

CHARACTERIZATION AND PRODUCTION OF LYSIN FROM MET P1-301  
BACTERIOPHAGE FOR BIOCONTROL APPROACHES AGAINST  
PATHOGENIC *ESCHERICHIA COLI*

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**CHARACTERIZATION AND PRODUCTION OF LYSIN FROM  
MET P1-301 BACTERIOPHAGE FOR BIOCONTROL APPROACHES  
AGAINST PATHOGENIC *ESCHERICHIA COLI***

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## ABSTRACT

### CHARACTERIZATION AND PRODUCTION OF LYSIN FROM MET P1-301 BACTERIOPHAGE FOR BIOCONTROL APPROACHES AGAINST PATHOGENIC *ESCHERICHIA COLI*

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Today, one of the most common foodborne pathogens is *E. coli*, which is Gram negative, rod shaped, non-spore forming bacteria. However, the increasing antibiotic resistance of *E. coli* has been reported worldwide. For this reason, new searches for alternatives to antibiotics have begun. It was thought that bacteriophages which are viruses that infect and lyse living bacterial cells could be an alternative to antibiotics. Bacteriophages use their lytic enzymes, called lysins, to lyse the host cell. Lysins are produced during intracellular proliferation of phages and cause cell death by disrupting the integrity of the host cell wall. Besides, unlike the bacteriophages, lysins act quickly since they do not need to bacterial replication. Moreover, they target the conserved areas of cell wall therefore the possibility of resistance development by bacteria against lysins are low. In this study, the genome of bacteriophage MET P1-301 against *E. coli* O104:H4 was whole genome sequenced using both Illumina and Oxford Nanopore Technologies. A putative lysin from bacteriophage MET P1-301, LysEc301, belonging to the lysozyme-like superfamily, was cloned, produced, and characterized. Recombinant LysEc301 (rLysEc301) is made up of a single domain with a molecular weight of approximately 17 kDa. The

yield of rLysEc301 was around 85 ng/ $\mu$ L. In Tris-HCl buffer, the bactericidal activity of rLysEc301 against *E. coli* O104:H4, *E. coli* O157:H7, EPEC, ETEC and *Salmonella* Typhimurium was determined. It was observed that rLysEc301 alone reduced both *E. coli* O104:H4 and *E. coli* O157:H7 concentration by 3 log(CFU/mL) and *Salmonella* Typhimurium concentration by 2.5 log(CFU/mL). Besides, combination of rLysEc301 and EDTA reduced the concentration of both EPEC and ETEC by approximately 2.5 log(CFU/mL). Further analysis should be performed to investigate the stability of rLysEc301 in potential food applications.

**Keywords:** Phage-derived lysin, *E. coli*, Biocontrol, Recombinant protein

## ÖZ

### **PATOJENİK *ESCHERICHIA COLI*'YE KARŞI BİYOKONTROL YAKLAŞIMLARI İÇİN MET P1-301 BAKTERİYOFAJINDAN LİZİN KARAKTERİZASYONU VE ÜRETİMİ**

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Günümüzde gıda kaynaklı hastalıklara neden olan en yaygın patojenlerden biri Gram negatif, çubuk şekilli, spor oluşturmeyen bir bakteri olan *E. coli*'dir. Ancak dünya genelinde *E. coli*'nin antibiyotik direncinin arttığı rapor edilmiştir. Bu nedenle antibiyotiklere alternatif olarak yeni arayışlar başlamıştır. Canlı bakteri hücrelerini enfekte eden ve parçalayan virüsler olan bakteriyofajların antibiyotiklere alternatif olabileceği düşünülmüştür. Bakteriyofajlar, konakçı hücreyi parçalamak için lizin adı verilen litik enzimlerini kullanırlar. Lizinler, fajların hücre içi çoğalması sırasında üretilir ve konakçı hücre duvarının bütünlüğünü bozarak hücre ölümüne neden olur. Ayrıca, bakteriyofajlardan farklı olarak lizinler, bakteriyel replikasyona ihtiyaç duymadıkları için hızlı hareket ederler. Bunun yanı sıra, lizinler hücre duvarının korunan alanlarını hedef alırlar ve bu nedenle, bakterilerin lizinlere karşı direnç geliştirme olasılığı düşüktür. Bu çalışmada, *E. coli* O104:H4'ü enfekte eden bakteriyofaj MET P1-301'in genomu, hem Illumina hem de Oxford Nanopore Technologies kullanılarak sekanslandı. Lizozim benzeri süper aileye ait olan MET P1-301 bakteriyofajından lizin LysEc301 klonlandı, üretildi ve karakterize edildi. Rekombinant LysEc301 (rLysEc301)'in, moleküler ağırlığı yaklaşık 17 kDa'dur ve tek bir domaini bulunmaktadır. rLysEc301'in saflaştırıldıktan sonraki

