Master's Thesis of Science in Agriculture

Characterization of the Antibiotic Substance Produced by *Weissella* sp. SNUL2 Isolated from Korean Traditional Food and the Estimation Based on Probiotics Guidelines

한국 전통 식품 분리 *Weissella* sp. SNUL2 균주 생산 항균물질의 특성과 프로바이오틱스 가이드라인에 따른 평가

February 2022

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Characterization of the Antibiotic Substance Produced by *Weissella* sp. SNUL2 Isolated from Korean Traditional Food and the Estimation Based on Probiotics Guidelines

A thesis

Submitted partial fulfillment of the requirements to the faculty

of Graduate School of International Agricultural Technology

for the Degree of Master of Science in Agriculture

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February 2022

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For the Degree of Master of Science in Agriculture

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Abstract

After Fleming's discovery of penicillin in 1928, mankind became more safety from bacterial infection. After this discovery, research on antibiotics began to gain momentum, and the role of antibiotics is still very important to this day. However, it took only one year between the introduction of penicillin in 1940 and the discovery of penicillin-resistant bacteria. The widespread emergence of antibiotic resistance in only 80 years since antibiotics were introduced into clinical practice, is causing treatment failure of various infectious diseases. Antibiotic abuse is the decisive reason for these bacteria to evolve to become resistant to antibiotics. Since these bacteria are literally resistant to antibiotics, it is impossible to kill bacteria during clinical treatment, which is a huge problem. Currently, to reduce antibiotic resistance, the need for alternative antibiotics is necessary. Therefore, many researchers are studying to find substances that can replace antibiotics, and one of the alternative antibiotics to solve the problem is probiotics.

However, existing probiotics have a narrow range of antibacterial properties, so they are mostly used as health functional foods rather than for the purpose of treating bacterial infections, which is considered the biggest weakness. Thus, to develop into therapeutic probiotics, it is essential to have a broad antibacterial spectrum, and at the same time, several criteria must be met. The criteria that aim for are as follows: Broad spectrum antibacterial properties; Biodegradable; Probiotics Guidelines. Here, we try to find a strain that meets this criterion and propose it as one of the antibiotic alternative candidates.

In Chapter 2, strains that can be used as an alternative to antibiotics were isolated and various characteristics of antibacterial substances were confirmed. The strain, *Weissella* sp. SNUL2, was isolated from Korean traditional fermented food was identified through 16s rRNA sequencing. The optimum temperature and pH of the strain were confirmed, and the correlation between $O.D_{600}$ and CFU was confirmed and applied to subsequent experiments. In the study of probiotics for therapeutic purposes, the most important antibacterial test was conducted on a total of 70 strains, and as a result, it was confirmed to have broad spectrum antibacterial properties than previous known probiotics. To know the characteristics of the antibacterial substance

secreted in the supernatant, an enzyme test was conducted, and as a result, it was confirmed that it was a proteinaceous substance. Partial purification was performed to further subdivide this antibacterial protein, and as a result of LC-MS/MS analysis, two proteins were found: C39 family protein that produces bacteriocin, a well-known antibacterial protein, and a peptidoglycan endopeptidase that degrades peptidoglycan, a cell wall component.

Chapter 3 confirmed whether the *Weissella* strains met the probiotic conditions according to the FAO and WHO probiotic guidelines. *Weissella* sp. SNUL2 performed whole genome sequencing through PacBio sequencing and acquired genetic information for one chromosome and four plasmids. There were 2,291 CDSs on one chromosome, and as a result of comparing them with the toxic metabolite pathway, several related genes could be identified. *Weissella* sp. SNUL2 was determined to have both acid tolerance and bile tolerance, in addition, as a result of the hemolytic activity, it was confirmed that hemolytic activity that destroys red blood cells did not occur, and although it has antibiotic resistance to streptomycin and kanamycin, using the tool called Plasmid Finder 2.1, it was confirmed as a characteristic of bacteria its selves, not resistance acquired from outside thus *Weissella* sp. SNUL2 met all probiotic trials. As it is a strain with broad range of antibacterial properties, it is thought that it can be used as probiotics for treatment purposes in the future. In addition, it is expected to be used as a substitute for antibiotics, thereby avoiding the risk of antibiotic resistance.

Keyword: *Weissella* sp. SNUL2; Antimicrobial peptide; Antibiotic substance; Antibiotic resistance; Korean traditional fermented food; Probiotics

Student number: 2020-24099

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List of Abbreviations

Chapter 1.

DDDs: defined daily dose, unit of measurement of drug consumption: the assumed average maintenance dose per day for a drug used for its main indication in adults.

AMR: Antimicrobial resistance

Chapter 2.

CFU: Colony forming unit

CFS: Cell free supernatant

LAB: Lactic acid bacteria

NGS: Next generation sequencing

SMRT: Single molecule real time

O.D: Optical density

CDS: Coding sequence

HGP: Human genome project

HMP: Human microbiome project

PBS: Phosphate buffer saline

Chapter 3.

GRAS: Generally Recognized as Safe

BSL: Bio safety level

KCTC: Korean collection for type cultures

ATCC: American type culture collection

NB: Nutrient broth

TSB: Tryptic soy broth

LB: Luria-Bertani broth

MRS: de Man, Rogosa and Sharp

CV: Column volume

BSA: Bovine serum albumin

Chapter 1.

Research background

1. Antibiotics

Antibiotics are antimicrobial agents that kill bacteria and other microbes. Because antibiotics are the most prevalent type of antibacterial agent, they are widely used in the treatment and prevention of bacterial infections. They have antibacterial properties in a way that kills bacteria or inhibit their growth. The first person to directly document the use of molds to treat infections was John Parkinson (1567-1650). After Alexander Fleming (1881-1955) developed modern day penicillin in 1928, the substance revolutionized medicine in 20th century, whose widespread usage proved to be significantly beneficial during wartime.

2. Antibiotics resistance

Antibiotic resistance is a kind of antimicrobial resistance that bacteria may live and proliferate in the presence of antibiotics at therapeutic levels (Ancillotti et al., 2021). The resistance is accelerated by the overuse of antibiotics to humans, animals, and the environment (European Centre for Disease et al., 2017). As a result, bacteria develop a defense against the medicines used to treat them, or some types of germs that have a natural resistance to antimicrobials become far more common than those that are readily defeated with medication. According to CDC report, it takes an average of 6 years for bacteria to become resistant to certain antibiotics (Fig. 1.1.). There are three major mechanisms by which bacteria develop the resistance against bacteria (Fig. 1.2.). The first is to inactivate the drug by producing

an enzyme that degrades the antibiotic. For example, β-lactam antibiotics act by inhibiting the cross-link of peptidoglycan which forms bacteria cell wall, induing cell wall collapse. Bacteria resistant to these antibiotics produce β-lactamase that hydrolyzes the β-lactam ring. The second is to block the synthesis of proteins necessary for cell activity. This method is called alteration binding site. Every bacterium should synthesize the protein, which is necessary to their cell viability, but antibiotic resistant bacteria produce ribosomal protection protein which binds with bacteria cell ribosome, change its conformational shape. A third method is to reduce the accumulation of antibiotics. This is the method to prevent the accumulation of antibiotics before they reach a certain concentration that is active. In antibiotic sensitive bacteria, antibiotics permeate into the cell then damage its cell. The resistant bacteria, However, decrease drug permeability or increase active efflux to pump drugs out of the cell.

3. The risk of antibiotic resistance bacteria

For more than a century, antibiotics have given protection against life-threatening bacterial infections. However, indiscriminate use of antibiotics and organism evolution have led to the emergence of multi drug resistance organism, at times resistant to most or even all currently available antibiotic classes. Antibiotic resistance is a serious emerging global health treat (Aslam et al., 2018). Indeed, as shown in Figure 1.4, global consumption of antibiotics increased by 65% between 2000 and 2015, from 21.1 to 34.8 billion DDDs (Defined daily dose), while the antibiotic consumption rate increased 39% from 11.3 to 15.7 DDDs per 1,000 inhabitants per day over the study period (Klein et al., 2018). According to OECD health statistics 2019, Korea recorded a DDDs per 1,000 population, per day of 26.5 in 2017, which is the third highest among 31 OECD nations. As the prescription of antibiotics has increased around the world, antibiotic resistant bacteria have also naturally increased. Annual mortality from antibiotic abuse in the United States increased by 336%, from about 20,000 in 1999 to 67,367 in 2018 (Fig 1.5), (Centers for Disease Control and Prevention, CDC Wonder online database, 2019).

According to KDCA (Korea disease control and prevention agency), in addition, in Korea, the infection of antibiotic resistant bacteria increased by as little as 23.2% and as high as 1135.9% from 2011 to 2014 (Fig 1.6.). The threat of AMR (Antimicrobial resistance) became gradually obvious among the science and medical community due to increased prevalence of sporadic drug resistant infection outbreaks in health care settings even among high income countries as well as middle, low income countries (Inoue, 2019). In fact, cancer is leading cause of death worldwide each year, and UK department of health predicted that AMR is expected to be major cause of death that makes 10 million people annually by 2050 (Annual report of the chief medical officer 2011 volume 2. London: UK department of Health.2013).

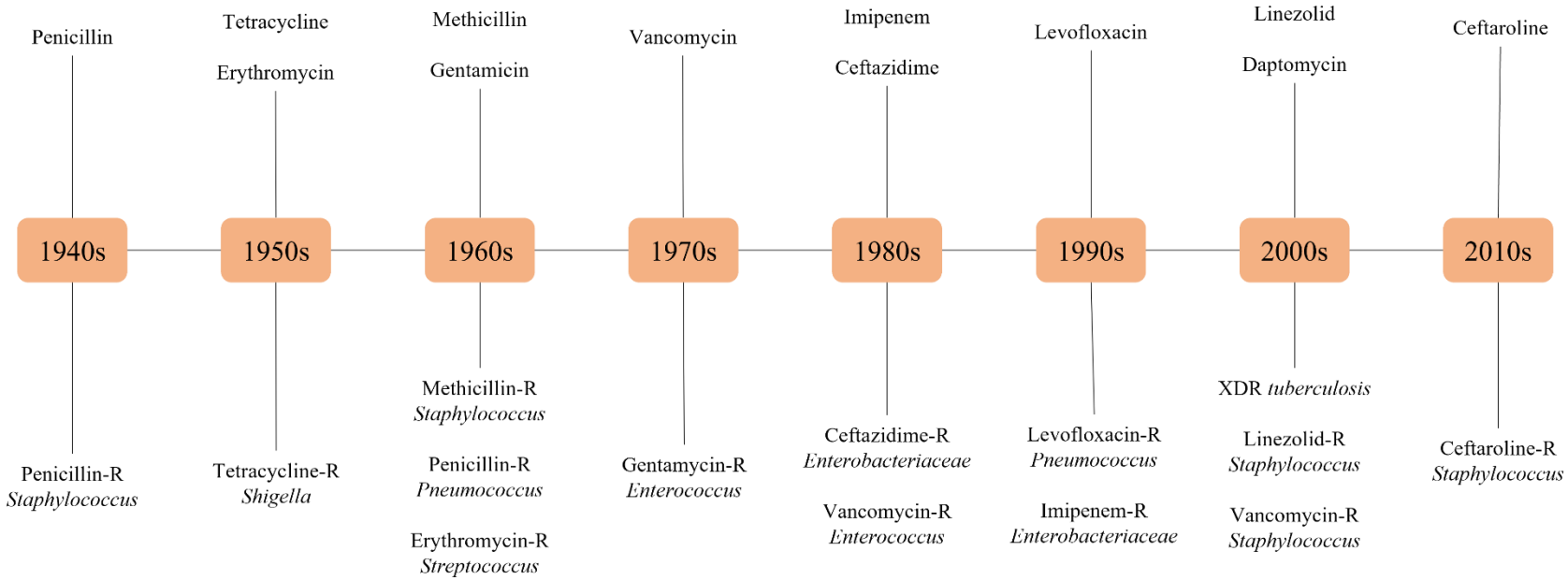


Fig 1.1. The period between the prevalence of antibiotics and the development of antibiotic resistance (CDC, 2019).

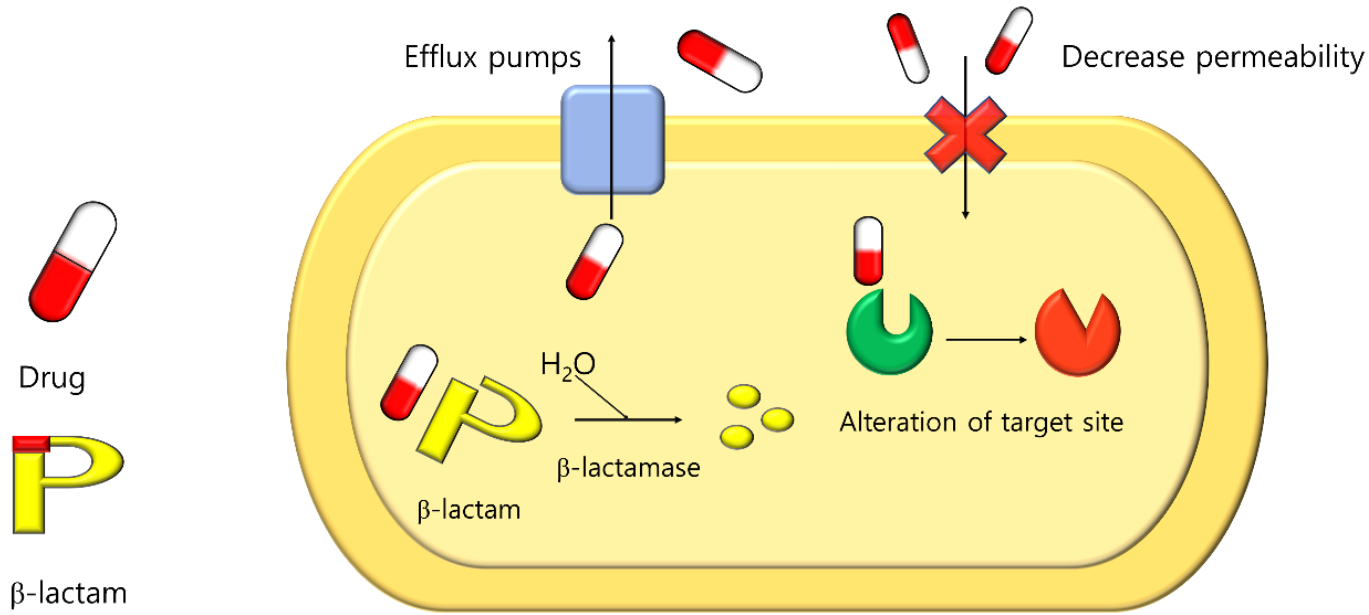


Fig 1.2. Mechanism of antibiotic resistant bacteria.

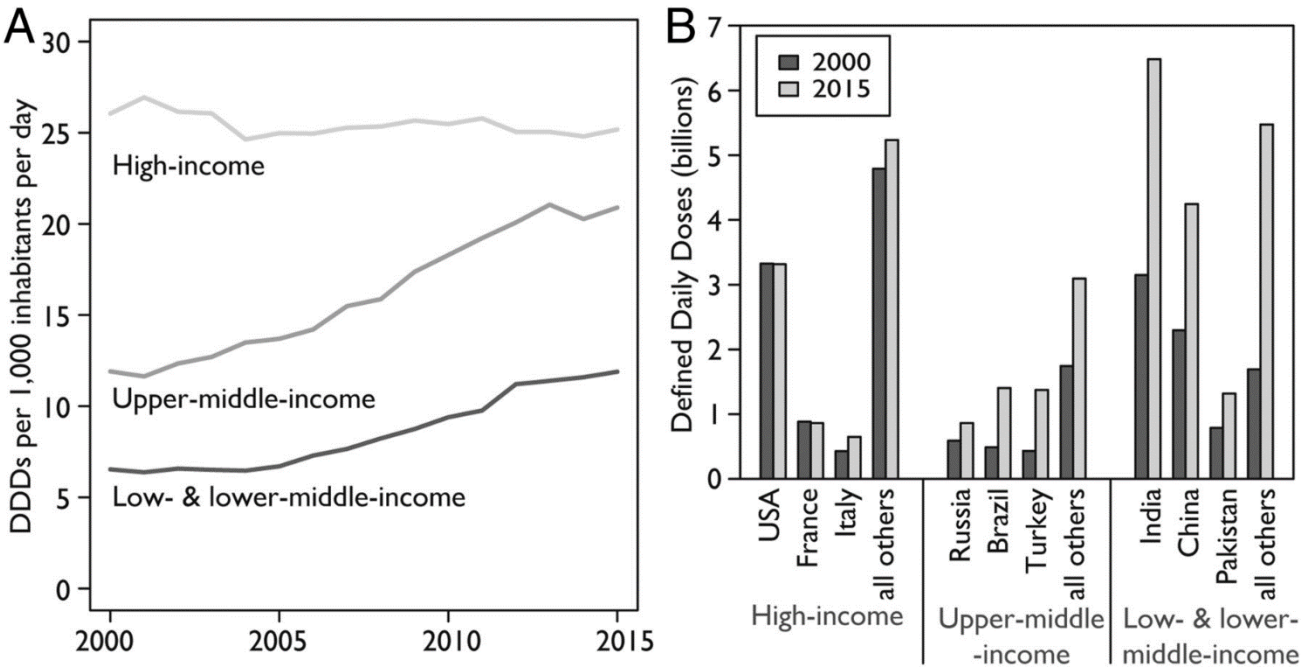


Fig. 1.3. Global antibiotic consumption by country income classification: 2000-2015 (Klein et al., 2018).

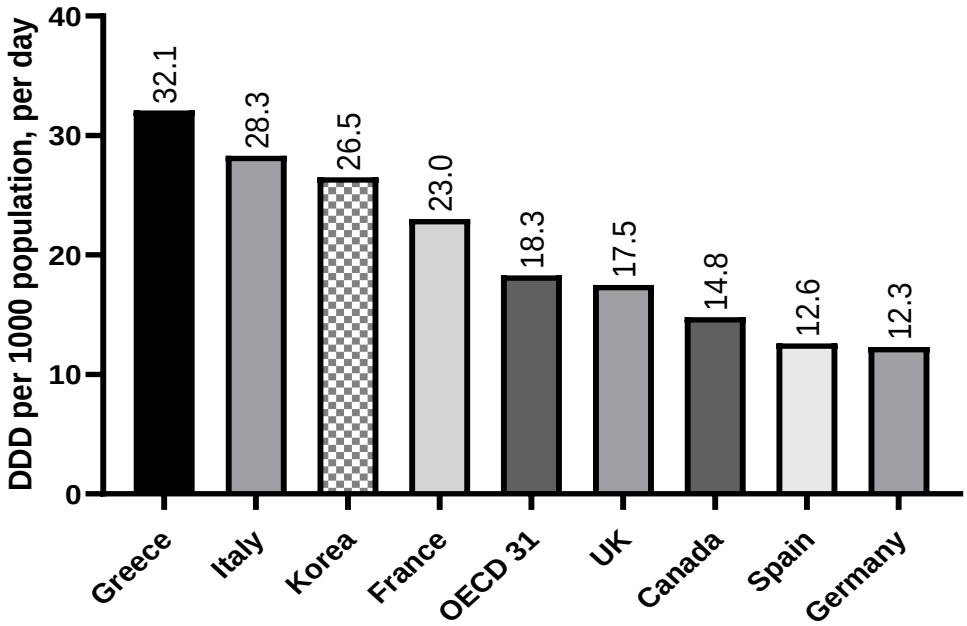


Fig. 1.4. Overall volume of antibiotics prescribed, 2017 (OECD, 2019).

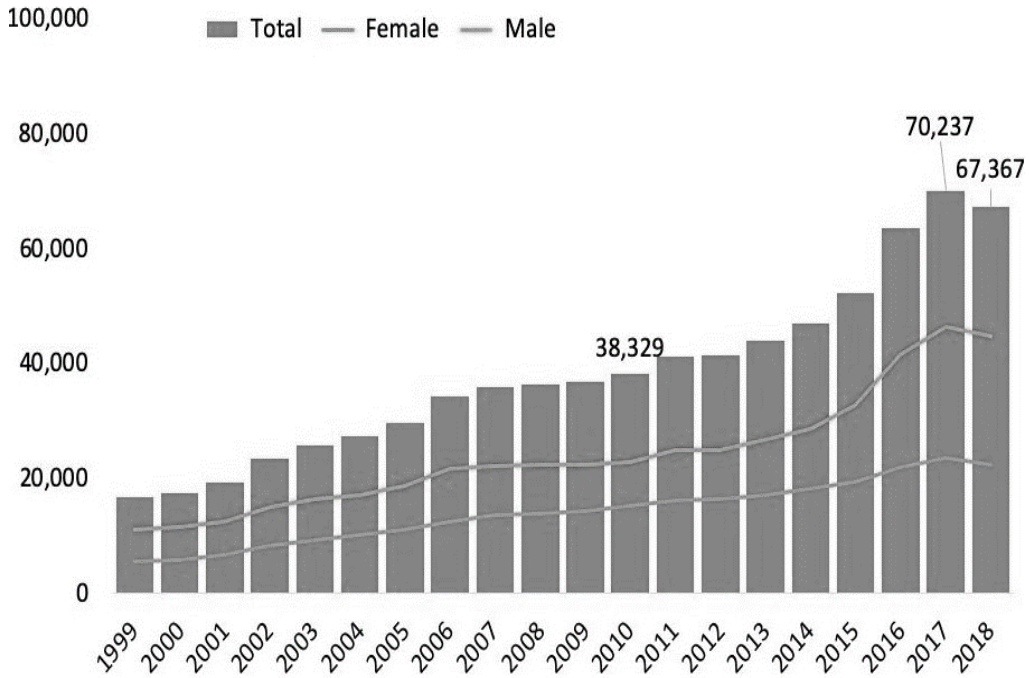


Fig. 1.5. Annual death by antibiotic overuse in USA (CDC, 2019).

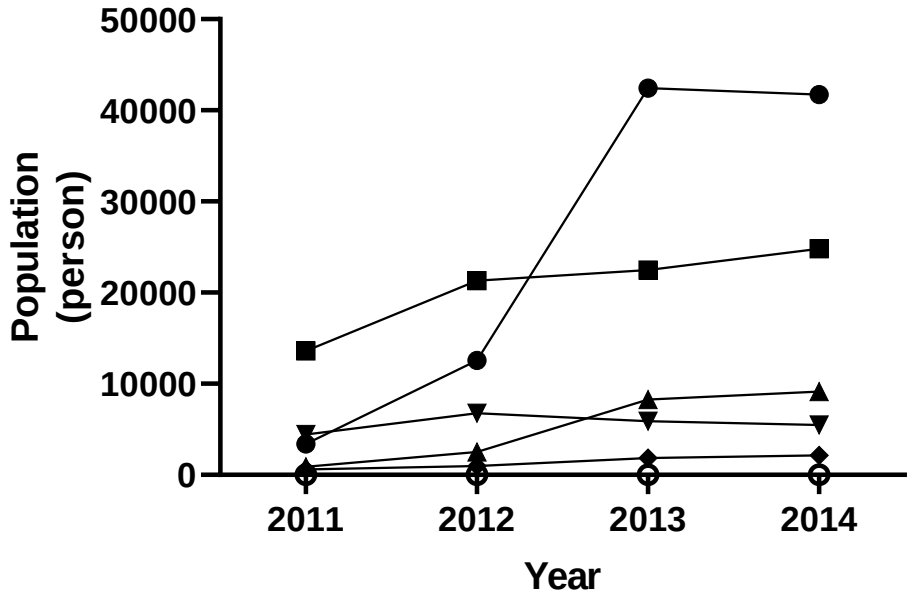


Fig. 1.6. Infection of antibiotic resistant bacteria in Korea (KDCA, 2019). (●), MRSA; (■), MRAB; (▲), VRE; (▼), MRPA; (♦), CRE; (○), VRSA.

4. Antibiotics prescribed diseases

However, despite the risk of antibiotic resistance, there are five diseases (Respiratory infection, Dermatitis, Otitis media, Urinary tract infection, Bacterial infection) that require antibiotic prescription. Since the following five diseases are mainly caused by bacterial infection, they must be treated with antibiotics. However, in the situation where the risk of antibiotic resistance mentioned above is increasing day by day, the need for antibiotic substitutes or supplements is constantly emerging.

5. Candidates of antibiotics alternative

Many antibiotics alternative candidates are being researched around the world, and among them, phage therapy, lysin, antibodies and probiotics are mainly being studied (Ghosh et al., 2019). Candidate groups each have their own strengths and weaknesses, and the clinical trial status for therapeutic purposes is summarized in the table. In this study, we focused on probiotics that can be grafted into the most diverse places. Probiotics are live microorganisms that are intended to have health benefits when consumed or applied to the body. Most of their antibacterial activity is due to the antimicrobial protein, and a representative example is bacteriocin. Unlike antibiotics, which are secondary metabolites, antimicrobial proteins can self-degradable and are known to have high antibacterial activity even in small amounts. However, due to the small amount, it consumes a lot of cost when purifying the antimicrobial protein in the scale-up step. But still, these proteins have a relatively narrow spectrum antimicrobial activity. To compensate for these problems, this study aimed to use live bacteria itself, not the use of antimicrobial protein alone.

Table 1. Candidates of antibiotics substitutes (Ghosh et al., 2019).

