128.

76. Kim, Ji-Sun, Ji-Ho Jeong, and Yongae Kim. "Design, characterization, and antimicrobial activity of a novel antimicrobial peptide derived from bovine lactophoricin." Journal of microbiology and biotechnology 27.4 (2017): 759-767.

77. Mazumdar, Aninda, et al. "Characterization and in vitro analysis of probiotic-derived peptides against multi drug resistance bacterial infections." Frontiers in Microbiology 11 (2020): 1963.

78. Han, Hyo Mi, Ramamourthy Gopal, and Yoonkyung Park. "Design and membrane-disruption mechanism of charge-enriched AMPs exhibiting cell selectivity, high-salt resistance, and anti- biofilm properties." Amino Acids 48 (2016): 505-522.

79. Ko, Su Jin, et al. "Macropis fulvipes venom component Macropin exerts its antibacterial and anti-biofilm properties by damaging the plasma membranes of drug resistant bacteria." Scientific Reports 7.1 (2017): 1-14.

80. Khan, Md Maruf, et al. "Reduction of oxidative stress through activating the Nrf2 mediated HO-1 antioxidant efficacy signaling pathway by MS15, an antimicrobial peptide from Bacillus velezensis." Antioxidants 9.10 (2020): 934.

81. Ko, Su Jin, et al. "Bee venom-derived antimicrobial peptide melectin has broad-spectrum potency, cell selectivity, and salt-resistant properties." Scientific reports 10.1 (2020): 10145.

82. Park, Seong-Cheol, et al. "Anti-biofilm effects of synthetic antimicrobial peptides against drug- resistant Pseudomonas aeruginosa and Staphylococcus aureus planktonic cells and biofilm." Molecules 24.24 (2019): 4560.

83. Almaaytah, Ammar, et al. "The design and functional characterization of the antimicrobial and antibiofilm activities of BMAP27-melittin, a rationally designed hybrid peptide." International Journal of Peptide Research and Therapeutics 21 (2015): 165-177.

84. Rahman, Md Saifur, et al. "A novel antioxidant peptide, purified from Bacillus amyloliquefaciens, showed strong antioxidant potential via Nrf-2 mediated heme oxygenase-1 expression." Food Chemistry 239 (2018): 502-510.

85. Ko, Su Jin, et al. "Macropis fulvipes venom component Macropin exerts its antibacterial and anti-biofilm properties by damaging the plasma membranes of drug resistant bacteria." Scientific Reports 7.1 (2017): 1-14.

86. Mayer, L. D., M. J. Hope, and P. R. Cullis. "Vesicles of variable sizes produced by a rapid extrusion procedure." Biochimica et Biophysica Acta (BBA)-Biomembranes 858.1 (1986): 161- 168.

87. Pulido, David, et al. "Antimicrobial action and cell agglutination by the eosinophil cationic protein are modulated by the cell wall lipopolysaccharide structure." Antimicrobial agents and chemotherapy 56.5 (2012): 2378-2385.

88. Kong, Delong, et al. "Antimicrobial and Anti-Inflammatory Activities of MAF-1-Derived Antimicrobial Peptide Mt6 and Its D-Enantiomer D-Mt6 against Acinetobacter baumannii by Targeting Cell Membranes and Lipopolysaccharide Interaction." Microbiology Spectrum 10.5 (2022): e01312-22.

89. Legrand, Dominique, et al. "Surface nucleolin participates in both the binding and endocytosis of lactoferrin in target cells." European journal of biochemistry 271.2 (2004): 303-317.

90. Kang, Hee-Kyoung, et al. "The therapeutic applications of antimicrobial peptides (AMPs): a patent review." Journal of microbiology 55 (2017): 1-12.

91. Kim, Min Kyung, et al. "Antibacterial and antibiofilm activity and mode of action of magainin 2 against drug-resistant Acinetobacter baumannii." International Journal of Molecular Sciences 19.10 (2018): 3041.

92. Shi, Jingru, et al. "The antimicrobial peptide LI14 combats multidrug-resistant bacterial infections." Communications Biology 5.1 (2022): 926.

93. Han, Hyo Mi, Ramamourthy Gopal, and Yoonkyung Park. "Design and membrane-disruption mechanism of charge-enriched AMPs exhibiting cell selectivity, high-salt resistance, and anti- biofilm properties." Amino Acids 48 (2016): 505-522.

94. Amado, Carolina Medina, et al. "Bacteriocin enterocin CRL35 is a modular peptide that induces non-bilayer states in bacterial model membranes." Biochimica et Biophysica Acta (BBA)- Biomembranes 1862.2 (2020): 183135.

