PRODUCTION AND PURIFICATION OF ENDOLYSINS FROM *SALMONELLA* BACTERIOPHAGES

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ABSTRACT

PRODUCTION AND PURIFICATION OF ENDOLYSINS FROM *SALMONELLA* BACTERIOPHAGES

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Salmonella is one of the major bacteria causing foodborne diseases in the world. *Salmonella* is Gram-negative, non-spore forming and rod shape bacteria, and those with enteric fever and non-typhoidal salmonellosis cause gastroenteritis problems. Antibiotic resistance is a problem that threatens the whole world in recent years, and bacteriophages come first as a novel method. Bacteriophages are the viruses that infect bacterial cells. Bacteriophages are used against antibiotic resistance, but their use is limited because they are not reproducible. Using their burst mechanisms, endolysins can be used instead of bacteriophages. Endolysins, bacteriophage lytic enzymes, are enzymes produced during growth of bacteriophage in the cell. Endolysins cause cell death by disturbing cell wall and cell membrane of the bacteria and to release new forming viruses. Endolysins may be suitable for food applications because they are more sustainable and more stable to changes in pH and temperature.

In this study, bacteriophage (MET P1- 137), isolated from our previous study, was used which can infect multi-drug resistant *Salmonella* Kentucky (MET S1-007). By analyzing genome of the phage (MET P1- 137), regions producing endolysins were

selected. By using a plasmid vector, the encoding gene of endolysin was transferred to *E. coli* BL21 strain. After microbial growth, purification of endolysin was conducted and lytic activity of the endolysin on *Salmonella* Kentucky was investigated. As a result, it was observed that presence of purified endolysin reduced *Salmonella* Kentucky cells by 1 log CFU / mL. Future research will examine external parameters such ideal pH and temperature to increase concentration of endolysin, interactions with metal ions, and the permeability effects of organic acids on the outer membrane. Food applications will also be tested.

Keywords: *Salmonella*, bacteriophage, endolysin, recombinant DNA technology

ÖZ

SALMONELLA BAKTERİYOFAJLARINDAN ENDOLİZİN ELDESİ VE SAFLAŞTIRILMASI

Yetişkin, Segâh
Yüksek Lisans, Gıda Mühendisliği
Tez Yöneticisi: Prof. Dr. Yeşim Soyer Küçükşenel

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Salmonella, dünyada gıda kaynaklı hastalıklara neden olan başlıca bakterilerden biridir. *Salmonella,* Gram-negatif, spor yapmayan ve çubuk şeklinde bir bakteridir ve enterik ateş ve tifoidal olmayan salmonelloz olanlar gastroenterit problemlerine neden olur. Antibiyotik direnci, son yıllarda tüm dünyayı tehdit eden bir sorundur ve yeni bir yöntem olarak bakteriyofajlar ön plana çıkmaktadır. Bakteriyofajlar, bakteri hücrelerini enfekte eden virüslerdir. Bakteriyofajlar antibiyotik dirençliliğine karşı kullanılır, ancak tekrarlanabilir olmadıkları için kullanımları sınırlıdır. Bakteriyofajların patlatma mekanizmaları kullanılarak, bakteriyofajlar yerine endolizinler kullanılabilir. Endolizinler, bakteriyofaj litik enzimler, bakteriyofajın hücre içinde büyümesi sırasında üretilen enzimlerdir. Endolizinler bakterilerin hücre duvarını ve hücre zarını bozarak hücre ölümüne ve yeni oluşan virüslerin salınmasına neden olur. Endolizinler, daha sürdürülebilir ve pH ve sıcaklık değişikliklerine karşı daha kararlı olduklarından gıda uygulamaları için uygun olabilir.

Bu çalışmada, çoklu ilaca dirençli *Salmonella* Kentucky'yi (MET S1-007) enfekte edebilen, önceki çalışmamızdan izole edilen bakteriyofaj (MET P1-137) kullanıldı.

Bakteriyofajın (MET P1- 137) genomu analiz edilerek endolizin üreten bölgeler seçildi. Plazmit vektörü kullanılarak, endolizinin kodlayıcı geni, *E. coli* BL21 suşuna aktarıldı. Mikrobiyal büyümeden sonra, endolizinin saflaştırılması gerçekleştirildi ve endolizinin *Salmonella* Kentucky hücrelerini 1 log CFU/mL azalttığı görüldü. Gelecekteki araştırmalar, endolizinin metal iyonlarıyla etkileşimlerini, organik asitlerin dış zar üzerindeki geçirgenlik etkilerini ve endolizin konsantrasyonunu arttırmak için pH ve sıcaklık gibi dış parametreleri inceleyecektir. Gıda uygulamaları da test edilecektir.

Anahtar Kelimeler: *Salmonella*, bakteriyofaj, endolizin, recombinant DNA teknolojisi

To My Lovely Brother and Parents

and

My Roommate Tırtıl

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PFU	Plaque Forming Unit
CFU	Colony Forming Unit
GRAS	Generally Recognized as Safe
PCR	Polymerase Chain Reaction
WGS	Whole Genome Sequencing
Rpm	Revolutions per Minute
OD	Optical Density
NCBI	National Center of Biotechnology Information
BLAST	Basic Local Alignment Search Tool
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Foodborne pathogens have been one of the most important factors threatening human health for centuries. Bacteria such as *Salmonella*, *Shigella*, *Escherichia coli*, *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus*, viruses such as Hepatitis A and Norovirus, parasites such as *Toxoplasma* and *Cryptosporidium* are the leading foodborne pathogens (Bintsis, 2017). Foodborne diseases can occur when the pathogen is consumed with food or water and increases its number in the host, i.e., infection, or by consuming food containing toxin produced by the pathogen, i.e., intoxication (Bintsis, 2017). Compared to intoxication, symptoms of infection appear a little later. Common foodborne disease symptoms include diarrhea (which may be bloody depending on situation), constipation, abdominal pain, weakness, fever in case of severe infection, headache, and vomiting. In more serious cases, conditions such as kidney failure, respiratory failure, cognitive impairment can be observed (Hoffmann & Ahn, 2021).

According to the estimates of the World Health Organization (WHO), one out of every 10 people in the world gets sick after consuming contaminated food every year. More than 420,000 of these diseases can lead to death (WHO, n.d.). These are 125,000 deaths each year in children under the age of 5 and are usually caused by diarrhea. Foodborne diseases, which have become an increasing problem not only in low- and middle-income countries but developed countries, cause 600 million cases per year (WHO, n.d.). International trade, farm-to-fork food chains and climate change are the factors affecting the food contamination risk.

Salmonella is among the most common foodborne pathogens in the worldwide. *Salmonella*, which is Gram-negative, facultative anaerobe, rod shaped, has a motile

structure thanks to its flagella (Fàbrega & Vila, 2013). Optimum growing conditions are environments with 35-37 ℃, pH value range of 4-9 and water activity (a_w) of 0.94-0.99 (Graziani et al., 2017).

Salmonella has two species, *bongori* and *enterica*, where *enterica* is the type infecting human and other warm-blooded animals. Most of the approximately 2500 serotypes belong to *Salmonella enterica* subsp. *enterica* (Brenner et al., 2000). In addition, *Salmonella* serotypes are divided into two as typhoid and non-typhoid. *Salmonella* Typhi and *Salmonella* Paratyphi are examples of typhoidal *Salmonella* (TS) that cause severe infection, called septicemia, typhoid, and paratyphoid fever. In various cases, it can damage internal organs and be life-threatening (Ray & Ryan, 2014).

Although their distribution in the world differs from country to country, the most common non-typhoidal *Salmonella* (NTS) serotypes are *S*. Enteritidis, *S*. Typhimurium, *S*. Infantis, *S*. Kentucky, and *S*. Anatum (Cheng et al., 2019). Contamination of animal-derived food products such as chicken, pig, cattle, milk, egg, meat, contact with unhygienic personnel, unhygienic environment cause NTS contamination (Atterbury et al., 2020). Depending on the situation, the patient may recover without antibiotics or hospitalization, but it can cause major problems in children, the elderly, pregnant women, and people with low immune systems (WHO, 2018).

According to World Health Organization, *Salmonella* causes 94 million gastroenteritis, of which 155,000 deaths (Denyes et al., 2017). 1.14 % of foodborne infections are caused by NTS. While European Food Safety Authorization (EFSA) declared the number of *Salmonella*-related cases in Europe as 91,000 each year, Centers for Control Disease and Prevent (CDC) stated this number in America as 1.35 million, annually (CDC, 2022; EFSA, n.d.).

World Health Organization (WHO) states that the increase in resistance of *Salmonella* to antibiotics poses a threat (Alenazy, 2022). Fluoroquinones, which have been used against salmonellosis since the 1980s, are no longer useful as

Salmonella serotypes show resistance by chromosomal mutation (Klemm et al., 2018). In addition, studies show that about 100,000 of *Salmonella*-related diseases are caused by *Salmonella* being resistant to antibiotics (VT Nair et al., 2018). *Salmonella* Kentucky is one of the most problematic serotypes with antimicrobial resistance. *S.* Kentucky, especially seen in poultry, eggs, and broilers, is resistant to most antibiotic types such as β-lactams, aminoglycosides, tetracyclines and sulphonamides (Hawkey et al., 2019). Therefore, a method that can be used instead of antibiotics has become a necessity.

Antibiotics have been used for years to treat foodborne illnesses. Antibiotics, which have a very important place in the treatment of infectious diseases and the reduction of deaths since their discovery in 1928, have become a threat to human health in recent years due to their misuse and overuse (Laure & Ahn, 2022). The resistance of microorganisms to antibiotics makes it very difficult or impossible to treat the diseases caused by these microorganisms. Antibiotics can inhibit cell wall synthesis, damage the cytoplasmic membrane, cause inhibition of nucleic acid and protein synthesis, or specifically damage enzyme systems (Kohanski et al., 2010).

The resistance mechanism of bacteria to antibiotics differs due to the different cell wall structure of Gram (+) and Gram (-) bacteria. Gr (+) bacteria do not have a lipopolysaccharide (LPS) outer membrane that acts as a barrier outside their cell walls (Chancey et al., 2012). This structure is found in Gr (-) and resistance mechanisms such as restriction of drug intake, increased / decreased binding ability of penicillin-binding proteins, drug degradation and drug inactivation are observed (Reygaert, 2018a).

Studies show that antibiotic resistance, which is a global threat, is the cause of death of 4.95 million people, with bacterial antibiotic resistance playing a role (Murray et al., 2022). In fact, 1.27 million deaths were a direct result of AMR in worldwide. It is estimated that antibiotic resistance, which has reached alarming situation not only in terms of health but also in economic terms, will cause an economic loss of up to 100 trillion dollars by 2050 (Chokshi et al., 2019). Awareness of antibiotic resistance

has begun to increase, and new methods are sought for the prevention and treatment of diseases by foodborne pathogens.

Bacteriophages or phages, one of the most promising solutions to antibiotic resistance, are the most abundant entities in the world (Chibani-Chennoufi et al., 2004). Bacteriophages are bacterial specific and do not affect human and animal natural microflora and can be found in open oceans, soil, ocean sediments, in short, wherever there are host cells such as bacteria and archaea to grow (Merril et al., 2003). The characterization studies show that most bacteriophages contain double-strand DNA (dsDNA) and most of them have tails (Clokie & Kropinski, 2009). Structurally, they consist of protein capsid (head) and tail. While protein capsids protect their genetic material, their tails specifically recognize the host cell and integrate into the cell wall or cell membrane, allowing new bacteriophages to form (Ackermann & Prangishvili, 2012; Nobrega et al., 2018). The fibers in the tail cause them to have a narrower host range than antibiotics, although it is desirable to be host-specific (Kingwell, 2015).

The most common classification of bacteriophages is based on their life cycle and is divided into lytic (virulent) and lysogenic (temperate). In the lytic cycle, bacteriophages attach to the host and transfers its own DNA with bacterial DNA. When new bacteriophages are formed in the medium, by the help of host cell' enzymes, lysis of the host cell takes place. Unlike the lytic cycle, in the lysogenic cycle, the bacteriophage can remain in the host without lysis (Motlagh et al., 2015). When environmental conditions deteriorate, the lysogenic cycle can evolve into the lytic cycle (Carvalho et al., 2017).

Recently, in the food industry or other fields use lytic bacteriophages. In addition, even though they are promising, it is known that bacteriophages following the lysogenic life cycle increase resistance mechanism in bacteria by horizontal gene transfer (Chibani-Chennoufi et al., 2004). Thus, lysogenic bacteriophages may be an obstacle in this regard. That's why, new method which will not cause bacteria to gain resistance is needed.

Endolysins are enzymes that cause hydrolysis of peptidoglycan produced by bacteriophages following the lytic cycle towards the end of this cycle. Endolysins play role in degrading the peptidoglycan structure of bacteria and after cell lysis of cell occurs, progeny virions are released (Schmelcher & Loessner, 2016). Although endolysins differ structurally in their targeting of Gr (+) or Gr (-) bacteria, they generally contain two conserved protein domains: the N-terminal enzymatically active domain (EAD) and the C-terminal cell wall binding domain (CBD) (Loessner, 2005; Villa & Crespo, 2010). EAD is the protein domain needed to catalyze the cell wall and classified by where they cleave peptidoglycan. CBD is the part of the cell wall that enables the recognition of target substrates and the host specificity of endolysins (Oliveira et al., 2013). Since endolysins do not spoil the microflora, are specific and do not increase bacterial resistance unlike bacteriophages, they have recently been highly preferred in the food industry for issues such as pathogen detection, biofilm control and food safety.

The structure of the cell walls of Gr (+) and Gr (-) bacteria is different from each other. While the peptidoglycan of Gr (+) bacteria is more layered, it is single layered in Gr (-) (Silhavy et al., 2010). On the other hand, the cell wall of Gr (-) bacteria contains outer membrane which acts as a barrier and prevents endolysins from reaching peptidoglycan. Therefore, to act on Gr (-) bacteria in general, besides endolysin, there is another protein required, called holin having active role in bursting the Gr (-) cells (Xu et al., 2005). Holins are small membrane proteins responsible for making holes in the inner membrane. Thanks to the holes opened, endolysin reaches the peptidoglycan and breaks its down, and disruption of the osmotic balance causes the cell to destroy (Young, 1992).

Studies show that in the absence of holin, various organic acids, chemicals or assistive technologies have a synergistic effect with endolysin. Organic substances such as citric acid, malic acid, EDTA increase the permeability of the outer membrane, so endolysin can degrade peptidoglycan without the need for a holin (Briers et al., 2008; Chang, et al., 2017; Oliveira et al., 2016). In some studies, the

concomitant use of endolysins in food procedures with high hydrostatic pressure (HHP) has resulted in better results (Misiou et al., 2018).

The aim of the study was primarily to produce proteins encoded by bacteriophage against antimicrobial resistance foodborne pathogens. Firstly, the gene regions encoding endolysin were selected from the bacteriophage (MET P1-137) which was previously isolated and whole genome sequenced. The gene encoding this protein was found, amplified by Polymerase Chain Reaction (PCR) and transferred to *Escherichia coli* BL21 strain by vector pET-28a (+) by using of recombinant DNA technology. After that, protein expression was performed and lytic activity of the endolysin against *Salmonella* was observed.

CHAPTER 2

LITERATURE REVIEW

2.1 Foodborne Pathogens

In 460 BC, Hippocrates realized that food consumption was associated with making people sick (Hutt & Hutt, 1984). Foodborne illness, also known as food poisoning, occurs when foodborne pathogens such as bacteria, viruses, parasites, and toxins, chemical or other substances produced by these organisms, contaminate food or water and a person consumes these products (FDA, 2020). If two or more people consuming the same food product show similar symptoms of illness, it is called a foodborne outbreak (CDC, 2012).

Foodborne illnesses can occur in different ways. If a pathogen is ingested with food and the pathogen attaches the gastrointestinal (GI) barriers and cell, it is called a foodborne infection. The incubation period of this type of disease is slightly longer, the symptoms are observed a little later. If a toxin-containing food product is consumed and symptoms are observed, it is called foodborne intoxication. Symptoms of intoxication occur relatively quickly (Bintsis, 2017). The symptoms of the two types of foodborne illness are generally similar, with nausea, vomiting, diarrhea (bloody diarrhea may be observed in some cases), abdominal pain, headache, loss of appetite, malaise, fever in case of infection (FDA, 2012). Even though the symptoms can be mild, it can cause serious problems in people with low immunity, children, the elderly, and people who are exposed to high doses of the toxin / organism (CDC, 2012).

One of the groups that causes foodborne diseases is viruses. As a result of studies, more than 100 types of enteric viruses cause foodborne diseases. The most common

are Hepatitis A and Noroviruses (Bintsis, 2017). Norovirus alone is known to cause approximately 5 million foodborne illnesses each year in the United States. It is one of leading viral gastroenteritis in the world (Scallan et al., 2011). These types of viruses are generally transmitted due to the contamination of the waters in which shell food such as oysters, mussels and clams grow, with human feces. Eating such food products raw or undercooked may also cause viral infections (Pradhan et al., 2019). The morbidity and mortality rates of foodborne viruses are not to be underestimated. The rate of foodborne diseases caused by viruses is 7 % (Mead et al., 1999). To reduce and control this rate, food and personal hygiene, good agricultural practices (GAP), post-harvest controls should be implemented and followed (Tuladhar et al., 2015).

Another causative agent group of foodborne outbreaks is bacteria. In fact, about 34% of the outbreaks reported in 2015 were caused by bacteria, with viruses taking the first place with 20 % in 2014 (EFSA & ECDC, 2016). While the first cause of these bacterial outbreaks is *Salmonella* with 22 %, *Campylobacter* spp. is in the second place with 9 %. Studies show that most of reported outbreaks were animal origin, particularly from egg and egg products, pork, chicken, cheese, fish and fish products, milk, and dairy (EFSA & ECDC, 2016).

Bacteria come in different shapes, types, and features. For example, some types of bacteria can form spores such as *Clostridium botulinum*, *Bacillus cereus*, *Bacillus subtilis*, and if their spores are heat-resistant, heat treatment may not be enough to eliminate them (Bacon & Sofos, 2003). Or some bacterial strains produce toxins that can be heat-resistant such as *Staphylococcus aureus*. Some pathogens are mesophilic, that is, those with optimum growth temperature range of 20-45 ℃, they do not grow in cold environment; however, psychrotrophs like *Yersinia enterocolitica* and *Listeria monocytogenes* can grow and multiply in refrigerator temperature and make people sick (Bacon & Sofos, 2003). The fact that they are in such different structures and increase in diversity makes it clear that bacterial foodborne outbreaks are very common.

According to the report prepared by EFSA, an average of 258.000 foodborne cases are reported annually in Europe between 2016-2021, and of these cases, 22.000 of them are hospitalized and 230 of them cause death (EFSA, 2020). In Figure 2.1, number of cases of foodborne illnesses in Europe between 2010-2021 is given.

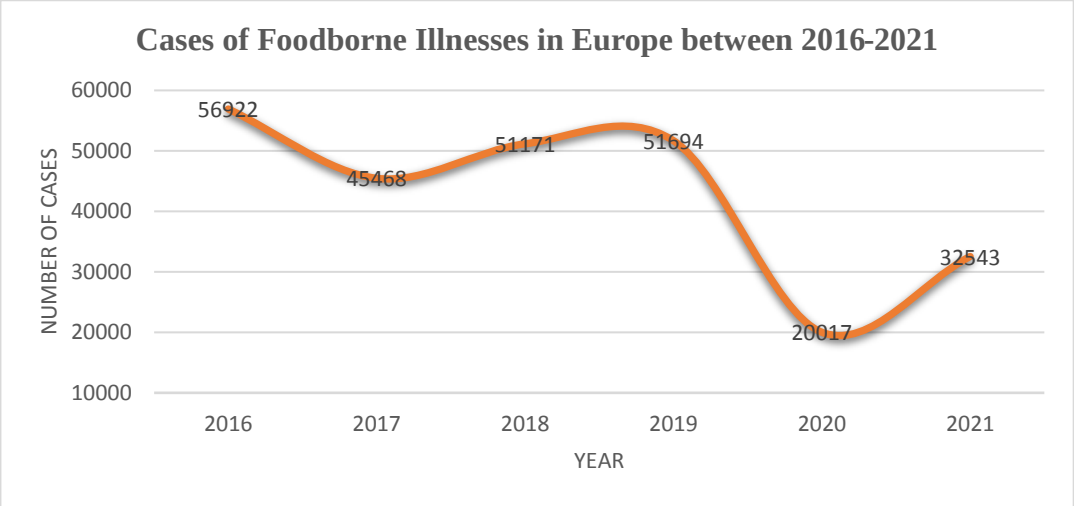

Figure 2.1 Cases of Foodborne Illnesses in Europe between 2016-2021 (EFSA, 2020).

According to Centers for Disease Control and Prevention (CDC) reports covering the years between 2017-2020, it was announced that the number of foodborne cases in the United States resulted in 7659 diseases, 2044 of which caused hospitalization and 41 resulted in death (CDC, 2022a). In Figure 2.2, number of cases of foodborne illnesses in US between 2017-2021 is given.

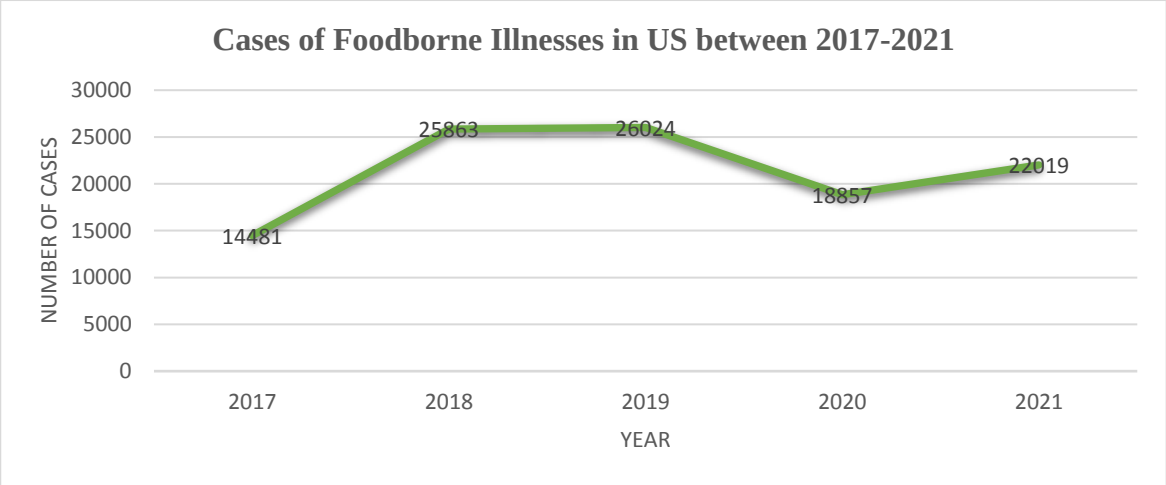

Figure 2.2 Cases of Foodborne Illnesses in US between 2017-2021 (CDC, 2022a).

Between 2016-2020, the reported case of food poisoning in Türkiye was around 500. The number of people affected by these cases was estimated to be 27.000 (Başaran, 2021). These numbers do not contain a definite result and it can be said that there were more cases than reported, considering that some cases recovered without going hospital or taking any medical treatment or notifying the relevant authorities. In Figure 2.3, number of cases of foodborne illnesses in Türkiye between 2016-2020 is given.