Strategy	Advantages over conventional antibiotics	Possible disadvantages	Phase (status)
Phage therapy	• Self-replicating pharmaceuticals • Amenable to genetic engineering	• Releases of bacterial endotoxins • Inadequate preparations failure to remove endotoxins and pyrogenic substances	• 14 approved • Burn infections • Prophylaxis of inflammatory infections
Lysins	• Amenable to genetic engineering • Not prone to resistance development	• Production • Lack of sufficient knowledge	-
Antibodies	• Selective towards specific strains of bacteria • Do not damage the microflora	• High cost of production • Poor shelf life	• 3 FDA approved • *C. difficile*-associated diarrhea • Anthrax
Probiotics	• Not prone to resistance development • Broad-spectrum activity	• Expensive large-scale production • Susceptible to proteolysis	• 0 approved, 4 Phase III, 9 phase II • Atopic dermatitis • Recurrent *C. difficile* infection • Bacterial infection - Preclinical

6. Bacteriocin

Consequently, there is a demand for the development of new antimicrobials that may be utilized in therapeutic settings. Plant-derived chemicals (Savoia, 2012), bacteriophages (Burrowes et al., 2011), and antimicrobial peptides (Li et al., 2012) have been investigated for antibiotic alternatives and bacteriocin is one of the candidates as an antimicrobial peptide.

Bacteriocin is a natural protein or peptide-based antibacterial agent released by a variety of microorganisms with the aim of suppressing the growth of germs that are distinct from or similar to bacteriocin-producing bacteria in a particular growth environment (Biswas et al., 1991). Antibiotics are secondary metabolites and, when administered to humans, may cause side effects due to accumulation in the body. Bacteriocin, on the other hand, is biosynthesized directly from its own gene, and is readily degraded by proteolytic enzymes because it is constructed of amino acids. Thus, it is non-toxic to humans and known as has a high stability against pH and heat.

6.1. Classification of bacteriocin

Bacteriocin is used to classify into class i peptides, which undergo post-translational modification, and class ii, which are largely unmodified (Cotter et al., 2005). Bacteriocins are initially classed as bacteriocins from Gram positive bacteria and bacteriocins from Gram negative bacteria. Gram negative bacteriocins are categorized based on their size. Microcin is a protein with a molecular weight of less than 20 kDa, whereas colicin from *Escherichia coli* refers to a protein between 20 and 90 kDa. Gram positive bacteriocins were originally divided into four classes. The fourth class of bacteriocins, which consisted of massive complexes including carbohydrate or lipid moieties, was abandoned, and renamed bacteriolysins. Thus, bacteriocins are classified majorly into three classes. Class i bacteriocins are made up of 19-50 amino acids and go through extensive post-translational modification, resulting in non-standard amino acids such lanthionine. Class i is further subdivided

into Class ia (lantibiotics), Class ib (labyrinthopeptins) and Class ic (sanctibiotics). Nisin is a class i bacteriocin that is frequently used. Bacteriocins of class ii are small heat-stable proteins. There are five subclasses within this class. Class iia bacteriocins have a high anti-*Listeria* activity and broad spectrum of activity, thus, have a large potential for use in food preservation and medical applications. The most well-known example of Class iia is pediocin PA-1. Class iib activity requires the use of two distinct peptides. Lactococcin G is one such example since it permeabilizes cell membranes for monovalent sodium and potassium cations but not for divalent cations. Almost all of these bacteriocins have GxxxG patterns. This motif also appears in transmembrane proteins, where it aids in the formation of helix-helix interaction. As a result, the bacteriocin with GxxxG motifs can interact with the motifs in the membranes of the bacterial cells, killing the cells. (Nissen-Meyer et al., 2009). Class iic encompasses cyclic peptides, in which the N-terminal and C-terminal regions are covalently linked. A typical example is Enterocin AS-48. Class iid cover single peptide bacteriocins, which are not post-translationally modified. The best example of this group is highly stable Aureocin A53. The bacteriocin is stable under highly acidic conditions and high temperature. Class iii bacteriocins are large, heat labile protein. There are two subclasses within this class. Class iiia kill bacterial cells by degrading their cell walls, resulting in cell lysis. Class iiib, in contrast, comprises those peptides that do not cause cell lysis, instead, killing the target cells by disrupting plasma membrane potential (Kumariya et al., 2019).

6.2. Synthesis of bacteriocin

The genes for active bacteriocin synthesis are generally found in operon clusters in the genome and plasmid. These operons are inducible, and the presence of auto-inducer peptides is required for induction (Uzelac et al., 2015). Nisin is expressed by activating two-component regulatory system, providing an auto-inducer on its own. Some bacteriocins, in contrast, such as the Class ii bacteriocin LsbB, have a special regulatory system which increase RNA stability and thus increase its expression (Uzelac et al., 2015). Bacteriocins are synthesized as precursors which

are then processed and post-translationally modified (Morton et al., 2015). Then, bacteriocins are transported and cleaved to produce the mature form. The modification and export genes are close to the bacteriocin biosynthesis gene. Modification varies depending on the bacteriocin type. Bacteriocin secretion is carried out by transporters such as ABC transporters and sec-dependent exporters.

6.3. Mechanism of bacteriocin

Many bacterial bacteriocins are cationic proteins that may bind to anionic regions of the bacterial surface. These cationic bacteriocins attack anionic lipids in the cell membrane, such as phosphatidylglycerol and cardiolipins, as well as anionic components in the cell envelope like lipopolysaccharide and lipoteichoic acid (Rashid et al., 2016). A commonly known mechanism of action of bacteriocin is membrane disruption. There are three models for example. In the 'barrel-stave' model, bacteriocin is inserted and dispersed in the lipid bilayer to create a channel (Ehrenstein & Lecar, 1977). In this process, the hydrophobic part of cationic bacteriocin faces the lipid bilayer, while the hydrophilic part creates a pore (Baumann & Mueller, 1974). According to the 'toroidal model', protein molecules are predominantly parallel to the membrane. In this case, a core of water molecule is formed in the center of the pore with the antimicrobial peptides and lipid head forming the wall of the pore (Ludtke et al., 1996). However, the peptides in the 'carpet model' do not produce holes. Instead, they bind parallel to the membrane surfaces, forming a carpet with other peptide monomers. The bilayers are disrupted at a specific concentration of peptide, breaking the membrane structure in a detergent condition (Pouny et al., 1992). Such like this, the positively charged groups engage electrostatically with the negatively charged bacterial cell surface, while the hydrophobic surfaces are oriented toward the membrane and cross the lipid bilayer. The peptides self-associate or polymerize after penetrating the lipid bilayer to form complexes (Shahnawaz & Soto, 2012). Bacteriocin promote permeabilization of the target bacterium cell membrane, most likely by creating ion selective pores which cause dissipation of the proton motive force and depletion of intracellular ATP (Christensen & Hutkins, 1992), leakage of intracellular substrates,

and eventual death (Minahk et al., 2000). In general, to interact with the cell membranes, bacteriocin needs a docking molecule like lipid ii or mannose permease of the phosphotransferase system (Hechard & Sahl, 2002).

7. Overall objectives

After Alexander Fleming (1881-1955) developed modern day penicillin in 1928, the substance revolutionized medicine in 20th century. Since then, numerous antibiotics have been discovered, and in fact, humans have gained freedom from bacterial infection. However, at the same time as the rapidly advancing antibiotics discovery, antibiotics have evolved to become resistant to survival. Bacteria that are resistant to antibiotics are called antibiotic-resistant bacteria, and this is because not simply the use of antibiotics, but overuse and misuse of antibiotics by people around the world. These antibiotic-resistant bacteria make it difficult to treat infectious disease, and the emergence of many antibiotic-resistant bacteria and multidrug-resistant bacteria causes serious clinical problems. Nevertheless, antibiotics are still the undisputed number one as a treatment for infectious diseases because there is a lack of suitable alternatives. Therefore, many studies are being conducted to find alternatives or complements to antibiotics. There are four representative antibiotic alternative candidates that are being studied a lot such as phage therapy, lysins, antibodies and probiotics.

Each of them has their own pros and cons. In this study, we focused on probiotics that can be applied not only to treatment but also to dairy products, fermented foods, and health functional foods at therapeutic level. The antibacterial properties of probiotics are due to several factors, but most of them are achieved through secreted antimicrobial proteins. Unlike antibiotics, which are secondary metabolites, antimicrobial proteins are biodegradable and have great antibacterial properties even in very small amounts.

However, when using the antimicrobial protein alone, when compared to using the probiotics itself, at least four processes such as extraction, filtration, purification, and analysis are added in the scale-up step. In this process, a fairly high cost of processing the organic solvent occurs, and as mentioned above, there are several limitations because only a very small amount is secreted during fermentation. In addition, the use of antimicrobial protein alone is mostly for those with a narrow range of antibacterial

properties compared to antibiotics and the conditions to permission by FDA are very strict, so only some are used.

To compensate for these problems, this study focused on the use of the beneficial bacteria itself, not the use of the antimicrobial protein alone.

In Chapter 2, the isolation of strains from Korean traditional fermented food, which called salted squid, ‘Jeotgal’ was conducted and at the same time optimal condition was confirmed. To estimate broad spectrum of antibacterial properties of the strain, antibacterial test was conducted against various pathogens through broth and agar conditions. In addition, various experiments were conducted with the aim of what the antibiotic substance in the supernatant was, through enzyme test and time-kill assay. Also, the antibiotic substance in the cell free supernatant was partially purified through size exclusion chromatography called gel filtration and ion-exchange chromatography. Partially purified fraction was conducted with SDS-PAGE for molecular weight analysis and identification was performed through LC-MS/MS.

In Chapter 3, various experiments were conducted to determine the safety of the strain under FAO/ WHO probiotics guideline. To determine the viability when administered into human intestine, acid tolerance and bile tolerance tests were performed. In addition, hemolysis activity test and antibiotic resistance test was conducted. Finally, gene annotation was performed through whole genome sequencing using the PacBio SMRT sequencer and analyzed presence of toxic metabolite genes.

Chapter 2.

Antibiotic properties of *Weissella* sp. SNUL2 isolated from Korean traditional fermented food

1. Introduction

Since the discovery of penicillin, antibiotics, which have made rapid development, have become an essential element that protects mankind from bacterial infections today. However, antibiotics cannot be used indefinitely because of antibiotic resistance. Due to the misuse of antibiotics, the world is exposed to the risk of antibiotic resistance, and bacterial infections cannot be cured, causing serious problems. Nevertheless, the need for an alternative to antibiotics is steadily rising due to diseases that require the use of antibiotics, and high-income countries and many researchers are studying various substances as candidates for antibiotics. Among them, probiotics have antibacterial properties due to antibacterial proteins. Unlike antibiotics, which are secondary metabolites, antimicrobial proteins are biodegradable and have good antibacterial effects even in small amounts. To find probiotics that secrete antibacterial substances, in Chapter 2, strains were isolated from Korean traditional food and the antibacterial properties of the strains were confirmed through several experiments. It was confirmed that it had antibacterial properties against various indicator bacteria, and through an enzyme test, it was confirmed that the antibacterial substances secreted by probiotics were proteinaceous substances. Also, antibacterial protein candidates were identified through partial purification.

2. Materials and Methods

2.1. Sample isolation

The strain *Weissella* sp. SNUL2 was isolated from Korean traditional fermented food called jeotgal. To obtain a sample for isolating the strain, samples of Korean traditional fermented were obtained from four regions of Gangwon-do (Yangyang(YY), Gangneung(G), Jumunjin(J), Samcheok(S)) and one region of Gyeongsangbuk-do (Daegu(D)). The samples are listed in Table 2. All samples are grinded with a mortar and then cultured 24 h in MRS medium (BD Difco™, Franklin lakes, USA) at 30 ℃, 100 rpm. Each culture was centrifuged at 4200 rpm, 4 ℃ for 20 m and the supernatant was discarded to collect the cell pellet. The harvested cells were washed with phosphate buffer saline (PBS) twice and resuspended the pellet using 1% peptone water. One hundred μl of resuspended cells are inoculated in 2% MRS agar with spreading at 37 ℃, overnight. Then the colonies are streaked again to isolate a single colony and each colony is sent Macrogen (Seoul, Korea) for 16s rRNA sequencing.

Table 2. The list of Korean traditional fermented food from Gangwon-do and Gyeongsangbuk-do.

Region	No.	Korean fermented food	Region	No.	Korean fermented food	No.	Korean fermented food
Yangyang	YY-16	Pollock Sikhae	Daegu	D-1	Salted clam	D-28	Green onion Kimchi
	YY-18	Cod roe		D-2	Salted small octopus	D-29	Green onion Kimchi
	YY-19	Salted anchovy		D-3	Salted pollock intestine	D-30	Radish water Kimchi
	YY-20	Salted herring scallop		D-4	Salted squid	D-33	Malted rice
	YY-21	Salted squid		D-5	Salted pollock intestine	D-34	Malted rice
Gangneung	G-1	Pollock Sikhae		D-6	Salted squid	D-35	Malted rice
	G-2	Flounder Sikhae		D-7	Salted small octopus		
	G-5	Cod roe		D-8	Salted damselfish		
	G-6	Salted small octopus		D-9	Salted anchovy		
	G-7	Salted small octopus		D-10	Cod roe		
Jumunjin	J-17	Flounder Sikhae		D-11	Mt. Palgong Makgeolli		
	J-22	Cod roe		D-14	Daegu raw rice wine		
	J-24	Salted small octopus		D-16	Young radish water Kimchi		
	J-25	Salted squid		D-17	Radish water Kimchi		
Samcheok	S-3	Frozen-dried pollock Sikhae		D-18	Cabbage Kimchi		
	S-4	Flounder Sikhae		D-19	Cabbage Kimchi		
	S-8	Cod roe		D-20	Cabbage Kimchi		
	S-9	Cod roe		D-21	Cabbage Kimchi		
	S-10	Cod roe		D-22	Cabbage Kimchi		
	S-11	Cod roe		D-23	Cabbage Kimchi		
	S-12	Salted small octopus		D-24	Cabbage Kimchi		
	S-13	Salted squid		D-25	Cabbage Kimchi		
	S-14	Salted pollock intestine		D-26	Cabbage Kimchi		
	S-15	Salted cutlassfish		D-27	Cabbage Kimchi		

2.2. Fermentation and growth conditions

Weissella sp. SNUL2 was cultured in MRS broth for seed culture at 30 ℃ for 12 h in shaking incubator at 100 rpm. The fresh inoculum was obtained by the seed culture after centrifugation at 4 ℃, 4200 rpm for 20 m. After centrifugation, the supernatant was discarded then washed the pellet twice with PBS buffer and resuspended in MRS medium. The main culture was inoculated with a fresh 1% (v/v) inoculum and propagated in the 100 ml MRS medium 30 ℃ for 24 h. The temperature was controlled to 30 ℃ and 37 ℃. The samples were collected every 4 h to measure $O.D_{600}$. The pH was confirmed by inoculating *Weissella* sp. SNUL2 in MRS adjusted to pH 3-8 using 1 N NaOH and HCl to determine the optimal growth pH. $O.D_{600}$ was measured and all $O.D_{600}$ values were converted to CFU/ml.

2.3. Antibacterial test against 60 bacteria with microplate

To confirm whether *Weissella* sp. SNUL2 has antibacterial properties, an antibacterial test was performed on 60 strains. After culturing *Weissella* sp. SNUL2, the supernatant was recovered with centrifugation. A supernatant from which cells were removed through filtration was called CFS (Cell free supernatant). This CFS was cultured with 60 indicator bacteria and media to confirm the range of inhibition of the growth of these indicator bacteria. The group without *Weissella* sp. SNUL2 CFS was set as a control, and the group containing CFS of *Pediococcus acidilactici* K10, which is used in many studies due to its good antibacterial properties, was set as a positive control. In each well, CFS and media was loaded with 1:1 ratio, and $O.D_{600}$ was measured every 4 h with microplate reader.

2.4. Antibacterial test against *E. coli*

The antibacterial property was investigated by cell free supernatant (CFS) of *Weissella* sp. SNUL2 against *Escherichia coli* as indicator strain. To obtain CFS from *Weissella* sp. SNUL2, 1×10^8 CFU/ml of the strain was inoculated in fresh MRS broth and incubated at 30 ℃ for overnight. After the incubation, the culture was centrifuged at 4200 rpm, 4 ℃ for 20 m. The supernatant was separated to different tube and pass through a sterile filter (pore size 0.2 μm; Satorious, Goettingen, Germany). The filtered supernatant, which called CFS, was stored in another 50 ml falcon tube. To get indicator cell from *E. coli*, 1×10^8 CFU/ml of the strain was inoculated in fresh LB (BD Difco™, Franklin lakes, NJ, USA) broth and incubated at 37 ℃ for 24 h. After the incubation, the culture was centrifuged at 4200 rpm, 4 ℃ for 20 m. The supernatant was discarded, and the pellet was washed with a sterile PBS twice.

The 96-well plate was filled with 160 μl of LB broth with 1×10^8 CFU/ml of *E. coli* inoculum. Rest of 40 μl was filled with the CFS of *Weissella* sp. SNUL2. Each of the CFS was adjusted to pH 4, pH 5 and pH 6 respectively with 0.1N NaOH. The sample without CFS treatment are assessed as control. The 96-well plate was incubated at 37 ℃ for 24 h and $O.D_{600}$ was measured by microplate reader (VERSAmax, San Jose, CA, USA) every 4 h.

2.5. Antibacterial test against pathogens

As mentioned, the antimicrobial ability against *E. coli* was previously confirmed using CFS. In addition, to check whether the antibiotic substances in CFS exhibit antimicrobial activity against pathogens, the test was conducted using 6 different types of pathogens. Bio safety level (BSL) 2 grade bacteria were distributed from

Korean collection for type cultures (KCTC, Joengeup, Korea) and American type culture collection (ATCC, Manassas, VA, USA) and used as indicator pathogens. Pathogens used in the antimicrobial test are listed in Table 3. Pathogens were seed cultured under each optimal condition for 12 h in shaking incubator at 100 rpm. The fresh inoculum was obtained by the seed culture after centrifugation at 4 ℃, 4200 rpm for 20 m. After centrifugation, the supernatant was discarded then washed the pellet twice with sterile PBS buffer and resuspended with MRS medium. The main culture was inoculated with a fresh 1% (v/v) inoculum and propagated in the 100 ml of each medium for overnight. Each culture was measured $O.D_{600}$ by spectrophotometer (Shimadzu, Kyoto, Japan) to calculate the inoculum.

Each of these indicator bacteria was categorized into five groups: (1)'Control' (pH 6); (2)'Control' (pH 4); (3)'L2 CFS' treated (pH 6); (4)'L2 CFS' treated (pH 4); (5)'duramycin treated'. (1)'Control' (pH 6) was composed of 50 ml of bacteria + media and 50 ml of MRS media (pH 6). (2)'Control' (pH 4) was composed of 50 ml of bacteria + media and 50ml of MRS media (pH 4) adjusted by 1 N HCl. (3)'L2 CFS treated' (pH 6) was composed of 50 ml of bacteria + media and 50 ml of *Weissella* sp. SNUL2 CFS (pH 6) adjusted by 1 N NaOH. (4)'L2 CFS treated' (pH 4) was composed of 50 ml of bacteria + media and 50 ml of *Weissella* sp. SNUL2 CFS (pH 4). (5)'duramycin treated' was composed of 50 ml of bacteria + media and 50 ml of 2 μM duramycin solution. Among commercial bacteriocins, duramycin (Sigma-Aldrich, St. Louis, MO, USA), having antimicrobial activity against Gram-negative bacteria was used as a positive control. Duramycin solution was made by adding 10 mg of duramycin to 0.1 N HCl. Each group was incubated for 24 h in shaking incubator at 100 rpm and $O.D_{600}$ value was measured every 4 h.

Table 3. Pathogens used as indicator strain in antimicrobial test with *Weissella* sp. SNUL2 CFS.

Indicator strain	Medium	Temperature	BSL
Salmonella enterica (ATCC 13076)	NB	37 ℃	2
Bacillus cereus (ATCC 14579)	NB	30 ℃	1
Staphylococcus epidermidis (KCTC 1917)	NB	37 ℃	2
Staphylococcus aureus (ATCC 25923)	TSB	37 ℃	2
Salmonella enterica (ATCC 43971)	TSB	37 ℃	2
Vibrio fluvialis (KCTC 2473)	LB	30 ℃	2

2.6. Antibacterial test against beneficial microbes

The same experiment was conducted to confirm how this antibacterial substance, which inhibits the growth of pathogens, affects the growth of microbes known as beneficial bacteria. 4 types of LABs were used as indicator of beneficial microbes. Bacteria used as indicator are listed in Table 4. These indicator bacteria were cultured overnight under optimal conditions and centrifuged. After centrifugation at 4200 rpm, 4 ℃ for 20 m, the supernatant was discarded, only cells were recovered. Recovered cell pellets were washed twice with sterile PBS buffer before inoculating. To obtain CFS from *Weissella* sp. SNUL2, the strain was cultured overnight under optimal conditions. After 24 hours culture, the culture was centrifugated at 4200 rpm, 4 ℃ for 20 m. The pellet was discarded, and the supernatant was recovered in another 50 ml falcon tube. The supernatant was filtered with 0.2 μm pore size sterile filter. CFS was added to the culture medium inoculated with indicator bacteria at a 1:1 ratio and co-cultured for 24 h, and 500 ml flasks were used with a total volume of 100 ml. $O.D_{600}$ value was measured every 4 h using spectrophotometer (Shimadzu, Kyoto, Japan). Each indicator strain was divided into three groups: the negative control group (no CFS treatment); the group with CFS treatment; the positive control group (with nisin solution). Nisin was added as a positive control because it is well known for its excellent antimicrobial properties against gram-positive bacteria among commercial bacteriocins. Nisin (Sigma-Aldrich, St. Louis, MO, USA) was added into 0.02 N HCl at a final concentration of 1 mg/ml to prepare a solution to have an activity of 1000 U/ml. It was also added at the same rate as CFS.

Table 4. Beneficial microbes used as indicator in antimicrobial test with *Weissella* sp. SNUL2 CFS.

Indicator strain	Medium	Temperature	BSL
Lactobacillus plantarum (KCTC 3108)	MRS	30 ℃	1
Leuconostoc mesenteroides (KCTC 3100)	MRS	25 ℃	1
Lactobacillus sakei (KCTC 3598)	MRS	30 ℃	1
Lactobacillus casei (ATCC 393)	MRS	30 ℃	1

2.7. Minimum inhibition concentration (MIC) test

The MIC test was conducted to determine the minimum inhibition concentration required for *Weissella* sp. SNUL2 to inhibit the indicator bacteria. Minimum inhibition concentration is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation against indicator strains. The test was performed on 6 strains known to be harmful (Table. 3.1.). Ampicillin, known as penicillin-based antibiotic, was used as a control group, and compared with *Weissella* sp. SNUL2 MIC against 6 indicator strains. The initial concentrations of *Weissella* sp. SNUL2 CFS and ampicillin were set at 0.2 mg/ml, and the two-fold dilution was repeated 6 times to dilute to 1.5% of the initial concentration. $O.D_{600}$ was measured for 24 h using a microplate, and 100 μl of medium + indicator bacteria and 100 μl of *Weissella* sp. SNUL2 CFS (ampicillin) were mixed in each well. Indicator bacteria were inoculated by 5×10^6 CFU/ml in each well.

2.8. Hourly antibiotic substance production

The test, so called Time kill assay, was conducted to determine when the antibiotic substance was produced while the strain was incubated. First, to obtain a *Weissella* sp. SNUL2 CFS, a flask inoculated by 1×10^8 CFU/ml in 100 ml of MRS (BD Difco™, Franklin lakes, NJ, USA) medium was cultured at 30 ℃, 100 rpm. In order to obtain CFS over time (4h, 8h, 12h, 16h, 20h, 24h), 6 different flasks were incubated at 4 h interval. After culturing for different times, the obtained culture solution was rotated in a centrifuge at 4200 rpm, 4 ℃ for 20 m. All the supernatants were separated to different tube and pass through a sterile filter (pore size 0.2 μm; Satorious, Goettingen, Germany). The filtered supernatant, which called CFS, was stored in another 50 ml falcon tube.

To get indicator cell from *E. coli*, 1×10^8 CFU/ml of the strain was inoculated in fresh LB (BD Difco™, Franklin lakes, NJ, USA) broth and incubated at 37 ℃ for 24 h. After the incubation, the culture was centrifuged at 4200 rpm, 4 ℃ for 20 m. The supernatant was discarded, and the pellet was washed with a sterile PBS twice.

The 96-well plate was filled with 100 μl of LB broth with 1×10^8 CFU/ml of *E. coli* inoculum. Rest of 100 μl was filled with the CFS (4 h, 8 h, 12 h, 16 h, 20 h, 24 h) of *Weissella* sp. SNUL2. The 96-well plate was incubated at 37 ℃ for 24 h and $O.D_{600}$ was measured by microplate reader (VERSAmax, San Jose, CA, USA) every 4 h.