95. Park, Chan Bae, Hun Sik Kim, and Sun Chang Kim. "Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions." Biochemical and biophysical research communications 244.1 (1998): 253- 257.

96. Choi, Yun Hee, et al. "A novel multifunctional peptide oligomer of bacitracin with possible bioindustrial and therapeutic applications from a Korean food-source Bacillus strain." PloS one 12.5 (2017): e0176971.

97. Regmi, Sudip, et al. "Antimicrobial peptide isolated from Bacillus amyloliquefaciens K14 revitalizes its use in combinatorial drug therapy." Folia microbiologica 62 (2017): 127-138.

98. Regmi, Sudip, et al. "Antimicrobial peptide isolated from Bacillus amyloliquefaciens K14 revitalizes its use in combinatorial drug therapy." Folia microbiologica 62 (2017): 127-138.

99. Kwon, Ju Young, et al. "Mechanism of action of antimicrobial peptide P5 truncations against Pseudomonas aeruginosa and Staphylococcus aureus." AMB Express 9 (2019): 1-15.

100. Alanis, Alfonso J. "Resistance to antibiotics: are we in the post-antibiotic era?." Archives of medical research 36.6 (2005): 697-705.

101. Wu, Xiaozhe, et al. "Synergistic effects of antimicrobial peptide DP7 combined with antibiotics against multidrug-resistant bacteria." Drug design, development and therapy (2017): 939-946.

102. Feng, Q., et al. "Functional synergy of α-helical antimicrobial peptides and traditional antibiotics against Gram-negative and Gram-positive bacteria in vitro and in vivo." European Journal of Clinical Microbiology & Infectious Diseases 34 (2015): 197-204.

103. Ko, Su Jin, et al. "Macropis fulvipes venom component Macropin exerts its antibacterial and anti-biofilm properties by damaging the plasma membranes of drug resistant bacteria." Scientific Reports 7.1 (2017): 1-14.

104. Kang, Hee-Kyoung, et al. "The therapeutic applications of antimicrobial peptides (AMPs): a patent review." Journal of microbiology 55 (2017): 1-12.

105. Zhu, Xin, et al. "Characterization of antimicrobial activity and mechanisms of low amphipathic peptides with different α-helical propensity." Acta biomaterialia 18 (2015): 155-167.

106. Saravanan, Rathi, et al. "Design of short membrane selective antimicrobial peptides containing tryptophan and arginine residues for improved activity, salt-resistance, and biocompatibility." Biotechnology and bioengineering 111.1 (2014): 37-49.

107. Chou, Shuli, et al. "Short, multiple-stranded β-hairpin peptides have antimicrobial potency with high selectivity and salt resistance." Acta Biomaterialia 30 (2016): 78-93.

108. Høiby, Niels, et al. "Antibiotic resistance of bacterial biofilms." International journal of antimicrobial agents 35.4 (2010): 322-332.

109. Park, Seong-Cheol, et al. "Anti-biofilm effects of synthetic antimicrobial peptides against drug-resistant Pseudomonas aeruginosa and Staphylococcus aureus planktonic cells and biofilm." Molecules 24.24 (2019): 4560.

110. Chung, Pooi Yin, and Ramona Khanum. "Antimicrobial peptides as potential anti-biofilm agents against multidrug-resistant bacteria." Journal of microbiology, immunology and infection 50.4 (2017): 405-410.

111. Høiby, Niels, et al. "Antibiotic resistance of bacterial biofilms." International journal of antimicrobial agents 35.4 (2010): 322-332.

112. Pontes, Janaína Teixeira Costa de, et al. "Antimicrobial peptides as an alternative for the eradication of bacterial biofilms of multi-drug resistant bacteria." Pharmaceutics 14.3 (2022): 642.

113. Mohamed, Mohamed F., et al. "A short D-enantiomeric antimicrobial peptide with potent immunomodulatory and antibiofilm activity against multidrug-resistant Pseudomonas aeruginosa and Acinetobacter baumannii." Scientific Reports 7.1 (2017): 1-13.

114. Huigens, Robert W., et al. "Inhibition of Pseudomonas aeruginosa biofilm formation with bromoageliferin analogues." Journal of the American Chemical Society 129.22 (2007): 6966- 6967.

115. Khara, Jasmeet Singh, et al. "Designing α-helical peptides with enhanced synergism and selectivity against Mycobacterium smegmatis: Discerning the role of hydrophobicity and helicity." Acta biomaterialia 28 (2015): 99-108.