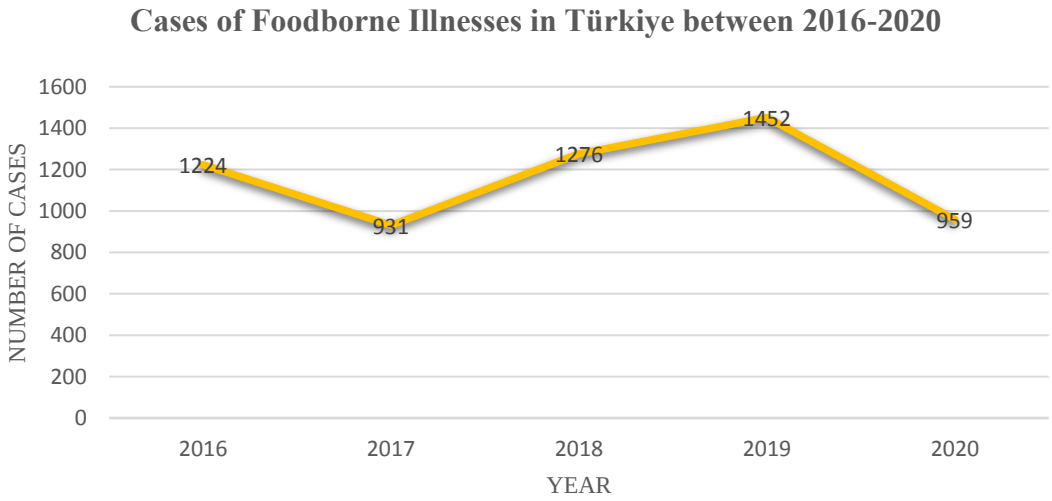

Figure 2.3 Cases of Foodborne Illnesses in Türkiye between 2016-2020 (Başaran, 2021).

2.1.1 *Salmonella*

In 1880, the first imaging of *Salmonella* from the spleens of typhoid patients was made by the German pathologist Karl Eberth (Eberth, 1880). Four years later, in 1884, Theodor Gaffky managed to grow them in pure culture (Hardy, 1999).

But *Salmonella* was first discovered in 1885, when Theobald Smith, an assistant in Bureau of Animal Industry, United States Department of Agriculture, thought that *Salmonella* Choleraesuis was the causative agent of swine cholera. Thus, *Salmonella* was isolated for the first time from the intestine of infected pigs (Eng et al., 2015).

Gram-negative, facultative anaerobe, rod shaped *Salmonella*, belonging to the *Enterobacteriaceae* family, is one of the most disease-causing foodborne pathogens in the world (WHO, 2018). *Salmonella* is a non-spore forming bacterium with diameters ranging from 0.7-1.5 μm and lengths between 2-5 μm (Graziani et al., 2017a). They are mostly motile, with a structure called flagella found throughout the cell body that enables them to move (Fàbrega & Vila, 2013).They use organic sources and derive their energy from oxidation-reduction reactions. Since they can produce ATP in the presence or absence of oxygen, they are known as facultative anaerobes (Lopes et al., 2016). Mostly, *Salmonella* serotypes grow in the temperature range of 5-47 ℃, although their optimum temperature is 35-37 ℃. The pH range required for their growth is 4-9, and the optimum range is between 6.5-7.5. They need very high water activity (a_w) to grow, 0.94-0.99. Generally, temperature values above 70 ℃, pH values lower than 4 and water activity values lower than 0.94 are sufficient to kill or prevent their growth (Graziani et al., 2017).

Salmonella contains 3 main antigens, flagellar (H), somatic (O) and Vi antigen (Graziani et al., 2017b). H antigens are heat-stable and are found in the flagella of bacteria. Its tasks to provide immune activation of the bacterium. In some *Salmonella* spp., flagella proteins are encoded by two separate genes, but bacteria can express one at a time. For this reason, they are called phase I and phase II, that is, diphasic (McQuiston et al., 2008). Bacteria can switch from one phase to another (Graziani

et al., 2017a). Bacteriophage I H antigen expresses the immunological identity of the serotype, which is specific, while phase II H antigen can be expressed by many serotypes, unlike phase I, which is non-specific (McQuiston et al., 2008). Like other Gram (-) bacilli bacteria, *Salmonella* has a complex structure called lipopolysaccharide (LPS), which consists of three components: outer O-polysaccharide, middle part (R core) and inner lipid A coat. The R core in the middle shows the common structure among other Gram (-) bacteria. In this way, when the produced antibody is directed to R core, it can provide protection from bacterial infection and reduce its lethality (Graziani et al., 2017). O antigens (O-specific polysaccharides or O-side chains) are heat-stable, another component of lipopolysaccharide, and is on the outer membrane surface of the cell. Their structures and compounds can be quite diverse, enabling the classification of serovars (Hu & Kopecko, 2003). Another antigen is the Vi antigen located on the O antigen and has been observed in few serovars. The Vi antigen is virulence capsular polysaccharides and is produced by typhoidal *Salmonella* (TS) serotypes Typhi and Paratyphi C (Keestra-Gounder et al., 2015).

More than one suggestion has been put forward for the nomenclature of *Salmonella*, and currently the Center for Disease Control and Prevention (CDC) names *Salmonella* with the system recommended by World Health Organization (WHO) (Popoff et al., 2003). According to the system proposed by WHO, 16S rRNA sequence analysis was used and it was determined that the genus *Salmonella* has two species, *Salmonella enterica* and *Salmonella bongori*. In addition, considering biochemical similarities or differences, genomic affinities, it was figured out that there are six subspecies of *Salmonella enterica* and are indicated with roman numerals (Reeves et al., 1989). These subspecies are I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamea*, IIIa, *S. enterica* subsp. *arizonae*, IIIb, *S. enterica* subsp. *diarizonae*, IV, *S. enterica* subsp. *houtenae* and VI, *S. enterica* subsp. *indica* (Eng et al., 2015).

Apart from subspecies classification, serotyping is done according to the scheme developed by Kaufman and White (Brenner et al., 2000). This classification is based

on the serological identification of the three main antigens (H, O and Vi) contained by *Salmonella*. For example, most laboraties choose reactions using antibodies specific for the O antigen to classify them as serogroups. The classification is crutial for epidemiological studies and the investigation and detection of an outbreak (Wattiau et al., 2011). The number of *Salmonella* serotypes defined according to the serological classification based on antigens is over 2500 (Guibourdenche et al., 2010).

More than half of the 2500 *Salmonella* serotypes identified are *Salmonella enterica* subsp. *enterica* and are mostly found in mammals. Almost all *Salmonella*-induced infections observed in humans and other warm-blooded animals belong to this serotype (Brenner et al., 2000). On the other hand, other species, *Salmonella bongori*, is isolated from environment or cold-blooded animals and is rare to infect humans (Farmer III et al., 1984).

Salmonella serotypes can be divided two as typhoidal and non-typhoidal (Okoro et al., 2012). The transmission route of the typhoidal serotypes is from human to human, and causes foodborne infection, typhoid fever and paratyphoid fever. As a result of typhoid fever, blood circulation can be blocked, and if it spreads to the body, it can invade the internal organs and produce and secrete endotoxin. Therefore, Typhoidal *Salmonella* infection can become life-threatening and require intensive care (Ray & Ryan, 2014). *Salmonella* Typhi, *Salmonella* Paratyphi A, B and C are given as examples of Typhoidal *Salmonella*, and only observed in human. Fever caused by the disease because of *S*. Typhi and *S*. Paratyphi serovars is called "enteric fever" (Connor & Schwartz, 2005). Disease can occur if people consume food and water contaminated by the waste of infected people. Among the most common symptoms are enteric fever, diarrhea or constipation, headache and abdominal pain (Bhan et al., 2005). These symptoms, which is known to have a long incubation period, has been observed to last for a month or more if left untreated (Patel et al., 2010). In some patients, the infection caused spleen and liver enlargement, bradycardia, and myalgia (Kuvandik et al., 2009).

The reservoir of Non-typhoidal *Salmonella* (NTS) serovars, unlike the other, is animals, not humans. The route of transmission is diverse, can be transmitted from animal to animal, animal to human or human to human. Direct contact with poultry, especially chicken, pig, cattle, consumption of contaminated food with animal origin such as milk, egg, meat, poor hygiene conditions, dry environments play a major role in the transmission of NTS serovars (Atterbury et al., 2020). As a result of NTS infections, symptoms such as vomiting, diarrhae without blood, headache and abdominal cramps are observed (Acheson & Hohmann, 2001). The incubation period is shorter, 6-12 hours, and recovery process is faster, 7-10 days as the symptoms observed are self-limiting (Crump et al., 2008). Even in some cases, recovery is observed without antibiotic treatment. However, it could be life-threatening in young children, the elderly, or people with compromised immunity (WHO, 2018). Despite the fact that it might manifest as mild symptoms, according to World Health Organization (WHO), salmonellosis causes approximately 94 million gastroenteritis and 155,000 deaths worldwide each year (Denyes et al., 2017). Moreover, WHO has declared that since 2010, 1.14 % of people infected in the world are caused by NTS serovars (Kirk et al., 2015). According to Centers for Disease Control and Prevention (CDC) reports, NTS serovars cause 1.35 million infections, approximately 27,000 hospitalizations, and 420 deaths in United States (CDC, 2022). Reported *Salmonella* cases in Europe are over 91,000 (EFSA, n.d.).

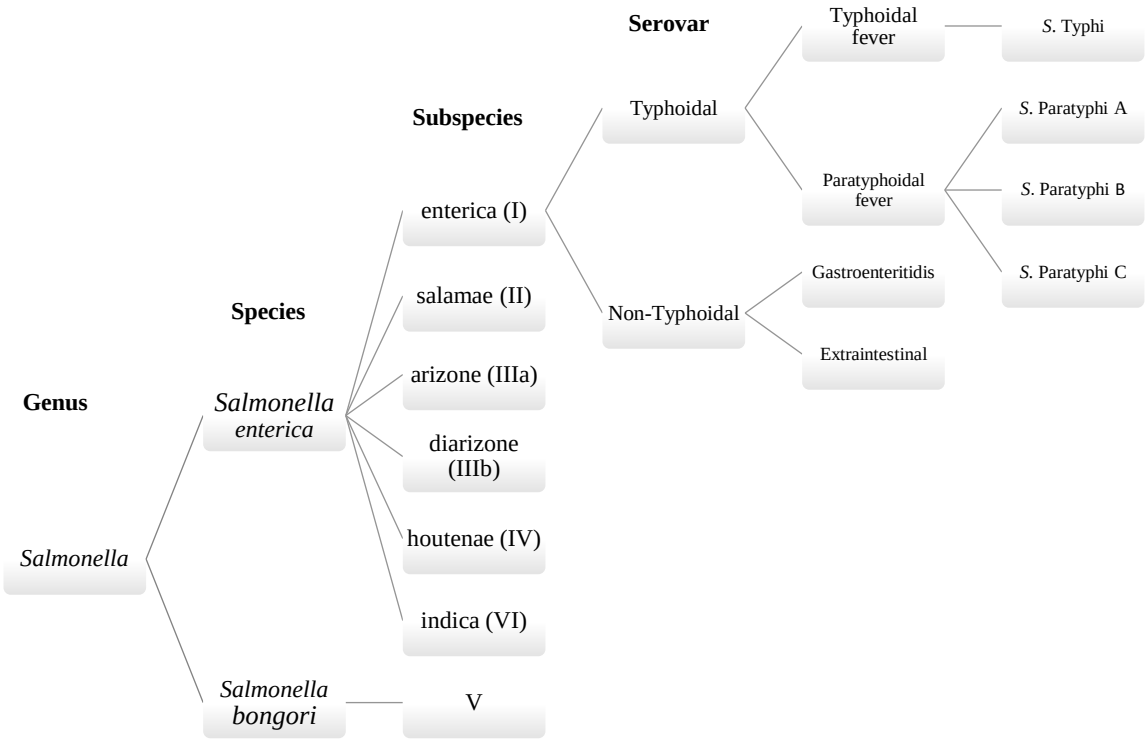

Figure 2.4 Classification of *Salmonella*. The figure was adopted by Hurley et al. (Hurley et al., 2014).

According to data presented to the World Health Organization (WHO), 20 *Salmonella* serotypes from 49 countries, 20 of which were isolated from both human and non-human sources, 21 from only human and 8 from non-human sources, were processed into data banks. Over a 3-year period (2000-2002), the most isolating serotype from humans was *Salmonella* Enteritidis, globally. In 2002, while *Salmonella* Enteritidis constituted 65 % of isolates, *S.* Typhi was 12 % and *S.* Newport was 4 %. Similarly, in 2002, looking at the serotypes isolated from humans, 84 % of the countries reported that *S.* Enteritidis and *S.* Typhimurium serotypes were the most common. Half of the countries stated that *S.* Infantis and *S.* Typhi were

among the 10 most common serotypes. On the other hand, *S*. Typhimurium was the most isolated serotype from non-human sources for 3 years (17 %). This number for *S*. Heidelberg was 11 % and for *S*. Enteritidis 9 % (Galanis et al., 2006). Worldwide, disease rates have not been consistently distributed, but studies show that most common serotypes for human salmonellosis are *S*. Typhimurium and *S*. Enteritidis (Hendriksen et al., 2011).

2.2 Antibiotic Resistance

Antibiotics are therapeutic substances used to slow, complicate, or stop bacterial growth and proliferation against infections caused by bacteria in humans and animals (EFSA, n.d.). Since its discovery (in 1928 by Alexander Fleming), it has made an enormous contribution to the treatment of infectious diseases and to the reduction of deaths (Armstrong et al., 1999). However, misuse and overuse of antibiotics affects the present and the future, as they cause bacteria to show resistance and become a global problem (Capita & Alonso-Calleja, 2013).

Antibiotic resistance is the capacity of microorganisms to survive the application of antibiotics (EFSA, n.d.). Because of this, it is very difficult or impossible to deactivate the resistant bacteria. Bacteria may show hereditary antibiotic resistance or subsequent acquisition. For example, Gram (+) bacteria show natural resistance to the colistin or the *Enterobacteriaceae* family has inherited resistance genes against glycopeptides and linezolid (MacGowan & Macnaughton, 2017). Subsequent acquisition is achieved by horizontal gene transfer mechanisms. In particular, this resistance can be acquired by conjugation, transformation and transduction (von Wintersdorff et al., 2016). In bacterial conjugation, small DNA is transferred from one bacterial cell to another through structures in cell membranes (Cabezón et al., 2017). When bacteria die, they can release some parts, including their DNA, into the environment. These parts can be taken into its own chromosome by a living bacterium in the environment. If there is an antibiotic resistance gene in the fragmented DNA and another bacterium takes it for itself, the bacterium now

contains the antibiotic resistance gene. This mechanism called transformation (von Wintersdorff et al., 2016). Transduction can occur when bacteriophages integrate the genome of a host cell with their own genome, while carrying the antibiotic resistance gene from that bacterium to a new host. Considering the rates of bacteriophages in biosphere, it is obvious that antibiotic resistance has increased considerably (Clokie et al., 2011).

Antibiotic resistance mechanism is divided into four, including restriction of drug intake, changing the drug target with an increase or decrease in the binding ability of penicillin-binding proteins (PBS), inactivation of the drug by fragmentation of the drug or addition of chemical groups, and active drug efflux (Reygaert, 2018). Acquired resistance may be due to alteration of drug target, drug inactivation, and drug efflux. Intrinsic resistance can be seen through drug intake restriction inactivation and drug efflux (Cox & Wright, 2013). Resistance mechanism differ in Gram (+) and Gram (-) bacteria. Gr (+) bacteria do not have a lipopolysaccharide (LPS) outer membrane, and therefore they cannot make much use of the restriction of the drug intake among the mechanism. On the other hand, Gr (-) bacteria use all four mechanisms. The presence of LPS acts as a barrier against the uptake of antibiotics (Chancey et al., 2012).

Antibiotic resistance causes death of 1.27 million people worldwide. According to the Antibiotic Resistance Threat Report published by CDC in 2019, 2.8 million antibiotic resistance infections are encountered in the United States every year. More than 35,000 of these infections result in death (CDC, 2021). In Europe, bacteria that resistance to antimicrobials cause 25,000 deaths every year (CDC, 2021). The overuse or misuse of antibiotics has become a concern all over the world and leads to significant uses not only in health but also economically (Laure & Ahn, 2022). Studies show that if the necessary precautions are not taken, approximately 10 million people will die every year due to antibiotic resistance infections by 2050. As a result, there will be an economic loss of almost $100 trillion (Chokshi et al., 2019).

Figure 2.5 shows the global distribution of deaths forecasted to occur by 2050 due to antimicrobial resistance.

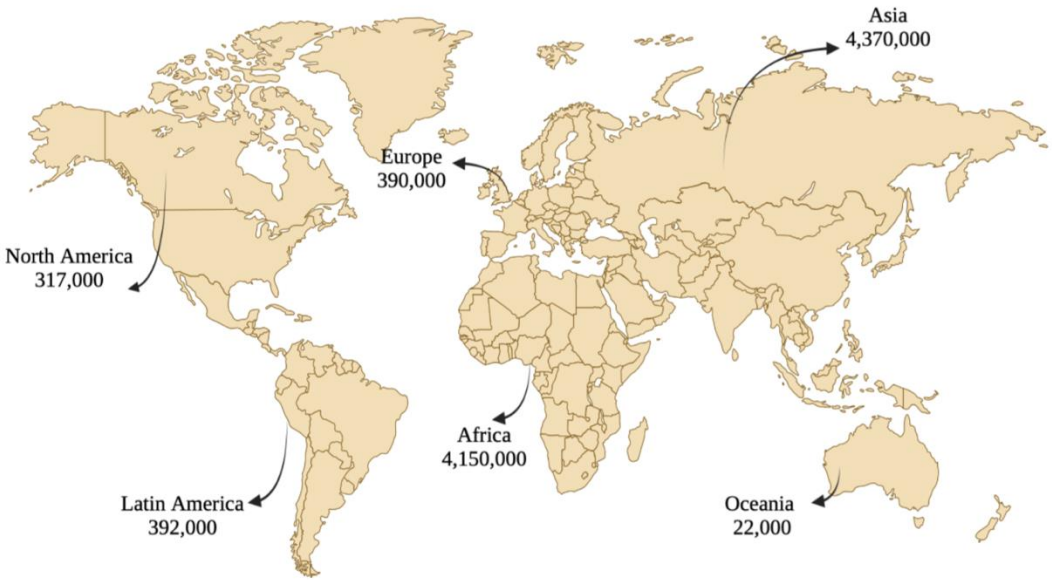

Figure 2.5 Global distribution of deaths forecasted to occur by 2050. This figure was drawn using BioRender (https://www.biorender.com) adopted by Tripathy et al. (Tripathy et al., 2017).

Measures have been taken against it around the world because of increasing awareness of antibiotic resistance. In 2006, the non-medical antibiotic used in animal feed to accelerate growth was banned in Europe (European Commission, 2005). Even though this ban is considered as an important step in reducing this threat, there are still countries in the world that use antibiotics for non-medical purposes.

In line with the prohibitions and the measures taken, alternative methods have begun to be sought instead of use of antibiotics (Laure & Ahn, 2022).

2.3 Bacteriophages

Bacteriophages, phages for short, which are the most abundant organisms on the planet and estimated to be approximately 10^{31}, are considered to be the most

promising alternative route to antibiotics (Chibani-Chennoufi et al., 2004). Bacteriophages are organisms that infect or kill the bacteria. Since they are host-specific properties and harmless to animals and humans, it is considered that they will have an important place in the treatment of bacterial infections and in reducing / eliminating completely the use of antibiotics (Carvalho et al., 2017).

In 1915, when British bacteriologist Frederick Twort was trying to grow smallpox vaccine virus he was working on *in vitro*, he noticed that cultures were not growing and had a different appearance than usual. In the agar tubes he inoculated, he observed "glassy and transparent" regions unlike normal, and he made the assumption that these regions he observed were formed by the destruction of bacterial cells (Keen, 2015). Not knowing exactly what caused these areas, Twort proposed three different hypotheses: I) A result of the bacteria's unconventional life cycles, II) Autocatalytic enzyme produced by the bacteria or III) Bacterial virus (Taylor & Taylor, 2014). However, Twort did not confirm any of these hypotheses.

While trying to stop the locust infestation, the French-Canadian bacteriologist Felix d'Herelle realized that the organism that infects the locusts cause diarrhea (dysentery) (Summers, 2012). He isolated the causative agent from insects and observed "clean" areas in the culture as the bacteria grew. Disregarding this at first, he tried to prevent caused by locusts by using isolated *Bacillus*. Felix d'Herelle first noticed bacteriophages while researching the dysentery epidemic during World War I. After isolating and characterizing the *Bacillus* that causes dysentery he realized "holes" in the cultures. He later understood that these areas could be filtered out and "plaque" formed, and he called these formations "bacteriophages", that is, "bacteria-eating" (Taylor & Taylor, 2014).

It is known that bacteriophages are the most abundant entities on the biosphere. This approach is based on the fact that bacteriophages require a host cell to grow / proliferate. This means that the abundance and distribution of bacteriophages will be similar to that of host cells. On Earth, bacteria and archaea are found in open oceans, in soil, in ocean sediments. Moreover, DNA staining has suggested that there are

approximately 10 bacteriophages for each bacterial or archaeal cell found in marine waters (Suttle, 2005). Similar numbers have been considered for freshwaters, but since nothing can be said for certain for other environments, an approximation of the exact bacteriophage count has not been made (Ashelford et al., 1999).

Besides being the most common organism, bacteriophages also vary in complexity, size, or shape. Genome sizes can vary between 3 kb and 500 kb (Keen, 2015). The bacteriophage with the smallest known genome size is the *Escherichia coli* bacteriophage carrying ssRNA with a genome size of about 3300 bp, and the *Bacillus megaterium* bacteriophage with the largest known genome size, carrying dsDNA with 500 kb (Hatfull & Hendrix, 2011). According to studies, the smallest of the tailed bacteriophages containing dsDNA are *Mycoplasma* bacteriophage P1, a member of the *Podoviridae* family with a genome size of approximately 11.5 kb, *Lactococcus* bacteriophage c2, a member of the *Siphoviridae* family with a size of 21 kb, and *Pasteruella* bacteriophage F108, a member of the *Myoviridae* family with a size of 30 kb (Campoy et al., 2006; Lubbers et al., 1995; Tu et al., 2001).

Their great diversity and abundance have made bacteriophages a very important biological, environmental, and evolutionary element. In a study by Danovaro et al., bacteriophages kill an average of 20 %-25 % of bacteria in the ocean every day. As a result, it is put forward that oxygen productions, phytoplankton productivity rates, the ratio of particles to dissolved carbon, climate and weather changes are affected (Danovaro et al., 2011). In addition, bacteria tend to develop defense mechanism, i.e., evolve, to escape the lethality of bacteriophages. Especially bacteriophages following the lysogenic life cycle are important agents for horizontal gene transfer, triggering bacterial evolution (Chibani-Chennoufi et al., 2004).