2.9. Antibiotic substance assay

To confirm whether the antibiotic substance was a peptide-based material, the sensitivity of the antibiotic substance to enzymes was tested using the CFS of *Weiseslla* sp. SNUL2, which was treated with following enzymes: trypsin (Sigma-Aldrich, St. Louis, MO, USA) at 37 ℃ and pH 7.8; pepsin (Sigma-Aldrich, St. Louis, MO, USA) at 37 ℃ and pH 2.2. The trypsin solution was prepared at a final concentration of 1 mg/ml in 50 mM potassium phosphate buffer (pH 7.8) made of 1M KH_2PO_4 9.2% (v/v) + 1M K_2HPO_4 90.8% (v/v). Thereafter, a solution prepared by adding 1 mg/ml trypsin solution and *Weissella* sp. SNUL2 CFS in a 1:1 ratio was activated at 37 ℃ for 4 h at pH 7.8. After trypsin was activated after 4 h, heat treatment was performed at 100 ℃ for 10 m to prevent additional enzymatic activity, and the pH was adjusted to 6. The pepsin solution was prepared at a final concentration of 1 mg/ml in 50 mM glycine-HCl buffer (pH 2.2) made of 0.1 M glycine 50 ml + 0.1 N HCl 32.4 ml + D.W 17.6 ml. After that, a solution prepared by adding 1 mg/ml trypsin solution and *Weissella* sp. SNUL2 CFS in a 1:1 ratio was activated at 37 ℃ for 4 h at pH 2.2. After pepsin was activated after 4 h, heat treatment was performed at 100 ℃ for 10 m as same reason in trypsin and the pH was adjusted to 6. *E. coli* cultured for 24 h was used as the indicator strain. Of the total volume of 200 μl in one well, LB medium containing 1×10^8 CFU/ml of *E. coli* was added to 100 μl and the enzyme solution adjusted to pH 6 was added to the remaining 100 μl. Two controls were used: only *E. coli*; *E. coli* with *Weissella* sp. SNUL2 CFS treatment. The growth of *E. coli* was measured by $O.D_{600}$, using a microplate reader, and was measured at 4 h intervals for 24 h.

2.10. Kirby-Bauer disk diffusion assay

Kirby-Bauer disk diffusion assay, also known as the agar well diffusion test, is a culture-based microbiology assay used in diagnostic and drug discovery laboratories. The assay is used to determine the susceptibility of antibiotic substance against certain strain. In this study, the assay was conducted with slight modification to determine how antimicrobial substances affect specific strain in agar condition. In general, the assay was carried out by treating purified antibiotic substances, but to observe whether a small amount of CFS from *Weissella* sp. SNUL2 has a visible inhibitory ability, 6 pathogens listed in Table 3. were conducted. Three kinds of medium were used: MRS (de Man, Rogosa and Sharp); NB (Nutrient broth); TSB (Tryptic soy broth). Each broth was made into agar plate by adding 2% (v/v) of agar. Each indicator strain was incubated for 24 h at different optimum condition, then diluted to 1×10^8 CFU/ml. Diluted strain was spread in spot-on-lawn method with sterile swab. After inoculation, punch the position where the CFS will be loaded using sterile cork borer (5 mm). Without any concentration or pretreatment, 40 μl of CFS was loaded in each spot and sealed. Every plate was stationary cultured for overnight in stationary incubator.

2.11. Partial purification

2.11.1. Ethyl acetate extraction

Ethyl acetate extraction is a kind of liquid-liquid extraction. This extraction is a method of selectively extracting a specific sample based on its partition between immiscible aqueous and organic solution. Ethyl acetate was purchase from Duksan Pure Chemical (Ansan, Korea). To obtain CFS, *Weissella* sp. SNUL2 was grown in MRS medium at 30 ℃ 100 rpm for 24 h. After incubation, supernatant of the culture was filtrated with syringe filter (pore size 0.2 μm; Satorious, Goettingen, Germany) to remove cell debris. CFS from *Weissella* sp. SNUL2 and ethyl acetate were poured using a funnel in a 1:1 ratio into a separatory funnel with the stopcock submerged. The separatory funnel was mixed with gentle agitation. Mixed for 20 s, the cock was briefly opened, and a little ventilation was repeated 3 times. The separatory funnel was placed upright in the ring clamp to allow the layers to fully separate. After separation complete, the bottom layer was drained into a clean Erlenmeyer flask and the top layer was poured out from the top of the separatory funnel to minimize re-mixing the solution. Each layer was concentrated using a vacuum rotary evaporator at 78 ℃ to evaporate ethyl acetate.

2.11.2. Gel filtration

Gel filtration was conducted to further refine the antibiotic substances in *Weissella* sp. SNUL2 CFS. *Weissella* sp. SNUL2 was grown in 300 ml of MRS medium (1% v/v) at 30 ℃, 100 rpm for 24 h. After centrifugation at 4200 rpm, 4 ℃ for 20 m, 290 ml of supernatant was obtained. This supernatant passed through the sterile syringe filter (pore size 0.2 μm; Satorious, Goettingen, Germany), and 288 ml of CFS was recovered. As gel filtration resin, Sephadex G-50 fine was purchased from

Cytiva (Marlborough, MA, USA). Five grams of Sephadex G-50 column was swollen in 30 mM Tris-HCl buffer (pH 8.0) for 3 h. 50 ml of Gel slurry equilibrated in 30 mM Tris-HCl buffer (pH 8.0) was loaded on 87 × 300 (ø × mm) glass column. After packing complete, equilibrated gel slurry was washed by 5 column volumes (CV) of same buffer as equilibrium buffer. Then, 2 ml of 10 x concentrated CFS was loaded to the top of gel bed, which called meniscus. Elution buffer, 30 mM Tris-HCl buffer (pH 8.0) was flowed at a flow rate of 0.5 ml/m, and fractions were received by 4 ml.

2.11.3. Bradford assay

Among the method of protein quantification, Bradford assay has become the preferred method for quantifying protein in many laboratories because of its simpleness. In addition, compared to the Lowry method, it is subjected to less interference by common reagents. For the calibration curve, volumes of 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.0 mg/ml of Bovine serum albumin (BSA) in sterile PBS buffer were used. Each concentration of BSA solution was made up to 800 µl, with BSA solution: diluted water as 10:790 ratio. Then 200 µl of Bio-Rad Bradford dye reagent (Coomassie brilliant blue G-250) was added to 800 µl of diluted BSA solution. Total 1 ml of mixed solution was measured O.D at 595 nm, and the blank was 10 µl of PBS instead of BSA. The fraction that gel filtration completed was concentrated to 4X its original concentration using speed vac (EYELA Co. Tokyo, Japan) at 1300 rpm for 6 h. After concentration, 10 µl of the sample was loaded to the cuvette, measured $O.D_{595}$.

2.11.4. UV assay with gel filtrated CFS

For each fraction after the Sephadex G-50 column was completed, absorbance was measured at a wavelength of 280 nm to check the protein concentration. The amino acids tyrosine and tryptophan show a unique absorption at 280 nm, allowing for protein concentration determination. Because of its simplicity, convenience of use, and cost, UV absorbance at 280 nm is commonly employed in labs to measure protein content.

2.11.5. Kirby-Bauer disk diffusion assay with gel filtrated CFS

The inhibitory activity of antibiotic substance is determined by Kirby-Bauer disk diffusion assay as well as spot-on-lawn assay. Each fraction after the Sephadex column was completed, Kirby-Bauer disk diffusion assay was conducted as described in 2.10., using *Salmonella enterica* ATCC 13076 as indicator strain. Total fractions were concentrated by speed vac (EYELA Co., Japan) at 50 ℃ for 6 h until 4× concentration. Indicator strain was incubated in Nutrient broth at 37 ℃ for overnight. The indicator culture was inoculated in Nutrient agar (2% v/v) with spot-on-lawn assay using sterile swab. To load the gel filtrated CFS on Nutrient agar plate, the spot had to be cut using sterile cork borer (5 mm). Before use, cork borer was sterilized with autoclave, and after cut the spot, 40 μl of gel filtrated CFS (2×) was loaded. Without CFS group was estimated as control.

2.11.6. Ion-exchange chromatography

After gel filtration with Sephadex G-50 fine resin completed, Ion-exchange chromatography was done using a Amberlite CG50 column (Sigma-Aldrich, St. Louis, MO, USA). Resin was swollen in 5mM Sodium phosphate buffer (pH 6.0) prepared with NaH_2PO_4 and Na_2HPO_4 in a ratio of 87.7:12.3 for 24 h. After swollen completed, Resin was packed in Econo-Pac Disposable chromatography column (Bio-rad, Hercules, CA, USA) (1.5 × 12 cm) and equilibrated another 3 h. If gel bed was ready, the sample was loaded. For the loading sample, the fraction showing the antimicrobial activity after the gel filtration was concentrated 4 times using speed vac was loaded. When the loaded sample passes through the column, 5 times the volume of the column (CV) was flowed with 5 mM Sodium phosphate buffer (pH 6.0) to wash away proteins which were not bounded to the column. Then, to elute the desire fraction, the elution buffer was loaded. As elution buffers, 6 types were used according to the concentration gradient of NaCl: 5 mM Sodium phosphate buffer + 100 mM NaCl; 5 mM Sodium phosphate buffer + 200 mM NaCl; 5 mM Sodium phosphate buffer + 400 mM NaCl; 5 mM Sodium phosphate buffer + 600 mM NaCl; 5 mM Sodium phosphate buffer + 800 mM NaCl; 5 mM Sodium phosphate buffer + 1 M NaCl. Each fraction was collected by flowing the elution buffer as 5 CV.

2.11.7. Kirby-Bauer disk diffusion assay with Ion-exchanged CFS

The inhibitory activity of antibiotic substance is determined by Kirby-Bauer disk diffusion assay as well as spot-on-lawn assay. Each fraction after the Ion-exchange chromatography was completed, Kirby-Bauer disk diffusion assay was conducted as described in 2.10., using *Salmonella enterica* ATCC 13076 as indicator strain. Total fractions which eluted in 6 different NaCl gradient were concentrated by speed vac (EYELA Co., Tokyo, Japan) at 50 ℃ for 6 hours until 4× concentration. Indicator strain was incubated in Nutrient broth at 37 ℃ for overnight. The indicator culture was inoculated in Nutrient agar (2% v/v) with spot-on-lawn assay using sterile swab. To load the Ion-exchanged fractions on Nutrient agar plate, the spot had to be cut using sterile cork borer (5 mm). Before use, cork borer was sterilized using autoclave, and after cut the spot, 40 μl of the fractions were loaded. Without CFS group was estimated as control.

2.12. SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

The molecular weight of the antimicrobial substance was determined by SDS-page (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to the method of Laemmli (Laemmli, 1970). The partially purified antimicrobial substance was concentrated 4 times by speed vac and mixed with a sample buffer 1:2. The sample buffer was used by mixing Tricine sample buffer (Bio-rad, Hercules, CA, USA) and β-mercaptoethanol in a 98:2 ratio. After that, the sample and the sample buffer were gently mixed with pipette and denatured at 100 ℃ for 3 m. The sample was loaded to 16.5% Tris-Tricine Gel (Bio-rad, Germany) for 30 μl in each well. At the same time, running buffer which composed by 1.2 M Triethanolamine, 0.8 M Tricine and 2% (w/v) SDS was filled in electrophoresis equipment and electrophoresis was conducted under 200 V, 100 mA for 70 m. As protein marker, Color Marker Ultra-low Range (Sigma-Aldrich, St. Louis, MO, USA) was used.

2.13. Gel extraction

Gel extraction was performed to obtain the protein in the band from the protein band after the SDS-page. After electrophoresis, the gel was washed with distilled water for 5 m and repeated three times. Washed gel was stained with stain solution which composed by 40% (v/v) Ethanol, 10% (v/v) acetic acid and 0.1% (w/v) of Coomassie brilliant blue R-250 (Thermo Fisher Scientific, Rockford, USA). Staining was carried out total 3 hours, and then destaining was performed for 3 h using destain solution which composed by 20% (v/v) Ethanol and 5% (v/v) acetic acid. After destaining, the band was cut using a razor blade, and polyacrylamide was cut out as much as possible. The band was washed with 500 µl of 50% (v/v) Acetonitrile (ACN) and 0.1% (v/v) Trifluoroacetic acid solution (TFA) in centrifuge tube. After that, put it in 100mM NH_4HCO_3 (ammonium bicarbonate) with 50% (v/v) ACN and proceed with destaining again at 37 ℃ for 45 m, repeated twice. Dehydration was done after destaining with 100 µl of 100% (v/v) ACN for 5 m at room temperature. The band was dried with speed vac at room temperature for 10 to 15 m. After all these processes were completed, the digestion process was prepared. Trypsin was dissolved in 50 mM acetic acid to make a solution at 1 mg/ml and diluted to 0.02 mg/ml with 40 mM NH_4HCO_3 (ammonium bicarbonate) and 10% (v/v) ACN solution. Dehydrated gel was incubated at room temperature for an hour in 20 µl of trypsin solution to re-hydration. Re-hydrated gel was incubated at 37 ℃ for 24 h in digestion buffer which composed by 40 mM ammonium bicarbonate and 10% (v/v) ACN. The cultured gel was first extracted by vortexing in distilled water and then put into a solution of 50 µl of 50% ACN and 5% TFA, incubated for an hour, extracted again. The extracted liquid was stored to different tubes, and the extract was dried for 2-4 h using a speed vac.

2.14. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was performed through nano ACQUITY UPLC and LTQ-orbitrap-mass spectrometer (Thermo Electron, San Jose, CA). The column used BEH C-18 1.7 µm, 100 µm × 100 mm column (Waters, Milford, MA, USA). The mobile phase A for the LC separation was 0.1% formic acid in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile (ACN). The chromatography gradient was set up to give a linear increase from 10% B to 40% B for 21 m, from 40% B to 95% B for 10 m. The flow rate was 0.5 µl/min. Mass spectra were collected utilizing data-dependent acquisition with a complete mass scan (300-2000 m/z) followed by MS/MS scans for tandem mass spectrometry. Each MS/MS scan acquired was an average of one microscans on the LTQ. The temperature of the ion transfer tube was controlled at 275 ℃ and the spray was 2.0 kV. For MS/MS, the normalized collision energy was set at 35%. Individual MS/MS spectra were processed using the SEQUEST software (Thermo Quest, San Jose, CA, USA), and the resulting peak lists were utilized to query an internal database with the MASCOT program (Matrix Science Ltd., London, UK). For MS analysis, we used the modifications Carbamidomethyl (C), Deamidated (NQ), and Oxidation (M), with a peptide mass tolerance of 10 ppm. MS/MS ion mass tolerance was 0.8 Da, allowance of missed cleavage was 2, and charge states (+2, +3) were taken into account for data analysis. We took only significant hits as defined by MASCOT probability analysis.

3. Results

3.1. Sample isolation

A total of 54 fermented food samples were collected from four regions (Yangyang, Gangneung, Jumunjin, Samcheok) in Gangwon-do and one region (Daegu) in Gyeongsangbuk-do (Fig. 2.1). As a result of analyzing the sample with 16s rRNA sequencing, *Pediococcus acidilactici* was detected in YY-16 and the genus *Weissella* in YY-21. The genus *Lactobacillus* was detected in the J-22 sample from Jumunjin, and *Bacillus* was detected from the G-7 sample from Gangneung. Also, the genus *Enterococcus* was found in S-9, 10, 11 samples from Samcheok. On the other hand, the analyzed fermented foods in Gyeongsangbuk-do were mainly fermented foods using plants such as cabbage, not using seafood, unlike in Gangwon-do. There are various factors such as the type of fermented food, the method of fermentation and region, but in the sample in Gyeonsangbuk-do, two species of *Pediococcus* and *Weissella confusa* were found in D-3 and *Weissella* sp. in D-4. Commonly, *Weissella* sp. was detected as the main beneficial microorganism in fermented food with cabbage as the main ingredient (D-17, 18, 24, 25, 30). Among these results, *Weissella* sp. detected in YY-21, the fermented food, which was made by salted squid, was selected as a target species and after isolation, various characteristics were tested.

3.2. Fermentation and growth conditions

The strain was cultured in 500 ml flask with 100 ml of MRS at 30 ℃ and 37 ℃ to find optimum temperature of the strain. Figure 2.2. shows the growth results obtained under two different temperatures. The highest O.D (Optical density) at 600 nm in the curve of 30 ℃ was observed at the hour 24, indicated 6.18. The initiation of stationary phase in growth curve was observed at the hour 12 in the curve of 30 ℃ and the hour 16 in the curve at 37 ℃. The difference in growth between the two graphs was about 43% based on 24 h, and the graph 30 ℃ recorded 6.18 and the graph 37 ℃ recorded 4.32, respectively. Through this result, it was confirmed that the strain showed better growth at 30 ℃. As shown in Fig. 2.2, the strain proliferates at pH 5, 6, 7, 8. Under pH 3, 4 condition, the strain did not show any growth. Optimum pH was measured by O.D at 600 nm, and under pH 7 condition, the strain recorded best growth. For reference in future experiments, a standard curve was prepared. After setting the strains according to different $O.D_{600}$, which cultured at 30 ℃, 100 rpm in MRS for 24 h, diluted the sample with distilled water. $O.D_{600}$ was assessed using UV-160 spectrophotometer (Shimadzu, Kyoto, Japan) and set 0.2, 0.4, 0.6, 0.8, 1.0. The set strain of each $O.D_{600}$ were inoculated to MRS agar plate after serial dilution from 10^{-1} to 10^{-8}. The complete standard curve of CFU:$O.D_{600}$ are described in Fig. 2.3. The curve shows that 1×10^{8} CFU/ml of the strain were numerically similar as $O.D_{600}$ 0.1 and its R^2 is around 0.9909.

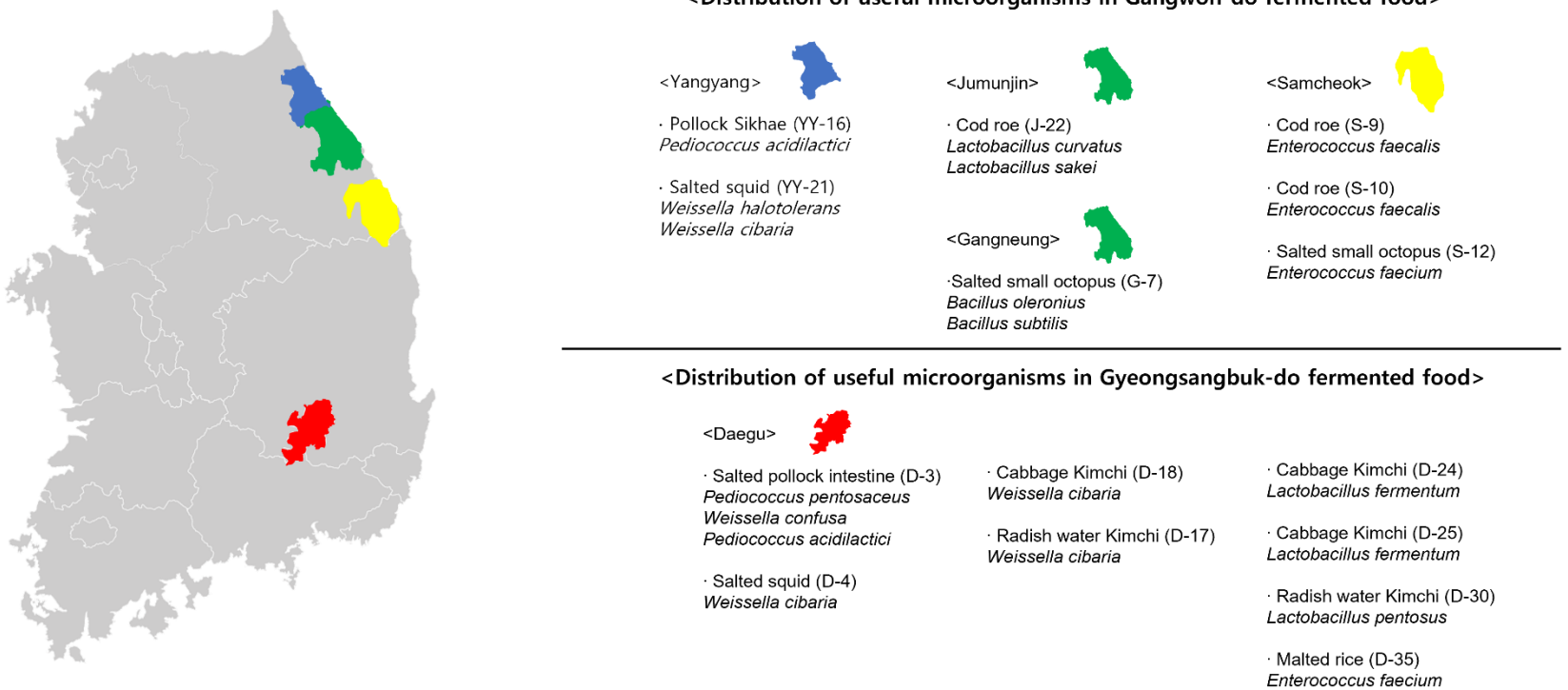


Fig. 2.1. Distribution of beneficial microbes in traditioinal fermented food samples in Gangwon-do and Gyeongsangbuk-do. The region with blue is describes as Yangyang; green, Gangneung and Jumunjin; yellow, Samcheok(Gangwon-do); red, Daegu(Gyeongsangbuk-do).

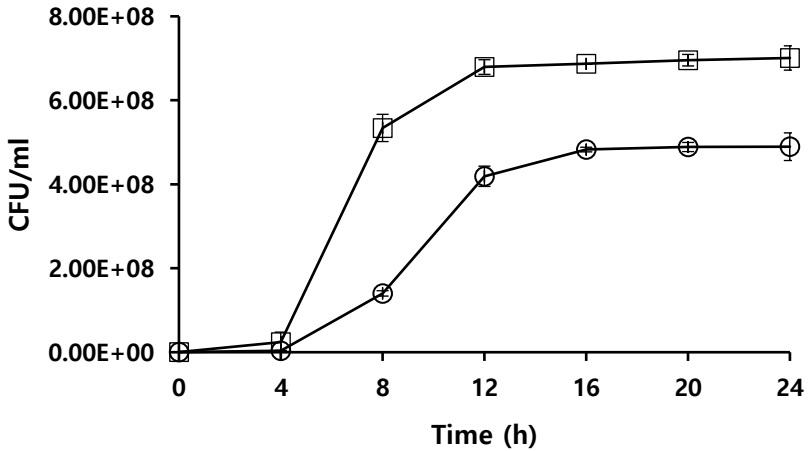


Fig. 2.2. Growth curves of *Weissella* sp. SNUL2 at different temperature during cultivation. (□), 30 ℃; (○), 37 ℃. All values indicated the averages of each fermentation and error bars depicted standard deviations.

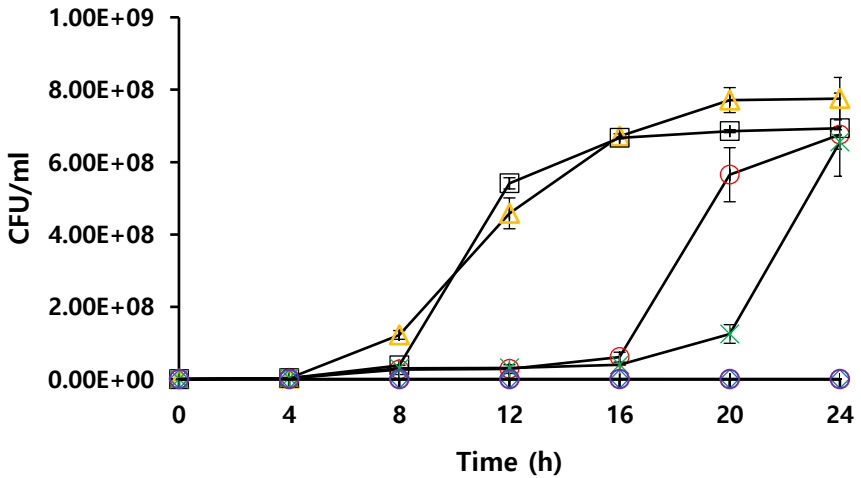


Fig. 2.3. Growth curves of *Weissella* sp. SNUL2 at different pH during cultivation. (○), pH 3; (◇), pH 4; (x), pH 5; (□), pH 6; (△), pH 7; (○), pH 8. All values indicated the averages of each fermentation and error bars depicted standard deviations.

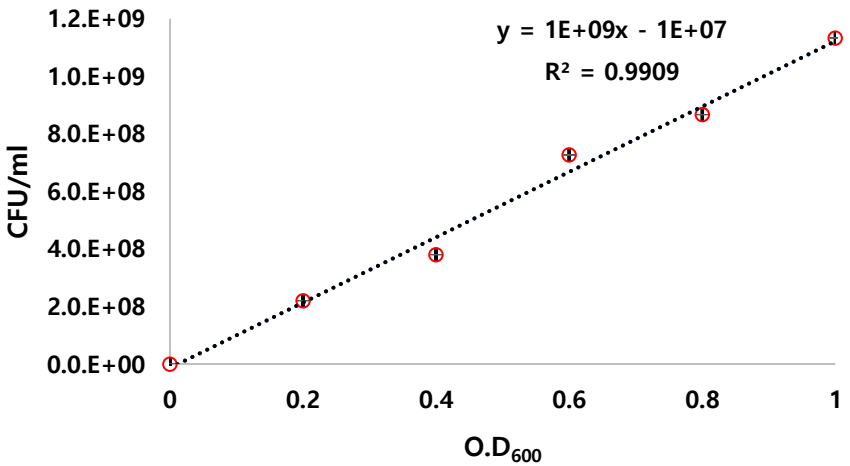


Fig. 2.4. CFU:O.D$_{600}$ standard curves of *Weissella* sp. SNUL2

3.3. Antibacterial test against 60 bacteria with microplate

An antibacterial test was performed to observe the degree of inhibition when *Weissella* sp. SNUL2 CFS (Cell free supernatant) was added to 60 Gram-negative and positive bacteria (Table 5.). CFS refers to the supernatant obtained after culturing *Weissella* sp. SNUL2 for 24 h to remove cell fragments through filtration. As a result, 59 out of a total of 60 indicator strains were inhibited when *Weissella* sp. SNUL2 CFS was added, and 13 strains were inhibited when *Pediococcus acidilactici* K10 CFS was added. As *Pediococcus acidilactici* K10 CFS is known to have good antibacterial properties, it was confirmed that *Weissella* sp. SNUL2 CFS had a broad spectrum of antibacterial properties.