116. Kim, Jieun, et al. "Development of a novel short 12-meric papiliocin-derived peptide that is effective against Gram-negative sepsis." Scientific Reports 9.1 (2019): 3817.

117. Rahman, Md Saifur, et al. "Glycin-rich antimicrobial peptide YD1 from B. amyloliquefaciens, induced morphological alteration in and showed affinity for plasmid DNA of E. coli." AMB Express 7 (2017): 1-11.

118. Chongsiriwatana, Nathaniel P., and Annelise E. Barron. "Comparing bacterial membrane interactions of antimicrobial peptides and their mimics." Antimicrobial Peptides: Methods and Protocols (2010): 171-182.

119. Li, Yi, et al. "Antimicrobial activity, membrane interaction and stability of the D-amino acid substituted analogs of antimicrobial peptide W3R6." Journal of Photochemistry and Photobiology B: Biology 200 (2019): 111645.

120. Han, Hyo Mi, Ramamourthy Gopal, and Yoonkyung Park. "Design and membrane-disruption mechanism of charge-enriched AMPs exhibiting cell selectivity, high-salt resistance, and anti- biofilm properties." Amino Acids 48 (2016): 505-522.

121. Ebbensgaard, Anna, et al. "Comparative evaluation of the antimicrobial activity of different antimicrobial peptides against a range of pathogenic bacteria." PloS one 10.12 (2015): e0144611.

122. Teixeira, Vitor, Maria J. Feio, and Margarida Bastos. "Role of lipids in the interaction of antimicrobial peptides with membranes." Progress in lipid research 51.2 (2012): 149-177.

123. Moore, RICHARD A., NANCY C. Bates, and R. E. Hancock. "Interaction of polycationic antibiotics with Pseudomonas aeruginosa lipopolysaccharide and lipid A studied by using dansyl- polymyxin." Antimicrobial agents and chemotherapy 29.3 (1986): 496-500.

124. Espeche, Juan C., et al. "Membrane permeability and antimicrobial peptides: Much more than just making a hole." Peptide Science (2023): e24305.

125. Zhong, Chao, et al. "Antimicrobial peptides conjugated with fatty acids on the side chain of D-amino acid promises antimicrobial potency against multidrug-resistant bacteria." European Journal of Pharmaceutical Sciences 141 (2020): 105123.

126. Papo, Niv, and Yechiel Shai. "Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes?." Peptides 24.11 (2003): 1693-1703.

127. Seo, Min-Duk, et al. "Antimicrobial peptides for therapeutic applications: a review." Molecules 17.10 (2012): 12276-12286.

128. Lee, Tzong-Hsien, Kristopher N Hall, and Marie-Isabel Aguilar. "Antimicrobial peptide structure and mechanism of action: a focus on the role of membrane structure." Current topics in medicinal chemistry 16.1 (2016): 25-39.

129. Ciumac, Daniela, et al. "Membrane targeting cationic antimicrobial peptides." Journal of colloid and interface science 537 (2019): 163-185.

130. Lin, Liming, et al. "Membrane-disruptive peptides/peptidomimetics-based therapeutics: Promising systems to combat bacteria and cancer in the drug-resistant era." Acta Pharmaceutica Sinica B 11.9 (2021): 2609-2644.

131. Zhong, Chao, et al. "Antimicrobial peptides with symmetric structures against multidrug- resistant bacteria while alleviating antimicrobial resistance." Biochemical Pharmacology 186 (2021): 114470.

132. Ko, Su Jin, et al. "Bee venom-derived antimicrobial peptide melectin has broad-spectrum potency, cell selectivity, and salt-resistant properties." Scientific reports 10.1 (2020): 10145.

133. Ma, Lingman, et al. "Potent antibacterial activity of MSI-1 derived from the magainin 2 peptide against drug-resistant bacteria." Theranostics 10.3 (2020): 1373.

6. List of Publications

1. **Suchi, S.A.,** Nam, K.B., Kim, Y.K., Tarek, H. and Yoo, J.C., 2023. A novel antimicrobial peptide YS12 isolated from *Bacillus velezensis* CBSYS12 exerts anti-biofilm properties against drug resistant bacteria. Bioprocess and Biosystems Engineering, pp.1-16.

2. Tarek, H., Nam, K.B., Kim, Y.K., **Suchi, S.A**. and Yoo, J.C., 2023. Biochemical Characterization and Application of a Detergent Stable, Antimicrobial, and Antibiofilm Potential Protease from *Bacillus siamensis.* International Journal of Molecular Sciences, 24(6),p.5774

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