There is no universal method for classifying viruses, and bacteriophage classification has begun to change with the discovery of new bacteriophages and the addition of new families and genera (Clokie & Kropinski, 2009). Bacteriophages may contain double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA). The most seen structure of nucleic

acid is dsDNA and the least common is dsRNA in bacteriophages (Clokie & Kropinski, 2009). The majority of viruses, 96 %, have tails, while the other virions are cubic, filamentous, or pleomorphic (rare). The word "cubic" refers to an icosahedron with cubic symmetry. Lipids in envelopes are a component of some kinds' or interior parts (Clokie & Kropinski, 2009).

Most bacteriophages are structurally proteinaceous capsid (or head) and capsid-bound tail (Ackermann & Prangishvili, 2012). Capsids have a regular protein structure that stores the bacteriophage's genetic material. They are mostly icosahedral (20 triangular faces) and fibrous. Icosahedral capsids are formed by multiple repetition of a small number of protein types and the stability and complexity of such capsids vary according to their subunit components, copy numbers and arrangement (Tama & Brooks III, 2005). In some bacteriophages the capsids show expanding to disclose protein binding sites which helps to enhance capsid stability (Qin et al., 2010). The maturing capsid diameters can vary between 43 nm – 160 nm (Donelli et al., 1975). The filamentous bacteriophages, also knowns as having helical array, are rod-shaped and can range from 800 to 2000 nm in length and 6.5 to 7.5 nm in diameter. Capsid length can vary with genome size (Xu et al., 2019). For filamentous capsids to form, five bacteriophage structural proteins (β-tulip, OB-fold, β-Tadpole, Ig-like, knotted α-helix) must be anchored to the host cell's inner membrane and assemble around the genome during translocation (Feng et al., 1997). Some bacteriophages obtain lipid envelopes from the phospholipids of their host cells and use these envelops externally to protect their genomes (e.g., family *Cystoviridae*) (Mäntynen et al., 2018). In addition, there are bacteriophage families that contain lipid membranes that cover the capsid from inside, such as *Corticoviridae*, *Sphaerolipoviridae* or *Tectiviridae* (Abrescia et al., 2004; Kivelä et al., 2002; Pawlowski et al., 2014).

The tails of bacteriophages are involved in the recognition of host cells, their penetration into the cell wall or cell membrane of the host cell, and the formation of new bacteriophage particles (Nobrega et al., 2018). At the end of the bacteriophage tails, there are tail fibers, also called spikes. These tail fibers bind to receptors on the

surface of bacteria such as lipopolysaccharide (LPS), porin transmembrane proteins, teichoic acid, organelles (Silva et al., 2016; Dunne et al., 2018; Maffei et al., 2021). The interaction of the tail fibers with the host cell is specific, so these fibers determine the host cell specificity and bacteriophage infecting process (Dams et al., 2019; Sant et al., 2021). Given the specificity of receptors, the bacteriophage can be classified as monovalent (able to infect only a single bacterial species or strain) and polyvalent (able to adhere to bacterial surfaces and infect a variety of bacterial species or even genera) (Motlagh et al., 2016). Although this specificity is used for therapeutic purposes without harming the natural microbiota since it is host-directed, it causes bacteriophages to have narrower host range, unlike antibiotics (Kingwell, 2015; Merril et al., 2003). Figure 2.6 shows the structure of typical bacteriophage.

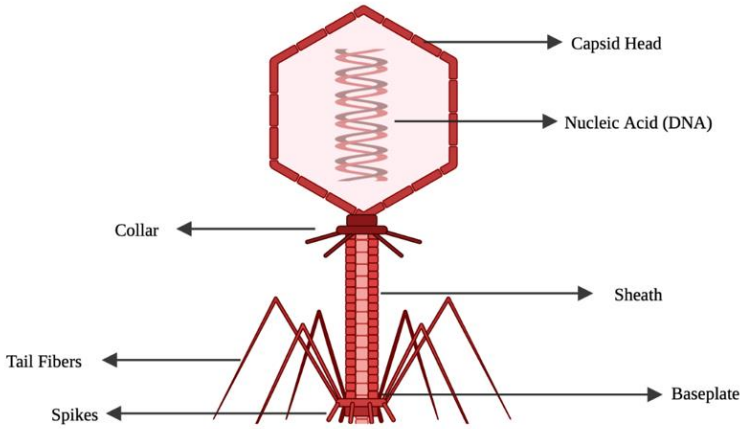

Figure 2.6 Structure of typical bacteriophage. This figure was drawn using Biorender (https://www.biorender.com), adopted by Jamal et al. (Jamal et al., 2019).

Prior to the activities of the Bacterial Viruses Subcommittee of the International Committee on Virus Taxonomy, proposed in 2021 and approved in 2022, it was possible to classify bacteriophages according to their morphology. Representing the largest group of bacteriophages, hence viruses, the order *Caudovirales* included the three families of bacterial viruses according to their tail morphology (Bamford &

Zuckerman, 2021). *Siphoviridae* which is the most abundant with long non-contractile tails that can infect bacteria and archaea, *Myoviridae* which is the second most abundant with long contractile tails, *Podoviridae* with short non-contractile tails. The collection of bacteriophages with tail is enormous, and they have a wide range of virion, genome, and replication characteristics (Fauquet et al., 2005).

With the new regulation, the *Podoviridae*, *Siphoviridae* and *Myoviridae* order in bacteriophage taxonomy was removed and binomial nomenclature was introduced. It has been suggested that classification according to morphological features does not fully specify the evolutionary background of bacteriophages, they should be classified according to their genomic features (Turner et al., 2023).

According to their life cycle, bacteriophages are divided into two main groups as lytic (virulent) and lysogenic (temperate) (Kutter & Sulakvelidze, 2004). In the lytic cycle, firstly, the lytic bacteriophage attaches to the host cell. It penetrates through the cell wall of the bacterium and injects its own DNA into the bacterial cell. As the bacterial DNA begins to degrade, the bacteriophage DNA starts to replicate, and its proteins are synthesized. As new bacteriophages are released into the environment, lysis of the host cell takes place (Motlagh et al., 2015). On the contrary, in order to follow lysogenic life cycle, temperate bacteriophages must integrate their genomes with the host cell's genome and form host cells containing bacteriophage genomes known as prophages (Motlagh et al., 2015). In the lysogenic cycle, bacteriophage can remain in bacteria without bursting the cell. However, bacteriophage can switch from the lysogenic cycle to lytic in the presence of required environmental triggers (Carvalho et al., 2017). In general, lytic (virulent) bacteriophages are used for therapeutic purposes. However, lysogenic bacteriophages can also be used for therapeutic purposes to transfer gene in order to sensitize bacteria to antibiotics or other factors (Lu & Collins, 2009). Against antibiotic resistance, bacteriophages, bacteriophage therapy and bacteriophage derivatives such as bacteriophage-based proteins have gained importance (Carvalho et al., 2017b).

2.3.1 Bacteriophage Applications in the Food Industry

Since bacteriophages are specific to the bacterial host, do not harm humans or animals, and do not disturb the natural microflora, it has become a solution that has started to attract a lot of attention as an alternative to antibiotic treatment. Its application has begun to increase not only in the field of food industry, but also in different fields such as medicine, agriculture, biotechnology, nanotechnology, and vaccine development (Hagens & Loessner, 2007).

Although the use of bacteriophages in the food industry is relatively new, it has become applicable throughout the "farm to fork" concept (Garcia et al., 2008). In the food industry, aim of the usage of bacteriophages to prevent and reduce diseases in farm animals, disinfect surfaces and equipment used in food processing plants, decontaminate raw materials such as fruits and vegetables, and extend the shelf life of foods with its biocontrol feature (Leverentz et al., 2003a).

In 2005, Fiorentin et al., in their experiment on broilers, observed that bacteriophages isolated from free-range chickens were effective on *Salmonella* Enteritidis. Broilers were infected by *S.* Enteritidis and a mixture of 3 different bacteriophages were given to treat. At the end of 5, 10, 15, 20 and 25 days of treatment, samples were taken from infected (and subsequently bacteriophage-treated) broilers, decrease in the concentration of colony-forming *S.* Enteritidis was observed. As a result of this study, it was concluded that foodborne pathogens infecting poultry can be reduced by bacteriophages (Fiorentin et al., 2005).

In another study conducted on Cheddar cheese, the presence of *S.* Enteritidis was investigated when bacteriophage was applied during manufacture, ripening and storage stages of Cheddar cheese obtained from raw and pasteurized milk. A 1-2 log cycle decrease in *S.* Enteritidis concentration was observed for two types of milk. In addition, *Salmonella* was found in bacteriophage containing raw milk cheese after a 99-day storage period after production, but not in pasteurized milk cheese containing bacteriophage (Modi et al., 2001).

The emergence of the *Salmonella* problem in fresh products such as vegetables and fruits has led to an invitation to bacteriophage treatment in these products. A cocktail containing 3 bacteriophages was tested on lettuce with *Salmonella* Enteritidis and *Salmonella* Typhimurium. Approximately 4 and 2 log CFU/g reduction were observed in *S.* Typhimurium and *S.* Enteritidis, respectively (Spricigo et al., 2013). In another lettuce experiment, bacteriophage was tested at different multiplicity of infection (MOI) values and a decrease in the concentration was observed (Huang et al., 2018).

Bacteriophage applications against *Salmonella*, which have also been tested on raw and cooked beef, fruits such as melons and apples, production, and storage of delicatessen products such as sausages, bacteriophages have been observed to be quite effective in reducing concentration of *Salmonella*, that's why bacteriophages have been a beacon of hope for food safety (Bigwood et al., 2008; Leverentz et al., 2001; Whichard et al., 2003).

Atterbury et al. (2005) conducted a study on *Campylobacter jejuni* bacteriophage in broilers. Broiler chickens selected from 90 flocks were treated with bacteriophage and compared with control group which samples only containing *Campylobacter jejuni*, a decrease in *C. jejuni* concentration of about 2 log CFU/g was observed between two groups (Atterbury et al., 2005). Other bacteriophage treatment study on *C. jejuni* did not show 100 % clearance but significant reductions in concentration were observed (Loc Carrillo et al., 2005).

Another bacteriophage study against *C. jejuni* was infection of chicken skin. It was observed that effect of bacteriophages applied at room temperature was quite high (95%) and the same experiment was performed with higher MOI values at 4 ℃ and -20℃ and seen that the concentration decreased (Goode et al., 2003).

Oral administration of bacteriophages has also been attempted for *Campylobacter* colonization. Bacteriophages applied to drinking water and feed reduced concentration of intestinal *Campylobacter* in broilers (Carvalho et al., 2010).

In order to reduce the contamination of *Escherichia coli* O157:H7, which is one of the leading foodborne pathogens, Abuladze et al. conducted a study on broccoli stored at 10℃ by using bacteriophage formulation. Bacteriophage treatment applied to broccoli stored for 24 h, 120 h and 168 h reduced contamination by 99.5 %, 99 % and 97 %, respectively. Similar results were observed in spinach and tomato samples (Abuladze et al., 2008).

When bacteriophage solution was applied to freshly cut and contaminated with *E. coli* O157:H7 lettuce, it was observed that the *E. coli* concentration on the lettuce surface decreased by 2 log CFU/cm^2 (Sharma et al., 2009). In another study, lettuce was infected with *E. coli* and kept at 4 ℃ for 5 days, and the bacteriophage applied by spraying resulted in a reduction of 87 % (Carter et al., 2012).

The fact that ruminants are reservoirs for *E. coli* leads studies on the oral application of the bacteriophage. It is aimed to reduce the risk of pathogen contamination during milking or slaughtering of these animals. In one study on the oral application of bacteriophage in sheep, a 2-log unit reduction of *E. coli* in intestinal concentration was observed (Raya et al., 2006).

In 2013, McLean et al., added bacteriophage cocktails containing 2 bacteriophages and 3 bacteriophages upon *E. coli* infection of both raw and UHT-treated milk, separately. *E. coli* concentrations were too low to be measured in the samples to which 3 bacteriophage containing cocktails were added and kept at 4 ℃ and 25 ℃. On the other hand, *E. coli* growth was observed in cocktails containing 2 bacteriophages. The reason for this, although not certain, is that the increase in the bacteriophage type will force the bacteria more and it needs more mutations to develop resistance (McLean et al., 2013).

ListexTM P100 is a commercial bacteriophage preparation developed against *Listeria monocytogenes*, approved by the US Food and Drug Administration (FDA) and Department of Agriculture (USDA) (Akhtar et al., 2017). The effect of this preparation against *Listeria monocytogenes* in fruits and vegetables was investigated. When the growth of *L. monocytogenes* in melon, apple and pear juices

and slices was examined in 2014, 1.5 log CFU per plug decrease in melon slices and 1 log CFU per plug decrease in pear slices were observed, but reduction in bacteriophage population in apple slices with very low pH value. For this reason, it was concluded that a combination of bacteriophages and other antimicrobials could be performed to provide *L. monocytogenes* reduction, especially in low pH food products (Oliveira et al., 2014). In fact, in 2003, the combination of bacteriocin nisin with bacteriophage was applied and its effects were evaluated on freshly cut apple and melon slices. Even though a decrease in bacteriophage titers was observed due to low pH, the synergistic effect of nisin and bacteriophage helped of declining concentration of *L. monocytogenes* in these fruits (Leverentz et al., 2003).

2.4 Structure of Peptidoglycan

Peptidoglycan, also known as murein, is a polymer that surrounds the cytoplasmic membrane found in bacteria other than mycoplasmas (Weidel et al., 1960). The main task of peptidoglycan (PD), which is involved in vital processes such as cell growth and division, is to provide cell integrity by resisting turgor pressure (Garde et al., 2021). Mutation, specific degradation, and antibiotic-induced inhibition that may occur during cell division lead to lysis of the cell (Vollmer et al., 2008). Peptidoglycan acts as the skeleton that holds other cell envelope components together, such as protein and teichoic acid, thus maintaining the shape of the cell and protecting against environmental threats (Dramsi et al., 2008; Neuhaus & Baddiley, 2003).

Peptidoglycan consists of glycan strands composed of repeating disaccharides and short peptide chains and form a reticulated sac surrounding the cytoplasmic membrane (Vollmer, 2015). Each of the glycan strands contains *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) residue linked by a β-1,4 glycosidic bond. It is the MurNAc moiety that is covalently bound to the peptides (Weidel et al., 1960).

In Gr (-) bacteria, L-alanine (L-ala), ɣ-D-glutamate (D-glu), meso-diaminopimelic acid (mDAP), and D-alanine (D-ala) form pentapeptide chains (Schleifer & Kandler, 1972). There is an isopeptide bond between $mDAP^3$ and $d\text{-}glu^2$. On the other hand, Gr (+) positives have L-lysine in the third position (Garde et al., 2021). $mDAP^3$ allows to form crosslinks with other peptides. In Gram (-) bacteria, it is cross-linked with the help of $d\text{-}ala^4$ and $mDAP^3$ or with the help of two $mDAP^3$, while in Gram (+) bacteria it is cross-linked by the presence of a bridging peptide with different amino acid length and composition (Garde et al., 2021).

The basis for the differentiation of Gram (+) and Gram (-) bacteria is the peptidoglycan structure. Peptidoglycan is more layered in Gr (+) ones and is exposed extracellularly by teichoic acids. In Gr (-) bacteria, there is an additional lipid layer known as the outer membrane, with a monolayer of peptidoglycan (Silhavy et al., 2010).

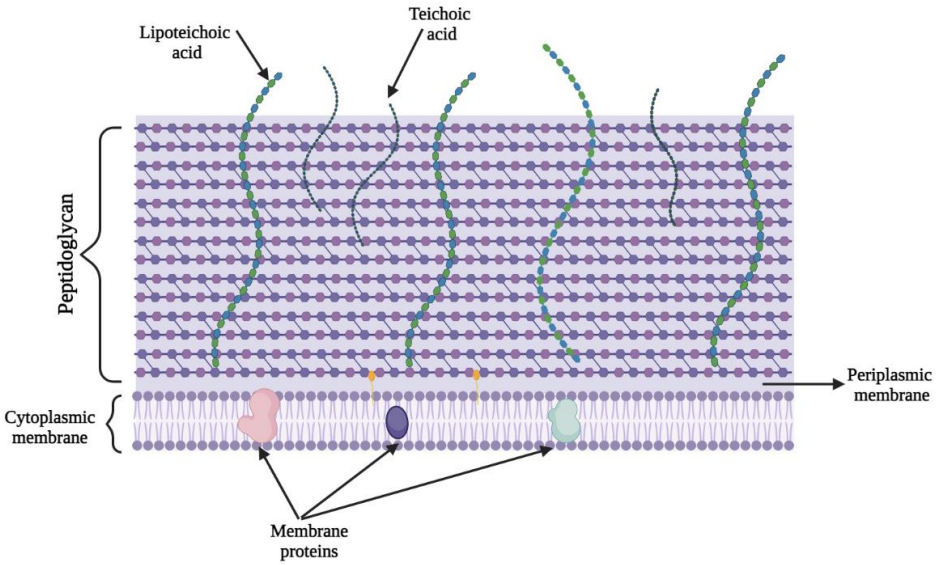

Figure 2.7 Cell wall structure of Gram (+) bacteria. This figure was drawn using Biorender (https://www.biorender.com), adopted by Pajerski et al. (Pajerski et al., 2019).

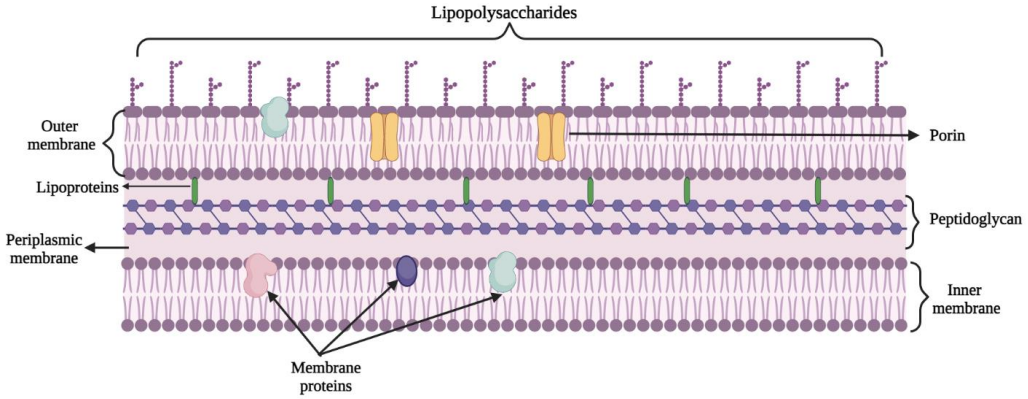

Figure 2.8 Cell wall structure of Gram (-) bacteria. This figure was drawn using Biorender (https://www.biorender.com), adopted by Pajerski et al. (Pajerski et al., 2019).

Microscope, reversed- phase high performance liquid chromatography (RP-HPLC), ultra-performance liquid chromatography are tools used to examine the peptidoglycan structure and chemical composition, and it has been observed that bacteria vary throughout their evolution thanks to these technologies (Desmarais et al., 2015). Modifications can be seen in the peptidoglycan structure to strengthen and resist antimicrobials and to prevent degradation of bacteria. It is known that there are variations in the glycan strand, peptide chain and cross-links (Vollmer et al., 2008).

2.5 Endolysin

Towards the end of the lytic life cycle, some of the bacteriophages produce an enzyme called “endolysin”, which causes the enzymatic degradation of the cell wall peptidoglycan (murein) of both Gram (+) and Gram (-) bacteria by releasing progeny virions (Schmelcher & Loessner, 2016).

Endolysins targeting Gram (+) and Gram (-) bacteria differ structurally from each other. Gram (+) targeting endolysins have a modular structure and two conserved protein domains: N-terminal enzymatically active domain (EAD) which hydrolyses peptidoglycan to enable lysis of the host cell and the C-terminal cell wall binding

domain (CBD), which is required to recognize the substrate (Loessner, 2005; Villa & Crespo, 2010). Enzymatically active domain (EAD) catalyzes the cell wall, and their classification is based on cleavage sites in peptidoglycan in Gram (+) and Gram (-) bacteria. Accordingly, there are for types of EADs: glycosidases, endopeptidases, lytic transglycosylase and amidohydrolase (Nelson et al., 2012).

Known as one type of glycosidase, N-acetylglucosaminidase acts by the reductive side of GlcNAc and is tasked with cleaving the glycan components present in peptidoglycan, an activity seen in autolysins. Another glycosidic activity is N-acetylmuramidase, which acts on the reducing site of MurNAc and cleaves the glycan component (Nelson et al., 2012).

Another group included in the classification according to enzyme activities is endopeptidases, also known as proteases. This enzyme is responsible for breaking the peptide bonds between two amino acids. Endopeptidases can be involved in the interpeptide bridge or stem peptides (Loessner et al., 1995a; Navarre et al., 1999).

Unlike other hydrolases, the third group, lytic transglycosylase, do not need water for catalysis of peptidoglycan. They are responsible for breaking the β (1→4) linkages between the N-acetylmuramyl and N-acetylglucosaminyl residues. In this respect they were similar to muramidase. The reason why lytic transglycosylases are classified in different group is that they form N-acetyl-1,6-anhydro-muramyl fragment residue while glycosidic cleavage occurs (Höltje & Tomasz, 1975).

The last group of classified for peptidoglycan hydrolase is the amidohydrolases. N-acetylmuramoyl-l-alanine amidase is involved in cleaving an essential amide bond between the glycan and peptide portions of peptidoglycan. By breaking this amide bond, the peptidoglycan becomes highly destabilized compared to hydrolysis of other bonds and is therefore evolutionarily preferred by bacteriophages (Nelson et al., 2012).

As mentioned in section 2.4, Gram-positive and Gram-negative bacteria differ in their peptidoglycan structure. Figure 2.9 below shows the points at which endolysins cleave in two different peptidoglycan structures.