Table 5. Antibacterial test against 60 bacteria with microplate. (+) shows growth inhibition of indicator strain, (-) shows no growth inhibition.

Indicator strain	Growth inhibition		Indicator strain	Growth inhibition	
	+ *Weissella* sp. SNUL2 CFS	**+ *Pediococcus acidilactici* K10 CFS**		**+ *Weissella* sp. SNUL2 CFS**	**+ *Pediococcus acidilactici* K10 CFS**
Bacillus cereus ATCC 14579	+	-	*Bacillus thuringensis* ATCC 35646	+	-
Bacillus cereus ATCC 10987	+	-	*Bacillus thuringensis* ATCC 35866	+	-
Bacillus cereus DSM 102	+	-	*Bacillus thuringensis* KCTC 1510	+	-
Bacillus cereus ATCC 21768	+	-	*Bacillus thuringensis* ATCC 35679	+	-
Bacillus cereus KCTC 1094	+	-	*Bacillus mycoides* ATCC 6462	+	-
Bacillus cereus ATCC 10876	+	-	*Bacillus mycoides* ATCC 21929	+	-
Bacillus cereus ATCC 11778	+	-	*Bacteroides fragilis* KCTC 3688	+	-
Bacillus cereus ATCC 10702	+	-	*Escherichia coli* ATCC 10536	+	-
Bacillus subtilis ECE 22	+	-	*Escherichia coli* ATCC 9637	+	-
Bacillus subtilis DSM 102	+	-	*Escherichia coli* ATCC 11775	+	-
Bacillus subtilis ECE 139	+	+	*Escherichia coli* O157:H7 932	+	-
Bacillus pseudomycoides KCTC 3862	+	-	*Escherichia coli* O104 NCCP 13721	+	+
Enterococcus faecalis KCTC 3206	-	-	*Escherichia coli* O26 NCCP 13580	+	+
Enterococcus faecalis KFRI 354	+	-	*Escherichia coli* O121 NCCP 12551	+	+
Enterococcus faecium KFRI 132	+	-	*Escherichia coli* O111 NCCP 13581	+	-
Enterococcus faecium KFRI 131	+	-	*Listeria grayi* ATCC 19120	+	+

Indicator strain	Growth inhibition		Indicator strain	Growth inhibition	
	+ *Weissella* sp. SNUL2 CFS	**+*Pediococcus acidilactici* K10 CFS**		**+ *Weissella* sp. SNUL2 CFS**	**+ *Pediococcus acidilactici* K10 CFS**
Listeria innuocua ATCC 33090	+	-	*Staphylococcus aureus* ATCC 25923	+	-
Listeria ivanovii subsp. *ivanovii* ATCC 19119	+	-	*Staphylococcus aureus* KCTC 1621	+	-
Listeria monocytogenes ATCC 19111	+	-	*Staphylococcus aureus* subsp. *aureus* ATCC 33591	+	-
Listeria seeligeri ATCC 35967	+	+	*Staphylococcus aureus* subsp. *aureus* ATCC 33593	+	-
Listeria welshimeri ATCC 35897	+	+	*Staphylococcus epidermidis* ATCC 12228	+	-
Salmonella enteritidis ATCC 13076	+	-	*Staphylococcus epidermidis* KACC 13234	+	-
Salmonella enteritidis KCCM 12021	+	+	*Staphylococcus hyicus* KTCC 13249	+	+
Salmonella enterica subsp.*enterica* ATCC 43971	+	-	*Streptococcus thermophilus* KCTC 5092	+	-
Salmonella typhimurium ATCC 19586	+	-	*Streptococcus thermophilus* KCTC 1621	+	-
Salmonella typhimurium ATCC 43174	+	-	*Streptococcus mutans*	+	-
Salmonella typhimurium DT104 killer cow	+	-	*Vibrio parahaemolyticus* ATCC 27969	+	+
Salmonella typhimurium ATCC 14028	+	+	*Vibrio parahaemolyticus* ATCC 17803	+	-
Staphylococcus aureus ATCC 6538	+	-	*Vibrio vulnificus* KCCM 13234	+	+
Staphylococcus intermedius KACC 13247	+	-	*Yersinia enterocolitica* KCCM 41657	+	+

3.4. Antibacterial test against *E. coli*

An antimicrobial test was conducted to verify that the supernatant of the strain had antimicrobial properties. The CFS was obtained from *Weissella* sp. SNUL2 and indicator strain was *E. coli* top 10. The test was conducted in three different pH condition. As shown in Fig.2.5., at pH 4, the control group (no CFS treatment) showed a log phase from 0 to 4 h and went through a stationary phase from 4 h after incubation start at which time the $O.D_{600}$ value was 0.77. By the way, another group (with CFS treatment) did not show any growth, and growth was stopped for 24 h during incubation, and the $O.D_{600}$ value was 0.12. Likewise, in Fig. 2.6., at pH 5, the control group showed a log phase from 0 to 12 h and went through a stationary phase from 12 hours after incubation start at which time the $O.D_{600}$ value was 0.45. In the contrast, another group showed slight growth during incubation, but it appeared that growth was significantly inhibited compared to the control group. At pH 6, it also showed inhibitory ability above a certain level, compared to the control group (Fig. 2.7.). As a results, at pH 4, 5, 6, the CFS inhibited growth of *E. coli* by 84.4%, 62.2%, 38.9% respectively based on the stationary phase.

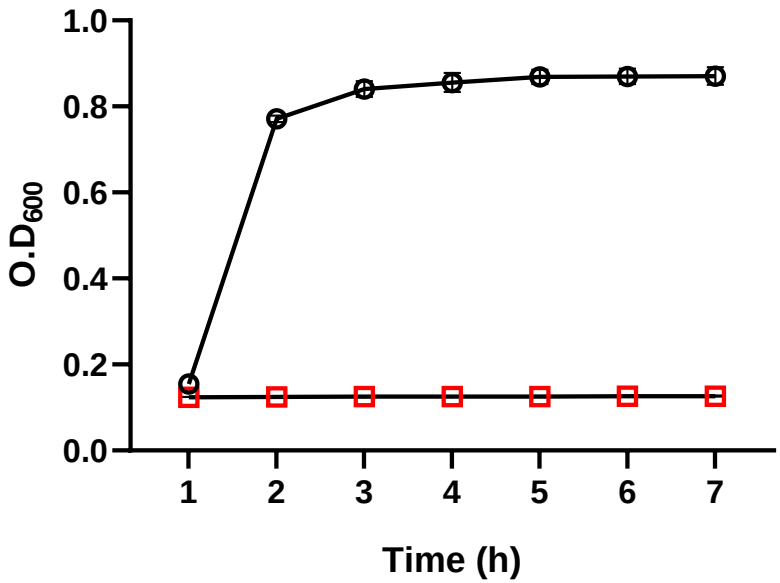


Fig. 2.5. The growth curve of *E. coli* which cultured with the cell free supernatant (CFS) of *Weissella* sp. SNUL2 at pH 4. (○), control (no CFS treatment); (□), *E. coli* with CFS treated. All values indicated the average of each fermentation and error bars depicted standard deviations.

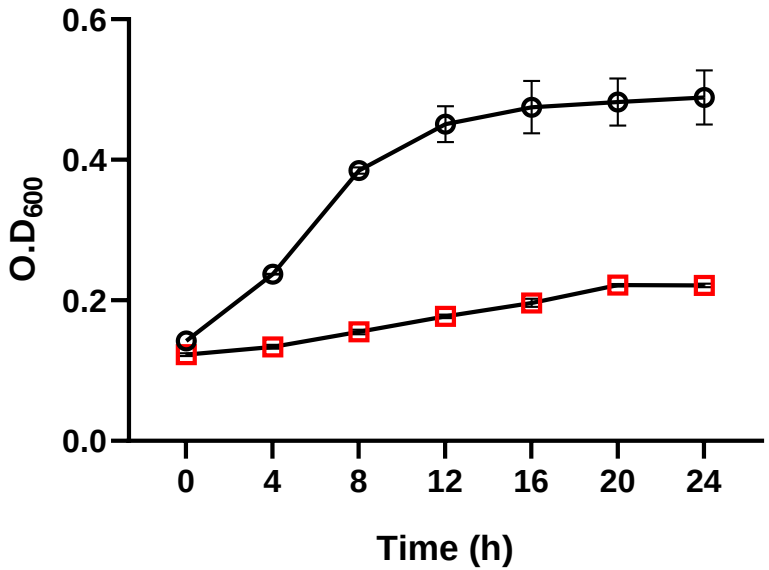


Fig. 2.6. The growth curve of *E. coli* which cultured with the cell free supernatant (CFS) of *Weissella* sp. SNUL2 at pH 5. (○), control (no CFS treatment); (□), *E. coli* with CFS treated. All values indicated the average of each fermentation and error bars depicted standard deviations.

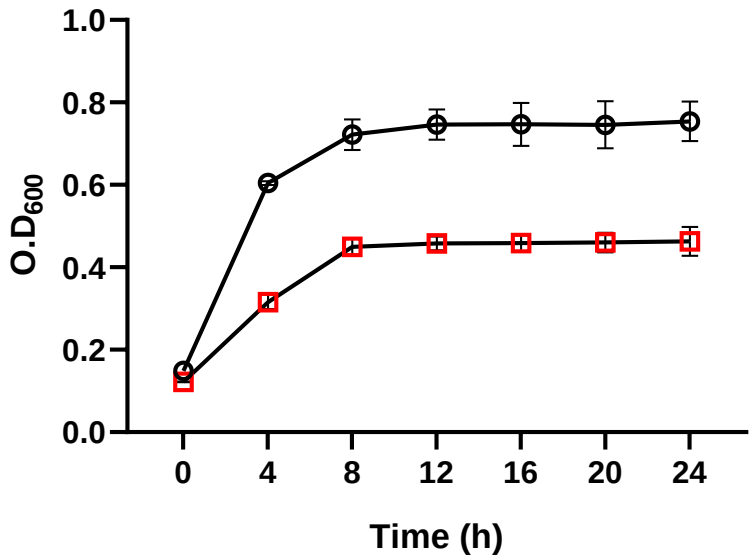


Fig. 2.7. The growth curve of *E. coli* which cultured with the cell free supernatant (CFS) of *Weissella* sp. SNUL2 at pH 6. (○), control (no CFS treatment); (□), *E. coli* with CFS treated. All values indicated the average of each fermentation and error bars depicted standard deviations.

3.5. Antibacterial test against pathogens

Weissella sp. are classified as LAB and generally presents in Korean traditional food especially Kimchi and Jeotgal. The genus *Weissella* was created in 1993 by Collins et al., collecting 5 species of *Leuconostoc paramesenteroides* and *Lactobacillus*, which are different from other species of *Leuconostoc*, and proposing a new genus with 6 species (Collins et al., 1993). LAB can produce antibacterial substances such as bacteriocin and lactic acid, and *Weissella* is also known to produce these substances, but compared to other LAB, very little information is known. The antimicrobial test was conducted with 6 pathogens, which are BSL grade 2. To rule out the antimicrobial properties caused by lactic acid, one group, the CFS from *Weissella* sp. SNUL2, was neutralized at pH 6 and another group was conducted at pH 4, which showed optimal activity. The result was shown in Fig. 2.8. First, when looking at the pH 6, the growth of indicator strain treated with CFS was inhibited by 29.53%, 28%, 25.43%, 29.8%, 13.9% and 91.16% respectively of *Salmonella enterica* ATCC 13076, *Bacillus cereus* ATCC 14579, *Staphylococcus epidermidis* KCTC 1917, *Salmonella enterica* ATCC 43971, *Staphylococcus aureus* ATCC 25923, *Vibrio fluvialis* KCTC 2473, compared to the each control group. On the other hand, the group treated with pH 4 CFS showed clear inhibition compared to pH 4 the control group that not treated with CFS. The inhibition was 89.19%, 97.47%, 95.4%, 90.13%, 94.25% and 80.96% respectively. As positive control, duramycin (Sigma-Aldrich, St. Louis, MO, USA), which is commercial bacteriocin, was used. Duramycin is a kind of lantibiotics that bacterially produced peptidic natural products that can inhibit the growth of other bacteria. In groups treated with duramycin, *Salmonella enterica* ATCC 13076, *Bacillus cereus* ATCC 14579 and *Vibrio fluvialis* KCTC 2473 showed significant inhibitory ability ($O.D_{600}$ = 0.19, 0.17, 0.13) similar to that of L2 CFS (pH 4) group. However, Staphylococcus epidermidis KCTC 1917, *Salmonella enterica* ATCC 43971 and *Staphylococcus aureus* ATCC 25923 did not suppressed by duramycin

treatment and showed similar growth to the control group. Thus, L2 CFS treated group showed a broad-spectrum inhibitory ability than duramycin for these 6 pathogens.

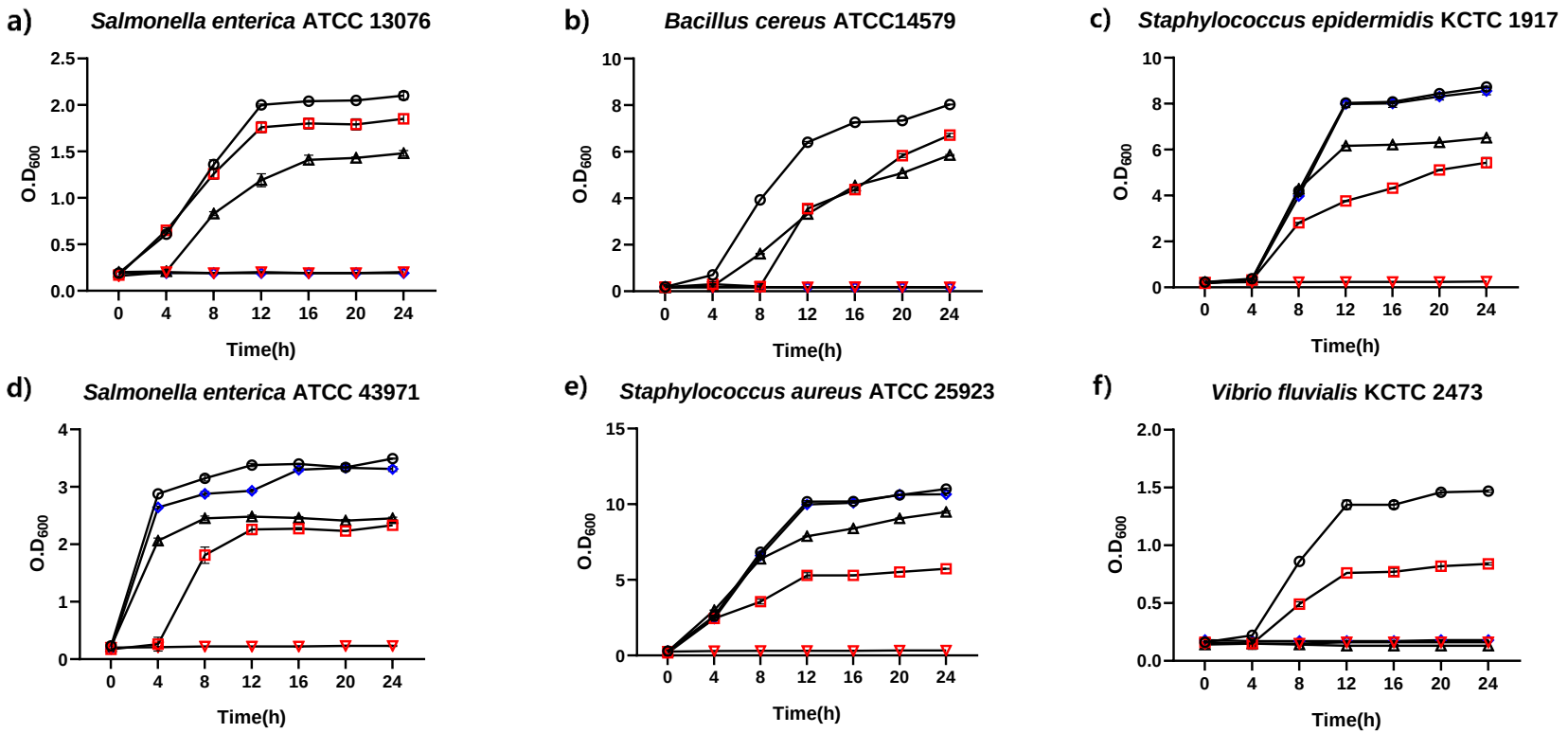


Fig. 2.8. Antibacterial spectrum of CFS obtained from *Weissella* sp. SNUL2 against pathogens. (○), Control (pH 6); (△), + L2 CFS (pH 6); (□), Control (pH 4); (▼), + L2 CFS (pH 4); (♦), + duramycin.

3.6. Antibacterial test against beneficial microbes

As shown in Table. 3., four beneficial microbes used as indicator strain. Likewise, the growth inhibition test was conducted with *Weissella* sp. SNUL2 CFS treatment. As positive control, nisin (Sigma-Aldrich, St. Louis, MO, USA), which are most well-known commercial bacteriocin, was used. As well as its popularity, nisin was used because its inhibitory property against Gram-positive bacteria. Generally, beneficial microbes such as LAB, *Bifidobacterium* were categorized Gram-positive bacteria and thus, the test was performed to determine whether *Weissella* sp. SNUL2 CFS has inhibitory property against four LAB. In the case of *Lactobacillus plantarum* KCTC 3108, the initial $O.D_{600}$ of the control group increased from 0.29 ± 0.01 to 12.58 ± 0.17 after 24 h and its stationary phase starts from 16 h. In the group treated with L2 CFS, the initial $O.D_{600}$ increased from 0.29 ± 0.00 to 9.95 ± 0.21 after 24 h incubation. The initial $O.D_{600}$ treated with nisin was 0.19 ± 0.00 and the final $O.D_{600}$ was 0.15 ± 0.00 confirming that growth was reduced or maintained under this treated condition (Fig.2.9.a). *Lactobacillus sakei* KCTC 3598 showed a similar growth pattern as *Lactobacillus plantarum* KCTC 3108. The initial $O.D_{600}$ of the control group increased from 0.32 ± 0.02 to 7.01 ± 0.25 after 24 h. In the group treated with L2 CFS, the initial $O.D_{600}$ increased from 0.30 ± 0.00 to 5.19 ± 0.06 after 24 h incubation. The initial $O.D_{600}$ treated with nisin, however, was 0.21 ± 0.00 and the final $O.D_{600}$ was 0.16 ± 0.00 (Fig.2.9.b). In the case of *Lactobacillus casei* ATCC 393, stationary phase was started after 12 h. The initial $O.D_{600}$ of the control group (○) was 0.23 ± 0.00 and increased to 12.18 ± 0.14 after 24 h incubation. In the group treated with L2 CFS, the initial $O.D_{600}$ was 0.24 ± 0.00 and the final $O.D_{600}$ was 10.14 ± 0.12. By the way, the initial $O.D_{600}$ treated with nisin was 0.17 ± 0.00 and the final $O.D_{600}$ was 0.15 ± 0.00 (Fig.2.9.c). As shown in Fig.2.9.d, *Leuconostoc mesenteroides* KCTC 3100 showed similar growth pattern as other three indicator strain. . The initial $O.D_{600}$ of the control group was 0.35 ± 0.00 and increased to 5.60 ± 0.10 after 24 h incubation. In the group treated with

L2 CFS, the initial $O.D_{600}$ was 0.28 ± 0.01 and the final $O.D_{600}$ was 4.19 ± 0.02. The initial $O.D_{600}$ treated with nisin, however, was 0.17 ± 0.00 and the final $O.D_{600}$ was 0.20 ± 0.00. When the CFS was treated compared to the control group of each strain, in the order Fig.2.9.a to d, the inhibition rate of growth was 20.91%, 25.97%, 16.75% and 25.18% respectively.

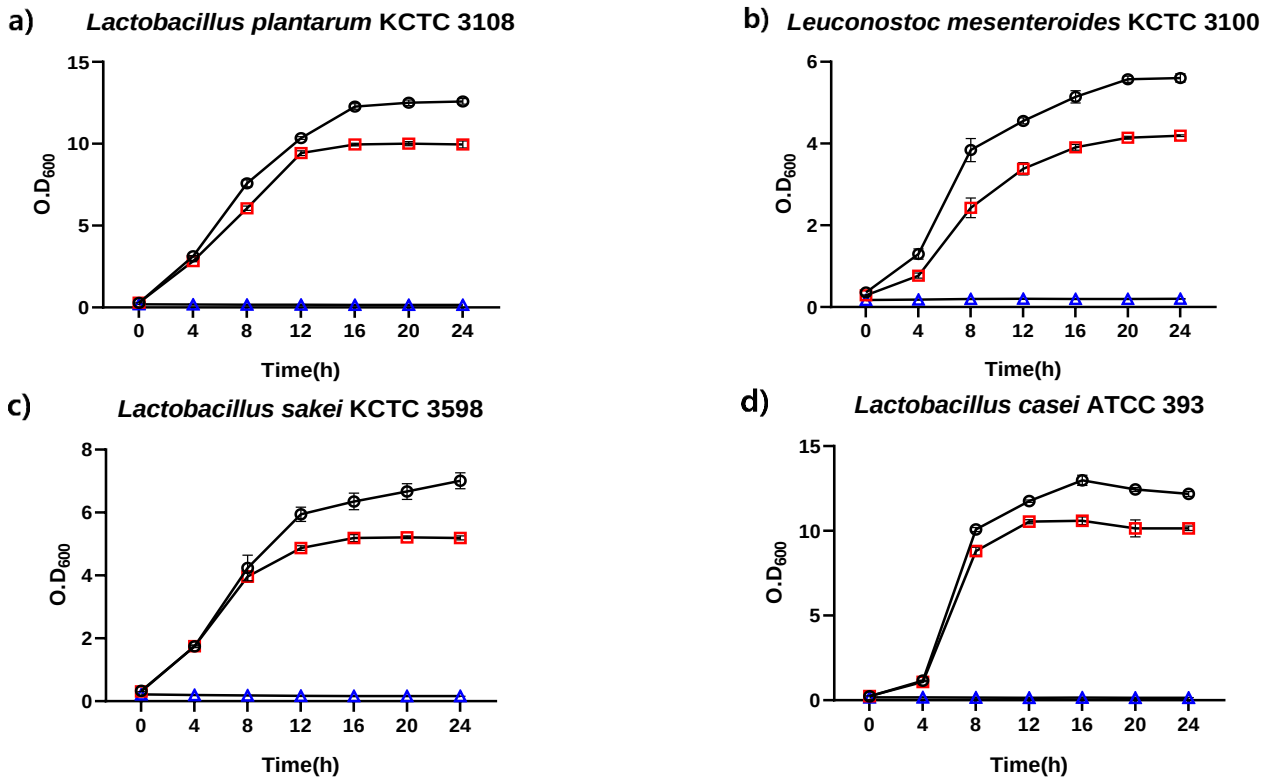


Fig. 2.9. Antibacterial spectrum of CFS obtained from *Weissella* sp. SNUL2 against beneficial microbes. (○), Control; (□), + L2 CFS; (△), + Nisin.

3.7. Minimum inhibition concentration (MIC) test

As mentioned above, MIC is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation against indicator strains. Schematic illustration of two-fold dilution is shown in Fig. 2.10. As shown in Fig. 2.11., 6 indicator strains are cultured overnight with two types of antimicrobial agent, *Weissella* sp. SNUL2 CFS and ampicillin using microdilution assay. Yellow boxes in Fig. 2.11. show MIC of two types of antimicrobial agents against each indicator strain. The results of MIC test are described. Naturally, ampicillin, a purified antibiotic, was good for the MIC for most indicator bacteria. However, among them, *Weissella* sp. SNUL2 CFS showed a better MIC than ampicillin against *Bacillus cereus* and recorded a similar MIC to that of ampicillin against *Vibrio fluvialis*.