konsantrasyonu 100 ng/ $\mu$ L civarındaydı. Tris-HCl tamponunda rLysEc301'in *E. coli* O104:H4, *E. coli* O157:H7, EPEC, ETEC ve *Salmonella* Typhimurium'a karşı bakterisidal aktivitesi belirlendi. rLys301'in tek başına hem *E. coli* O104:H4 hem de *E. coli* O157:H7 konsantrasyonunu yaklaşık 3 log(KOB/mL) ve *Salmonella* Typhimurium konsantrasyonunu ise yaklaşık 2,5 log(KOB/mL) azalttığı gözlemlendi. Ayrıca, rLysEc301 ve EDTA kombinasyonu, hem EPEC 6 hem de ETEC konsantrasyonunu yaklaşık 2,5 log(KOB/mL) azalttı. İleri çalışmalar, potansiyel gıda uygulamalarında rLysEc301'in stabilitesini araştırmaya odaklanmalıdır.

**Anahtar Kelimeler:** Faj türevi lizin, *E. coli*, Biyokontrol, Rekombinant protein

To my family

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## LIST OF ABBREVIATIONS

### ABBREVIATIONS

<b>PFU</b>	Plague Forming Unit
<b>CFU</b>	Colony Forming Unit
<b>EFSA</b>	European Food Safety Authority
<b>WHO</b>	World Health Organization
<b>CDC</b>	Center for Disease Control and Prevention
<b>NCBI</b>	National Center of Biotechnology Information
<b>BLAST</b>	Basic Local Alignment Search Tool
<b>EAEC</b>	Enterotoxigenic <i>Escherichia coli</i>
<b>EHEC</b>	Enterohemorrhagic <i>Escherichia coli</i>
<b>EIEC</b>	Enteroinvasive <i>Escherichia coli</i>
<b>EPEC</b>	Enteropathogenic <i>Escherichia coli</i>
<b>ETEC</b>	Enterotoxigenic <i>Escherichia coli</i>
<b>DAEC</b>	Diffusively adherent <i>Escherichia coli</i>
<b>HUS</b>	Hemolytic Uremic Syndrome
<b>STEC</b>	Shiga toxin-producing <i>Escherichia coli</i>
<b>LB</b>	Luria Bertani

## CHAPTER 1

### INTRODUCTION

Foodborne infections and foodborne deaths are a major global health concern, and bacterial pathogens are primarily responsible for the transmission of disease through contaminated food products. A foodborne outbreak is defined as the case in which two or more people experience a disease with similar symptoms because of consuming the same food (Bean et al., 1990). According to European Food Safety Authority (EFSA), in 2022, the European Union (EU) experienced 5,763 foodborne outbreaks, a 44% increase over 2021 (EFSA, 2022). The Food and Drug Administration (FDA) states that ingesting the two most prevalent forms of foodborne pathogens, viruses such as norovirus or hepatitis A; and bacteria such as *Salmonella*, *Listeria*, or *E. coli*, causes numerous outbreaks and individual cases of foodborne illness (FDA, 2023). Among these, strains of *Escherichia coli* (*E. coli*) such as O157:H7 and O104:H4 are especially dangerous because of their strong pathogenicity and link to widespread outbreaks (Griffin & Tauxe, 1991). Undercooked beef and raw vegetables have been linked to outbreaks of *E. coli* O157:H7, which is known to induce severe diarrhea and hemolytic uremic syndrome (HUS) (Ferens & Hovde, 2011). By contrast, *E. coli* O104:H4 is recognized for its distinct combination of enteroaggregative and Shiga toxin-producing capacities, which result in severe gastrointestinal infections and problems (Mora et al., 2011). To avoid future outbreaks, these infectious agents must be effectively managed in food supply chains. Strict cleanliness standards are performed at every level of food production, processing, and distribution as part of the current preventive measures. A range of techniques designed to inhibit or eradicate pathogen growth during food processing are used as precaution against foodborne diseases. A non-thermal food preservation technique called high hydrostatic pressure (HHP) uses incredibly high pressure to inactivate microbiological foodborne pathogens without significantly

impacting the nutritional and sensory qualities of the food. However, it is an expensive process (San Martín et al., 2002). Another efficient technique is irradiation, which uses X-rays, electron beams, or gamma rays to sterilize the food product or package. However, consumer adoption of radiation is weak because of false beliefs about the quality and safety of the food after radiation (Ravindran & Jaiswal, 2019). Although chemical treatments, such as the use of preservatives and disinfectants, can successfully inhibit the bacterial growth, they can also change the sensory properties of food. In addition, consumers also have concerns about long-term use of chemicals and their possible health risks (Shim et al., 2011).