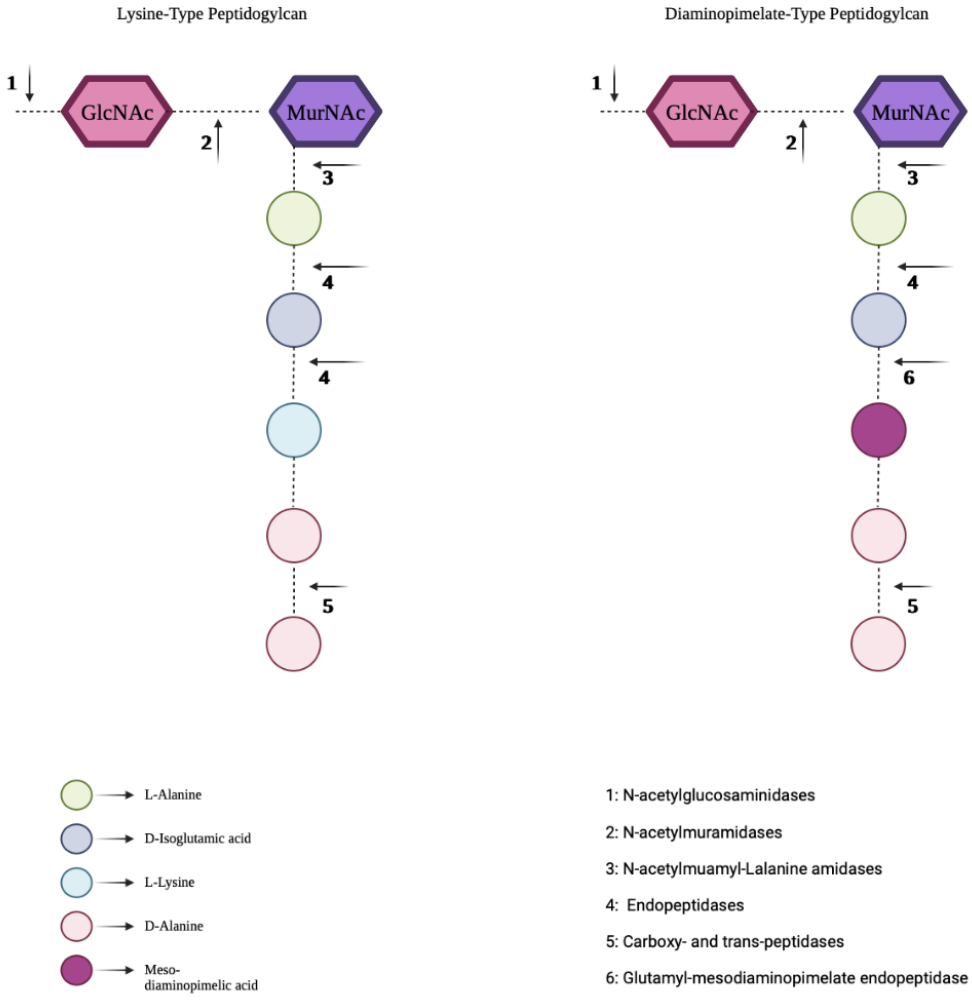

Figure 2.9 Cleavage sites of endolysins in lysine-type and diaminopimelate-type peptidoglycan structures. The left figure shows lysine-type peptidoglycan and has L-alanine at position 3, while the right one shows diaminopimelate-type peptidoglycan and has meso-diaminopimelic acid at position 3. The figure was drawn using BioRender (https://www.biorender.com), adopted by Humann and Lenz (Humann & Lenz, 2009).

Cell wall binding domain (CBD) are attached to the C-terminus by a short and flexible linker, and it recognizes the substrates bound to the specific ligand molecules in the cell wall. It is CBD that determines the specificity of endolysins because they target specific bonds on the surface of the cell wall (Oliveira et al., 2013). According to Becker et al. (Becker et al., 2008), interactions between EAD and CBD also increase specificity. The fact that they are specific, do not spoil the microflora unlike antibiotics, the possibility of bacterial resistance to endolysins is low, and do not have any adverse effect on humans and animal are proof of the high popularity of endolysins (Nelson et al., 2012). However, their narrow specificity, being serovar-specific, can make diagnosis difficult in cases where multiple bacterial infection (Dong et al., 2015).

Usage of endolysins in Gr (-) bacteria is limited due to the outer membrane (OM) structure of their cell walls (Lai et al., 2020). Peptidoglycan is located under the OM, and it prevents endolysins from reaching peptidoglycan, as OM forms a barrier. Therefore, CBD is not needed to bind to cell debris after cell lysis has taken place. A study conducted in 2002 revealed that if the CBD is semi-irreversibly bound to the bacterial cell wall, degradation of other host cells caused by endolysin is prevented (Loessner et al., 2002). These two explains the globular structure of Gram (-) targeting endolysins with a single enzymatically active domain (Schmelcher et al., 2012). However, the number of modular structures of endolysins produced by bacteriophages targeting Gr (-) bacteria has also increase, and it has been observed that these modular structures are especially defined in jumbo bacteriophages (Briers et al., 2007). PVP-SE1gp146, SPN1S_0028 and Gp110 lysins which are encoded by *Salmonella* bacteriophages, OBPgp279, KZ144 and EL188 lysins which are encoded by *Pseudomonas* bacteriophages can be given as examples (Briers et al., 2007, 2009; Park et al., 2014; Rodríguez-Rubio et al., 2016; Walmagh Maarten & Briers, 2012).

Modular Gr (-) endolysins are different from Gr (+) endolysins. Gr (-) modular endolysins contain a peptidoglycan binding domain (PBD) at the N-terminus, as well as EAD at C-terminus, which recognizes peptidoglycan composition in Gr (-) (Briers et al., 2009).

When comparing the modular and globular endolysins targeting Gr (-), it was observed that the activity of the modular ones was higher than the globular ones. This is explained by the fact that modular endolysins are responsible for the peptidoglycan binding domain that keeps enzymes close to peptidoglycan substrate. Another explanation is the increased targeting of EAD to the PG substrates in the experiment performed on globular and modular endolysins obtained from *Pseudomonas* bacteriophages (Walmagh et al., 2013; Walmagh Maarten & Briers, 2012). Even though the effect of modular endolysins is greater than that of globular ones, very few Gr (-) targeted endolysins are of modular type, for example, bacteriophage 201phi2-1-229 of *Pseudomonas chlororaphis*, bacteriophage BcepNY3, phiE202, phiE52237, Bcep78, Bcep43 and Bcep1 of *Burkholderia*, bacteriophage phiRSA1 of *Ralstonia* (Briers et al., 2009). This can be explained by the high evolutionary pressure to maintain the substrate binding site.

Compared to Gram (+) targeted endolysins, Gram (-) targeted endolysins have slower bacteria-killing kinetics. According to the study conducted in 2018, endolysin obtained bacteriophage PlyE146, an *Escherichia coli* targeted lysin, showed a killing effect after 1 hour of incubation and 3.6 log reduction after 2 hours (Larpin et al., 2018). The effect of endolysin was observed in *E. coli* cells only after 30 minutes of exposure, whereas in Gr (+) bacteria-targeted endolysins, it was observed after 5 minutes (Pastagia et al., 2013). Slow killing kinetics were explained by peptidoglycan hydrolysis following the slow first penetration of the outer membrane, that is, a two-step mechanism. Sykilinda et al. (2018) and Defraine et al. (2016) have shown that the killing effect of endolysin against *Acinobacter baumannii* starts in a few minutes and reaches a maximum in 1-2 hours (Defraine et al., 2016; Sykilinda et al., 2018). However, some studies have shown that Gr (-) endolysins can also partially kill quickly. PlyPa03 and PlyPa91 lysins affecting *Pseudomonas aeruginosa* reduced the bacterial population at 5 and 20 minutes, respectively (Raz et al., 2019).

The rapid killing kinetics of endolysins have also been associated with the internal osmotic pressure of the bacteria (Lai et al., 2020).In the study conducted against

Acinobacter baumannii, it was observed that the bacteria degraded immediately in the low ionic strength buffer system, while it was observed that the cells in the high ionic strength buffer system took a spherical shape and gradually fragmented before the cell ruptured (Defraine et al., 2016). This means that buffer choices affect the results, especially *in vitro* use of Gr (-) targeted endolysins.

Bacteriophages containing single-stranded RNA or DNA perform host lysis by inhibiting peptidoglycan synthesis (Young et al., 2000). Bacteriophages containing dsDNA, on the other hand, require a protein called holin during the lysis of the host cell to degrade peptidoglycan, in addition to endolysin (Ackermann, 2003). Holins are small membrane proteins tasked with making holes in the membrane to allow endolysins to reach its aim, while trying to cleave peptidoglycan glycosidic bonds, amide bonds or peptide bonds (Xu et al., 2005; Young et al., 2000). Following the lytic life cycle, bacteriophages produce endolysin by the holin-endolysin system towards the end of this cycle and collet in the cytoplasm. Holins create holes in the cytoplasmic membrane, through which endolysin reaches its substrate. After the peptidoglycan is broken down, there is an osmotic imbalance in the cell and cell lysis occurs (Young, 1992).

The holin-endolysin system is known as the lambda paradigm and is devoid of secretory signals (Loessner, 2005). As an alternative to the lambda paradigm, the host Sec mechanism can be used. The Sec mechanism is a mechanism that ensures the transport of bacterial proteins across the cytoplasmic membrane (Jiang et al., 2021). If N-terminal signal sequences in endolysins use the Sec mechanism, they can reach and degrade peptidoglycan without the need for holin (Fischetti, 2010).

Another alternative is the single-arrest-release (SAR) system, where the endolysins involves the indivisible N-terminal type II signal anchor (Xu et al., 2004). In this system, the N-terminal type II signal anchor is not in active form and is embedded in the inner cell membrane. But at this point, endolysins need pinholins to provide membrane depolarization to reach the host cell wall (Xu et al., 2005). In the SAR-

endolysin system, cell lysis can occur without the need any other protein such as holin (Park et al., 2007).

Due to the structure of the cell walls of Gram (-) bacteria, endolysins may not be as effective as Gram-positives ones. The outer membrane of Gr (-) bacteria is bound to the lipopolysaccharide (LPS) structure. LPS structurally contains lipid A and a highly negative charge in its inner core, and since it is polyanionic it binds cations (Vaara, 1992). The cation binding sites of LPS are electrostatically bound by holding divalent cations such as Mg^{2+} and Ca^{2+} in the outer membrane. Chemicals such as EDTA can chelate Mg^{2+} ions and destabilize the outer membrane, causing the cell wall to become permeable (Barnett et al., 2006).

Although endolysins act externally when permeabilizers are not used, studies have proven that such permeabilizers increase the effect of endolysin (Guo et al., 2017). For example, in a study conducted in 2019, it was observed that lytic activity of endolysin on *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinobacter baumannii* was better in the presence of EDTA (Antonova et al., 2019). Some endolysins have been found to function only in the presence of chemicals that will increase the permeability of the outer membrane. According to study by Bai et al., it was reported that endolysins did not show any effect in *Salmonella* and *E. coli* cells that were not treated with EDTA (Bai et al., 2019).

Apart from EDTA, it has been proven by various studies that better lysis activities of endolysins are obtained by interacting with other auxiliaries such as organic acids (malic acid and citric acid), essential oils, high hydrostatic pressure (HHP) (Briers et al., 2008; Chang et al., 2017; Oliveira et al., 2016). For example, oregano oil, containing carvacrol, was tested on *E. coli* and cleaved the outer membrane, allowing endolysin to reach peptidoglycan (Díez-Martínez et al., 2013). Similar effect of endolysins with oregano oil was also observed on *S. aureus* (Chang et al., 2017).

2.5.1 Endolysin Applications

Although bacteriophages are a promising method in the food industry, they may have some disadvantages. For example, a bacteriophage should be selected that will prevent transduction, which is one of the mechanisms that cause antibiotic resistance in bacteria (Shannon et al., 2020). In addition, they pose a threat to starter cultures in the dairy industry by slowing down fermentation and deteriorating the quality of the product (Brüssow, 2001). Therefore, in the food industry, endolysins can be used as antimicrobial candidates instead of bacteriophages. Endolysins have started to be used not only in the food industry, but also in fields such as agriculture, veterinary medicine, and medicine.

Antibiotic resistance also poses a problem in agriculture (McManus et al., 2002). Phytopathogenic bacteria threaten food security in agriculture. For this reason, endolysins have been used as a solution to ensure food safety and prevent bacterial diseases (Strange & Scott, 2005).

It has been observed that targeted endolysins of Gram (-) bacteria, *Agrobacterium tumefaciens*, have the ability to degrade this bacterium (Attai et al., 2017).

It showed lytic activity by synergistic combination of endolysins LysPN09 with EDTA, which acts against *Pseudomonas syringae* pv. *actinidiae* (Psa), that is a problem for kiwifruit production (Ni et al., 2021).

Clavibacter michiganensis, a type of bacteria that can cause cancer, was inhibited by transgenic tomatoes with CMP1 bacteriophage endolysins (Hausbeck et al., 2000). In a similar study, it was noted that transgenic potatoes were resistant to the rot-causing bacteria *Pectobacterium carotovora* (Düring et al., 1993). From this point of view, it can be said that transgenic plants can maintain food safety without need for antibiotics with the prediction mechanisms of endolysins.

Some strains of *Xanthomonas oryzae* pv. *oryzae* bacterium, which cause leaf blight in rice, have antibiotic resistance. As a result of a study conducted in 2006, it was

understood that Lys411 endolysin has lytic activity against *Xanthomonas* (Dow et al., 1996).

Staphylococcal and streptococcal bacteria pose a problem in the livestock and dairy industry (Donovan et al., 2006). It causes inflammation of the mammary gland (bovine mastitis) in cows, reducing milk quality and safety. Therefore, in 2015, Schmelcher et al. administered streptococcal bacteriophage endolysins to bovine mastitis mice (Schmelcher et al., 2015). Endolysins injected into the murine mammary duct were observed to reduce concentration of the streptococcal strains used. Likewise, to prevent mastitis caused by *Staphylococcus aureus*, mice were treated with bacteriocin lysostaphin, similar in modular structure to bacteriophage endolysins, and reductions in bacterial concentration were observed (Schmelcher, et al., 2012). The use of mice as a model and the administration of endolysins to cows may not give exactly the same results. That's why, in another study, it was stated that transgenic cows secreted lysostaphin and showed resistance to mastitis caused by *S. aureus* (Wall et al., 2005).

According to one study, when an endolysin purified from streptococcal bacteriophage was administered orally to mice, no streptococci were observed after 2 hours (Nelson et al., 2001). In a similar study, pneumococcal bacteriophage endolysin, Pal, applied as an enzyme treatment on colonized mice, killed 15 pneumococcal serotypes, including strains that were even resistant to penicillin (Loeffler et al., 2001).

The use of endolysin in the food industry is given in detail below.

2.5.1.1 Endolysin Applications in the Food Industry

2.5.1.1.1 Identification and Reduction of Pathogens

Identification of pathogens has a very important place in disease treatment and prevention. Pathogens such as *Salmonella*, *Escherichia coli*, *Listeria*

monocytogenes, *Campylobacter jejuni*, *Staphylococcus aureus*, which are quite common in the food industry, need to be determined by using inexpensive and effective methods that do not take time, and appropriate precautions should be taken (Kretzer et al., 2007). Although traditional methods or PCR are time-consuming and labor-intensive, they may not always give accurate results (Hagens & Loessner, 2007). For example, detection by PCR does not provide information about whether the cell is dead or alive. Even if the pathogen on the processed food is not viable, it will still give positive result and cause false interpretations (Rahman et al., 2021).

Cell wall binding domains (CBD) are one of the protein domains found in endolysins targeting Gram (+) bacteria and provide binding by specifically recognizing the host. Based on this, in a study conducted in 2007, the presence of *L. monocytogenes* cells was detected by using of magnetic beads coated with CBD of *Listeria* endolysins (Kretzer et al., 2007). *Listeria* cells in various artificially contaminated food samples were detected at the even serotype level and captured by magnetic beads. Meanwhile, the presence of different microorganisms in the medium did not affect the activity of the CBD-containing beads. Three years after this experiment, the detection of *Listeria* on artificially contaminated milk and cheese was attempted by binding *Listeria* endolysin CBDs to different colored fluorescent proteins (FP) (Schmelcher et al., 2010). The CBD-FP construct allowed the staining of different *Listeria* cells in culture to be detected by fluorescent microscopy. In fact, different serovars in culture could be distinguished due to specificity of the CBDs. By using CBDs constructed with colored fluorescent proteins, the presence of multiple different microorganisms in a food product can be detected without the need for conventional methods or PCR (Bai et al., 2016).

In another study, the specificity of CBDs was used for *Bacillus cereus*. By using CBD with surface plasmon resonance (SPR), *B. cereus* could be detected between 10^5 and 10^8 CFU/ml. This limit can be as low as 10^2 CFU/ml when cells are pre-incubated with CBD (Kong et al., 2015). In 2017, Kong et al., experimented with a nitrocellulose-based lateral flow test combining *Bacillus* CBDs with colloidal gold nanoparticles to develop a less costly detection method (Kong et al., 2017). This

biosensor they used was able to detect *B. cereus* et the level of 10^4 CFU/ml in a short time. A magnetic enrichment immunoassay, also based on CBD, was developed for *Staphylococcus aureus* (Yu et al., 2016). Immunomagnetic particles were coated with IgG antibodies that bind to staphylococcal protein A and were observed to capture *S. aureus* cells. Cells in contaminated milk could be detected at the $4*10^3$ CFU/ml.

Even if the presence of pathogen can be detected with antibodies, their binding specificity may not be as high as CBD and production costs are not as low as CBD. In addition, studies also support that using CBD together with different fusion proteins or fluorescent proteins is also an advantage. On the other hand, methods based on CBD are a more suitable option for Gram (+) bacteria since they don't have an outer membrane. Therefore, more studies are needed to detect Gram (-) bacteria by using endolysins (Schmelcher & Loessner, 2016).

Even though can be considered as a new method, there are studies on endolysins against various bacteria in various foods. In 2012, LysH5 endolysin against *Staphylococcus aureus* was applied in cow's milk. Compared with the control group, it was observed that the *S. aureus* concentration in milk supplemented with endolysin decreased by 8 CFU/ml (Obeso et al., 2008). In fact, it has a synergistic effect with antibacterial peptide nisin, significant decreases in bacterial concentration have occurred when both were used at the same time (García et al., 2010). Another *S. aureus* study used purified and essential oil, lean beef, skim milk and whole milk. Remarkable reductions in bacterial concentration were noted with LysSA97 endolysin used together with carvacrol, again showing a synergistic effect. Especially, against skim milk, *S. aureus* cells were below the detection limit (Chang et al., 2017). In 2017, Chang et al. applied the Methicillin-resistant *Staphylococcus aureus* (MRSA) targeted endolysin LysSA11 to both milk and ham, as well as to stainless steel blades and polypropylene plastic cutting boards. Experiments carried out at 4 ℃ and 25 ℃ showed that there was a decrease in the number of bacteria in milk and ham (2 log CFU/ml and ∼3 log CFU/ml, respectively). On the stainless-

steel blades and cutting boards, after 30 minutes endolysin application, all the bacteria were killed (Chang et al., 2017).

Listeria monocytogenes endolysins have also shown synergistic effects with other processes. For example, in 2018, a study used *Listeria* endolysin Plyp825 in combination with high hydrostatic pressure (HHP) against milk, mozzarella and smoked salmon (Misiou et al., 2018). When endolysin and HHP were used together in milk, they showed 2 log cycles more inhibition than used alone. In mozzarella, the combined use resulted in a 4 log cycles reduction in total cell count, ∼1 log more than the two treatments alone reduced. Both in combination and separately, the reduction in smoked salmon was not as effective as in milk and mozzarella. The combination of PlyP825 and HHP reduced *L. monocytogenes* concentration by only 1.6 log cycles (Misiou et al., 2018). In another study, LysZ5 endolysin reduced the concentration of *L. monocytogenes* in soy milk by 4 log CFU/ml (Zhang et al., 2012). The fact that *Listeria* is a psychotropics creates quite a problem during most food production and processing. Another *L. monocytogenes* targeted endolysin, PlyP100, was tested on fresh cheeses with delicate texture to address this problem and it has been observed that it inhibits the growth for 3 log cycles (Van Tassell et al., 2017). In addition, as a result of the application of three different endolysins used on lettuce contaminated with *L. monocytogenes*, it was observed that the number of *L. monocytogenes* decreased by 2.4 log after 6 days storage at 6 ℃ and 12 ℃ (Schmelcher et al., 2012).

In a study conducted in 2010, it was concluded that endolysin Ctl1L decreased the concentration of *Clostridium tyrobutyricum* in milk and lysed *Clostridium sporogenes* cells (Mayer et al., 2010).

As a result of fermentation of bacteria such as *Lactococcus lactis* and *Lactobacillus* spp., which are starter culture, endolysin can be produced and secreted (Chang, 2020). Apart from that, they can be added to food externally.

2.5.1.1.2 Reduction of Biofilm Formation

Biofilms are bacterial communities in the self-produced extracellular matrix on surfaces that threaten the food industry (Chang, 2020). Bacteria in biofilms show greater resistance to antimicrobials such as antibiotics and disinfectants that those that are not (Abee et al., 2011). Because endolysins target the cell walls of pathogens and perform bacterial lysis and do not produce resistant bacteria, they offer an alternative solution to biofilms (Vukotic et al., 2020).

In 2007, Sass and Bierbaum obtained that the *Staphylococcus aureus* targeted endolysin Phi11 and SAP-2 reduced biofilms formed on the polystyrene surface (Sass & Bierbaum, 2007). Another *Staphylococcus aureus* endolysin, PlyGRCS, caused the degradation of Methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms (Linden et al., 2015) Biofilm formation on surfaces such as polystyrene, glass and stainless steel was reduced by approximately 85 % with LysCSA13, a staphylococcal endolysin (Cha et al., 2019).

Similar results have been observed in some *Streptococcus* strains. For example, the biofilms of LysSMP, *S. suis* endolysin, 32 biofilm forming strains were more degraded than antibiotic and bacteriophage treatment (Meng et al., 2011). In another study, *Streptococcus pyogenes* targeted endolysin PlyC in biofilm with antibiotic resistance destroyed biofilm formation (Shen et al., 2013).

Endolysin produced by the bacteriophage vB_LmoS_293, a bacteriophage of *Listeria monocytogenes*, has been observed to prevent biofilm formation on abiotic surfaces (Pennone et al., 2019).

In another study, it was stated that *Salmonella* endolysin Lys68 reduced biofilm formation by 1 log CFU by showing a synergistic effect with malic acid and citric acid (Oliveira et al., 2014). Based on this, it can be said that endolysins also act against biofilms formed by Gram (-) bacteria.

The safety and stability of using endolysin during food production and processing have also been investigated, with promising results. In 2018, safety and toxicity studies were conducted on Cpl-1 and Pal, which are pneumococcal endolysins. In the light of the studies, no changes in physical or behavioral or fecal microbiomes, allergic reactions and hypersensitivity were found in endolysin injected mice (Harhala et al., 2018). Table 2.1 shows the applications of endolysins applied to selected foodborne pathogens.

Table 2.1 Endolysin Food Applications to the Most Common Foodborne Pathogens

Target Pathogen	**Endolysin**	**Application**	**Results**	**Reference**
Staphylococcus aureus	LysH5	Milk	After 4 h, pathogen was not detected	(Obeso et al., 2008)
	LysH5	Milk	Combination with nisin, synergistic effect was observed	(García et al., 2010)
	HydH5Lyso, HydH5SH3b, CHAPSH3B	Milk	CHAPSH3b showed the highest lytic effect both in raw and pasteurized milk	(Rodríguez-Rubio et al., 2013)
	LysSA97	Milk, Beef	Combination with carvacrol, synergistic effect was observed	(Chang et al., 2017)
	LySA11	Milk, Ham	~ 4 log CFU / cm^3 reduction was observed	(Chang, et al., 2017)
Listeria monocytogenes	PlyP825	Milk, Mozzarella	Combination with HHP, synergistic effect was observed	(Misiou et al., 2018)

Table 2.1 con't.

	Ply100	Cheese	~ 3.5 log CFU / g reduction was observed	(Van Tassell et al., 2017)
	Ply500	Iceberg Lettuce	~ 4 log CFU reduction was observed	(Solanki et al., 2013)
	LysZ5	Soya Milk	4 log CFU / mL reduction was observed	(Zhang et al., 2012)
	293-amidase	Biofilm formation	No biofilm formation was observed in polystyrene microtiter plates	(Pennone et al., 2019)

Endolysin applications against *Salmonella* is given in detail below.