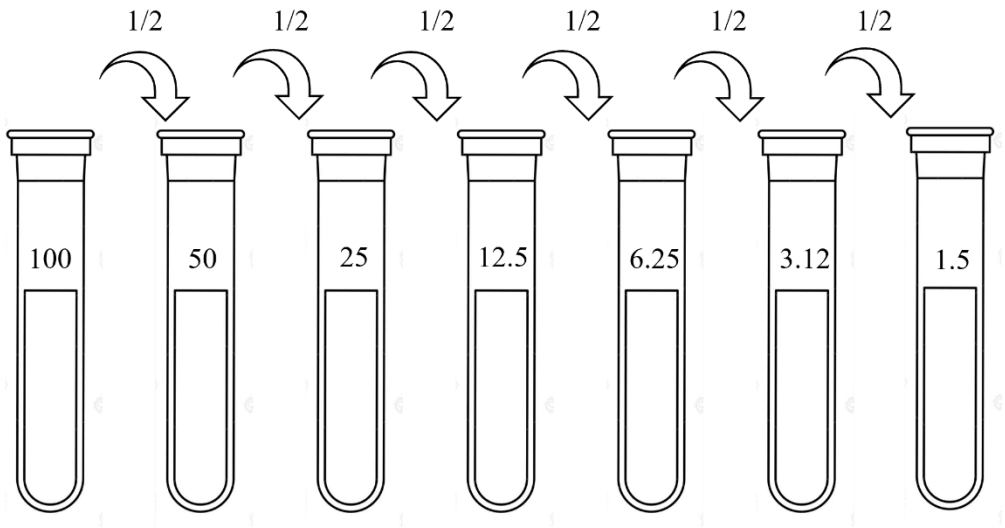


Fig. 2.10. Schematic illustration of two-fold dilution. Number means percentage of initial sample concentration.

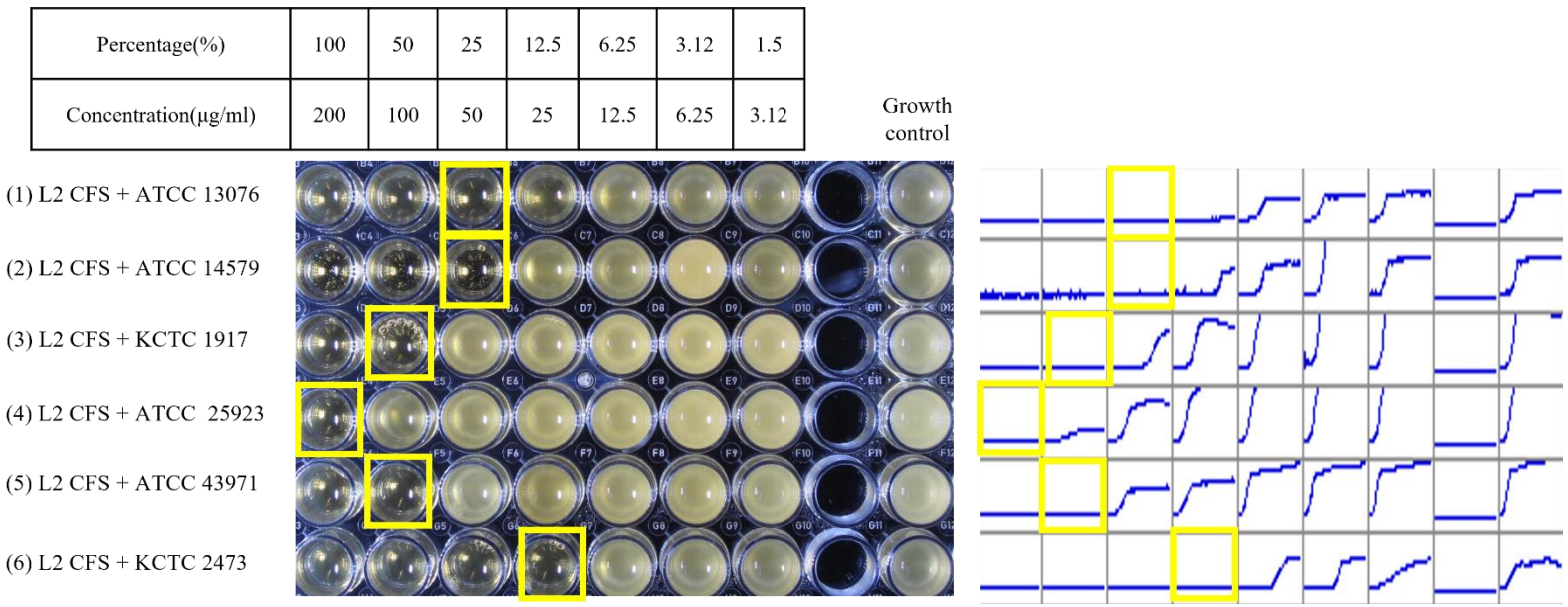


Fig. 2.11. Microdilution assay of 6 indicator strains added with *Weissella* sp. SNUL2 CFS and its growth curve. Yellow boxes mean minimum inhibition concentration of *Weissella* sp. SNUL2 CFS.

Table 6. Minimum inhibition concentration of *Weissella* sp. SNUL2 (L2) CFS and ampicillin against 6 indicator strains.

Indicator strain	L2 CFS MIC (μg/ml)	Ampicillin MIC (μg/ml)
Salmonella enterica (ATCC 13076)	50	0.38
Bacillus cereus (ATCC 14579)	50	200
Staphylococcus epidermidis (KCTC 1917)	100	50
Staphylococcus aureus (ATCC 25923)	200	0.19
Salmonella enterica (ATCC 43971)	100	1.56
Vibrio fluvialis (KCTC 2473)	25	12.5

3.8. Hourly antibiotic substance production

To determine when the antibiotic substance was produced while the strain was incubated, so called Time-kill assay was conducted. Generally, the assay is used to study activity of an antimicrobial agent against a bacterial strain and can determine the bactericidal activity of an agent over time. As mentioned in Table 3., 6 CFSs cultured for different times (4h, 8h, 12h, 16h, 20h, 24h) were used. Based on 12 h from the beginning of the stationary phase, the *E. coli* culture co-cultured with the *Weissella* sp. SNUL2 CFS after 4 h ($O.D_{600}$=0.447) of incubation had 31.5% of inhibitory effect compared to the control group ($O.D_{600}$=0.652) (Fig.2.12.). However, the absorbance of the *E. coli* culture to which the CFS was added after 8 h of incubation, was 74% suppressed compared to the control group. The absorbance of the *E. coli* culture co-cultured with the CFS after 12 h or more of incubation was also similar to the result of 8 h, and the absorbance is as follows: L2 CFS (8 h) =0.172; L2 CFS (12 h) =0.173; L2 CFS (16 h) =0.173; L2 CFS (20 h) =0.169; L2 CFS (24 h) =0.168.

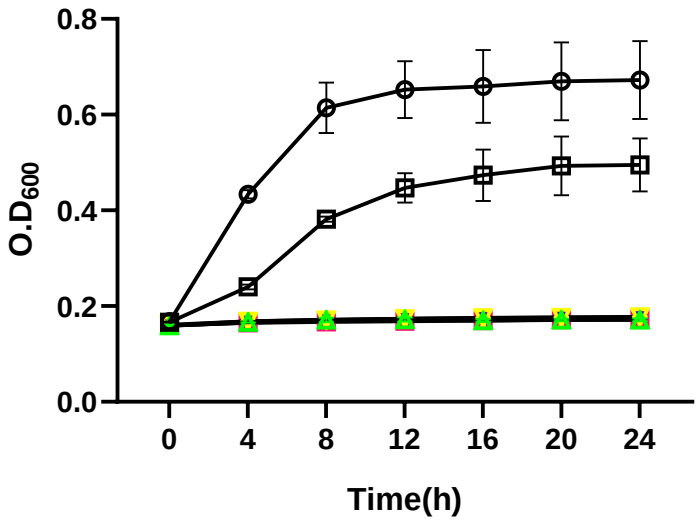


Fig. 2.12. Time-kill assay of *E. coli* using *Weissella* sp. SNUL2 CFS for 24 h. (○), Control; (□), *E. coli* with 4 h incubated CFS; (△), *E. coli* with 8 h incubated CFS; (▽), *E. coli* with 12 h incubated CFS; (◆), *E. coli* with 16 h incubated CFS; (x), *E. coli* with 20 h incubated CFS; (□) *E. coli* with 24 h incubated CFS.

3.9. Antibiotic substance assay

To determine whether the antibiotic substance was a peptide-based material, the antibiotic substance was treated with proteinase such as trypsin and pepsin (Aran et al., 2015). The test was designed with the idea that if the target antibiotic substance in *Weissella* sp. SNUL2 supernatant is a proteinaceous substance, it will be degraded and lose its antimicrobial properties when a proteolytic enzyme is added. The 'control' group is a group in which CFS is not added to the *E. coli* culture and 'L2 CFS' is a group in which CFS is added to the *E. coli* culture. Looking at these two groups cultured for 24 h, the control group showed an absorbance of 0.623 based on 24 h, whereas L2 CFS group showed an absorbance of 0.127 at 600 nm. It was confirmed that the growth of L2 CFS group was inhibited by 79.6% compared to the control group. In the case of the 'L2 CFS + trypsin and L2 CFS + pepsin' in which protein components were decomposed by activating trypsin and pepsin, 0.589 and 0.586 of absorbance were respectively shown.

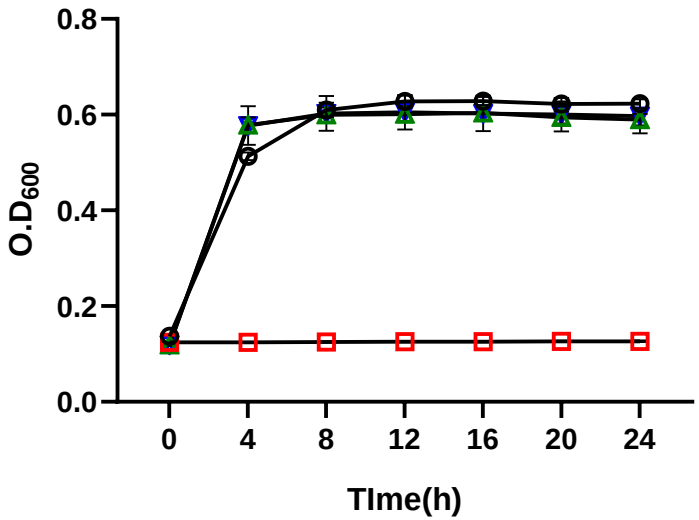


Fig. 2.13. The growth curve of *E. coli* co-cultured with CFS from *Weissella* sp. SNUL2 with two types of proteinase. (○), Control; (□), *E. coli* with *Weissella* sp. SNUL2 CFS; (△), *E. coli* with *Weissella* sp. SNUL2 CFS after trypsin treatment; (▽), *E .coli* with *Weissella* sp. SNUL2 CFS after pepsin treatment.

3.10. Kirby-Bauer disk diffusion assay

Inhibition zone is an area of media where bacteria are unable to grow, due to presence of a antibiotics that impedes their growth. Generally, the zone is measured in millimeters by a ruler from the center of the disk to the edge of area with zero growth. In this study, the center and edge of punched spot was measured instead of the disk. The diameter of the inhibition zones was measured and interpreted as (+) weak inhibition (6-9 mm), (++) intermediate inhibition (10-13 mm), (+++) strong inhibition (14 mm-16 mm) and (++++) very strong inhibition (>17 mm). Inhibition zone (mm) was observed in three indicator strains treated with the CFS: *Salmonella enterica* ATCC 13076; *Bacillus cereus* ATCC 14579; *Vibrio fluvialis* KCTC 2473. Although antibiotic substance in the CFS was not purified or concentrated, inhibition zone (mm) of three indicator strains was observed 14.00 ± 0.00, 13.00 ± 0.82 and 12.50 ± 0.70 respectively. In the remaining three indicator strains: *Staphylococcus aureus* ATCC 25923; *Salmonella enterica* ATCC 43971; *Staphylococcus epidermidis* KCTC 1917, no inhibition zone (mm) was observed. As mentioned before, in the experiment using the broth, 50 ml of the CFS was added out of the total volume of 100 ml. However, in Kirby-Bauer disk diffusion test using agar plate, 40 µl of the CFS was added out of the total volume of 20 ml medium, thus there may be a difference in inhibitory ability.

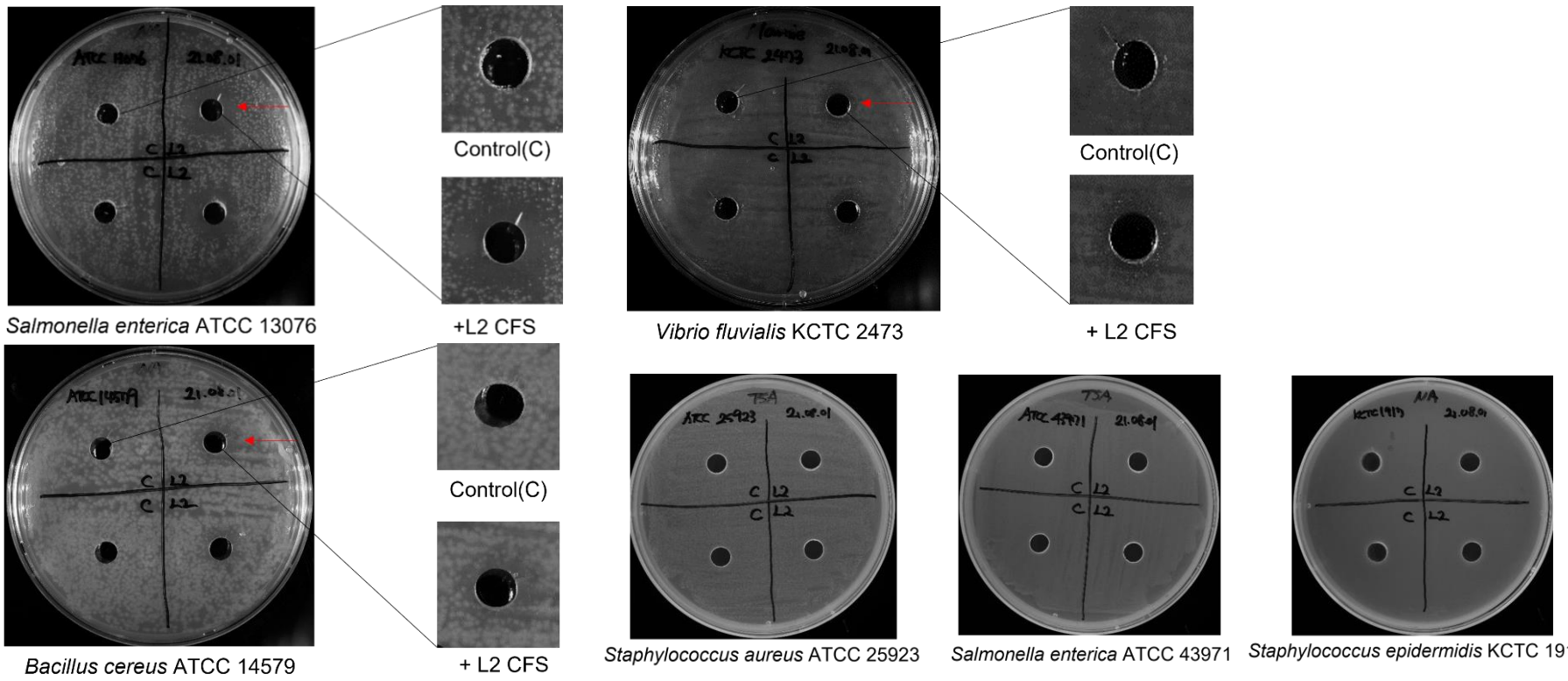


Fig. 2.14. Inhibition zone of pathogens treated with CFS from *Weissella* sp. SNUL2 by Kirby-Bauer disk diffusion test.

Table. 7. Inhibition zone (mm) and antimicrobial activity of pathogens treated with CFS from *Weissella* sp. SNUL2 by Kirby-Bauer disk diffusion test. The diameter of the inhibition zones was interpreted as (+) weak inhibition (6-9 mm); (++) intermediate inhibition (10-13 mm); (+++) strong inhibition (14-16 mm), (++++) very strong inhibition (>17 mm).

Indicator strain	Medium	Inhibition zone (mm)	Antimicrobial activity
Salmonella enterica (ATCC 13076)	NA	14.00 ± 0.00	+++
Bacillus cereus (ATCC 14579)	NA	13.00 ± 0.82	++
Vibrio fluvialis (KCTC 2473)	LB	12.50 ± 0.70	++
Salmonella enterica (ATCC 43971)	TSA	-	-
Staphylococcus aureus (ATCC 25923)	TSA	-	-
Staphylococcus epidermidis (KCTC 1917)	NA	-	-

3.11. Partial purification

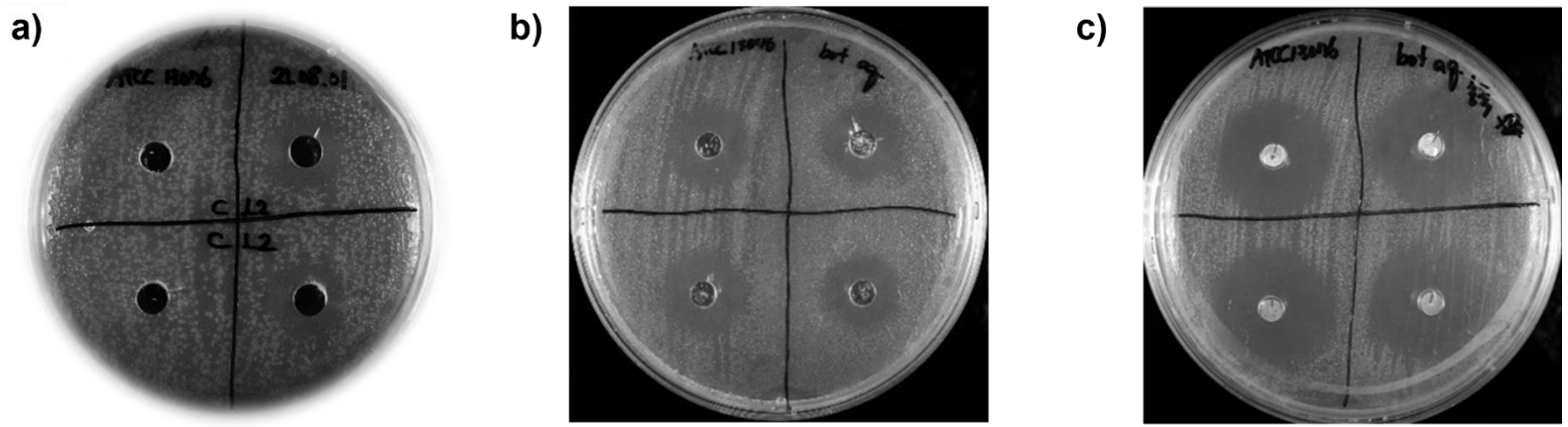
3.11.1. Ethyl acetate extraction

To extract antibiotic substances based on its partition between immiscible aqueous and organic solution selectively, Ethyl acetate extraction was conducted. Total 100 ml of the CFS was extracted and this extracted CFS was tested with Kirby-Bauer disk diffusion test. As a result of the experiment, the inhibition zone (mm) was not seen in the organic layer loaded plate. By the way, the 17 mm of inhibition zone was seen in the aqueous layer loaded plate (Fig. 2.15.b). When normal CFS was loaded, the inhibition zone was 14 mm, and it increased by 4 mm after extraction. Through this, it could be predicted that the antibiotic substances were in the aqueous layer. The inhibition zone (mm) of the agar plate loaded by 2× concentrated aqueous layer was 25 (Fig. 2.15.c.), which was increased by 8 mm compared to the previous one.

3.11.2. Gel filtration

Gel filtration was conducted to partially purify antibiotic substances from the CFS. In this filtration, 10× concentrated extracted CFS was loaded to Sephadex G-50 fine resin packed in 87 × 300 (ø × mm) glass column. Total 23 fraction was collected, and each fraction was collected in sterile 15 ml falcon tube. These fractions were measured for optical density at a wavelength of 280 nm to determine the amount of protein contained in the fraction, and at the same time quantified through Bradford assay. Finally, each fraction was subjected to Kirby-Bauer disk diffusion test after two-fold concentration. As shown in Fig. 2.16., only two fractions: No.14; No.15

showed inhibition zone (mm) against indicator strain. Except two fractions, indicator strain grew as normal condition. Inhibition zone (mm) was observed at both of No.14, No. 15 fraction, 22 mm. According to optical density at 280 nm wavelength, No.7 and No.11 fraction showed the highest, but did not show any inhibitory property.



Loaded sample	Normal CFS	Aqueous layer of ethyl acetate extracted CFS	Aqueous layer of ethyl acetate extracted CFS (2×)
Inhibition zone (mm)	14 ± 0.00	17 ± 0.00	25 ± 0.00

Fig. 2.15. Inhibition zone (mm) of three different types of CFS from *Weissella* sp. SNUL2 treated indicator pathogens. (a) normal CFS; (b) aqueous layer of ethyl acetate extracted CFS; (c) aqueous layer of ethyl acetate extracted CFS (2×)

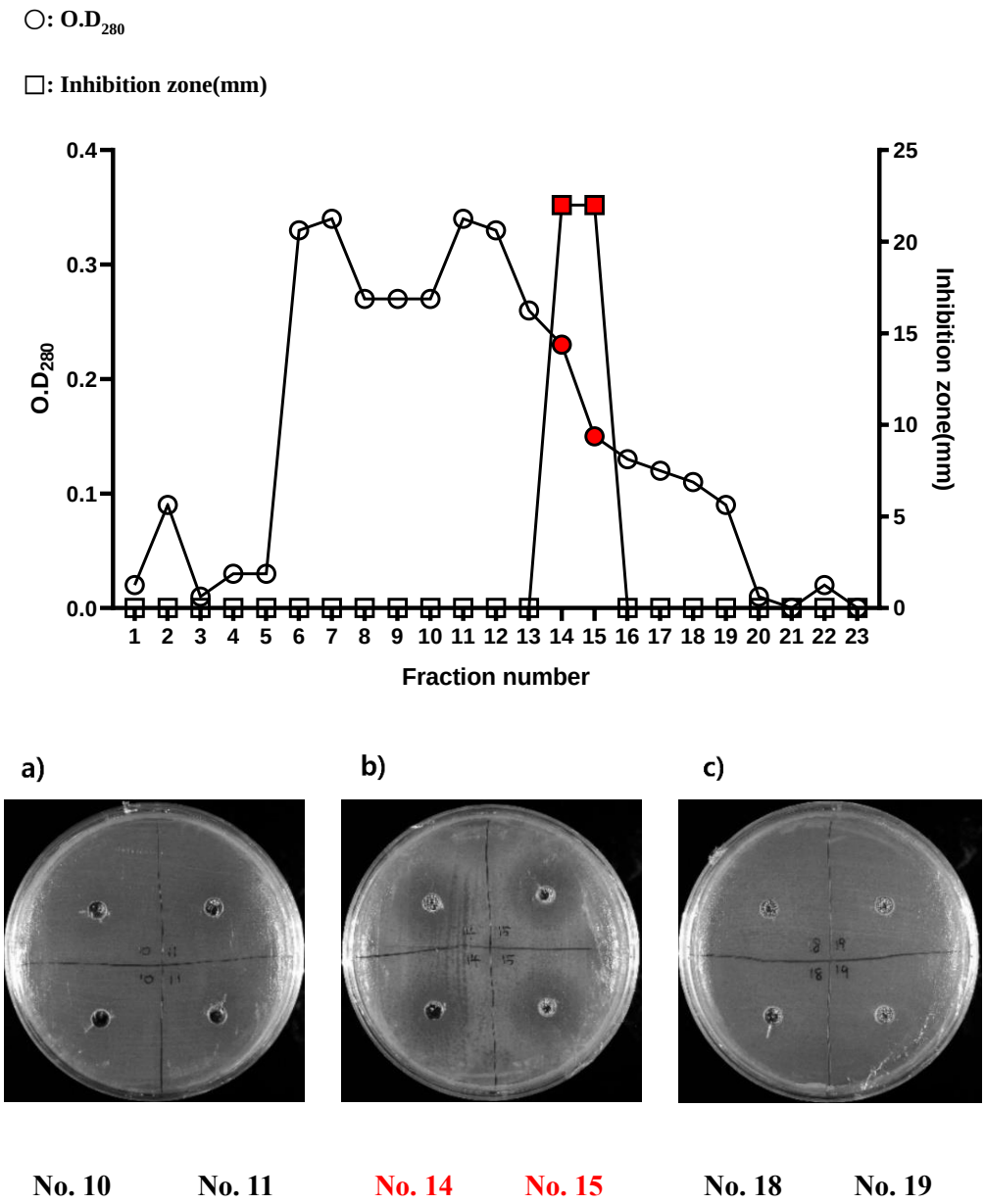
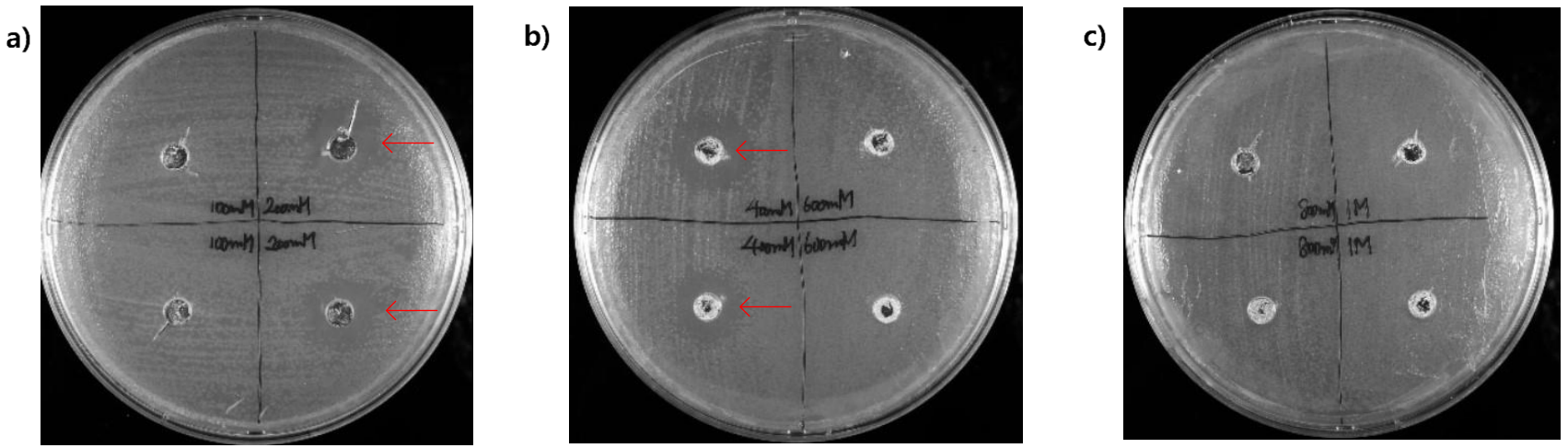


Fig. 2.16. Elution profile of partially purified antibiotic substances from a Sephadex G-50 column equilibrated with 30 mM Tris-HCl buffer (pH 8.0). a), fraction No. 10, 11; b), fraction No. 14, 15; c), fraction No. 18, 19.