2.5.1.1.3 Endolysin Applications Against *Salmonella*

Endolysin studies have been carried out to prevent *Salmonella* spp., one of the leading problems of the food industry and food safety.

In 2012, it was observed that *Salmonella* Typhimurium endolysin OBPgp279, used together with EDTA, could reduce concentration of cells 1 log in 30 minutes application (Walmagh & Briers, 2012).

According to results of another study, when used together with citric or malic acid, the thermostable *Salmonella* endolysin Lys68 killed not only *Salmonella* but also other Gr (-) bacteria such as *E. coli* O157:H7, *Shigella*, *Pseudomonas* (Oliveira et al., 2014). In fact, endolysin with organic acids has been observed to be more effective (approximately 5 log CFU/ml reduction) than its combination with EDTA.

Endolysin LysSP1 from *Salmonella* bacteriophage SLMP1, combined with 5mM EDTA, showed lytic activity against both Gr (-) and Gr (+) (Jiang et al., 2021). ε-poly-L-lysine (EPL), known as edible polymer, also makes the outer membrane of bacteria permeable (Wang et al., 2021). In a study conducted in 2019, it was stated

that LyS15S6 endolysins used together with EPL provided 2.56 log reduction at 25 ℃ and 3.14 log at 8 ℃ (Han et al., 2019).

The broader lytic activity of endolysins in combination with weak organic acids was determined by the fact that *Acinobacter baumannii* endolysin ABgp46 killed *Salmonella* Typhimurium and *Pseudomonas aeruginosa* in addition to *A. baumannii*, with the presence of 3.65 mM citric acid and 4.55 mM malic acid (Oliveira et al., 2016).

In another *Salmonella* endolysin study, encapsulation was performed by using dipalmitoylphosphatidylcholine, cholesterol and hexadecylamine (Bai et al., 2019). BSP16Lys endolysin was encapsulated and a 2.2 log CFU/ml reduction was observed in *Salmonella* Typhimurium cells, without using any other substance to increase permeability of the outer membrane.

In 2016, characterization of *Salmonella* endolysin, Gp110, which shows very high lytic activity not only against *Salmonella* but also other Gr (-) bacteria, was performed (Rodríguez-Rubio et al., 2016). Gp110 is a thermostable, modular endolysin with N-acetylmuramidase lysis activity.

In a study by Zhang et al. in 2021, a high lytic activity of endolysin LysSTG2 was observed in *S.* Typhimurium cells treated with chloroform and incubated in the presence of NaCl (Zhang et al., 2021). At the same time, biofilm formation was also reduced by 1.2 log after 1 hour of application. In the study, also, endolysin and slightly acidic hypochloric water were used in combination, and as a result of the use of these two, 99 % destruction was noted in the cells forming the biofilm (Zhang et al., 2021).

Liu et al. identified two endolysins from the LPST10 bacteriophage and studied the effect on lettuce contaminated with *S.* Typhimurium (Liu et al., 2019). It was observed that LysWL59, one of the endolysins used, was more stable and stayed active in the pH range of 6-10 and the temperature range 4-90 ℃. It was also stated

that when 0.5 mmol/L EDTA was used as the outer membrane permeabilizing agent, the cell concentration on the lettuce decreased by 93 %.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

3.1.1 Chemicals

For this study, all materials and kits were selected carefully. In Appendix A and B, the list of chemicals, materials and kits with commercial manufacturers are given.

3.1.2 Bacterial Strains and Bacteriophage

Salmonella enterica subp. *enterica* (*Salmonella*) Kentucky (MET S1-007) used in this study was isolated from chicken meat in 2015 and resistant to Kanamycin, Streptomycin, Tetracycline, Ampicillin, Sulfisoxazole and Nalidixic acid (Acar, 2015). *Escherichia coli* BL21 strain used for transformation was kindly received from METU, Department of Biology, Prof. Dr. Gülay Özcengiz's laboratory. Bacteriophage, MET P1-137, used in this study was selected from 10 different genome sequenced bacteriophages, isolated from TÜBİTAK project number 119O345. Selection criteria was based on the presence of the endolysin gene in the pure bacteriophage, detailly explained in the bioinformatic analysis section. MET P1-137 was isolated from a wastewater facility with *Salmonella* Kentucky host (MET S1-007) in 2020 (Güzel, 2022).

3.2 Methods

3.2.1 Bioinformatic Analysis of Bacteriophage and Endolysin

Bacterial and Viral Bioinformatics Resource Center (BV-BRC) tool was used for advanced bioinformatics analysis of phage MET P1-137 whose whole genome sequenced. With the help of this tool, gene regions on the phage genome were identified. In addition, virulence factor, antibiotic resistance, coding sequences (CDS) and number of tRNAs were determined. The physical and chemical properties of the endolysin such as molecular weight, amino acid composition, isoelectric point, instability index, aliphatic index, hydrophilic coefficient, extinction coefficient were estimated with online tool ProtParam (https://web.expasy.org/protparam/). Basic Local Alignment Search Tool (BLASTP) database, embedded in National Center for Biotechnology Information (NCBI) was used to find the previously identified amino acid sequence similar to amino acid sequence of the endolysin. The conserved domain of endolysin was estimated using the Pfam database (https://www.ebi.ac.uk/interpro/). The secondary structure and solvent accessibility of the protein were predicted with I-Tasser online tool. The tertiary structure of the protein was predicted with I-Tasser and Swiss-Model online tool. These online tools help to understand or predict that applicability of the endolysin.

3.2.2 Bacteriophage DNA Isolation

For further analysis of bacteriophage, bacteriophage titer was used greater than 10^8 in order to amplify to the target site. For titer determination, bacteriophage host *Salmonella* Kentucky (MET-S1-007) was inoculated into the BHI broth and incubated at 37 ℃ overnight. The next day, 100 µL of the bacteriophage solution at 4℃ was taken and placed in a 1.5 mL Eppendorf tubes containing 900 µL 0.9 % sterile NaCl. Serial dilutions were made up to 10^{-8}. A double plaque assay was performed from the last 3 dilutions. For this, 100 µL of diluted bacteriophage and

100 µL of host incubated overnight were mixed in 1.5 % semi-solid LB agar. It was then poured onto LB agar. After solidification, the Petri dishes were incubated at 37 ℃ overnight. The next day, the plaques formed were counted and multiplied by the dilution factor to determine bacteriophage titer in the main solution.

For the isolation of bacteriophage DNA, Bacteriophage Isolation Kit (NORGEN, ON, Canada) procedure was applied. 1 mL of bacteriophage with titer 10^8 was transferred to a sterile 15 mL falcon tube. To eliminate the host DNA, 10 µL of RNase-free DNase I was added and incubated for 15 minutes at room temperature. Then, it was incubated at 75 ℃ for 5 minutes for DNase inactivation. 500 µL Lysis Buffer B included in the kit was added and vortexed for 10 seconds. To increase DNA yield, 4 µL of Proteinase K was added and incubated at 55 ℃ for 15 minutes. It was then incubated at 65 ℃ for 15 minutes. During this time, the tube was inverted 2-3 times. 320 µL of isopropanol was added and vortexed. The spin column provided by the kit was placed in the collection tube and 650 µL of lysate was transferred to the column and centrifuged at 8000 rpm for 1 minute. The supernatant was discarded. The same procedure was repeated by adding 650 µL of lysate. After discarding the supernatant again, it was centrifuged at 8000 rpm for 2 minutes. 400 µL Wash Solution A was added and centrifuged at 8000 rpm for 1 minute. This process was performed 3 times in total. Then, it was centrifuged again at 14000 rpm for 2 minutes. The spin column was placed in a sterile 1.5 mL Eppendorf tube and 75 µL of Elution Buffer B was added. Centrifugation was done at 8000 rpm for 1 minute. Purified bacteriophage DNA was stored at -20 ℃ and used for bacteriophage PCR.

3.2.3 Vector Construction

3.2.3.1 Primer Designing with Restriction Enzyme and PCR Amplification

In order to amplify the insert DNA, a primer design was made by adding restriction enzymes to the beginning and end of the target region. Restriction enzymes were selected based on the enzymes found in the multiple cloning sites of the pET-28a (+)

vector, and *Eco*RI (5'-GAATTC- 3') and *Bam*HI (5'-GGATCC- 3') were used. A leader sequence, which is used to cut enzymes more efficiently, was added for both forward and reverse primers while primer was being designed. *Bam*HI was added to forward primer and *Eco*RI was added to reverse primer. The start codon, ATG, was used in forward, the stop codon, TGA, was used in reverse primer. The forward and reverse primers designed accordingly are given below.

Forward (5'→3'): AGT**GGATCC**ATGAGTTTTAAAT

Reverse (5'→3'): GTAGAATTAGTCTGA**GAATTC**TGC

The first underlined base sequence of forward primer represents the leader sequence. In reverse primer, the last 3 base sequences underlined are leader sequence. Bold sequences indicate restriction enzymes. The base sequence that is not underlined is the first 10 nucleotides of endolysins for the forward primer and the last 10 nucleotides of the endolysins for the reverse primer.

The GC content, melting temperature (T_m), self and dimer probabilities of the designed primer were checked using the Multiple Primer Analyzer tool. This information of primers is given in Appendix C. Then, using the SnapGene application, it was checked that the designed primers did not bind elsewhere in the whole genome of the bacteriophage.

Table 3.1 Master Mix Reagents for 25 μL of PCR

Component	**Volume (μL)**
ddH_2O	16.5
Buffer	2.5
$MgCl_2$	2
dNTPs	0.5
Forward	1
Reverse	1
Taq DNA polymerase	0.5
DNA template	1
Total	25

PCR conditions are as follows:

Table 3.2 PCR Conditions

Temperature (℃)	**Time**	**Number of Cycles**
95	5 min	1
95	30 sec	
55.5*	30 sec	35
72	30 sec	
72	5 min	1
4	∞	Until stop

***:** Annealing temperature (55.5 ℃) was determined by considering oligonucleotide synthesis report.

The amplified insert DNA was run in the prepared 1.5 % agarose gel at 110 V for 50 minutes. Then, the gel, which was kept in Ethidium Bromide (EtBr) solution for 5

minutes and in ddH_2O for 40 minutes, was visualized with the BIO-RAD GelDoc Go Imaging System and the results were recorded.

3.2.3.2 DNA Recovery and Purification from Gel

The purchased Qiagen QIAquick® Gel Extraction Kit (Hilden, Germany) procedure was applied for DNA purification from the gel. First of all, the gel, which was placed under UV light, was cut with the help of a sterile scalpel by taking the minimum amount of gel without damaging the DNA and transferred to an empty, sterile Eppendorf tube that was pre-weighed. Eppendorf was then weighed again with gel in it and the gel weight was determined. QG Buffer was added to 3 times the gel weight. The gel was incubated at 50 ℃ for 10 minutes. During this time, the tube was vortexed every 2-3 minutes. After the gel was completely dissolved and its color was yellow, 1 gel volume amount of isopropanol was added and mixed by inverting the tube several times. The entire mixture in Eppendorf was transferred into the spin column provided by the kit and placed in a 2 mL collection tube and centrifuged at 13000 rpm for 1 min. 500 µL of QG Buffer centrifuged at 13000 rpm for 1 min. The liquid in the collection tube was poured again and 750 µL of PE Buffer was added to the top of the column and centrifuged at 13000 rpm for 1 min. After centrifugation, the liquid in the collection tube was poured and centrifuged again for 1 min. The column was placed in a sterile 1.5 mL Eppendorf tube and 50 µL of EB Buffer was added. After waiting for 1 minute, it was centrifuged for 1 min at 13000 rpm. For checking purity and concentration, Thermo Scientific™ Multiscan Sky Microplate Spectrophotometer µDrop Plate (Waltham, MA, USA) was used. 2 µL of EB Buffer was used as a blank, and 2 µL of DNA was taken in 3 replicates. The purified DNA was stored at -20 ℃.

3.2.3.3 DNA Isolation of the Plasmid Vector

The protocol of the WizPrep™ Plasmid DNA Mini Kit (Wizbio Solutions, Republic of Korea) was applied for plasmid DNA isolation. The vector stored at -80 ℃ was inoculated on BHI agar containing kanamycin and incubated at 37 ℃ overnight. The next day, a colony was selected and placed in BHI broth and incubated overnight at 37 ℃. 1.5 mL of culture was taken from the broth and put into a sterile Eppendorf tube. It was centrifuged at 13000 rpm for 1 minute and the supernatant was discarded. 200 µL of PD1 Buffer with added RNase was taken and added by pipetting onto the pellet. Then, 200 µL of PD2 Buffer was added and the tube was mixed by inverting. After waiting for 2 minutes at room temperature, 300 µL of PD3 Buffer was added and the tube was mixed by inverting again. Centrifugation was done at 13000 rpm for 10 minutes. The spin column provided by the kit was placed in a 2 mL collection tube and the supernatant after centrifugation was added to spin column. It was centrifuged at 13000 rpm for 1 minute and the supernatant was poured out. 600 µL of Wash Buffer with ethanol was added to spin column and centrifuged at 13000 rpm for 1 minute. Centrifugation was done at 13000 rpm for an extra 2 minutes to completely dry the column. The spin column was placed in a sterile 1.5 mL Eppendorf tube and 50 µL of Elution Buffer was added on top. After waiting for 3 minutes, it was centrifuged at 13000 rpm for 1 minute. After checking for purity and concentration, plasmid DNA was stored at -20 ℃.

3.2.3.4 Digestion of the Plasmid Vector

The vector purified was digested using restriction enzymes. For this procedure, the protocol of the company New England Biolabs (NEB), from which the enzymes, *Eco*RI and *Bam*HI (New England Biolabs, Ipswich, MA, USA) were purchased, was applied. Components and quantities to be added for 50 µL reaction are given in Table 3.3.

Table 3.3 Components for 50 µL of Digestion

Component	**Quantity**
Vector	1 µg
10x NEB buffer	5 µL
*Eco*RI	1 µL
*Bam*HI	1 µL
Nuclease free water	To 50 µL

The reaction mixture prepared in a sterile 1.5 mL Eppendorf tube was incubated at 37 ℃ for 15 minutes. It was then incubated 65 ℃ for 20 minutes for *Eco*RI inactivation.

3.2.3.5 Ligation

For ligation of insert and vector, T4 ligase enzyme (Vazyme Biotech Co., Ltd., Nanjing, China) was used. For this procedure, the protocol provided by the company was applied. The components and quantities required for the ligation process are given in Table 3.4.

Table 3.4 Components for Ligation

Component	**Quantity**
Nuclease free water	10 µL
T4 ligase buffer	1 µL
Insert	0.3 pmol*
Vector	0.03 pmol*
T4 ligase enzyme	1 µL

The pmol to µL conversion was applied to determine the quantity of vector and insert:

$$\mu g\ DNA\ x\ \frac{pmol}{660\ pg}\ x\ \frac{10^6 pg}{1\ \mu g}\ x\ \frac{1}{N} = pmol\ DNA \quad (1)$$

Where N is length of DNA.

After the ligation mixture was prepared in a sterile 1.5 mL Eppendorf tube, it was incubated at 16 ℃ overnight.

3.2.4 Preparation of Competent Cells

Competent cell preparation protocol was implemented based on the protocol Li et al. with minor modifications (Li et al., 2010). *Escherichia coli* BL21 strain stored at -80 ℃ was inoculated on LB agar and incubated at 37 ℃ overnight. The next day, one colony was selected on agar and placed in 10 mL of LB broth in a 15 mL sterile falcon tube. It was incubated overnight in a shaking incubator at 37 ℃. 50 mL of LB broth was added to sterile Erlenmeyer and 250 µL of *E. coli* BL21 was placed on it. *E. coli* BL21 cells were incubated for approximately 3-4 hours in a 37℃-shaking incubator until the absorbance reached to 0.5-0.6 at OD_{600}. During this time, measurements were made with the help of a spectrophotometer so that mid-log phase would not be exceeded. As a blank LB broth was used. After the absorbance reached to 0.5, *E. coli* BL21 cells were placed in a sterile 50 mL falcon tube. After centrifugation at 4 ℃, 4000 rpm for 10 minutes, the supernatant was poured out and the tube was placed on ice. 15 mL of the $CaCl_2$ solution on ice was taken and added to the pellet by pipetting. It was kept on ice for 20 minutes. After centrifugation at 4 ℃, 4000 rpm for 10 minutes, the supernatant was discarded, and the mixture of 4 mL of the $CaCl_2$ and 15 % glycerol, kept on ice, was added to the tube. The mixture was k dispensed as 200 µL into sterile 1.5 mL Eppendorf tubes on ice. Competent cells were stored at -80 ℃ (Li et al., 2010).

3.2.5 DNA Transformation

For DNA transformation, the NEB protocol was slightly modified and applied (NEB, 2015). Competent cells from -80 ℃ were placed on ice. The ligated insert and vector, incubated at 16 ℃ overnight, were incubated at 65 ℃ for 20 minutes and then placed on ice. 50 µL of competent cells were added to 2 sterile 1.5 mL Eppendorf tubes, one as a control. 5 µL of ligated insert and vector were added to the non-control Eppendorf tube. The mixture was incubated on ice for 20 minutes. After incubation for 60 seconds in a 42 ℃ water bath, the tubes were put on ice again and left for 2 minutes. 950 µL of LB Broth was added to the tubes and incubated for 45 minutes in a 37 ℃ shaking incubator. Then, 100 µL of the mixture was taken on LB agar plates containing kanamycin and spread plate was made. Petri dishes were incubated at 37 ℃ overnight.

3.2.5.1 Colony PCR

To screen *E. coli* BL21 with recombined plasmid, colony PCR for endolysin was conducted. A colony was taken from Petri dishes with kanamycin in which colony formation was observed with the help of sterile loop and put into sterile 1.5 mL Eppendorf tube containing 50 µL of LB broth, and it was used as a template for colony PCR. Components and quantities for master mix preparation are as is section 3.2.3.1. 1 µL of 50 µL template was used for colony PCR, and if band formation was observed, the remaining 49 µL was dropped into 6 mL LB broth containing kanamycin with a pipette tip and incubated overnight in a 37 ℃ shaking incubator. The next day, it was dispersed into sterile 1.5 mL Eppendorf tubes, frozen with glycerol, and stored at -80 ℃ for protein expression.

3.2.6 Production and Purification of the Endolysin

For endolysin expression of transformed *E. coli* BL21 cells, the method of Shen et al. was slightly modified and applied (Shen et al., 2023). First, transformed samples were inoculated on LB agar containing kanamycin and incubated at 37 ℃, overnight. A colony from transformed cells was selected and inoculated into 5 mL LB Broth containing kanamycin. Samples were incubated at 37 ℃, overnight. Transformed cells were added to 50 mL of LB Broth with antibiotics prepared in autoclaved Erlenmeyer. Approximately 2.5 hours after the samples placed in the shaking incubator at 37 ℃, absorbance values of samples were measured at OD_{600} nm and when absorbance values were around 0.4-0.6, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression. The mixtures were incubated in shaking incubator at 15 ℃ for 24 hours (Shen et al., 2023).

At the end of 24 hours, all samples were taken into 1.5 mL sterile Eppendorf tubes, centrifuged at 10920 for 10 minutes, and supernatant was discarded. Samples were resuspended by taking 400 μL of 50 mM/L Tris-HCl buffer. An equal volume of glass bead was added to resuspended samples and vortexed for 2-3 minutes in order to lyse cells. To eliminate the glass beads, the bottom of the Eppendorf tubes was perforated and taken into new Eppendorf tubes and centrifuged at 1560 for 10 seconds. Centrifuged samples were passed through 0.22 μm filter.

For purification of the endolysins in the filtered proteins, Thermo Scientific HisPur™ Ni-NTA Purification Kit (Rockford, USA) protocol was applied. First of all, Equilibration Buffer (10 mM imidazole), Wash Buffer (25 mM imidazole) and Elution Buffer (250 mM imidazole) were prepared within the scope of the protocol. Then, 200 μL of protein extract and 200 μL of Equilibration Buffer were mixed in a sterile Eppendorf tube. At this time, the bottom tab of the spin column in the kit gently removed and placed in an empty collection tube and centrifuged at 2720 rpm for 2 minutes. The supernatant in the collection tube was discarded. 400 μL of Equilibration Buffer was added to the spin column and centrifuged at 2720 rpm for

2 minutes. After pouring the supernatant, the mixture containing 200 μL of protein extract and 200 μL of Equilibration Buffer was added in a spin column and kept at 4 ℃ for 30 minutes. After 30 minutes, it was centrifuged at 2720 rpm for 2 minutes. Supernatant was discarded and 400 μL of Wash Buffer was added and centrifuged at 2720 rpm for 2 minutes. This step was repeated 3 times in total. After pouring the supernatant, 200 μL of Elution Buffer was added to spin column and centrifuged at 2720 rpm for 2 minutes. This step was also repeated 3 times. For each step, new, sterile Eppendorf tube was used. Proteins were put at -20 ℃ for lytic analysis.

3.2.7 Lytic Activity of the Endolysin

For the lytic activity assay, the method of Jiang et al. was applied with a slight modification (Jiang et al., 2021). *Salmonella* Kentucky cells were incubated in 50 mL of LB Broth at 37 ℃ until absorbance of 0.6 was reached at OD_{600}. Cells were obtained by centrifugation at 9000 rpm for 15 minutes and 50 mmol/L Tris-HCl was added. The concentration of *Salmonella* cells was adjusted to 10^8 CFU/mL.

100 µL of endolysin was added onto bacteria treated with 100 µL of 0.5 mmol/L EDTA. As a negative control, 100 µL of Tris-HCl was added instead of endolysin. In order to see effect of EDTA, for one tube 100 µL of Tris-HCl, 100 µL of endolysin and 100 µL of *Salmonella* cells were prepared. The mixtures were incubated at 37 ℃ for 2 hours. After incubation, for four different groups (control *Salmonella*, Tris-HCl + EDTA, Tris-HCl + endolysin and EDTA + endolysin), serial dilutions were made.10 µL of samples with 10^7, 10^5, and 10^3 concentrations were taken and spread plate was made on Xylose Lysine Deoxycholate (XLD) agar and incubated overnight at 37 ℃ and lytic activity was checked by counting viable cells on the plate.

3.2.8 Statistical Analysis

Three inoculations were made from three different dilutions of four different groups and the results were shown as mean value ± standard deviation. All experimental

data were evaluated with one-way analysis of variance and Tukey's multiple range tests on MiniTab. ($p<0.05$).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Bioinformatic Analysis of Bacteriophage

Salmonella infecting phage MET P1-137 was previously isolated and sequenced (Güzel, 2022). According to the results of the phage genome annotated by Bacterial and Viral Bioinformatics Resource Center (BV-BRC), total genome of phage is 112355 bp with 40% GC content. It contains 524 coding sequences (CDS) and 24 tRNAs. According to a study, it was revealed that the number of tRNA contained in the phage contributes to the virulence factor of the phage (Bailly-Bechet et al., 2007). Although phages use the host's mechanism, they tend to use their own tRNA for greater fitness. There was no virulance factor in the bacteriophage genome and 2 antibiotic resistance genes were determined. Genomic map of the bacteriophage was given in Appendix D.

4.2 Determination of Features of Phage Endolysin by Bioinformatic Tools

By using BV-BRC tool, the location of endolysin was determined between 97051-97464 upstream of the phage genome. The length of the endolysin was 414 bp and including 137 amino acids. The nucleotide and amino acid sequence are given in Appendix E.1 and E.2, respectively. As a result of the ProtParam tool, the weight of the endolysin was 15.2 kDa and isoelectric point (pI) was 6.84. Isoelectric point of the protein indicates the pH value at which the total charge of the protein is zero, i.e., neutral (Tokmakov et al., 2021). The isoelectric point is defined by the combination of the pKa values of the amino acids, while the pKa is the value that tells the extent to which the amino acids are dissociated (Tokmakov et al., 2021). If a protein is at pH below the pI value, it is positively charged, and at pH above the pI value, it is

negatively charged. The repulsive forces occur between molecules with similar charges, while the balancing of positive and negative charges at the pI value leads to a decrease in the repulsive forces and the dominance of the attractive forces. As a result, agglomeration and precipitation are observed (Novák & Havlíček, 2016).

The pI value is used for processes such as protein isolation, separation, purification, and crystallization. Since it is the value at which the proteins precipitate, the target protein can be obtained by precipitating other proteins in the solution at pI values instead of the target protein (Zellner et al., 2005). Amino acid composition of the endolysin is given in Table 4.1.

Table 4.1 Amino Acid Composition of the Endolysin

Amino Acid*	**Number of Amino Acid**	**Percentage (%)**
Ala (A)	13	9.5
Arg (R)	5	3.6
Asn (N)	5	3.6
Asp (D)	11	8.0
Gln (Q)	6	4.4
Glu (E)	8	5.8
Gly (G)	12	8.8
His (H)	3	2.2
Ile (I)	7	5.1
Leu (L)	9	6.6
Lys (K)	14	10.2
Met (M)	1	0.7
Phe (F)	8	5.8
Pro (P)	4	2.9
Ser (S)	9	6.6
Thr (T)	6	4.4
Trp (W)	3	2.2
Tyr (Y)	4	2.9
Val (V)	9	6.6

***: A:** Alanine, **R:** Arginine, **N:** Asparagine, **D:** Aspartic acid, **Q:** Glutamine, **E:** Glutamic acid, **G:** Glycine, **H:** Histidine, **I:** Isoleucine, **L:** Leucine, **K:** Lysine, **M:** Methionine, **F:** Phenylalanine, **P:** Proline, **S:** Serine, **T:** Threonine, **W:** Tryptophan, **Y:** Tyrosine, **V:** Valine

In terms of amino acid composition, lysine (Lys) was the most abundant in the endolysin. The side chains of lysine have a positively charged group at one end, while backbone has long carbon tails (NCBI, 2023). Lysine can be involved in covalent interactions, hydrogen bonds or salt bridges, thereby ensuring protein stability as well (Sokalingam et al., 2012). Alanine (A) comes in second place and has a hydrophobic and nonpolar structure (Wilson et al., 2005). Alanine has a methyl group in its side chains and is attached to the α-carbon atom, therefore it is an aliphatic amino acid (Patnaik, 2012). Glycine (G) contains a single hydrogen atom in its side chain and is the simplest amino acid and has a stable structure (Albrecht & Corey, 1939). Glycine is a protein precursor that plays a role in breaking or attaching a part of a molecule to another molecule. It can be activated by post-translational modification (Razak et al., 2017).

Instability index of the endolysin was found 31.18 by ProtParam. An instability index value of less than 40 indicates that the protein has a stable structure (Gamage et al., 2019). Peptide bond stability is thought to be based on covalent bonds formed between the carbon of one of the amides and the nitrogen of the other amide (Greenberg et al., 2000). Peptide bond stability may result from the resonance of amides (Kemnitz & Loewen, 2007). The interaction between the double bonds in the carbonyl group and the carbon-nitrogen bonds causes resonance. This resonance form allows to increase stability. The protein instability index is estimated by the instability weight values of the dipeptides formed by the amides, experimentally (Enany, 2014). Protein stability generally refers to whether proteins are naturally folded or denatured as a result of the balance of net forces (Gromiha, 2010). It has

an important role for studies such as protein expression, purification, formulation, and storage (Deller et al., 2016).

Protein stability might be also affected by protein expression in the host cell, as well as in the purification steps. During protein expression, the protein may be unstable, unfolded, or proteolytically cleaved (Deller et al., 2016).The factors that cause these affects might be vector structure used during recombinant expression, temperature, and protein-host cell interaction (Papaneophytou & Kontopidis, 2014). During purification of DNA, protein stability mainly be affected by incubation temperature.

The grand average of hydropathicity (GRAVY) index was found as -0.518. The hydropathy index, first proposed by Kyte et al. in 1982, is the number that tells whether the side chains of proteins or peptides are hydrophobic or hydrophilic (Di Rienzo et al., 2021). The hydropathy index shows the interaction between water and amino acids and is obtained by summing the hydropathy values of amino acids in the protein or peptide sequence divided by the number of residues (Enany, 2014). Calculating the hydropathy index for each amino acid in the sequence is used to determine the chemical, physical and structural properties of proteins. A positive sign of GRAVY shows that the protein is hydrophobic, while minus sign indicates that the protein is hydrophilic (Kyte & Doolittle, 1982). By considering GRAVY result of the endolysin, it is hydrophilic protein i.e., it is soluble in water.

The aliphatic index is the relative volume of the protein made up of aliphatic side chains, that is, structures composed of non-aromatic and open chains (Panda & Chandra, 2012). Alanine, isoleucine, leucine, valine are the amino acids that show aliphatic properties and play an important role in the thermostability of globular proteins (Gasteiger, 2005). A higher aliphatic index means that the protein is more thermally stable. The aliphatic index of the endolysin was found as 74.09 which can be considered as thermally stable.

Extinction coefficient (ε) is a number that indicates how much a protein absorbs light at a particular wavelength. In analytic chemistry, this unit, also called molar absorptivity, is calculated based on the concentration of the protein solution, the

distance the light travels through the solution, and the absorbance at a particular wavelength (Chang & Zhang, 2017). Even if concentrations down to 100 μg / mL are sufficient for UV light absorption of proteins, accurate results may not be obtained for complex solutions containing proteins with different absorption coefficients. Other compounds, such as nucleic acid, in complex solutions absorb UV light. Therefore, protein solutions are measured at 280 nm so that other potentially interfering compounds do not lead to false results (Gill & Von Hippel, 1989). The amino acid compositions of proteins affect the extinction coefficient in the UV spectrum. While the aromatic amino acids tryptophan (W) and tyrosine (Y) play a major role in the strong absorption at 280 nm, the amino acid cysteine (C) also contributes to a small extent. Phenylalanine (F) absorbs light at lower wavelengths, 240-265 nm (Gill & Von Hippel, 1989). A high extinction coefficient indicates that the proteins of Trp, Tyr and Cys. Generally, most proteins in 1 g / L solution have an A280 nm value between 0.4-1.6 (Pace et al., 1995). The extinction coefficient of the endolysin was found as 1.472 in this study that indicates the presence of Try and Trp.

BLASTp was used to identify similarities between the amino acid sequence of endolysin with previously identified amino acids in NCBI. When we BLASTp our endolysin, considering the significance value (E value) and total score results, the most similarity was observed with our endolysin between endolysin produced by *Salmonella* phage 100268_sal2 with 100.00% percent identity (E value 5e-96, total score 283 bit) (NCBI, n.d.). This was followed by *Salmonella* phage Stitch and StG2 phages with the same percent identity, 99.27% (NCBI, n.d.). The table containing sequences producing significant alignment with E- values, percent identity, etc. from NCBI website is given in Appendix E.3.

The secondary structure of our endolysin was estimated using the I-Tasser online tool. The predicted secondary structure result is given in Figure 4.1.

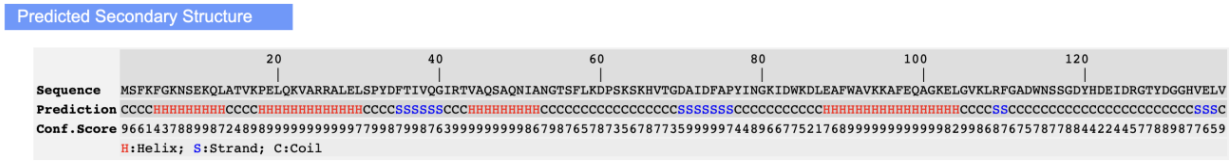

Figure 4.1 Predicted secondary structure of the endolysin.

Confidence score (3rd row) in I-Tasser ranges from 0 to 9 for each amino acid, and a higher score indicates a more confident estimation of the secondary structure. Considering the results, between the alpha helix and beta sheet structure, endolysin was mostly in the alpha helix structure (48 alpha helix, 18 beta sheet).

The secondary structure is explained by hydrogen bonds between the carboxyl oxygen and amino hydrogen atoms in the backbone of the polypeptides (Sun et al., 2004). In general, proteins have coiled or folded patterns of polypeptides. These patterns contribute to the shape of proteins (Rehman et al., 2021). The most common folds are alpha helix and beta sheet.

The alpha helix is a right-handed structure formed by hydrogen bonds between the carbonyl group and the four-residue amino group of amino acids, in which polypeptides twist like a spiral (Eisenberg, 2003). Most globular proteins have multiple alpha helix segments, while some fibrous proteins are composed entirely of alpha helices. Other secondary structure, found in the endolysin, is the beta sheet. Beta sheet consists of beta strands of different regions of polypeptide chains linked side by side by hydrogen bonds (Xu et al., 2019). Beta strands are polypeptide extensions that usually consists of 3-10 amino acids. The beta sheet structure is found in the core of the most globular proteins. Our results coincided with the literature.

A structure is formed that does not form a regular second structure except for the alpha helix and beta sheet, and therefore is not defined by any hydrogen bond model. These structures are called coils and can occur at N-terminus or C-terminus of the protein. Coils are generally 4-20 residues long and have polar or charged side chains. These evolutionarily poorly conserved regions are more prone to change (Gernert, 1994). The endolysin in this study, had 71 coil structure.

As another feature, solvent accessibility of endolysin was estimated. Solvent accessible surface area (SASA) is used to measure protein folding and stability and identifies buried or solvent exposed sites (Savojardo et al., 2021). In Figure 4.2, the predicted solvent accessibility result of endolysin is given.

Figure 4.2 Predicted solvent accessibility of the endolysin.

Solvent accessibility values range from 0 (buried residue) to 9 (exposed residue) and this property is evolutionarily conserved (Rost & Sander, 1994; Roy et al., 2010). According to the result, the solvent accessibility value of 15 amino acids of endolysin was between 5-9, while the rest was between 0-4. The region containing buried residues can be used to express the core region of the protein (Roy et al., 2010). In addition, some studies have used to solvent accessibility for the prediction of protein hydration sites (Ehrlich et al., 1998). According to a study by Chen and Zhou, it has been observed that the buried residues are sites of harmful mutations (Chen & Zhou, 2005). In this case, a mutation that may occur can alter the structure of the endolysin and affect its ability to lyse the bacteria. In another study, it was observed that the presence of proteins in the random coil structure between alpha helix and beta sheet affected solvent accessibility. It has been observed that proteins with more random coils have fewer exposed residues, which is in line with the results of this study (Khrustalev, 2020).

The tertiary structure of endolysin was estimated using the I-Tasser and Swiss – Model online tools. I- Tasser, which gives an average of 5 models, suggests that the models be analyzed according to the confidence score (C-score). The C-score is a value calculated to predict the quality of the models. The program use template alignments and structure assembly simulation convergences to calculate (Yang et al., 2015). The C-score is generally between -5 to 2, and it can be said that the model with a higher C-score is of better quality. The first model usually has a higher C-

score, and its TM-score is also considered for its estimation. The TM-score measures the structural similarity between the two structures (Zhang, 2008). The TM-score aims that the local modeling is less susceptible to possible errors and has a smaller root-mean-square deviation (RMSD) value that indicates average distance of all residue pairs in the protein structure. If the TM score is greater than 0.5, it indicates a correct topology model (Roy et al., 2010).

Figure 4.3 shows the predicted tertiary structure of the endolysin created by Swiss-Model, considering the C-score and TM-score. C-score of the model is 1.08 and TM-score of the model is 0.86 ± 0.07.

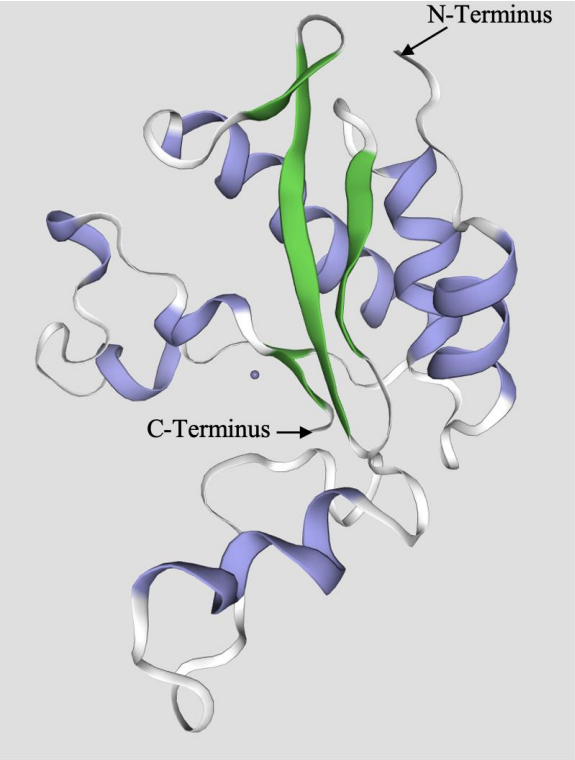

Figure 4.3 Predicted tertiary structure of the endolysin. Purple color represents α-helix, green color represents β-sheet and white color represents random coil.

According to Pfam analysis, endolysin belonged to the subfamily C of family M15 and has the conserved domain L-Ala-D-Glu peptidase, aka L-alanyl-D-glutamate endopeptidase.

The hydrolases that hydrolyze internal alpha-peptide bonds in polypeptide chains from the N-terminus or C-terminus are called endopeptidases (Rawlings & Barrett, 2014). Endopeptidases differ according to functional group in their active sites. Metallo (M) proteins represent proteins containing metal ion cofactor (Banci & Bertini, 2013). In order to maintain enzymatic activity, these proteins required divalent metal ions such as Zn^{2+}, Mn^{2+} and Ca^{2+} (Shen et al., 2023). The peptidases of the M15 family includes bacteriophage endolysins, zinc-dependent D-Ala-D-Ala carboxypeptidases and dipeptidases (Rawlings & Barrett, 1995). Peptidases in this family are involved in cell wall biosynthesis. The L-Ala-D-Glu peptidase belongs to subfamily C of the M15 family. The peptidoglycan structure consisting of short peptide chains is internally degraded by this peptidase family and lysis of the host cell takes place (Vollmer, 2015). With the rupture of the cell wall, new phages are released into the environment (Loessner et al., 1995b). Pfam tool uses the Ply118 endolysin of *Listeria* phage lysing *Listeria* cells as a reference endolysin that cleaves the cell wall between L-Ala and D-Glu residues. In this tool our endolysin shared the same family (M15C) and conserved domain (L-Ala-D-Glu peptidase) with the Ply118 endolysin. One can conclude that it is possible our endolysin use the same mechanism to lyse *Salmonella* cells (Gaeng et al., 2000).

The table below summarizes the results of the bioinformatic analysis of the endolysin.

Table 4.2 Results of Bioinformatic Analysis of the Endolysin

Features	Result
Length of the Endolysin (bp)	414
Weight of the Endolysin (kDa)	15.2
Number of the Amino Acids	137
Isoelectric Point (pI)	6.84
Instability Index	31.18
GRAVY Index	-0.518
Aliphatic Index	74.09
Extinction Coefficient (ε)	1.472
Protein Family	M15C
Domain	L-Ala-D-Glu Peptidase

Looking at the results, it can be said that it is suitable to use the endolysin in vitro studies, as it shows stable properties.

4.3 PCR Amplification of the Endolysin

As a result of PCR for amplification of the target region, a band of the desired size was observed. Figure 4.4 showed the PCR gel image of the amplified insert. Master mix was prepared for 6 samples and samples are shown as 1, 2, 3, 4, 5, and 6. The L represented the ladder used.

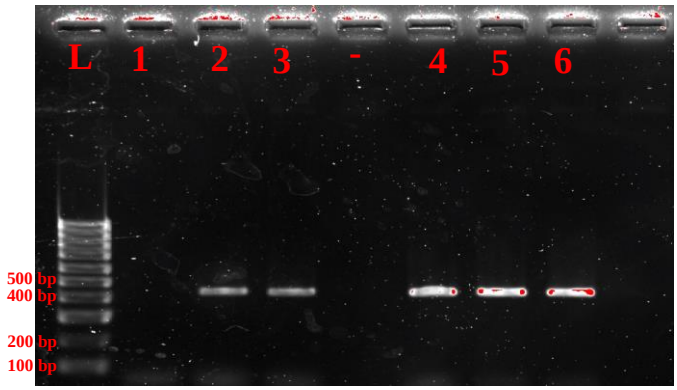

Figure 4.4 PCR gel image of amplified endolysin. 1, 2, 3, 4, 5, and 6 represented samples and L represented ladder.

According to the results of bioinformatic analysis, the size of the endolysin used in this study known as 414 bp and sum of the length of primers was 50 bp, total size of the target region was 464 bp. The ladder used was opened with 100 band sizes between 100 and 1000 bp. Accordingly, band formation was observed between the 4^{th} and 5^{th} bands. In this case, PCR was successful and the target gene region, endolysin with restriction enzymes, was amplified. The reason why no band was observed in the 1^{st} sample may be due to the insufficient amount of DNA in the PCR tube.

Negative control was placed between the 3^{rd} and 4^{th} samples. For the negative control, the master mix composition was the same, the only difference was that it didn't contain any DNA. The absence of any band indicates that there was no any foreign DNA that may cause contamination in the environment.

The gel placed under UV light was cut with the help of a clean scalpel and purified with the gel extraction kit. The DNA becomes visible under UV light due to Ethidium Bromide (EtBr). Fluorescent EtBr binds to DNA and glows under UV light (Sigmon & Larcom, 1996). The aim of the gel extraction kit was to obtain pure endolysin DNA by removing impurities from the gel and master mix. Thus, it was inserted into the vector by ligation during vector construction.

DNA concentration and purity values were measured with the help of NanoDrop. Absorbance values at A260 nm are acceptable between 0.1 and 1, with 0.1 equivalent to 5 μg/mL for double-stranded DNA (Lucena-Aguilar et al., 2016). According to the NanoDrop results, the DNA concentrations cut and extracted from the gel ranged from 2 to 6 μg/mL, which was low.

Low concentration varies according to the buffer ratios in the kit used, incubation temperatures, the size of the piece cut from the agarose gel, and the amount of DNA loaded into agarose gel (Sun et al., 2012). In this case, the recovery efficiency from extraction should be increased by optimization.

For further analysis, the DNA purity measured after extraction should be considered. The ratio of absorbance values measured at 260 and 280 nm (A260/A280) gives the DNA purity (Glasel, 1995). Having this ratio around 1.8 is an acceptable purity value for DNA. If this ratio is lower than 1.6, it indicates that there are impure substances such as protein or phenol in the environment (Rosline Hassan MMED et al., 2015). If it is more than 2, it indicates that there is RNA contamination, and DNA purity can be increased by treating it with RNAse (Manek, 2014).

Another purity measure is the A260/A230 ratio. Acceptable values of this ratio for DNA are between 2.0-2.2. If this value is less than 2, it indicates the presence of contaminants that absorb light at 230 nm, such as EDTA, lipid, salt, or phenol (Liu et al., 2009).

Since the extracted DNA of endolysin purity values were within acceptable ranges, it was used for cloning.

4.4 Vector Construction

In this study, pET-28a (+), one of the pet series expression plasmids, was used for vector construction. This plasmid series was described in the 1980s and is widely used for the production of recombinant proteins in *Escherichia coli* strains (Shilling et al., 2020).

In most of the studies, pET-28a (+) plasmid was used. With a length of 5369 bp, pET-28a (+) has the T7 promoter (binding site upstream of the gene to initiate transcription) and the lac operator sequence (Dubendorf & Studier, 1991). The T7 expression system consists of a T7 promoter and the gene that this promoter controls. The T7 promoter is recognized by the enzyme T7 RNA polymerase and is involved in the expression and detection of recombinant proteins in *E. coli* (Rong et al., 1998). T7 RNA polymerase is responsible for initiating transcription of the T7 promotor, which is under the control of the lac operator which is the negative regulatory region to which the lac repressor protein binds (Rosano & Ceccarelli, 2014). Transcription begins when the lac repressor is bound with an inducer and thus expression is not inhibited (Clark & Pazdernik, 2012).

pET-28a (+) also has the gene for resistance to the kanamycin, and it can be verified whether the transformation process was successful or not by this selectable marker (Shilling et al., 2020).

The map of the vector pET-28a (+) is given in Figure 4.5.

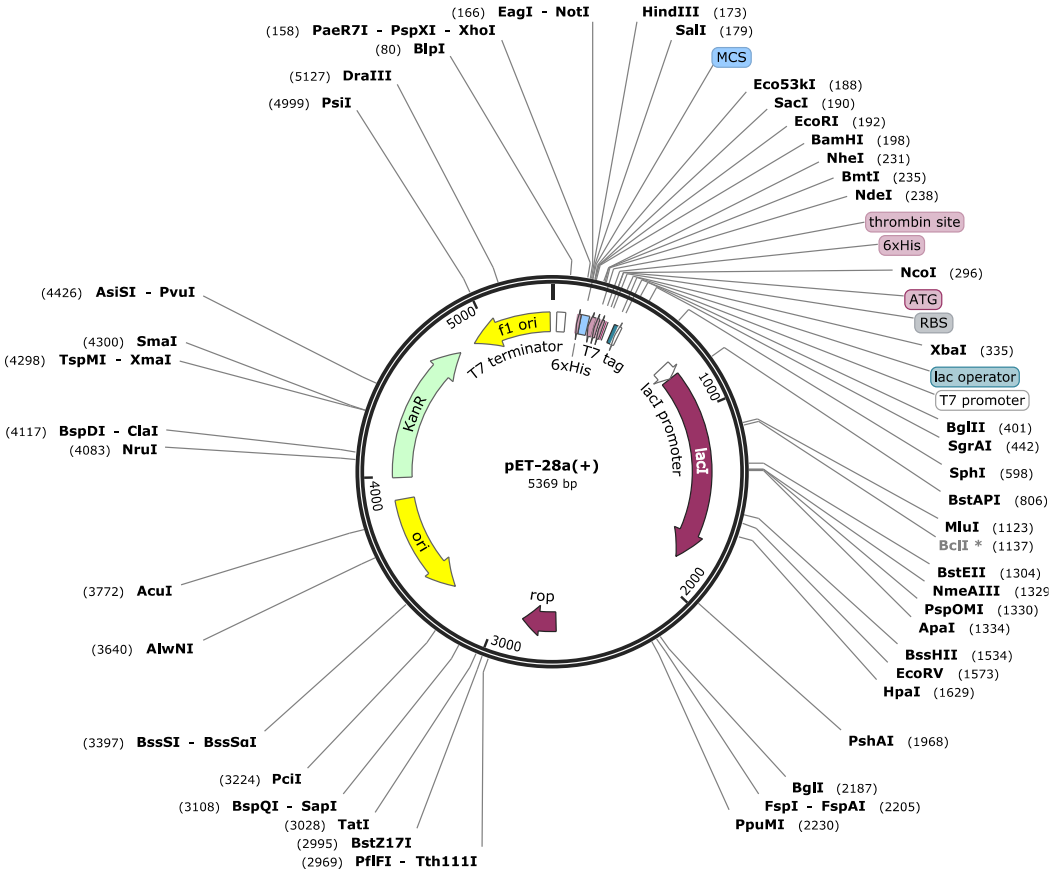

Figure 4.5 Map of the vector pET28-a (+). Retrieved from: https://www.snapgene.com/plasmids/pet_and_duet_vectors_(novagen)/pET-28a(%2B)

For the construction of the vector, two different restriction enzymes on the vector were selected. The use of two enzymes is important because it ensures that the target gene is inserted in the correct orientation after the vector has been cut, and that the plasmid prevents to bind to itself (Bello, 2016). Since the same restriction enzymes were used during primer design, it should be checked that they do not bind anywhere else in the whole genome of phage from the target region.