3.11.3. Ion-exchange chromatography

The size exclusion of the sample was performed by gel filtration chromatography, and the desired fraction was obtained. To purify the obtained fraction using the ion strength characteristics, ion-exchange chromatography was conducted. Amberlite CG50 column (Sigma-Aldrich, St. Louis, MO, USA)., an anion exchange resin, was used and as elution buffers, 6 types were used according to the concentration gradient of NaCl: 5 mM Sodium phosphate buffer + 100 mM NaCl; 5 mM Sodium phosphate buffer + 200 mM NaCl; 5 mM Sodium phosphate buffer + 400 mM NaCl; 5 mM Sodium phosphate buffer + 600 mM NaCl; 5 mM Sodium phosphate buffer + 800 mM NaCl; 5 mM Sodium phosphate buffer + 1 M NaCl. Each fraction was collected by flowing the elution buffer as 5 CV. The samples eluted with the buffer according to the NaCl gradient were quantified by Bradford assay, and the same time Kirby-Bauer disk diffusion test was conducted after concentration. As shown in Fig. 2.17., the fraction eluted by 5 mM Sodium phosphate buffer + 200 mM NaCl showed 16.15 ± 0.21 mm of inhibition zone, and the fraction eluted by 5mM Sodium phosphate buffer + 400 mM NaCl showed 17.50 ± 0.70 mm of inhibition zone against the indicator strain. In the fractions eluted by other elution buffers, no inhibition zone (mm) was shown, and it was confirmed that the desired antibiotic substances were eluted at a concentration between 200 mM and 400 mM NaCl.



NaCl gradient of elution buffer	100 mM	200 mM	400 mM	600 mM	800 mM	1 M
Inhibition zone (mm)	-	16.15 ± 0.21	17.50 ± 0.70	-	-	-

Fig. 2.17. Inhibition zone (mm) of ion exchanged CFS from *Weissella* sp. SNUL2 eluted according to the concentration gradient of the elution buffer. The red arrow indicates the inhibition zone (mm) of each agar plate.

3.12. SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

The molecular weight of the antibiotic substance was determined by SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to the method of Laemmli (Laemmli, 1970). As Fig. 2.16 shows, the protein marker was loaded in Lane 1 and normal CFS was loaded in Lane 2. Three clear bands were formed in Lane 3, and among these bands, same band (red arrow pointed) was formed in Lane 3, which was the partially purified CFS. According to the ladder, the molecular weight of it was 26.6 kDa or larger than 26.6 kDa because the band was formed at the beginning spot of the running gel.

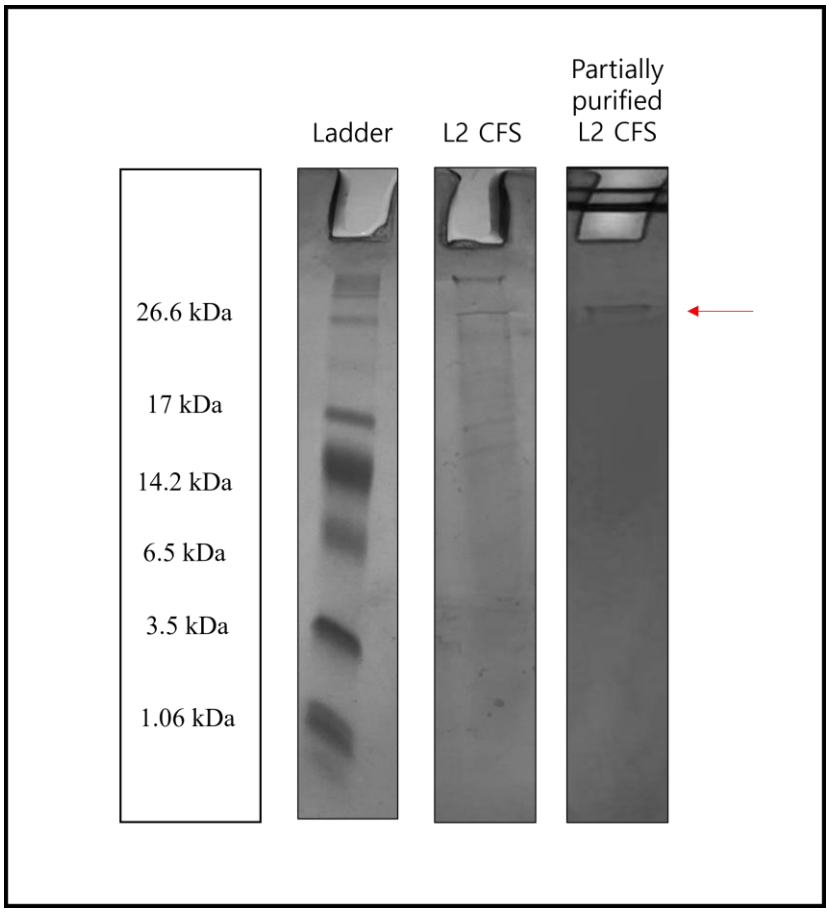


Fig. 2.18. SDS-PAGE analysis of the antibiotic substances from *Weissella* sp. SNUL2. Lane 1: molecular weight marker; Lane 2: the CFS from *Weissella* sp. SNUL2; Lane 3: Partially purified CFS from *Weissella* sp. SNUL2.

3.13. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

To identify the protein band from CFS produced by *Weissella* sp. SNUL2, LC-MS/MS analysis was performed through nano ACQUITY UPLC and LTQ-orbitrap-mass spectrometer (Thermo Electron, San Jose, CA). The individual spectra from MS/MS were proceed using SEQUEST software (Thermo Quest, San Jose, CA, USA) and the generated peak list were used to query in house database using the MASCOT program (Matrix Science Ltd., London, UK). According to MASCOT score histogram, individual scores larger than 14 indicate identity or extensive homology ($P<0.05$). As shown in Table. 8., Total 67 proteins were identified. Among them, there are 21 proteins with a score of 100 or higher. L2_1_01888, which recorded the highest score, is a C39 family protein and contains bacteriocin processing endopeptidases. C39 family peptidase is a peptide produced from precursors of various bacteriocins and is known to cleave the double glycine bond of peptidoglycan. A total of five C39 family proteins were identified, L2_1_1888, L2_1_1932, L2_1_1872, L2_1_2024 and L2_1_1847, and the scores were 10138, 240, 215, 134 and 44 respectively. Each protein was identified based on the sequences shown in the table, and the sum of the MASCOT scores of the peptide sequence is the score of the corresponding protein. In addition, as a result of identification, precursors related to peptidoglycan endopeptidase such as L2_1_01908 and L2_1_01871 were found, and precursors related to murein endopeptidase such as L2_1_01907 and L2_1_00062 were found. Peptidoglycan, an essential cell wall polymer, formed by glycan chains of β-(1-4)-linked-*N*-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), cross-linked by short peptide stems. Peptidoglycan endopeptidase cleaves β-(1-4) glycosidic bond between carbon 1 of N-acetylglucosamine and carbon 4 of N-acetylmuramic acid. According to this mechanism, as many endopeptidases of peptidoglycan have been identified, it is expected to play a significant role in degradation of other bacteria.

Table 8. Peptide summary report after LC-MS/MS analysis interpreted by MASCOT score histogram.

Locus_Tag	NCBI BLAST	Score	Mass	Sequence	Mass
L2_1_01888	C39 family protein	10138	72119	K.WVSQWGNK.Y	502.7474
				K.WYDFGSNK.T	508.7249
				K.RVTADPQFK.N	531.2953
				K.TYDLTNNLK.R	541.7686
				K.SFGIPVLDWR.E	595.3190
				K.TYDLTNNLKR.V	619.8198
				R.EGGYKVVTDANR.Q	436.8877
				K.LSENEHDLQVK.F	656.8187
				R.QYATNNALASGFK.N	682.8424
				R.WFENGKPYTGFR.L	751.8536
				R.QENKWVSQWGNK.Y	502.2417
				K.LVNGKWYDFGSNK.T	764.3759
				R.FYMGTYYWFEK.G	775.8341
				K.FTSNGWQTDESGNR.Q	800.3328
				K.TYNFGTNHTFFLR.-	809.8786
				K.TYYVATDGTTQEGIK.L	823.8926
				K.WYDFGSNKTYYAR.N	557.5883
				R.TAWNLKYYVDSEGR.A	567.9425
				K.SFGIPVLDWREGGYK.	575.2963
				R.MYMGAYYYFINGVR.Q	890.8861
				R.NFTQSGYLNTDQGWR.W	893.9062
				K.WVSQWGNKYYVGSDGR.S	634.6332
				K.NINGQGYFFNPSNNGLLK.G	999.4841
				K.IDGKTYNFGTNHTFFLR.-	508.5054
				K.GQAGYTFNQLADALTEVYK.S	1045.0070
				K.ASGYVSTPDGWLWIENGKR.Y	712.6840

				K.TYYVATDGTTQEGIKLVNGK.W	1080.0415
				K.LVNGKWYDFGSNKTYYAR.N	546.2710
				K.LVIYGVLPIPSYMGGDNLNVK.G	1139.6065
				R.GKASGYVSTPDGWLWIENGKR.Y	581.2922
				R.QENKWVSQWGNKYYVGSDGR.S	601.0353
				K.GKNQLAMAGGKTYYVATDGTTQEGIK.L	680.3351
				R.AVQGDGYNVDGTYYNFSHDGTFFLR.G	948.4228
				K.FTSNGWQTDESGNRQYATNNALASGFK.N	989.7750
				K.NAESLVLEIGVNDLNYSDNNLGYVQQR.L	1013.1620
				R.QQTLGDHEVHPTQETYANMAELMAQWMVDNSK.L	934.4135
				K.QNYPTLLASIIKPESMHAGYASSGSQISGNQNGPGDK.T	959.4570
L2_1_00882	Iron Transport-associated domain protein	1038	95518	R.FFNPTAK.L	412.7163
				R.FVTFEIPTK.D	541.2975
				K.NDITFNIPTK.D	581.8068
				K.IASFTSVPMSPSK.G	684.3440
				K.LVNNYYESTYR.M	711.3340
				K.TGTNNASAMNNYLK.T	749.8461
				K.LAVKGDTTDVTLSFK.D	532.2913
				K.SVTLASFTSVPMSPTK.G	834.9249
				K.TGTQEDSAMMSYVESK.G	890.3676
				K.NADGSYYGYLTTHTPK.L	894.4111
				K.GQSQMDMMTGFTVAGHTATK.S	716.3057
				R.FVTFEIPTKDLSPDDTNVAK.I	746.3785
L2_1_01908	Peptidoglycan DL-endopeptidase CwlO precursor	898	59707	R.NSWSIAAR.Q	453.2245
				R.YLFRNGVK.Q	499.2742
				R.QFDCSSFVR.W	573.2527
				K.FQGTYYMFNLR.D	728.3381
				R.WGTWYMYDETGR.C	782.8265
				R.TYLKHTNVYEPAR.W	531.2760
				R.AYAVPWYDIVDGVVR.R	861.9419

				R.EVTPSAQATHQSAAVVK.K	862.4467
				K.FQGTYYMFNLRDGR.M	595.2756
				K.GVQKFQGTYYMFNLR.D	623.3070
				R.VLTDVQPWAGGYYTFDAR.T	1029.9970
				K.ATNEAVWENGNEYWADGGGVVR.T	1198.0302
L2_1_01894	Chaperone protein DnaK	522	65296	R.GVPQIEVK.F	435.2559
				K.IAGLEVER.I	443.7509
				R.TTPSAVSFK.N	469.2510
				R.IPAVQESVK.G	485.7793
				R.FQLTDIPAAPR.G	614.8344
				K.DAGKIAGLEVER.I	419.8960
				R.AQFNQLTADLVK.R	674.3655
				K.LIDRNTTIPTSK.S	453.5898
				K.YAEDYLGETVDK.A	701.8188
				R.QAITNPDTILSIK.S	707.3985
				K.KYAEDYLGETVDK.A	510.9122
				R.TTPSAVSFKNGETQVGDTAK.R	680.3336
				R.TTPSAVSFKNGETQVGDTAKR.Q	549.5264
				K.SQVFSTAADNQPAVDIHVLQGER.S	828.0805
L2_1_01871	Peptidoglycan endopeptidase LytF precursor	398	37152	K.WENAWGMK.Y	511.2280
				K.MLGLPYAWGGK.T	596.8085
				R.QDSGWREAWGMK.Y	484.2209
				R.GKTSGYTDAGEGWK.W	486.2260
				R.QQNKWENAWGMK.Y	507.2390
				K.TWSGVDCAGYVALVR.S	551.9384
				R.EAWGMKYYTDNQGR.A	578.9197
				K.NVSGGTHYFGNDGTFFER.S	668.9615
				K.TSGYTDAGEGWKWYEGGQK.F	707.3107
L2_1_00448	Internalin-A precursor	336	86562	K.LTSLNFSK.D	455.2526
				K.TVGVATLMTTK.F	561.3127

				K.LTNLTTLSIGNNR.S	708.8891
				K.VGSNQITDFTPIAK.L	745.8961
				R.QALQSALGADVPLTK.S	756.4193
				K.VTSVPELGNLHQLR.E	521.6246
L2_1_01907	Murein DD-endopeptidase MepS/Murein LD-carboxypeptidase precursor	298	38865	K.YYVDNVGR.A	493.2377
				R.TTYEQAAYLR.A	608.3013
				K.TSGYTDAGQGWK.W	636.2780
				R.QDSGWREAWGMK.Y	484.2209
				K.WYENGQQFTGFR.F	766.8429
				K.SYYFGNDGTFFLR.D	793.8644
L2_1_01932	C39 family protein	240	40773	R.AVQGLQTINNK.K	593.3298
				K.DAYVPVNNSGWR.W	689.3275
				K.YYFGNDGTFYSR.A	745.3209
				K.VKDAYVPVNNSGWR.W	535.6069
				R.AVPGKPTYFSQWDGR.W	570.2835
L2_1_01872	C39 family protein	215	50074	K.YGVTGVIVK.L	468.2795
				K.TGNVTNNALAFR.N	639.8231
				K.SVVMVDDLEDSSTK.T	762.8557
				K.LTEHTTYVNPYAR.Q	522.2599
L2_1_00644	Xylulose-5-phosphate phosphoketolase	212	91993	K.ISNPTIFSR.M	517.7824
				R.YVNVVELHR.L	376.8749
				K.AEDLALPDWK.S	579.2914
				R.IFGPDETMSNR.L	641.7868
				M.AVDFDSKEYLAK.V	462.5662
				K.VMDQAIEDIQAIQK.D	539.9418
				K.GWGGPTHDQSGMPIEDSFR.A	697.3016
L2_1_00917	Methionine-tRNA ligase	203	74784	K.GEPIFPR.V	408.2212
				R.TADALAEVWK.L	552.2883
				R.VAAILLQPALTR.A	633.3950

				K.SHPEFIQPESR.M	442.8838
				R.VAEILEVTEVEK.S	679.8719
				R.VAEILEVTEVEKSNK.L	563.3043
L2_1_00297	Hypothetical protein	175	84321	K.LADAGALDATALK.T	615.3351
				K.ALDAIQLTSDGR.T	630.3304
				K.GNGAALNADVLATVAK.T	743.3972
L2_1_01489	Membrane lipoprotein TmpC precursor	164	39532	K.GGYDYFLSK.T	525.2485
				K.YALVDAQANPK.L	595.3119
				K.TQADFDTNFQQAQAAK.F	892.4127
L2_1_01501	Elongation factor G	141	78049	K.LAEEDPSFR.A	532.2539
				K.TAGAVILEPIMK.V	621.8578
				R.VYTGTLESGSYVLNTSK.G	909.9556
L2_1_02024	C39 family protein	134	36105	K.TYYLGADGR.S	508.2424
				R.QTNTWATEWGK.T	661.3101
				R.AVQGWQTINGIR.Y	671.8610
L2_1_00373	N-acetylmuramoyl-L-alanine amidase domain-containing protein precursor	132	28374	K.AVVLSAGTEYK.V	569.3082
				R.TISAAGIQFVH.-	572.3076
				K.LTPADKQDNNSASDALK.Q	596.6272
L2_1_00626	Neutral endopeptidase	132	70278	R.LQTADWLQPATR.E	700.3677
				K.VVLSQEESAEYAK.L	726.8619
				R.DALGTDPVKPLIAR.Y	489.2822
L2_1_02151	GTP-binding protein TypA/BipA	113	68463	R.MEWLVPSR.G	509.2620
				K.TTLVNELLK.Q	515.8097
				R.IDEPTLQMTFR.T	683.8334
				K.AFEQNLTPIVVVNK.V	786.4402
L2_1_00758	Hypothetical protein	106	68947	R.GLLSAAEYTK.A	526.7820
				-.MNIVLVGAGPR.N	563.8214
L2_1_00135	Pyruvate kinase	85	50386	K.VSDGLMIAR.G	481.2581
				K.TVGFLLDTK.G	497.2838

				K.AETALALNGR.H K.LIESGANVVR.F	508.2837 529.3013
L2_1_01527	Enolase	72	49011	M.SAITDIYAR.E K.GINSFYNIDK.T	505.2661 585.7903
L2_1_01891	Hypothetical protein	68	37837	K.VGLAYIANR.Y R.AVQGLQTIGGK.Q	488.7800 536.3094
L2_1_00062	Murein DD-endopeptidase MepH precursor	67	49117	R.QTIVTAALK.Y K.YAGQGIPYVWGGK.T	472.7902 698.3547
L2_1_00895	DNA gyrase subunit B	63	71949	R.LTQAILPIR.G R.FMLENPTVAK.Q	512.8273 575.3007
L2_1_01152	Hypothetical protein	63	50637	K.NALPNIFK.N K.NMYTSSLHFR.D R.IATTNLPADVDQGAYSR.I	458.7643 424.5303 896.4440
L2_1_01805	Putative transglycosylase lsaA precursor	59	26515	R.YGSVDGAVQFR.A	599.7909
L2_1_00256	Hypothetical protein	51	123794	K.NVATMALAIK.A R.LTAPENGTVIK.A	516.2971 572.3137
L2_1_02355	Elongation factor Tu	50	43456	K.SMQQGQAGDNIGALLR.G	837.9111
L2_1_02404	Glycine-tRNA ligase beta subunit	50	76035	K.VANFLELDVK.S	574.3200
L2_1_00391	Hypothetical protein	49	60560	-.MIQRDGTR.K R.TTLLQPGIR.T	497.2430 499.8000
L2_1_00296	Hypothetical protein	47	72444	K.ANPNVVDADTLTK.L	679.3474
L2_1_01523	Phosphoenolpyruvate-protein phosphotransferase	45	62652	K.LVDPDLSFDTR.T	639.3201
L2_1_01443	Hypothetical protein	45	128190	R.MALIIYGAR.D	512.2827
L2_1_01847	C39 family protein	44	27112	R.NAWGMTYYTDNQGR.A	838.8555
L2_1_02083	N-acetylmuramoyl-L-alanine amidase domain-containing protein precursor	43	29387	R.SLNAVITTYR.L	569.3149
L2_1_01882	Exo-glucosaminidase LytG precursor	42	41768	K.MTPAILADTSK.Y	574.3011

L2_1_01568	Lipoteichoic acid synthase 1	40	82512	K.IFLQDSAK.Y K.TTTGDKTVDGYVQTAR.Y	461.2519 571.6155
L2_1_01844	Hypothetical protein	39	65180	K.FYTVNVVEK.A K.AYPGYATPISQK.V	549.7919 648.3331
L2_1_01936	Glutamine-fructose-6-phosphate aminotransferase(isomerizing)	39	66064	R.VAQLEALVGTK.N	564.8308
L2_1_00586	ATP synthase subunit beta	34	50220	K.IGLFGGAGVGK.T	488.2817
L2_1_01515	Hypothetical protein	34	79319	K.VLQDLFTK.M	482.2772
L2_1_00243	Threonine-tRNA ligase	33	75145	K.ITFPDGAVK.E K.QIASENLPIVSR.E	474.2610 663.8701
L2_1_00452	Pyruvate oxidase	33	66918	K.VAPVEENAWWR.A	678.8342
L2_1_00828	Ornithine carbamoyltransferase, catabolic	30	38029	R.NIALLFEK.T	946.5462
L2_1_00010	Aspartate-tRNA ligase	26	66880	K.ITDEGITGPIAK.F K.AIVVPGGADNYSR.K	607.8320 659.8379
L2_1_01885	Hypothetical protein	25	66324	K.QLVADERR.T	493.7740
L2_1_01045	Hypothetical protein	24	89243	R.MSADPIAVVVDGK.T K.TVANVAAPAGTVDGLR.Y	659.3364 756.4095
L2_1_00049	Hypothetical protein	24	68032	K.FDPNFVYVAPK.V	648.8323
L2_1_00920	Ribonuclease M5	22	20431	R.LGLGYVNGK.Q	461.2537
L2_1_01226	Hypothetical protein	22	19372	M.KLGMFRLGMR.T	414.2282
L2_1_02169	Glycine betaine/carnitine transport ATP-binding protein GbuA	20	44005	K.QLSGGMQQRVGLAR.A	501.5954
L2_1_00199	Glycoxylate/hydroxypyruvate reductase A	20	34357	R.QVMLTNASGLK.S	393.2166
L2_1_01596	Glyceraldehyde-3-phosphate dehydrogenase 1	19	35756	K.AIGLVVPSVAGK.L	555.8483
L2_1_01477	Transcriptional regulatory protein SrrA	18	28468	M.KLLMVEDNTSVSEMMGMFFKK.E	833.3998

L2_1_01965	Hypothetical protein	18	22345	K.QAATQASKK.A	467.7453
L2_1_01223	50S ribosomal protein L9	18	16650	K.NKKAEPATNANVSAMR.G	573.9478
L2_1_01051	Hypothetical protein	17	-	-.MGADLSAATFGNTDK.T	749.8461
L2_1_00117	Isoleucine-tRNA ligase	17	104743	K.AVGPVIWR.K	449.2659
L2_1_02023	Aryl-phospho-beta-D-glucosidase BglA	17	54798	K.EDLALMGEMGFK.A	678.8083
L2_1_01978	Hypothetical protein	17	14365	R.KMRAGQTMSSIVR.E	494.2614
L2_1_01096	ATP-dependent C1p protease ATP-binding subunit C1pE	17	76368	R.VADFYLDNPDVK.Q	698.3398
L2_1_00865	Flavin monocleotide phosphatase Ybjl	17	29992	R.YPAALDDNNNDGVLLTLDEILR.K	612.5534
L2_1_02118	Polysaccharide pyruvyl transferase	17	30840	-.MFIQGANGNWKEK.L	770.3666
L2_1_01321	Proline-tRNA ligase	16	63892	K.VFIPTLR.E	423.2642
L2_1_02402	Inner membrane protein alx	15	35785	K.EEAQDLNDSFIIK.G	761.8668
L2_1_00401	N-acetylmuramoyl-L-alanine amidase sle1 precursor	15	29888	R.TIPTSGLLFIHQ.-	663.8707
L2_1_00999	MucBP domain protein	15	338215	K.QATSASFTQTDQK.L	707.3320
L2_1_01986	tRNA(adenine(22)-N(1))-methyltransferase	14	25553	R.LGDVLVPR.L	434.7636
L2_1_00535	tRNA N6-adenosine threonylcarbamoyltransferase	14	36513	K.SAVINLMHNADQR.G	743.3566
L2_1_00035	Hypothetical protein	14	13394	R.EANDIMANANTLLNDVNGK.V	679.3069
L2_1_01835	Hypothetical protein	14	36425	K.LGYNVVLQNRGPKIVQHVATK.V	468.2599
L2_1_00769	HTH transcriptional regulator BenM	14	33141	K.NLEEEIGAPLFHR.E	508.9317
L2_1_00978	Lysine-tRNA ligase	13	56177	K.HMVDLIK.E	436.2383
L2_1_02041	Hypothetical protein	13	15603	-.MSESGQTTEMNLFALTLLRDR.L	612.2951

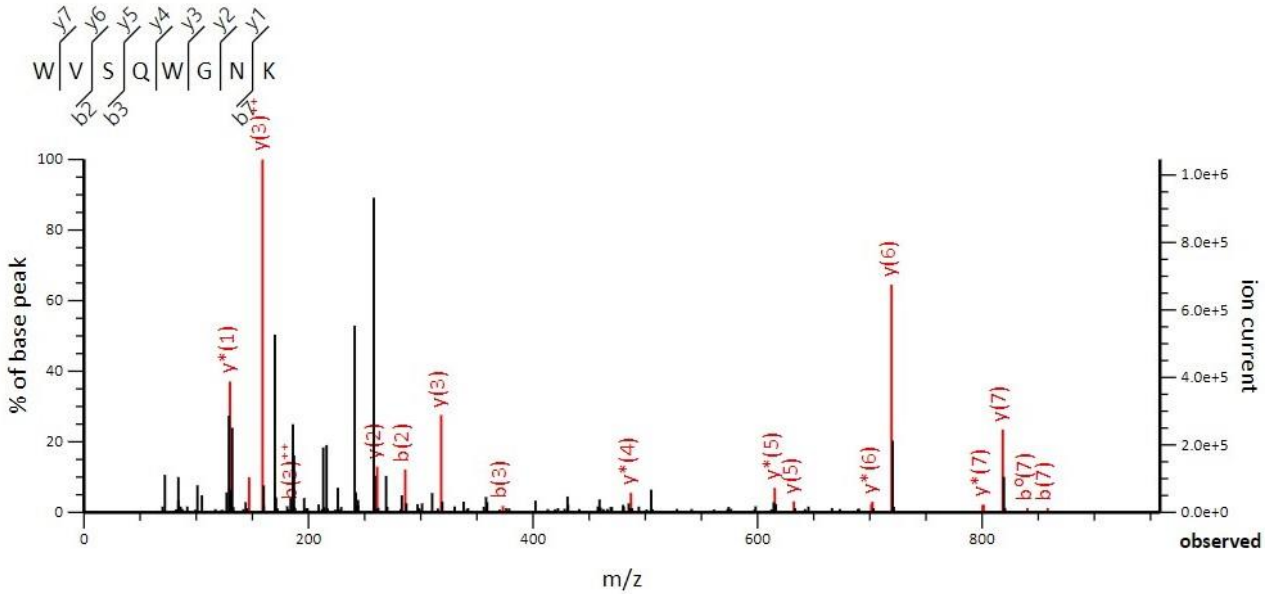


Fig. 2.19. MS/MS fragmentation of WVSQWGNK found in L2_1_01888.