4.5 Transformation of the Endolysin

After the vector was constructed, transformation into *E. coli* BL21 cells was performed. *E. coli* BL21 cells, normally susceptible to the kanamycin antibiotic, would grow on LB agar containing kanamycin if transformed by the plasmid vector which has kanamycin resistance gene. Petri dishes with colonies observed as a result of the transformation process are given in Figure 4.6.

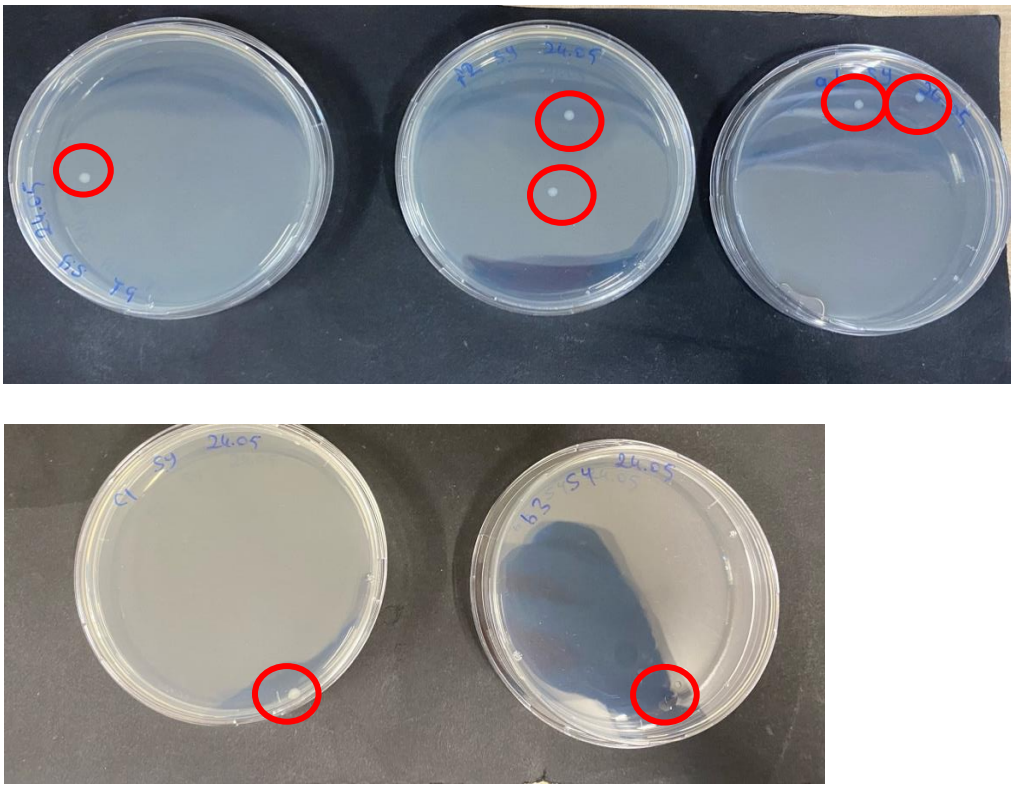

Figure 11 Petri dishes with colonies observed. Red circles represent colonies.

Each colony served as a template for colony PCR and *E. coli* BL21 cells were checked for endolysin gene. Colony observation on Petri dishes alone does not indicate that the transformation was successful, even if *E. coli* BL21 contains the vector, the vector may not be ligated with endolysin if there is a problem with the vector construction steps. Since the master mix components required for colony PCR were same as those used for the PCR amplification of the endolysin, it can be said

that transformation has occurred if a band size of the endolysin was observed. Colony PCR results are given the figure below.

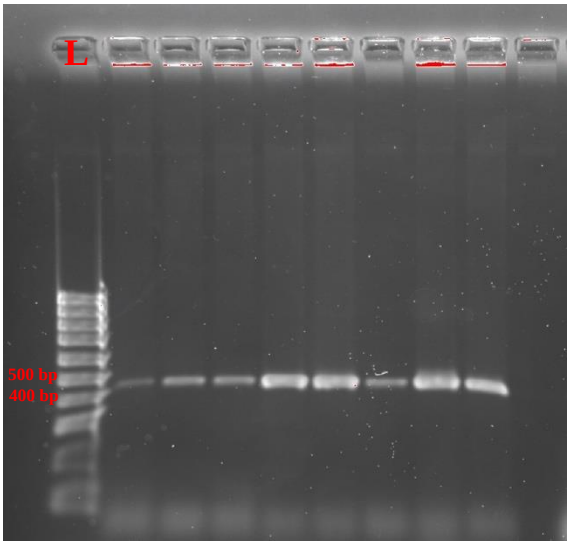

Figure 12.7 PCR gel image of colony PCR. L represents the ladder.

Samples with bands of endolysin size were grown by incubating overnight in LB Broth containing kanamycin and prepared for protein expression.

4.6 Production and Purification of the Endolysin

Transformed *E. coli* BL21 cells were incubated overnight in a LB Broth containing kanamycin for protein expression. Protein production is simply the process required for the synthesis of protein in living cells. For protein production, transcription of template DNA into mRNA and translation into protein with the information contained in the mRNA are required (Graslund et al., 2008).

In *E. coli* cells, the T7 promoter must be induced by a stimulator in order to express large amounts of proteins (Namdev et al., 2019). One of the most commonly used molecules to induce expression of recombinant proteins is isopropyl β-D-1-thiogalactopyronoside (IPTG). IPTG is a molecule that acts as a lac operon inducer.

Lac repressor proteins (LacI) are proteins that detect the presence of lactose in the environment. Normally, both the host cell (*E. coli* BL21 in this study) and the constructed vector (pET-28a (+) in this study) have lac repressor genes, and if there is no lactose in the medium, the lac repressor binds to the operator sequence in the DNA and inhibits the T7 RNA polymerase enzyme from accessing the promoter region (T7 promoter) (Hansen et al., 1998). In this case, protein expression may not occur.

If IPTG, which is structurally mimics lactose, is present in the medium, it binds to the lac repressor and the T7 RNA polymerase enzyme recognizes the T7 promoter inducing protein expression (Studier et al., 1990). The use of IPTG instead of lactose during protein expression is important in that IPTG does not appear to be a suitable substrate for lactose metabolic pathway in the cell (Tian et al., 2011). That is, IPTG cannot be used by the cell as an energy source and remains in the environment for induction.

One of the points to be considered during the study was the IPTG concentration to be added when the *E. coli* BL21 cells reached the mid-log phase. Expression levels of proteins can be regulated by testing different IPTG concentrations. For some proteins, a low concentration of IPTG increases the activity of the protein, while for others, a high concentration of IPTG is required (Sambrook et al., 1989). For *Salmonella* endolysin rLysJNwz, 0.2 mmol/L IPTG was used, and protein expression was achieved (Shen et al., 2023). On the other hand, 1 mmol/L IPTG was added, and expression was performed in two different studies (Baliga et al., 2022; Li et al., 2016). In a study conducted in 2022, different concentrations of IPTG (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mmol/L) were added for expression of the same endolysin. While no significant difference was observed between 0.2-1 mmol/L, it was observed that the yield was low at 0.1 mmol/L (Shen et al., 2022).

In this study, expression was performed with IPTG final concentration of 0.2, 0.5 and 1 mmol/L, but the lytic activity was observed at 0.5 mmol/L IPTG concentration.

0.2 mmol/L IPTG concentration may have been insufficient for the expression of endolysin produced in this study. On the other hand, 1 mmol/L IPTG may have been toxic to *E. coli* BL21 and may have affected the protein mechanism (Dvorak et al., 2015). This coincided with a study conducted in 2017. Inhibition was observed in *E. coli* BL21 cells when high amount of IPTG (1 mmol/L) was used (Browning et al., 2017).

Incubation temperature during protein expression is another factor affecting protein production. One of the most encountered situations during the production of recombinant proteins is the formation of inclusion body (IB) (Palmer & Wingfield, 2012). Generally, where protein expression occurs at high temperatures, the recombinant protein can misfold and form insoluble aggregations. These aggregations accumulate in the cell as inclusion bodies and their biological activity is quite low. Even if the inclusion bodies are eliminated in the purification steps, the protein activity may not be fully regained (Singh et al., 2015).

Using temperature below the optimum temperature during protein expression increases the solubility of polypeptides, reducing inclusion body inclusion (Vera et al., 2007). In a study conducted by Vera et al., a reduction in IB formation was observed when production of the recombinant protein in *E. coli* was performed below 37 ℃. It has even been stated that lowering the temperature does not reduce protein quality (Vera et al., 2007).

In this study, incubation was done at 15 ℃ for 24 hours to avoid IB formation for protein expression. This situation compatible with literature. Shen et al., incubated at 15 ℃ for 24 hours for the expression of *Salmonella*-targeted endolysin and no inclusion body formation was mentioned (Shen et al., 2023). In another study, the same conditions were applied to *Escherichia coli* targeted endolysins (Shen et al., 2022). The assays showed that the temperature conditions applied were suitable for protein production.

There are also studies in the literature showing the opposite of this situation. In the study of Sadeghi et al., in 2011, they achieved the best protein production of at 37 ℃

(Sadeghi et al., 2011). In a different study, a 4 hour incubation at 37 ℃ was applied for the production of *Salmonella*-targeted endolysin (Jiang et al., 2021). From this point of view, it can be said that various temperature-time combinations should be tried in order to achieve optimum production of endolysins.

After protein expression, HisPur™ Ni-NTA Purification Kit was used to purify endolysin from crude mixture. The vector pET-28a (+) has the His-tag motif consisting of 6 histidine residues at the N-terminus (Shilling et al., 2020). This DNA sequence is used when producing recombinant proteins because the histidine sequence interacts with immobilized metal ions such as nickel, cobalt, copper when the appropriate buffer is provided and helps in the separation of the target protein from the medium (Bornhorst & Falke, 2000). There are nickel ions in the spin columns of the kit and interact with the his-tag. During purification kit, imidazole agent is used to remove other proteins in the medium and increase the purity of the target protein (Hengen, 1995).

The concentration and purity (A260/A280) of the pure endolysin obtained after purification were measured with NanoDrop and measured as 12 μg/mL and 0.75, respectively. For proteins, an A260/A280 ratio of 0.6 is ideal, for values that are much higher than this value, it can be said that there might be contamination in the environment (Béguin, 2018). The concentration of endolysin was not high, a higher concentration can be obtained by changing factors such as IPTG concentration, incubation temperature and time mentioned above.

There are various methods to identify proteins, Bradford Assay, enzymatic activity assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page) are the most preferred methods (Raynal et al., 2014).

The Bradford method is widely used as quantitative method. This method considers the total protein in the sample, not the target protein, uses standards for calibration and the target protein may not match this calibration (Raynal et al., 2014). It also does not provide information about protein size. In contrast, the SDS-Page method is considered a more sensitive method compared to Bradford. Sodium dodecyl

sulfate is a detergent and has a significant effect on proteins (Hou et al., 2020). SDS molecules interact with the proteins in solution, in the presence of an electric field, change in the secondary structures of the proteins and separate them according to their size (Winogradoff et al., 2020). Western blotting method using specific antibodies can sometimes accompany SDS-Page. Protein separation can be done by using antibodies specific to the target protein in the solution (Mahmood & Yang, 2012).

The concentration of endolysin purified in this study was 12 μg/mL, a relatively low. Generally preferred minimum protein concentration for SDS-Page is 20 μg/mL. In some cases, higher concentration may be required based on the staining affinity of the protein (Mahmood & Yang, 2012).

Another way to measure the presence of protein is the activity assay. The activity analysis is specific to the target protein, if the target is purified, only that protein's existence can be tested (Raynal et al., 2014). Therefore, in this study, the lytic activity of endolysin on *Salmonella* was examined.

4.7 Lytic Activity of the Endolysin

As a result of the lytic activity assay, viable cell count was performed on XLD agar. Statistical analysis was performed and XLD agar results for all samples were given in Appendix F.1. In the figure below, lytic activity results of samples with only containing *Salmonella* cells, samples with Tris-HCl, EDTA and *Salmonella*, samples with Tris-HCl, endolysin and *Salmonella*, and samples with endolysin, EDTA and *Salmonella* were given. Detailed MiniTab results can be found in Appendix F.2.

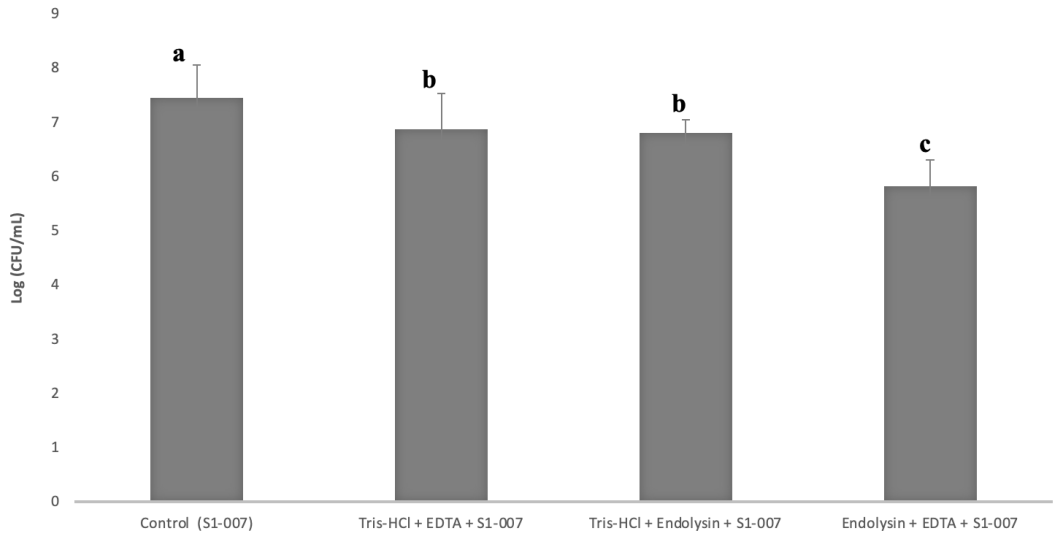

Figure 13 Lytic activity assay of endolysin. Each column represented different treatment for *Salmonella*. Each column was plotted with mean ± standard deviation of triplicate assay.

According to lytic activity assay results, a decrease in the number of viable cells was observed in the samples containing Tris-HCl + EDTA and samples containing Tris-HCl + endolysin compared to the control group containing only *Salmonella* (S1-007). The reduction for these two cases was not significantly different. On the other hand, the reduction in samples treated with endolysin + EDTA was 1 log CFU/mL and differed significantly from all samples.

The presence of phospholipids and lipopolysaccharides in the outer membrane of Gram-negative bacteria is difficult for endolysins to reach and lyse the peptidoglycan and therefore the presence of auxiliary substance is needed (Murray et al., 2021).

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent that binds to metal ions such as calcium, magnesium, lead, or iron (George & Brady, 2020). EDTA binds to the divalent cations (Ca^{2+} and Mg^{2+}) in the outer membrane to be permeable, ends when the cells become vulnerable and endolysin enters the cell, disrupting the peptidoglycan structure and causing lysis of the cell (Schmelcher et al., 2012).

EDTA is not accepted as a Generally Recognized as Safe (GRAS) according to Food and Drug Administration (FDA), but it can be used as a preservative, stabilizer, antioxidant, or texturizer in the food industry (FDA, 2022). Although it is used as a food additive there are various limitations and regulations. Since it is not considered as a GRAS, the synergistic effect of EDTA with endolysin may be limited in the food industry. Instead of EDTA, organic acids such as citric acid and malic acid, which change the outer membrane structure and ensure permeability, allow endolysins to reach peptidoglycan, can also be used (Oliveira et al., 2016).

The reason for the further reduction observed in the presence of EDTA may be that EDTA increases the permeability of the Gram-negative cell wall. In the absence of EDTA (c), even though there was a decrease in the number of *Salmonella* Kentucky, endolysin did not show much effect when used alone than with used with EDTA. The effect of endolysin in the presence of EDTA in this study was compatible with the literature. *Salmonella*-targeted endolysin showed no effect in the absence of EDTA, but showed a synergistic effect with EDTA, resulting in a 3 log reduction in viable cells (Jiang et al., 2021). In another study, endolysin was applied with different EDTA concentrations and the optimum result was achieved when 0.5 mmol / L EDTA was used (Shen et al., 2023). In a similar study, *E. coli* targeted endolysin rLysJN01 and 0.5 mmol / L EDTA were used together, and the lowest value was recorded from the OD_{600} measured absorbance values by turbidity assay (Shen et al., 2022).

The low concentration of endolysin obtained in this study may have caused the decrease in *Salmonella* concentration to be only 1 log CFU / mL. Changing the temperature-time combinations of IPTG concentrations during the production and purification steps, using other permeabilizer or optimizing the EDTA concentration for the lytic activity assay can provide more reduction.

CHAPTER 5

CONCLUSION

Misuse and overuse of antibiotics has led to a major health threat in the world in recent years and necessary precautions should be taken (Capita & Alonso-Calleja, 2013).

Bacteriophages have been proposed as an alternative solution to antibiotics. Especially those that follow a lytic life cycle become very preferred not only in the food industry, but also in the fields of medicine, agriculture, or veterinary medicine (Hagens & Loessner, 2007). However, studies show that bacteria have mechanisms to prevent viral infections by changing or losing receptors and secreting substances that prevent the phage from sticking to itself, or they can develop these mechanisms later (Principi et al., 2019). Therefore, bacteria acquire resistance to bacteriophages.

Lysogenic phages, on the other hand, can lead to the spread of antibiotic resistance genes by horizontal gene transfer when they integrate their DNA into bacteria (Chibani-Chennoufi et al., 2004). In this regard, more resistance bacteria strains may emerge. These problems cast doubt on the continued use of bacteriophages.

Endolysins, which are the enzymes produced by bacteriophages, affect the peptidoglycan structure of the bacteria. By cleaving of glycosidic bonds, hydrolyzing of peptide crosslinks, disrupting lattice structure of peptidoglycan, endolysin cause lysis of the bacteria (Young et al., 2000). Endolysins have recently gained a more important place as they have a wider range of activity compared to bacteriophages, are stable at a wider range of pH and temperature, are more resistant to enzymes or chemicals, and have a longer shelf life (Murray et al., 2021b).

This study showed that endolysin of *Salmonella* Kentucky phage MET P1-137 can be suitable for use as a result of analysis with the help of online tools. In addition, lytic activity analysis showed that endolysin had a synergistic relationship with EDTA and provides a 1 log reduction in pathogen *Salmonella* Kentucky cells.

For future studies, external factors such as optimum pH and temperature, interactions of endolysins with metal ions, different endolysin concentrations, the permeability effects of organic acids on the outer membrane will be investigated and food applications will be tried.

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APPENDICES

A. PREPARATION OF MEDIA

Table A.1 0.90% NaCl Solution

NaCl	2.25 g
dH_2O	250 mL

Table A.2 Brain Heart Infusion (BHI) Broth

BHI Medium	9.25 g
dH_2O	250 mL

Table A.3 Brain Heart Infusion (BHI) Agar

BHI Medium	18.5 g
Agar Bacteriological	7.5 g
dH_2O	500 mL

Table A.4 Luria-Bertani (LB) Broth

LB Medium	6.25 g
dH_2O	250 mL

Table A.5 Luria-Bertani (LB) Agar

LB Medium	12.5 g
Agar Bacteriological	7.5 g
dH_2O	500 mL

Table A.5 Semi-Solid Luria-Bertani (LB) Agar

LB Medium	5.25 g
Agar Bacteriological	1.5 g
dH_2O	250 mL

Table A.7 Luria-Bertani (LB) Agar with Kanamycin

LB Medium	5 g
Agar Bacteriological	3.75 g
dH_2O	250 mL
Kanamycin	0.25 mL

Table A.8 Xylose Lysine Deoxycholate (XLD) Agar

XLD Agar	26.75 g
dH_2O	500 mL

Table A.9 1.5 % Agarose Gel

Agarose	1.5 g
TBE Buffer	5 mL
ddH_2O	95 mL

Table A.10 0.5 mM Isopropyl β-D-1-thiogalactopyronoside (IPTG)

IPTG	0.12 g
ddH_2O	1 mL

Table A.6 50 mM Tris-HCl

Tris-HCl	7.88 g
ddH_2O	1 L

Table A.7 0.5 mM EDTA

EDTA	0.186 g
ddH_2O	1 L

B. CHEMICALS AND MATERIALS

Table B.1 The List of Chemicals and Materials with Their Suppliers

Chemicals	**Producers**
American Biological Agar	Condalab (Madrid, Spain)
Luria-Bertani Broth	Condalab (Madrid, Spain)
Brain Heart Infusion Broth	Condalab (Madrid, Spain)
Sodium chloride	Merck (Darmstadt, Germany)
Sterile, nuclease free water	Bioshop (Burlington, Canada)
Ethanol	ISOLAB (Eschau, Germany)
QIAquick® Gel Extraction Kit	QIAGEN (Hilden, Germany)
WizPrep™ Plasmid DNA Mini Kit	Wizbio (South Korea)
Bacteriophage DNA Isolation Kit	NORGEN (Thorold, Canada)
HisPur™ Ni-NTA Purification Kit	Thermo Scientific (Rockford, USA)

C. OLIGONUCLEOTIDE SYNTHESIS REPORT OF THE PRIMERS

Table C.8 Oligonucleotide Synthesis Report of the Primers

Primer	Sequence (5'-3')	Tm (℃)	GC (%)	nt	100 µM stok – µL TE	Self / Cross Dimer
RendF2	AGTGGATCCATGAGTTTTAAAT	53	32	22	529	Yes / No
RendR2	GCAGAATTCTCAGACTAATTCTAC	58	38	24	411	No / No

D. GENOMIC MAP OF THE PHAGE P1-137

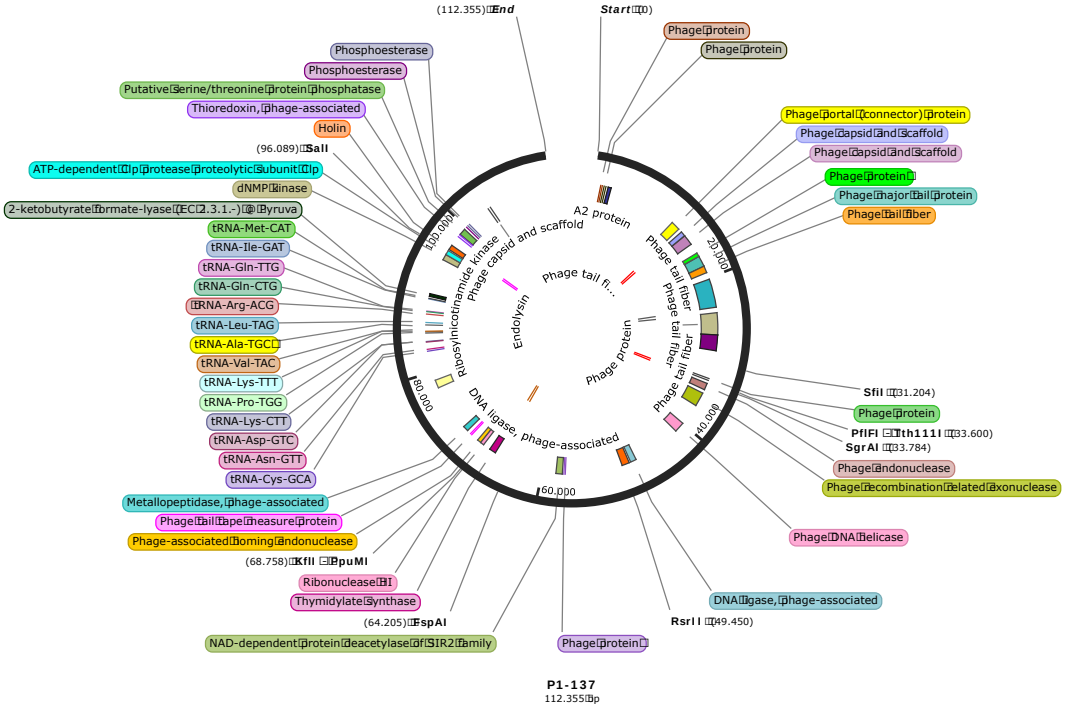

Figure D.14 Genomic map of the P1-137 drawn by SnapGene.

E. RESULTS OF BIONFORMATIC ANALYSIS OF THE ENDOLYSIN

1) Sequence of the nucleic acids of the endolysin

atgagttttaaatttggtaaaaatagcgaaaaacaattagcaactgttaagcccgagctacagaaggtagctcgtagggc
tttagaattatctccatatgatttcacaatcgtacagggtattcgtacagtagcacaaagtgcccaaaacattgctaatggta
cttcatttttgaaagaccctagcaaaagtaagcatgtaactggagacgctatcgattttgcaccatatattaatggtaagatt
gattggaaagatctggaagcattttgggctgttaagaaggcttttgaacaagctggtaaggaacttggtgtcaaacttcgt
tttggtgccgactggaatagttctggtgattatcatgatgagatcgatcgtggtacttacgacggtggtcacgtagaattag
tctga

2) Sequence of the amino acids of the endolysin

MSFKFGKNSEKQLATVKPELQKVARRALELSPYDFTIVQGIRTVAQSAQNI
ANGTSFLKDPSKSKHVTGDAIDFAPYINGKIDWKDLEAFWAVKKAFEQAG
KELGVKLRFGADWNSSGDYHDEIDRGTYDGGHVELV

3) BLASTp results of the endolysin

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
endolysin [Salmonella phage 100268_sal2]	Salmonella phage 100268_sal2	283	283	100%	5e-96	100.00%	137	YP_009320767.1
endolysin [Salmonella phage Stitch]	Salmonella phage Stitch	282	282	100%	9e-96	99.27%	137	YP_009145980.1
endolysin [Salmonella phage STG2]	Salmonella phage STG2	282	282	100%	9e-96	99.27%	137	YP_009815088.1
M15 family metallopeptidase [Salmonella enterica subsp. enterica serovar Derby]	Salmonella enterica subsp. enterica serovar Derby	281	281	100%	1e-95	99.27%	137	EDA1231321.1
endolysin [Salmonella phage S116]	Salmonella phage S116	281	281	99%	2e-95	100.00%	137	YP_009805236.1
endolysin [Salmonella phage Sepoy]	Salmonella phage Sepoy	281	281	100%	2e-95	99.27%	137	YP_009845242.1
L-alanyl-D-glutamate peptidase [Escherichia phage vB_EcoS-26175I]	Escherichia phage vB_EcoS-26175I	281	281	99%	3e-95	99.26%	137	QDJ99986.1
endolysin [Escherichia phage TrudiRoth]	Escherichia phage TrudiRoth	280	280	100%	4e-95	99.27%	137	QXV85369.1
TPA: M15 family metallopeptidase [Salmonella enterica]	Salmonella enterica	280	280	100%	7e-95	98.54%	137	HCH9411439.1
lysozyme [Salmonella phage falkor]	Salmonella phage falkor	280	280	100%	8e-95	98.54%	137	QIO01957.1
M15 family metallopeptidase [Salmonella enterica subsp. enterica serovar Hadar]	Salmonella enterica subsp. enterica serovar Hadar	280	280	99%	9e-95	99.26%	137	EDV1300475.1
endolysin [Salmonella phage S114]	Salmonella phage S114	280	280	99%	1e-94	99.26%	137	YP_009805075.1
lysozyme [Salmonella phage bobsandoy]	Salmonella phage bobsandoy	279	279	99%	1e-94	99.26%	137	QIO01452.1
endolysin [Salmonella phage bastian]	Salmonella phage bastian	279	279	99%	1e-94	98.53%	137	YP_009858684.1
lysozyme [Salmonella phage STWB21]	Salmonella phage STWB21	279	279	100%	1e-94	97.81%	137	QTJ63372.1
M15 family metallopeptidase [Salmonella enterica]	Salmonella enterica	279	279	99%	2e-94	98.53%	137	ECJ3451790.1
endolysin [Salmonella phage 2-3]	Salmonella phage 2-3	278	278	99%	2e-94	97.79%	137	YP_009851950.1
endolysin [Salmonella phage atrejo]	Salmonella phage atrejo	278	278	99%	3e-94	98.53%	137	YP_009859020.1
lysozyme [Klebsiella phage vB_Kpn_3]	Klebsiella phage vB_Kpn_3	278	278	100%	4e-94	97.81%	137	QXN67642.1
endolysin [Salmonella phage vB_SenS_UTK0009]	Salmonella phage vB_SenS_UTK0009	277	277	100%	7e-94	96.35%	137	WDR22125.1
endolysin [Escherichia phage T5]	Escherichia phage T5	276	276	100%	2e-93	96.35%	137	YP_006868.1
endolysin [Escherichia phage DT57C]	Escherichia phage DT57C	276	276	100%	3e-93	95.62%	137	YP_009149816.1
lysozyme [Escherichia phage vB_EcoS_ESCO40]	Escherichia phage vB_EcoS_ESCO40	276	276	100%	3e-93	95.62%	137	UPW38997.1
lysozyme [Salmonella phage MET_P1_100_107]	Salmonella phage MET_P1_100_107	276	276	100%	3e-93	96.35%	137	WFG41157.1
M15 family metallopeptidase [Salmonella enterica]	Salmonella enterica	275	275	100%	4e-93	95.62%	137	ECP6930427.1
endolysin [Escherichia phage vB_EcoS_AKFV33]	Escherichia phage vB_EcoS_AKFV33	275	275	100%	5e-93	95.62%	137	YP_006382340.1
M15 family metallopeptidase [Salmonella enterica]	Salmonella enterica	275	275	100%	8e-93	96.35%	137	EJT0117327.1
endolysin [Salmonella phage Stp1]	Salmonella phage Stp1	275	275	100%	9e-93	94.89%	137	ARQ96246.1
endolysin [Phage NBEco002]	Phage NBEco002	274	274	100%	1e-92	94.89%	137	YP_009856861.1
endolysin [Salmonella phage Th1]	Salmonella phage Th1	274	274	100%	2e-92	94.89%	137	YP_009849721.1
M15 family peptidase [Salmonella enterica subsp. enterica serovar Thompson]	Salmonella enterica subsp. enterica serovar Thompson	274	274	100%	2e-92	94.89%	137	EBX4970951.1
putative lysozyme [Escherichia phage JLBYU43]	Escherichia phage JLBYU43	273	273	100%	3e-92	94.89%	137	UGO55750.1
endolysin [Escherichia phage vB_EcoS_HdH2]	Escherichia phage vB_EcoS_HdH2	273	273	100%	3e-92	94.89%	137	YP_009843361.1
endolysin [Escherichia phage TrudiGerster]	Escherichia phage TrudiGerster	273	273	100%	5e-92	96.35%	137	QXV85196.1
endolysin [Salmonella phage S124]	Salmonella phage S124	272	272	100%	6e-92	94.89%	137	YP_009806171.1
M15 family metallopeptidase [Salmonella enterica]	Salmonella enterica	269	269	100%	1e-90	94.16%	137	EJB4242326.1
endolysin [Yersinia phage phiR2-01]	Yersinia phage phiR2-01	254	254	100%	1e-84	86.86%	137	YP_007237012.1
endolysin [Bacteriophage Eos]	Bacteriophage Eos	235	235	100%	3e-77	81.02%	137	QGH45158.1
endolysin [Klebsiella phage vB_Kpn_IME260]	Klebsiella phage vB_Kpn_IME260	221	221	100%	2e-71	75.18%	137	YP_009597415.1
endolysin [Klebsiella phage KPN4]	Klebsiella phage KPN4	220	220	100%	2e-71	75.18%	137	QEG11294.1
endolysin [Klebsiella phage Sugarland]	Klebsiella phage Sugarland	220	220	100%	3e-71	75.18%	137	YP_009620962.1
L-alanyl-D-glutamate peptidase [Klebsiella phage vB_KpnS_FZ41]	Klebsiella phage vB_KpnS_FZ41	219	219	100%	8e-71	74.45%	137	QCG76521.1
putative endolysin [Klebsiella phage Spivey]	Klebsiella phage Spivey	219	219	100%	1e-70	74.45%	137	QBX06998.1
endolysin [Klebsiella phage KpGranit]	Klebsiella phage KpGranit	219	219	100%	1e-70	74.45%	137	QEA03214.1
D-alanyl-D-alanine carboxypeptidase [Bacteriophage sp.]	Bacteriophage sp.	219	219	100%	1e-70	74.45%	137	UVX82006.1

endolysin [Klebsiella phage PWKp9S]	Klebsiella phage PWKp9S	218	218	100%	3e-70	74.45%	137	UJD05163.1
endolysin [Klebsiella phage vB_KpnS_Uniso31]	Klebsiella phage vB_KpnS_Uniso31	216	216	100%	1e-69	73.72%	137	UTN90347.1
endolysin [Pectobacterium phage DU_PP_V]	Pectobacterium phage DU_PP_V	205	205	100%	3e-65	65.69%	137	YP_009795235.1
endolysin [Pectobacterium phage My1]	Pectobacterium phage My1	205	205	100%	3e-65	67.15%	137	YP_006906290.1
M15 family peptidase [Salmonella enterica subsp. enterica serovar Bareilly]	Salmonella enterica subsp. enterica serovar Bareilly	198	198	100%	1e-62	66.42%	137	EBX7861961.1
M15 family peptidase [Salmonella enterica]	Salmonella enterica	198	198	100%	2e-62	66.42%	137	EAT0097321.1
L-alanyl-D-glutamate peptidase [Serratia phage vB_SmaM-Kamaji]	Serratia phage vB_SmaM-Kamaji	192	192	99%	3e-60	69.85%	137	URC22474.1
endolysin [Serratia phage Slocum]	Serratia phage Slocum	191	191	99%	9e-60	69.85%	137	QFR57638.1
endolysin [Salmonella phage Shivani]	Salmonella phage Shivani	191	191	100%	1e-59	66.42%	137	YP_009194685.1
endolysin [Salmonella phage NR01]	Salmonella phage NR01	190	190	100%	2e-59	66.42%	137	YP_009283472.1
endolysin [Escherichia phage HildyBeyeler]	Escherichia phage HildyBeyeler	188	188	100%	1e-58	65.69%	137	QXV80131.1
endolysin [Salmonella phage SP1a]	Salmonella phage SP1a	188	188	100%	1e-58	65.69%	137	ATI18557.1
endolysin [Salmonella phage Spc35]	Salmonella phage Spc35	187	187	100%	4e-58	64.96%	137	YP_004306522.1
endolysin [Escherichia phage Eps7]	Escherichia phage Eps7	167	167	58%	5e-51	98.75%	80	YP_001836966.1
M15 family metallopeptidase [Gammaproteobacteria bacterium]	Gammaproteobacteria bacterium	125	125	98%	1e-33	46.67%	132	MBS9781461.1
peptidase M15 [Gammaproteobacteria bacterium]	Gammaproteobacteria bacterium	124	124	98%	2e-33	41.73%	140	PID66617.1
M15 family metallopeptidase [Gammaproteobacteria bacterium]	Gammaproteobacteria bacterium	124	124	98%	4e-33	48.20%	145	MBS9780893.1
M15 family peptidase [[Haemophilus] felis]	[Haemophilus] felis	123	123	98%	6e-33	48.89%	137	NBI43856.1
hypothetical protein APT65_00062 [Aeromonas phage APT65]	Aeromonas phage APT65	120	120	98%	5e-32	46.67%	130	UZV39665.1
M15 family metallopeptidase [Gammaproteobacteria bacterium]	Gammaproteobacteria bacterium	120	120	98%	8e-32	45.93%	136	MBS9781097.1
M15 family metallopeptidase [Yersinia pekkanenii]	Yersinia pekkanenii	120	120	98%	1e-31	46.67%	138	WP_049615297.1
M15 family metallopeptidase [Plesiomonas shigelloides]	Plesiomonas shigelloides	120	120	99%	1e-31	47.79%	137	WP_115149345.1
M15 family metallopeptidase [Plesiomonas shigelloides]	Plesiomonas shigelloides	118	118	99%	7e-31	47.06%	137	WP_152109270.1
TPA: L alanyl D glutamate peptidase endolysin [Caudoviricetes sp.]	Caudoviricetes sp.	117	117	98%	1e-30	48.15%	135	DAS83452.1
TPA: M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	117	117	98%	1e-30	46.67%	137	HDR1007475.1
M15 family metallopeptidase [Serratia sp. DD3]	Serratia sp. DD3	117	117	97%	2e-30	45.52%	138	WP_023489981.1
TPA: L alanyl D glutamate peptidase endolysin [Caudoviricetes sp.]	Caudoviricetes sp.	117	117	98%	2e-30	48.89%	135	DAP65176.1
M15 family peptidase [Shigella sonnei]	Shigella sonnei	117	117	98%	2e-30	47.41%	137	EFX7053211.1
M15 family peptidase [[Haemophilus] felis]	[Haemophilus] felis	116	116	98%	3e-30	45.93%	137	NBI12512.1
peptidase M15 [Serratia sp. S1B]	Serratia sp. S1B	116	116	97%	4e-30	45.52%	138	PVZ79520.1
M15 family metallopeptidase [Plesiomonas shigelloides]	Plesiomonas shigelloides	116	116	99%	4e-30	46.32%	138	WP_084977912.1
TPA: L alanyl D glutamate peptidase endolysin [Caudoviricetes sp.]	Caudoviricetes sp.	116	116	98%	4e-30	48.15%	135	DAD64362.1
TPA: M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	116	116	98%	5e-30	45.93%	137	HDR1907255.1
TPA: M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	115	115	98%	5e-30	45.93%	137	HDR1216621.1
M15 family metallopeptidase [Serratia sp. JSRIV001]	Serratia sp. JSRIV001	116	116	97%	5e-30	47.76%	139	UAN43853.1
TPA: M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	115	115	98%	6e-30	45.93%	137	HDR1123389.1
M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	115	115	98%	6e-30	45.93%	137	WP_075266311.1
M15 family metallopeptidase [Gammaproteobacteria bacterium]	Gammaproteobacteria bacterium	115	115	98%	8e-30	43.17%	138	MBS9781218.1
M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	115	115	98%	8e-30	45.93%	137	WP_271344251.1
M15 family metallopeptidase [Serratia fonticola]	Serratia fonticola	115	115	97%	8e-30	45.52%	139	WP_179249003.1
M15 family peptidase [[Haemophilus] felis]	[Haemophilus] felis	115	115	98%	9e-30	45.93%	137	NBI40176.1
TPA: M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	115	115	98%	9e-30	45.93%	137	HDR1108745.1
M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	115	115	98%	1e-29	45.93%	137	WP_165544657.1
TPA: M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	115	115	98%	1e-29	45.93%	137	HDR1796630.1
M15 family metallopeptidase [Plesiomonas]	Plesiomonas	115	115	99%	1e-29	45.59%	137	WP_084976991.1
TPA: M15 family metallopeptidase [Serratia fonticola]	Serratia fonticola	114	114	97%	2e-29	46.27%	138	HBE9077628.1
M15 family metallopeptidase [Pasteurella oralis]	Pasteurella oralis	114	114	98%	2e-29	45.19%	137	WP_101774965.1

M15 family metallopeptidase [Plesiomonas shigelloides]	Plesiomonas shigelloides	114	114	99%	2e-29	45.59%	137	WP_207542759.1
M15 family metallopeptidase [Plesiomonas shigelloides]	Plesiomonas shigelloides	114	114	99%	2e-29	45.59%	138	WP_064977271.1
precorrin 3B synthase CobZ [Plesiomonas shigelloides]	Plesiomonas shigelloides	114	114	99%	2e-29	45.59%	137	SBT60190.1
M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	114	114	98%	2e-29	45.19%	137	WP_071523590.1
M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	114	114	98%	3e-29	45.19%	137	WP_151249012.1
M15 family metallopeptidase [Plesiomonas shigelloides]	Plesiomonas shigelloides	114	114	99%	3e-29	45.59%	137	WP_152117291.1
M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	114	114	98%	3e-29	45.19%	137	WP_126417815.1
TPA: M15 family metallopeptidase [Pasteurella multocida]	Pasteurella multocida	114	114	98%	3e-29	45.93%	137	HDR0673400.1

F. LYTIC ACTIVITY RESULTS OF ENDOLYSIN

1) XLD agar results of lytic activity assay

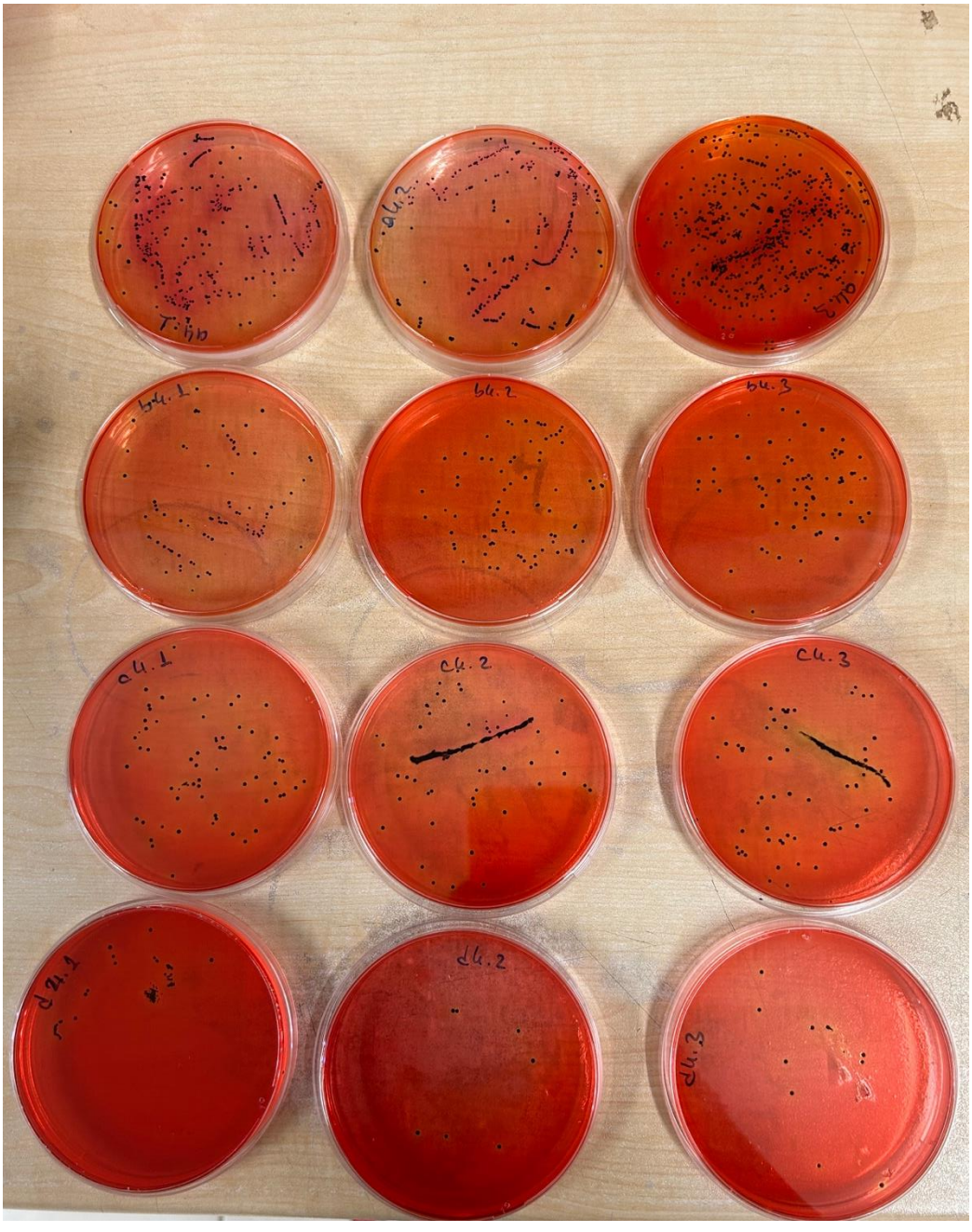

Figure F.15 XLD results. First row represents samples containing only *Salmonella*. Second row represents samples containing Tris-HCl, EDTA and *Salmonella*. Third

row represents samples containing Tris-HCl, endolysin and *Salmonella*. Last row represents samples containing endolysin, EDTA and *Salmonella*.

2) MiniTab results of lytic activity assay

Table F.9 Factor Information

Factor	Levels	Values
Sample	4	Control (S1-007), Tris-HCl+EDTA, Tris-HCl+Endolysin, Endolysin+EDTA

Table F.2 Means

	N	Mean	Std. Dev.
Control (S1-007)	3	7,45	0,60
Tris-HCl + EDTA + S1-007	3	6,88	0,66
Tris-HCl + Endolysin + S1-007	3	6,81	0,24
Endolysin + EDTA + S1-007	3	5,82	0,49

Table F.10 Tukey Pairwise Comparisons Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Control (S1-007)	3	7,45	A
Tris-HCl + EDTA + S1-007	3	6,88	B
Tris-HCl + Endolysin + S1-007	3	6,81	B
Endolysin + EDTA + S1-007	3	5,82	C

Means that do not share a letter are significantly different.