4. Conclusion

In Chapter 2, strains that can be used as an alternative to antibiotics were isolated and various characteristics of antibacterial substances were confirmed. The strain, *Weissella* sp. SNUL2, was isolated from Korean traditional fermented food was identified through 16s rRNA sequencing. The optimum temperature and pH of the strain were confirmed, and the correlation between $O.D_{600}$ and CFU was confirmed and applied to subsequent experiments.

In the study of probiotics for therapeutic purposes, the most important antibacterial test was conducted on a total of 70 strains, and as a result, it was confirmed to have broad spectrum antibacterial properties than previous known probiotics. To know the characteristics of the antibacterial substance secreted in the supernatant, an enzyme test was conducted, and as a result, it was confirmed that it was a proteinaceous substance.

Partial purification was performed to further subdivide this antibacterial protein, and as a result of LC-MS/MS analysis, two proteins were found: C39 family protein that produces bacteriocin, a well-known antibacterial protein, and a peptidoglycan endopeptidase that degrades peptidoglycan, a cell wall component.

Chapter 3.

Characteristics of *Weissella* sp. SNUL2 according to 'Probiotics guidelines'

1. Introduction

There is a large scientific consensus that, in order to assess the properties of probiotic bacterial strains, it is mandatory to perform a preliminary in vitro assessment according to 'Probiotics guidelines' in FAO/WHO (Food and Agriculture Organization of the United Nations/ World Health Organization) (FAO/WHO, 2002). This assessment has traditionally paid special attention to the ecological origin of bacteria, their tolerance to the hostile conditions of the stomach and the small intestine, and their ability to adhere to intestinal surface. Several papers suggest that a probiotic bacterial strain should be assessed according to the following scheme:

(i) It must survive during gastric transit

(ii) It must tolerate bile salts

(iii) It must adhere to gut epithelial tissue

In this study, the isolated strain from Korean traditional food was assessed according to 'Probiotics guidelines' from FAO/WHO and MFDS (Ministry of Food and Drug Safety) in Korea and the types of tests are as follows (FAO/WHO, 2002):

(i) Acid tolerance

(ii) Bile tolerance

(iii) Antibacterial properties against pathogens

(iv) EFSA MIC cut-off of antibiotics

(v) Hemolysis

(vi) Adherence to mucus and/or human cell lines

In addition, there are cytotoxicity tests such as MTT assay, and animal tests, but these tests are omitted because they are not a subject for evaluation as they are strains with food experience that have not been announced but correspond to the 3rd standard of the MFDS criteria.

2. Methods & Materials

2.1. Cell viability test

2.1.1. Acid tolerance test

In order to confirm whether the corresponding strain has resistance under specific conditions, an acid tolerance test was performed. For the acid tolerance test, the method of Conway was referenced with slight modification (Conway et al., 1987). The culture was grown in MRS broth at 30 ℃ overnight, then 1% (v/v) of inoculum was sub-cultured into 10 ml of fresh MRS broth and incubated for another 24 h. After incubation, the culture was centrifuged at 4200 rpm for 20 m at 4 ℃, the pellet washed twice in sterile PBS buffer. The washed pellet was resuspended in 1 ml of PBS. For the strain, 100 µl of culture suspension was added separately into tubes containing 2 ml of PBS, and each tube was set with various pH values. The pH values tested were 1, 2, 3, 4. Hydrochloric acid (1 N) was used to adjust the pH of the PBS. These tubes are incubated for 0, 0.5, 1, 2, 3 h and each incubation time, 100 µl was cultured on MRS agar plates with serial dilution (10^{-1}-10^{-6}) and viable colony forming units (CFU) are counted.

2.1.2. Bile tolerance test

The tolerance of the isolated strain to bile salts was evaluated as described by Guo et al, with slight modifications (Guo et al., 2012). The culture was grown in MRS broth at 30 ℃ for 24 h, then 1% (v/v) of inoculum was added into 10 ml of fresh MRS broth and incubated for another 24 h. After incubation, the culture was centrifuged at 4200 rpm for 20 m at 4 ℃, the cells are washed twice in sterile PBS buffer. The pellet was resuspended in 1 ml of PBS. For the strain, 90 µl ($O.D_{600}$=0.5)

of culture suspension was added separately into tubes containing 5 ml of MRS with various bile salts (Sigma-Aldrich, St. Louis, MO, USA) concentration. The tubes of 5 ml MRS broth with bile salts are made with 0.1% (w/v), 0.3%, 0.5% and without bile salts tube is assessed as control. The samples are incubated 0, 1, 2, 3, 24 h, and each tube is collected every hour and estimated the survivability during incubation time.

The survival rate (%) of the strain treated with acid or bile salts was calculated by counting the surviving cells (N_1) on the MRS plates compared to the initial cell numbers (N_0) before incubation as follows:

$$\text{Survival rate (\%)} = \frac{\log N_1}{\log N_0} \times 100$$

2.2. Whole genome sequencing

2.2.1. DNA extraction

The genomic DNA of *Weissella* sp. SNUL2 was extracted by the Genomic DNA Purification Kit (iNtRON, Seongnam, Korea) according to the manufacturer instructions. To harvest 1-2 ml of the cell ($O.D_{600}$=0.8-1.0), the culture was centrifugated at 13,000 rpm for 1 m and the supernatant was discarded. After removing the supernatant, the cell was completely resuspended by vortexing or tapping. Then, 300 µl of G-buffer within 250 µl of RNase A and 40 µl of Proteinase K solution was added to the tubes containing the pellet with gentle mixing. After incubating the tubes at 65 ℃ for 15 m, 250 µl of binding buffer was added to increase gDNA binding onto column resins. Then, cell lysates were loaded on column and centrifuged at 13,000 rpm for 1 m again. To wash the sample, 500 µl of washing buffer with absolute EtOH was added then centrifuged and this process

was repeated twice. After all these preparations, the G-spin column was placed in a clean 1.5 ml microcentrifuge tube and elution buffer were added directly on to the membrane. The mixture was centrifuged at 13,000 rpm for 1 m to gain purified genomic DNA. The quantity and purity of DNA were determined using the NanoVue Plus Spectrophotometer (GE Health Care Co., Nordrhein-Westfalen, Germany) and sent Macrogen (Seoul, Korea) for sequencing.

2.2.2. Genomic sequencing and assembly

The sample was prepared according to a protocol for sequencing on the PacBio Sequel system. The DNA templates were sequenced using PacBio RS II sequencer (Pacific Biosciences, Menlo Park, USA). *De novo* assembly was performed using HGAP3 software. It was done by mapping single pass reads to seed reads, which represented the longest part of the read length distribution. Following that, the mapped read's consensus sequence was produced, resulting in long and highly accurate fragments of the target genome. After that, reads were corrected and filtered since some reads did not provide extra information for constructing the genome. Also, the reads which had too high or low overlaps been filtered. With the overlapping data, they contained information of each contig, so contigs were constructed with higher quality through the self-mapping step. After complete genome was assembled, the locations of genes were predicted. Prokka, which is a software tool to achieve a reliable annotation of genomic bacterial sequences, was used to predict the location and their functions were annotated.

2.3. Toxic metabolite

With the rise of the market, research on the possible benefits of probiotics on the treatment and prevention of illnesses has progressed steadily over the last two decades, and various *Bifidobacterium* and *Lactobacillus* species of probiotics have been produced and commercialized (Ku et al., 2016). Because they indicate long-term safety in fermented foods or dairy products, these probiotics are generally regarded as safe (GRAS). However, the UN food and Agriculture Organization (FAO) and the World Health Organization (WHO) believe that a minimal safety evaluation, which includes antibiotic resistance and particular metabolite synthesis such as D-lactate, toxin production and potential hemolysis, is necessary (FAO/WHO, 2002). As such, one of the conditions to determine whether it can be used in the human body as a probiotic includes whether a toxic substance is produced from the strain. We analyzed the genes in the complete genome information of *Weissella* sp. SNUL2 to confirm the presence of proteins involved in production. Whole genome information was obtained through the PacBio sequencer, the pathway was confirmed using KEGG, and the obtained FASTA file was analyzed using RAST and Genious 6.0.6.

2.4. Hemolysis

To determine whether *Weissella* sp. SNUL2 induce hemolysis, which induce the rupturing of red blood cells and the release of their contents (cytoplasm) into surrounding fluid (blood plasma), hemolysis activity test was conducted. *Weissella* sp. SNUL2 was cultivated for 24 h on 5% sheep blood agar (Bandio, Pocheon, Korea). *Streptococcus mutans*, α-hemolysis control, and *Streptoccus aureus*, β-hemolysis control, were cultivated using streaking with same conditions. After 24 h incubation, to read the hemolytic reaction on a blood agar plate, the plate was held up to a light source and observed with the light coming from behind and compared to two different hemolysis control.

2.5. EFSA MIC cut-off value of antibiotics

As mentioned, The MIC (Minimum Inhibition Concentration) is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation against indicator strains. Unlike the previous MIC test, this test was conducted to determine the minimum inhibition concentration required for antibiotics to inhibit *Weissella* sp. SNUL2. Three antibiotics were used: Ampicillin; Kanamycin; Streptomycin and MIC cut-off value (mg/L) of antibiotics were cited by EFSA (European Food Safety Authority). All these antibiotics were set at 0.2 mg/ml and two-fold dilution was repeated 10 times to dilute to 0.1% of the initial concentration. $O.D_{600}$ was measured for 24 h using a microplate, and 100 μl of medium + *Weissella* sp. SNUL2 and 100 μl of antibiotics (0.2 mg/ml) were mixed in each well. *Weissella* sp. SNUL2 were inoculated by 5×10^6 CFU/ml in each well.

3. Results

3.1. Cell viability test

3.1.1. Acid tolerance test

To determine the cell viability under acidic condition, acid tolerance was conducted. The number of viable bacteria was counted using CFU/ml and was calculated by multiplying the number of CFU by the dilution factor. As shown as Table. 9., the $\log_{10}$ is displayed for easier viewing. At pH 1, viable bacteria ($\log_{10}$CFUml^{-1}) recorded 7.42 and standard deviation was 0.31 in the initial phase. And after 30 m of incubation at the same pH, it was observed that all cells died. At pH 2, the number of viable cells was 8.60 and all cells died from 30 m after incubation. From pH 3, there was some change in cell viability. At 0 h, the number of viable cells was observed as 8.84 and even after 3 h of incubation, the number was still recorded as 8.81. This means that the strain can survive under the pH 3 condition for 3 h, and the survival rate was 99.67%. Similarly, it showed a high survival rate even at pH 4, which observed as 8.83 at 0 h and 8.77 after 3 h of incubation. In this case, the survival rate was 99.27%. According to the previous research, the results of cell viability under various pH conditions were obtained. Commercial probiotics such as *Lactobacillus acidophilus* (A), *Lactobacillus casei* Shirota strains (B), *Lactobacillus acidophilus* + *Streptococcus thermophilus* + *Bifidobacterium* (C), *Lactobacillus acidophilus* + *Lactobacillus casei* + *Bifidobacterium* (D) were assessed acid tolerance as same method in this research (Conway et al., 1987). Based on the results obtained, at pH 1.5, the highest viable cell count ($\log_{10}$CFU/ml) commercial probiotics was (B) which recorded 8.53 ± 0.64 (0 h) and 0 (3 h). The lowest commercial probiotics was (A) which recorded 0 (0 h) and 0 (3 h). At pH 3, the highest commercial probiotics was (B) which recorded 9.06 ± 0.06 (0 h) and 9.04 ± 0.06 (3 h), survival rate was 99.78%. The

lowest commercial probiotics was (D) which recorded 5.25 ± 0.02 (0 h) and 3.59 ± 1.12 (3 h), survival rate was 68.3% (Sahadeva et al., 2011).

3.1.2. Bile tolerance test

Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host. This would help probiotic strains to reach the small intestine and colon and contribute in balancing to intestinal microflora (Tambekar & Bhutada, 2010). To determine the cell viability under acidic condition, bile tolerance was conducted. The number of viable bacteria was on counted using CFU/ml and was calculated by multiplying the number of CFU by the dilution factor. As shown as Table. 10., the $\log_{10}$ is displayed for easier viewing. MRS broth without bile salt treatment used as a control, whereas 0.1%, 0.3%, 0.5% bile salt concentrations in MRS broth were used to check the tolerance of *Weissella* sp. SNUL2 by measuring cell viability. As given in Table. 10., Control showed the highest survival rate (111.25%). 0.1% sample showed 102.42% of the survival rate which is intermediate between 0 h and 24 h. 0.3% sample showed 100.14% of the survival rate which means not much gap in growth between 0 h and 24 h. In the contrast, 0.5% sample was observed slight decrease in growth between 0 h and 24 h, recorded 91.91% of the survival rate. However, it is still good tolerance to bile salts. According to the previous research, LGG observed 81% of survival rate at 0.3% bile condition and *Lactobacillus fermentum* recorded 91% of survival rate at same condition (Mandal et al., 2016). Based on the results, it can be said that *Weissella* sp. SNUL2 has equal or even better bile tolerance than other well-known probiotics or potential probiotic microbes.

Table 9. Survival rate of *Weissella* sp. SNUL2 at various pH values determined by counts of viable bacteria over 1 hour interval. Each value in the table represents the mean value ± Standard Deviation (SD). Each data point is the average of three repeated measurements from 3 independently replicated experiments, n=3.

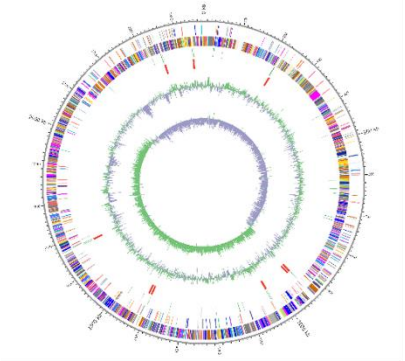
	Viable bacteria ($log_{10}CFUml^{-1}$)					**Survival rate (%)**		
						In this study	Conway et al., 1987	
pH	0 h	0.5 h	1 h	2 h	3 h	*Weissella* sp. SNUL2	Commercial probiotics A	Commercial probiotics B
1	7.42 ± 0.31	-	-	-	-			
2	8.60 ± 0.75	-	-	-	-			
3	8.84 ± 1.05	8.83 ± 1.10	8.83 ± 0.45	8.81 ± 0.63	8.81 ± 0.69	99.67	99.78	68.30
4	8.83 ± 1.05	8.81 ± 1.19	8.81 ± 0.32	8.78 ± 1.21	8.77 ± 0.69	99.27	-	-

Table 10. Survival rate of *Weissella* sp. SNUL2 at various bile salts concentration as determined by counts of viable bacteria. Each value in the table represents the mean value ± Standard Deviation (SD). Each data point is the average of three repeated measurements from 3 independently replicated experiments, n=3.

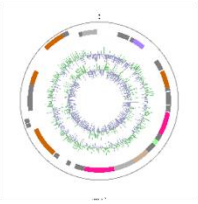
	Viable bacteria ($log_{10}CFUml^{-1}$)					Survival rate (%)		
						In this study	Mandal et al., 2016	
Bile salt	0 h	1 h	2 h	3 h	24 h	*Weissella* sp. SNUL2	*Lactobacillus rhamnosus* GG	*Lactobacillus fermentum*
w/o bile	8.39 ± 0.75	8.79 ± 0.00	9.07 ± 1.02	9.25 ± 1.28	9.34 ± 1.45	111.25	-	-
0.1%	8.43 ± 0.32	8.49 ± 0.80	8.39 ± 0.45	8.50 ± 0.84	8.64 ± 0.45	102.42	-	-
0.3%	8.26 ± 0.54	8.45 ± 0.15	8.49 ± 0.15	8.38 ± 0.45	8.27 ± 0.45	100.14	81.00	91.00
0.5%	8.46 ± 0.84	8.41 ± 0.75	8.33 ± 0.32	8.21 ± 0.54	7.77 ± 0.62	91.91	-	-

3.2. Genome sequencing and assembly

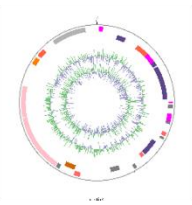
The whole genome sequence of *Weissella* sp. SNUL2 was generated using the PacBio RS II sequencer and *de novo* genome assembly was conducted with HGAP3 software. As shown in Figure 3.1., the complete genome of *Weissella* sp. SNUL2 is composed of one 2.475,587 bp complete circular chromosome and four circular plasmids (plasmid 1 [29,266 bp], plasmid 2 [23,800 bp], plasmid 3 [16,212 bp], plasmid 4 [16,212 bp]). GC contents were 45%, 38.8%, 39%, 38%, 38% respectively. The data was conducted with HGAP 3 software which made the linear DNA to circular one. Focusing on the chromosome in more detail, there are 2,291 of CDS, 86 of tRNA, 28 of rRNA. These data were annotated by Prokka, which is the pipeline that predict the location of protein expression genes, tRNA genes and rRNA genes and annotate its function. The extra information about whole genome information and annotation results are described in Chapter 3.



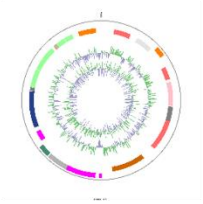
Chromosome (contig 1)



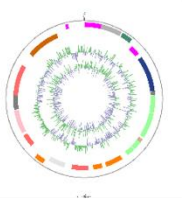
Plasmid 1



Plasmid 2



Plasmid 3



Plasmid 4

Contig Name	Bases(bp)	CDS	tRNA	rRNA	Alias
Contig 1	2,475,587	2,291	86	28	Chromosome
Contig 2	29,266	30	0	0	Plasmid
Contig 3	23,800	26	0	0	Plasmid
Contig 4	16,212	21	0	0	Plasmid
Contig 5	16,212	22	0	0	Plasmid
Total	2,561,077	2390	86	28	

Fig. 3.1. The circular genome graph of *Weissella* sp. SNUL2. As the result, the whole genome of *Weissella* sp. SNUL2 composed of five contigs include one chromosome and four plasmids. From the inner ring to the outer ring, the first circle describes the GC skew (G+C/G-C), which means the replication starts. The second circle depicts CG contents, and the third, fourth circle indicate rRNA, tRNA. The fifth and sixth circle indicate reverse coding sequence (CDS) and forward coding sequence respectively.

3.3. Toxic metabolite

The presence or absence of four toxic metabolites in *Weissella* sp. SNUL2 was compared with two strains known as probiotics. As a result, two genes related to D-lactate production were found in *Weissella* sp. SNUL2, two in *L. plantarum* WCFS1, and one in *L. rhamnosus* GG. D-lactate cannot be metabolized in the human body but when microorganisms convert L-lactate in the body to D-lactate using DL-lactate racemase, D-lactate accumulates and causes acidosis and headache, so it was selected as a toxic factor (Oh et al., 1985). Two biogenic amines, histamine causing abdominal pain and tyramine causing hypertension, were confirmed not to exist in all three strains (Bianchetti et al., 1982; Waldum & Sandvik, 1989). Proteins involved in the production of hemolysin that induce hemolysis (Muncie Jr & Campbell, 2009), which degrade red blood cells, were identified in *Weissella* sp. SNUL2 three, two in *L. plantarum* WCFS1 and one in *L. rhamnosus* GG. For the whole genome information of *Weissella* sp. SNUL2 and *L. plantarum* WCFS1, the FASTA file, the result of whole genome sequencing, was used and for the whole genome information of *L.rhamnosus* GG, T00987 from KEGG (assembly in GenBank: GCA_000026505.1) was referenced.

Table 11. The presence of toxic metabolites production related protein.

Toxic metabolite	Toxicity	*Weissella* sp. SNUL2			*Lactobacillus plantarum* WCFS1			*Lactobacillus rhamnosus* GG			Reference
		Number	Start	End	Number	Start	End	Number	Start	End	
D-lactate	Headache, hyperpnea	2	481,491 1,074,284	482,486 1,075,273	2	809,220 1,860,108	810,851 1,861,106	1	171,277	172,278	Oh et al., 1985
Histamine	Stimulates secretion of excess stomach acid	0	-	-	0	-	-	0	-	-	Waldum & Sandvik, 1989
Tyramine	Headache, high blood pressure	0	-	-	0	-	-	0	-	-	Bianchetti et al., 1982
Hemolysin	Hemolysis	3	310,383 953,932 955,391	311,036 955,302 956,755	2	2,372,626 2,925,988	2,373,993 2,926,626	1	395,755	396,189	Muncie Jr & Campbell, 2009

3.4. Hemolysis

As a result of the hemolysis activity test using blood agar, it was confirmed that no hemolysis was activated when the blood agar cultured with *Weissella* sp. SNUL2 was compared with the two controls. In addition, it was confirmed that the red color of the blood agar was maintained even after 48 h of incubation.

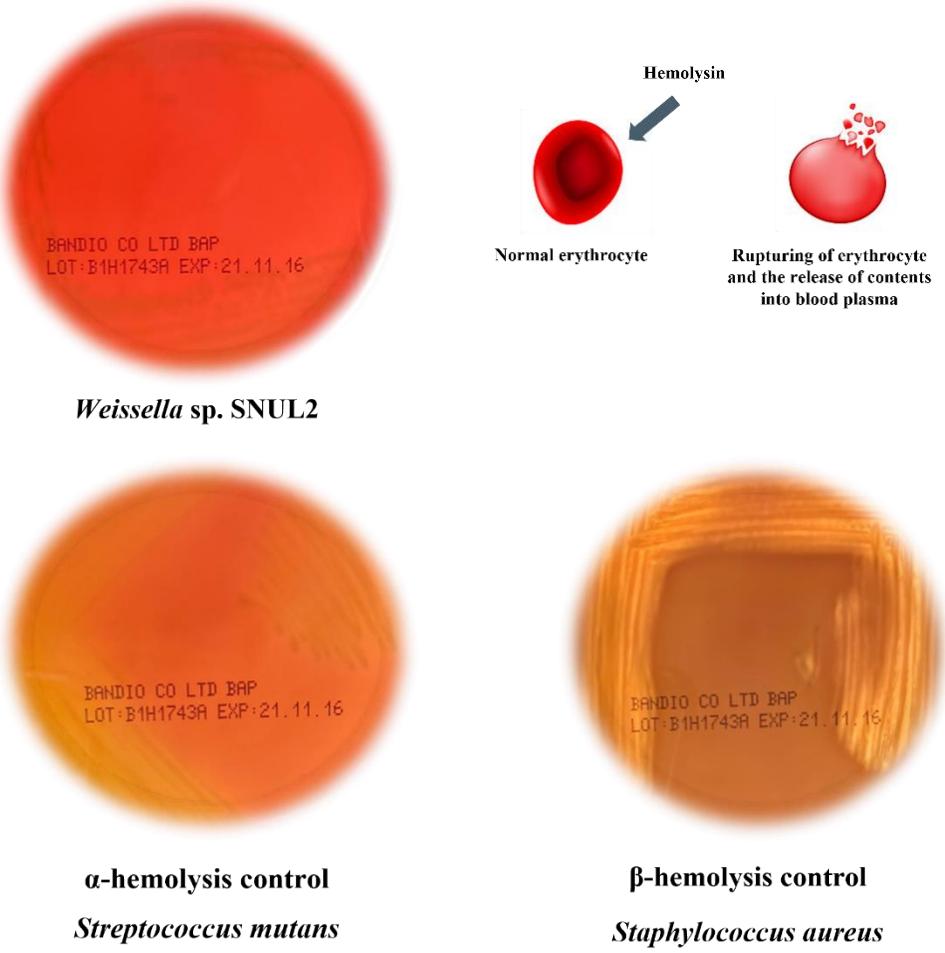


Fig. 3.2. Hemolysis activity test against *Weissella* sp. SNUL2, *Streptococcus mutans*, *Staphylococcus aureus*

3.5. EFSA MIC cut-off value of antibiotics

The EFSA MIC cut-off value of antibiotics test is an experiment to confirm antibiotic resistance. If it is higher than the standard, it is judged to have antibiotic resistance because it means that a larger amount of antibiotic is needed to suppress the corresponding bacteria. Conversely, if it is lower than the standard, it is judged that there is no antibiotic resistance, and thus the probiotic standard can be satisfied. The criteria for *Leuconostoc* spp. to which *Weissella* sp. SNUL2 belongs according to EFSA are listed in Table 12. According to the standard, it was confirmed that *Weissella* sp. SNUL2 had no resistance to ampicillin, and the MICs of kanamycin and streptomycin were 100.00 (mg/L) and 200.00 (mg/L), respectively, and resistance was confirmed. However, *Weissella* sp. JW15 and LGG (Jang et al., 2021) also showed no resistance to ampicillin, but showed resistance to kanamycin and streptomycin, which met the probiotic criteria. This is because it is estimated to be intrinsic antibiotic resistance, which is an inherent characteristic of bacteria, rather than acquired antibiotic resistance obtained from the outside. To confirm this, using a tool called Plasmid Finder 2.1, it was determined that there was no acquired antibiotic resistance-related genes for kanamycin and streptomycin shown in Table 13 (Aristimuno Ficoseco et al., 2018; Ouoba et al., 2008). Through this, it was confirmed that the resistance of *Weissella* sp. SNUL2 to the two antibiotics was an inherent characteristic of the strain.

Table 12. EFSA MIC cut-off value of antibiotics against three indicator strains.

Antibiotics	MIC cut-off value (mg/L) of antibiotics			
	EFSA, 2012	In this study	Jang et al., 2021	Jang et al., 2021
	***Leuconostoc* spp.**	***Weissella* sp. SNUL2**	***Weissella* sp. JW15**	***Lactobacillus rhamnosus* GG**
Ampicillin	2.00	0.78	0.10	0.29
Kanamycin	16.00	100.00	256.00	256.00
Streptomycin	64.00	200.00	56.00	96.00

Table 13. The presence of acquired antibiotic resistance-related genes in *Weissella* sp. SNUL2 through Plasmid Finder 2.1. The presence of related genes was interpreted as (+); the absence of related genes was interpreted as (-).

Antibiotics	Target Gene	Reference	*Weissella* sp. SNUL2	
			Chromosome	Plasmid
Kanamycin	*aph-III*	Ouoba et al., 2008	-	-
	ant-I	Ouoba et al., 2008	-	-
Streptomycin	*aadA*	Ouoba et al., 2008	-	-
	aadE	Aristimuno et al., 2018	-	-
	strB	Ouoba et al., 2008	-	-

4. Conclusion

Chapter 3 confirmed whether the *Weissella* strains met the probiotic conditions according to the FAO and WHO probiotic guidelines. In general, food passes through the strongly acidic stomach and moves into the intestine. The hydrochloric acid secreted from the stomach normally has a pH of 0.9, but when food is introduced, it rises from 2 to 3, and it takes about 2 to 4 h to pass through the stomach. During this time, an acid tolerate test was conducted to confirm that it survived under strong acidic conditions. As a result, *Weissella* sp. SNUL2 recorded a high survival rate of 99.67%, which is comparable to that of commercial probiotics. For a similar reason, a bile tolerance test was also performed, which showed a survival rate of 0.3% to 100.14%, which was higher than that of a commercial strain. *Weissella* sp. SNUL2 performed whole genome sequencing through PacBio sequencing and acquired genetic information for one chromosome and four plasmids. There were 2,291 CDSs on one chromosome, and as a result of comparing them with the toxic metabolite pathway, several related genes could be identified. In addition, as a result of the hemolytic activity, it was confirmed that hemolytic activity that destroys red blood cells did not occur, and although it has antibiotic resistance to streptomycin and kanamycin, using the tool called Plasmid Finder 2.1, it was confirmed as a characteristic of bacteria its selves, not resistance acquired from outside thus *Weissella* sp. SNUL2 met all probiotic trials.

5. Discussion

With the advent of antibiotic-resistant bacteria, mankind has become a serious problem in the treatment of infectious disease, which has raised the need for antibiotic substitutes. The purpose of this study is to propose an antibiotic substance produced by *Weissella* sp. SNUL2, a type of LAB, as an alternative of complement to antibiotics. Among the many antibiotic candidates, in this experiment, we focused on probiotics. Most of the antibacterial properties of probiotics occur through antibacterial protein. However, the existing antibacterial protein was produced only in a small amount, making it difficult to scale up and having a narrow range of antibacterial properties was a limitation. To compensate for this limitation, the goal was to use the bacteria itself, and genetic information was used to find a strain that satisfies the following criteria from Korean traditional fermented food. The criteria that aim for are as follows: Broad spectrum antibacterial properties; Biodegradable; Probiotics Guidelines. A strain that satisfies this standard was isolated from 'salted squid', Korean traditional fermented food, and named *Weissella* sp. SNUL2. As a result of purifying and identifying the antibiotic substance produced by *Weissella* sp. SNUL2, two proteins were found. As a result of experiments according to various criteria, *Weissella* sp. SNUL2 showed generally excellent results, and showed similar or better results when compared to commercial strains. As it is a strain with broad range of antibacterial properties, it is thought that it can be used as probiotics for treatment purposes in the future. In addition, it is expected to be used as a substitute for antibiotics, thereby avoiding the risk of antibiotic resistance.

References

Ancillotti, M., Nihlen Fahlquist, J., & Eriksson, S. (2021). Individual moral responsibility for antibiotic resistance. *Bioethics*.

Annual report of the chief medical officer 2011 volume 2, London: UK department of health (2013)

Aran, H., Biscola, V., El-Ghaish, S., Jaffres, E., Dousset, X., Pillot, G., Haertlé, T., Chobert, J.-M., & Hwanhlem, N. (2015). Bacteriocin-producing *Enterococcus faecalis* KT2W2G isolated from mangrove forests in southern Thailand: purification, characterization and safety evaluation. *Food Control*, *54*, 126-134.

Aristimuno Ficoseco, C., Mansilla, F. I., Maldonado, N. C., Miranda, H., Fatima Nader-Macias, M. E., & Vignolo, G. M. (2018). Safety and growth optimization of lactic acid bacteria isolated from feedlot cattle for probiotic formula design. *Front Microbiol*, *9*, 2220.

Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., Nisar, M. A., Alvi, R. F., Aslam, M. A., Qamar, M. U., Salamat, M. K. F., & Baloch, Z. (2018). Antibiotic resistance: a rundown of a global crisis. *Infect Drug Resist*, *11*, 1645-1658.

Baumann, G., & Mueller, P. (1974). A molecular model of membrane excitability. *J Supramol Struct*, *2*(5-6), 538-557.

Bianchetti, M., Minder, I., Beretta-Piccoli, C., Meier, A., & Weidmann, P. (1982). Effects of tyramine on blood pressure and plasma catecholamines in normal and hypertensive subjects. *Klinische Wochenschrift*, *60*(9), 465-470.

Biswas, S. R., Ray, P., Johnson, M. C., & Ray, B. (1991). Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. *Appl Environ Microbiol*, *57*(4), 1265-1267.

Burrowes, B., Harper, D. R., Anderson, J., McConville, M., & Enright, M. C. (2011). Bacteriophage therapy: potential uses in the control of antibiotic-resistant

pathogens. *Expert Rev Anti Infect Ther*, *9*(9), 775-785.

Christensen, D. P., & Hutkins, R. W. (1992). Collapse of the proton motive force in *Listeria monocytogenes* caused by a bacteriocin produced by *Pediococcus acidilactici*. *Appl Environ Microbiol*, *58*(10), 3312-3315.

Collins, M. D., Samelis, J., Metaxopoulos, J., & Wallbanks, S. (1993). Taxonomic studies on some leuconostoc-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. *J Appl Bacteriol*, *75*(6), 595-603.

Conway, P. L., Gorbach, S. L., & Goldin, B. R. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J Dairy Sci*, *70*(1), 1-12.

Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nat Rev Microbiol*, *3*(10), 777-788.

Ehrenstein, G., & Lecar, H. (1977). Electrically gated ionic channels in lipid bilayers. *Q Rev Biophys*, *10*(1), 1-34.

European Centre for Disease, P., Control, European Food Safety, A., & European Medicines, A. (2017). ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals: Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) Report. *EFSA J*, *15*(7), e04872.

FAO/WHO. (2002). Food and Agriculture Organization–World Health Organization. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food.

Ghosh, C., Sarkar, P., Issa, R., & Haldar, J. (2019). Alternatives to conventional antibiotics in the era of antimicrobial resistance. *Trends Microbiol*, *27*(4), 323-338.

Guo, C. F., Zhang, L. W., Han, X., Yi, H. X., Li, J. Y., Tuo, Y. F., Zhang, Y. C., Du, M., Shan, Y. J., & Yang, L. (2012). Screening for cholesterol-lowering probiotic based on deoxycholic acid removal pathway and studying its functional mechanisms in vitro. *Anaerobe*, *18*(5), 516-522.

Hechard, Y., & Sahl, H. G. (2002). Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie*, *84*(5-6), 545-557.

Inoue, H. (2019). Strategic approach for combating antimicrobial resistance (AMR). *Glob Health Med*, *1*(2), 61-64.

Jang, Y. J., Gwon, H. M., Jeong, W. S., Yeo, S. H., & Kim, S. Y. (2021). Safety evaluation of *Weissella cibaria* JW15 by phenotypic and genotypic property analysis. *Microorganisms*, *9*(12).

Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., Goossens, H., & Laxminarayan, R. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc Natl Acad Sci U S A*, *115*(15), E3463-E3470.

Ku, S., Park, M. S., Ji, G. E., & You, H. J. (2016). Review on *Bifidobacterium bifidum* BGN4: functionality and nutraceutical applications as a probiotic microorganism. *International journal of molecular sciences*, *17*(9), 1544.

Kumariya, R., Garsa, A. K., Rajput, Y. S., Sood, S. K., Akhtar, N., & Patel, S. (2019). Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. *Microb Pathog*, *128*, 171-177.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680-685.

Li, Y., Xiang, Q., Zhang, Q., Huang, Y., & Su, Z. (2012). Overview on the recent study of antimicrobial peptides: origins, functions, relative mechanisms and application. *Peptides*, *37*(2), 207-215.

Ludtke, S. J., He, K., Heller, W. T., Harroun, T. A., Yang, L., & Huang, H. W. (1996). Membrane pores induced by magainin. *Biochemistry*, *35*(43), 13723-13728.

Mandal, H., Jariwala, R., & Bagchi, T. (2016). Isolation and characterization of lactobacilli from human faeces and indigenous fermented foods for their potential application as probiotics. *Canadian journal of microbiology*, *62*(4), 349-359.

Minahk, C. J., Farias, M. E., Sesma, F., & Morero, R. D. (2000). Effect of enterocin CRL35 on *Listeria monocytogenes* cell membrane. *FEMS Microbiol Lett*, *192*(1), 79-83.

Morton, J. T., Freed, S. D., Lee, S. W., & Friedberg, I. (2015). A large scale prediction of bacteriocin gene blocks suggests a wide functional spectrum for bacteriocins. *BMC Bioinformatics*, *16*, 381.

Muncie Jr, H. L., & Campbell, J. S. (2009). Alpha and beta thalassemia. *American family physician*, *80*(4), 339-344.

Nissen-Meyer, J., Rogne, P., Oppegard, C., Haugen, H. S., & Kristiansen, P. E. (2009). Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by gram-positive bacteria. *Curr Pharm Biotechnol*, *10*(1), 19-37.

Oh, M. S., Uribarri, J., Alveranga, D., Lazar, I., Bazilinski, N., & Carroll, H. J. (1985). Metabolic utilization and renal handling of D-lactate in men. *Metabolism*, *34*(7), 621-625.

Ouoba, L. I., Lei, V., & Jensen, L. B. (2008). Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. *Int J Food Microbiol*, *121*(2), 217-224.

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Pouny, Y., Rapaport, D., Mor, A., Nicolas, P., & Shai, Y. (1992). Interaction of antimicrobial dermaseptin and its fluorescently labeled analogues with phospholipid membranes. *Biochemistry*, *31*(49), 12416-12423.

Rashid, R., Veleba, M., & Kline, K. A. (2016). Focal targeting of the bacterial envelope by antimicrobial peptides. *Front Cell Dev Biol*, *4*, 55.

Sahadeva, R., Leong, S., Chua, K., Tan, C., Chan, H., Tong, E., Wong, S., & Chan, H. (2011). Survival of commercial probiotic strains to pH and bile. *International Food Research Journal*, *18*(4).

Savoia, D. (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. *Future Microbiol*, *7*(8), 979-990.

Shahnawaz, M., & Soto, C. (2012). Microcin amyloid fibrils A are reservoir of toxic oligomeric species. *J Biol Chem*, *287*(15), 11665-11676.

Tambekar, D., & Bhutada, S. (2010). Studies on antimicrobial activity and characteristics of bacteriocins produced by *Lactobacillus* strains isolated from

milk of domestic animals. *The Internet Journal of Microbiology*, *8*(2), 1-6.

Uzelac, G., Miljkovic, M., Lozo, J., Radulovic, Z., Tosic, N., & Kojic, M. (2015). Expression of bacteriocin LsbB is dependent on a transcription terminator. *Microbiol Res*, *179*, 45-53.

Waldum, H. L., & Sandvik, A. K. (1989). Histamine and the stomach. *Scand J Gastroenterol*, *24*(2), 130-139.

Abstract in Korean

1928년 Fleming의 penicillin 발견 이후, 인류는 세균 감염으로부터 보호 받을 수 있게 되었다. 이 발견 이후, 항생제는 비약적인 발전을 이루었고 오늘날에는 없어서는 안 될 필수적인 요소가 되었다. 그러나, 1940년 penicillin의 도입 이후, penicillin 내성균이 발견되기까지 1년밖에 걸리지 않았다. 항생제가 임상에 적용된 80여년 동안, 전 세계적으로 발생하는 항생제 내성은 다양한 세균 감염 치료에 큰 제약인 동시에, 위협이 되었다. 항생제 내성균이 발생하는 주 원인은 항생제의 오남용인데, 항생제 내성은 말 그대로 항생제에 내성을 가진다는 의미이며 임상에서 세균을 사멸시키는 것을 불가능하게 한다는 의미를 가지기도 한다. 때문에 오늘날 항생제 내성을 줄이기 위해 항생제 대체제의 필요성이 끊임없이 대두되고 있고 실제로 많은 연구진들은 항생제를 대체할 만한 물질을 찾기 위해 다양한 연구를 진행하고 있고 후보군중 하나는 probiotics이다.

하지만 현존하는 probiotics는 좁은 범위의 항균성을 갖고 있는 한계점이 있으며 이 때문에 세균 감염 치료목적의 약 보다는 건강 기능 식품으로 사용되는 경우가 많다. 때문에 치료 목적의 probiotics로 개발하기 위해서 넓은 범위의 항균성을 갖는 것이 필수적인 동시에, 생분해성, probiotics guidelines 등의 기준들을 충족해야 한다. 본 논문에서는 이 기준들을 충족하는 균주를 찾아 항생제 대체재 후보로서 제안한다.

챕터 2에서는, 항생제 대체재 후보군으로 사용하고자 하는 균주를 분리하고, 항균물질의 특성을 확인했다. *Weissella* sp. SNUL2라는 strain을 한국 전통 식품에서 분리했고 16s rRNA sequencing을 통해 동정하였다. 이 균주의 최적온도와 pH를 확인하고, $O.D_{600}$과 CFU의 상관관계를 확인해서 이후 실험에 적용했다. 균주를 치료 목적으로 사용하기 위해 가장 중요한

실험인 항균성 실험을 총 70여가지 세균에 대해 진행했으며 그 결과 *Weissella* sp. SNUL2는 기존의 probiotics 보다 넓은 범위의 항균성을 갖는 것을 확인할 수 있었다. 균주의 배양과정에서, 상등액에 분비되는 항균물질의 특성을 확인하기 위해 효소 실험을 진행했고 항균성 단백질이라는 결과를 얻었다. 항균성 단백질이 어떤 물질인지 추가 정보를 얻기 위해 부분 정제를 진행했고 LC-MS/MS 결과 bacteriocin이라는 항균성 단백질을 생산하는 단백질인 C39 family protein과 세포벽 성분인 peptidoglycan을 분해하는 효소인 peptidoglycan endopeptidase, 두 가지 단백질이 동정되었다.

챕터 3에서는, 한국 전통 식품에서 분리한 *Weissella* sp. SNUL2라는 균주가 FAO와 WHO의 probiotics guideline을 충족시키는지 확인하기 위해 이 가이드라인에 따라 실험을 진행했다. 먼저 PacBio sequencing을 통한 *Weissella* sp. SNUL2 전장유전체 정보를 얻었고 그 결과 1개의 염색체와 4개의 plasmid로 구성된 것을 확인했다. 총 2,291개의 CDS가 염색체에 존재한다는 것을 확인했고 이 정보와 toxic metabolite pathway에 기반하여 체내에 악영향을 줄 수 있는 toxic metabolite를 생산하는지 비교, 분석하였다. 앞서 말한 probiotics guideline에 따라 내산성, 내담즙성과 적혈구를 파괴하는 용혈활성 여부 마지막으로 항생제 내성 여부를 확인한 결과 내산성, 내담즙성은 상용화된 probiotics에 상응하는 결과를 보여주었고, 용혈활성은 나타나지 않았다. 항생제 내성은 ampicillin에 대해선 없는 것으로 확인되었지만 streptomycin과 kanamycin에 대해선 내성이 있는 것으로 나왔다. 하지만 이 내성도 Plasmid Finder 2.1이라는 tool을 이용해 외부로부터 얻은 획득성 내성인지, 균주 고유의 특성인 내재성 내성인지 확인 결과 내재성이라는 결과를 얻었다. 한국 전통 식품에서 분리한 *Weissella* sp. SNUL2 균주가 넓은 범위의 항균성을 갖고 probiotics 기준을 충족하는 결과를 보여준 만큼 미래에 항생제 대체재로서 사용되어 항생제 내성의 위험으로 부터 벗어날 수 있기를 기대한다.

Acknowledgements

학부를 졸업하고, 차세대 pharmabiotics의 리더가 되고 싶다는 큰 꿈을 갖고 서울대학교 국제농업기술대학원 식품미생물소재 연구실에 입학한 지 어느새 2년이라는 시간이 지났습니다. 짧다면 짧고 길다면 긴 시간이지만 좋은 연구 환경에서 연구할 수 있음에 감사하고, 제 인생에서 정말 소중한 시간이었습니다.

돌이켜보면, 저는 인복이 많은 사람이었습니다. 학위기간동안 정말 많은 분들이 응원해주시고, 도움을 받았습니다. 이 자리를 빌려 모든 분들께 깊은 감사의 인사를 드리고자 합니다.

먼저 언제나 연구에 대해 아낌없는 지도와 따뜻한 조언 해주시는 지도교수님이신 김효진 교수님께 깊은 감사의 뜻을 전합니다. 부족한 제가 무탈하게 졸업을 하게 된 것은 교수님의 열정적인 지도와 가르침 덕분이라고 생각합니다. 실수를 하거나, 결과가 잘 나오지 않아도 교수님께서 주신 믿음과 따뜻한 격려는 교수님의 믿음에 보답하고자 제 마음을 다잡고, 더욱 연구에 매진할 수 있게끔 해주는 원동력이었습니다. 부족한 저를 묵묵히 잘 이끌어 주셔서 정말 감사드립니다. 교수님께 지도받은 2년이라는 시간을 자양분으로, 교수님의 꿈처럼 인류에 이바지하는 훌륭한 연구자가 되기 위해 앞으로도 정진하겠습니다.

또한 논문 심사를 맡아주신 김도만 교수님과 정동화 교수님께도 깊은 감사의 말씀드립니다. 두 분의 강의를 들으며 부족한 저를 채울 수 있었습니다. 교수님들께서 주신 날카로운 충고와 따뜻한 조언들 덕분에 제 연구를 보완하고, 완성도를 높일 수 있었습니다. 감사드립니다.

연구실 친구들에게도 감사한 점이 참 많습니다. 가장 먼저 제 마음 속 영원한 랩장인 준상이형! 아무것도 모르던 저에게 실험적으로나, 생활적

으로 많은 것을 알려주고 고민을 털어 놓을 때면 항상 따뜻하게 조언해줘서 감사합니다. 형이 잘 되어서 정말 좋고, 졸업하면 밥 한 끼 하러 세종으로 가겠습니다.

혜리한테도 정말 고마운 점이 많습니다. 처음 입학해서 도움을 정말 많이 받았습니다. 옆자리에 앉아 이야기도 정말 많이 하고 궁금한 거 물어보곤 했었는데, 그럴때마다 정말 잘 받아주고 친절히 알려줘서 감사하다는 말 전하고 싶습니다. 본인 일이 아니어도 본인 일처럼 축하해주고, 위로해주는 모습을 보며 동생이지만 많이 배웠습니다. 이외에도 감사한 일이 정말 많지만, 따로 전하도록 하겠습니다. 졸업하고 취업하여 맡은 일 잘 해내는 것 같아 보기 좋습니다.

숙이도 2년이라는 시간을 함께해서 정이 정말 많이 들었습니다. 항상 남을 먼저 챙겨주려고 하고, 공감해주는 모습을 보며 마찬가지로 동생이지만 많이 배웠습니다. 저랑 학교에 온 시기가 비슷해 먼저 떠나는 게 마음이 안 좋기도 하지만 지금까지 보여준 실험에 대해 열중하는 모습과 본인 실험에 대한 자신감이 있는 만큼 좋은 연구 결과로 졸업할 수 있을 거라 믿어 의심치 않습니다.

정빈이는 숙이 다음으로 입학해서 저에겐 두 번째 후배지만, 숙이가 동기로 느껴져서 첫 번째 후배 같은 마음입니다. 제 성격상 형이나 선배들을 대하기 더 편해하고, 동생이나 후배에게 어떻게 대해줘야 할 지 잘 몰랐는데, 다행히 첫 후배가 너무나 반듯하고 성격 좋은 친구라 감사했다는 말을 이 자리를 빌려 전합니다. 하던 대로 하면 좋은 결과가 있을 거라고 말해주고 싶습니다.

단열이도 힘든 시기를 보내고 우리 실험실에 들어와 잘 적응한 모습이 보기 좋습니다. 막내 동생 같은 느낌이지만 실험할 때는 누구보다 진지하기에 마찬가지로 좋은 결과 있었으면 좋겠고 미래에 박사과정까지 잘 마치길 바랍니다.

혜준씨도 함께한 시간이 길지는 않지만, 큰 결심을 하고 오신 만큼 잘 해내시리라 생각되고, 좋은 결과 있으셨으면 좋겠습니다.

마지막으로 희대형! 제 몇 안 되는 동기로, 입학한 지 얼마 안 되었을 때 서로 얘기도 정말 많이 하고 통하는 점도 많아 붙어 다녔는데 어느새 2년이 지나 제가 졸업하게 됐습니다. 형이 나갈 때 나눴던 대화들이 가끔 생각나곤 하는데 형도 꿈을 좇아간 만큼 좋은 결과 있었으면 좋겠습니다.

우리 연구실과 다름없었던 혜리, 지혜누나, 다영누나, 민정이, 승연씨, 성암이, 현민이, 대범이, 수연씨에게도 감사드립니다. 항상 저희 챙겨주시고, 따뜻하게 대해주셔서 감사합니다. 소중한 인연인 만큼 사회에 나가서도 연락 자주 하겠습니다. 다들 열심히 하는 사람들이란 걸 너무도 잘 알기에 목표하신 바 잘 이뤄낼 것이라고 생각합니다.

전공은 다르지만 박사과정으로서 아낌없는 조언해준 원준이형 에게도 감사 인사 전하고 싶습니다. 나가서도 연락 자주 할게요.

마지막으로, 저를 지금까지 한결 같은 사랑으로 키워 주신 부모님께 감사드립니다. 언제나 아들의 꿈을 지지해주시는 아버지, 티는 안내시지만 밤낮으로 제 걱정 해주시는 어머니. 부모님의 아들로 태어나 모든 순간이 행복했습니다. 언제나 변함없는 그 마음에 감사드리며 존경의 마음을 전합니다. 우애 좋은 제 동생들 선우, 연우에게도 감사의 인사 전하며 우리 가족 앞으로도 행복하고 좋은 일만 가득했으면 좋겠습니다. 사랑합니다.

2022년 2월

한재원 올림