Antibiotics have been used to treat bacterial illnesses and to encourage the growth of livestock. However, due to misuse and overuse, antibiotic resistance has become a severe problem, making certain pathogens resistant to standard antibiotic treatments. Antibiotic resistance claims the lives of 700,000 people each year (Clifford et al., 2018). As a result of a study commissioned by the United Kingdom government, it is predicted that if nothing is done about antimicrobial resistance, 10 million people will die annually by the year 2050 (O'Neill, 2014). Furthermore, in developing nations like our own, antibiotic resistance is a more widespread problem. Antimicrobial resistance in *E. coli* has been identified all over the world. Treating *E. coli* infections has become harder due to the emergence of resistance to most first-line antimicrobial medications (Benklaouz et al., 2020). A novel antibiotic substitute is required given the current state of increasing antibiotic resistance.

The increasing inadequacy of conventional antibiotics has driven researchers to consider bacteriophages (phages for short) as a potential substitute. Bacteriophages are the viruses that infect bacteria and reproduce within the bacteria. Due to their ability to specifically target and eliminate bacteria, phages are currently being reconsidered as an antimicrobial agent. They provide specificity. Therefore, unlike broad-range antibiotics, bacteriophage therapy does not affect beneficial bacteria (Golkar et al., 2014).

Bacteriophages have two types of life cycle: lytic and lysogenic. Applications of phages against bacterial infections use phages that follow the lytic cycle (Matsuzaki et al., 2005). A lytic cycle, sometimes referred to as the virulent life cycle, is the process by which bacteriophage first penetrate the cell then multiplies and lyse its host cell at the end of cycle (Sabour & Griffiths, 2010). Phage therapy does not, however, come without difficulties. Due to their specificity, phages need to be precisely matched to their target bacteria; also, bacteria may become resistant to phages (Górski et al., 2019).

A recently developed area of study in bacteriophage research is the application of lysins, which are enzymes produced by phages. Lysins, or bacteriophage lytic enzymes, are produced when phages multiply inside of cells. These enzymes cause damage to the cell wall especially peptidoglycan (PG) layer of cell wall, which results in cell death and the release of freshly produced viruses (Rodríguez-Rubio et al., 2016). Lysins target the conserved cell wall regions. Due to this characteristic, bacteria are less likely than antibiotics to become resistant to lysins (Pastagia et al., 2013). Lysin has no effect on humans or animals because eukaryotic cells lack the peptidoglycan layer that lysins targets (Nelson et al., 2012). Furthermore, lysins have the ability to efficiently target both Gram positive and negative bacteria (Rodríguez-Rubio et al., 2016).

In this study, it was aimed to identify the lytic enzyme of previously isolated phage (MET P1-301) against *E. coli* O104:H4, to produce the lysin using recombinant DNA technology, to purify the enzyme and finally to examine its inhibitory effect on foodborne bacteria. For this purpose, the genome of bacteriophage MET P1-301 was whole genome sequenced using both Illumina and Oxford Nanopore Technologies (ONT). Bioinformatic analysis was done to annotate the whole phage genome and to find open reading frame region belonging to the lysin enzyme. A putative lysin from bacteriophage MET P1-301, LysEc301, was found and analyzed bioinformatically to obtain functional information about LysEc301 and to predict the

structure of LysEc301. LysEc301 was recombined, produced, and characterized. The lysin gene was recombined to *E. coli* BL21 via a pET-28a(+) vector. The high lytic activity of rLysEc301 against *E. coli* O104:H4, *E. coli* O157:H7, EPEC, ETEC and *Salmonella* Typhimurium was observed.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Foodborne Pathogens

Since ancient times, there has been knowledge of the connection between human diseases and food consumption. Hippocrates (460–470 BC) was the first to record this connection (Pappas et al., 2008). Numerous different types of microorganisms can be found in food. Some of them preserve food's natural life activities, some are employed in food production such as fermented food production, on the other hand, plenty of them lead to food spoiling or food-borne illnesses (Lorenzo et al., 2018). Foodborne pathogens are hazardous bacteria, viruses, parasites, or fungus that contaminate food and cause foodborne illness, sometimes known as food poisoning. These pathogens can enter the food supply chain at any point, from production and processing to preparation and serving (Martinović et al., 2016). Foodborne diseases are clinical problems that are typically associated with the consumption of foods contaminated with microbes that are pathogenic or toxins produced by bacteria and are characterized by gastrointestinal symptoms. Common symptoms of foodborne illnesses include nausea, vomiting, abdominal cramps, diarrhea, fever, and dehydration (Ermenlieva et al., 2018).

Intoxication is a disease caused by toxins produced by pathogens that contaminate food in a variety of ways and accumulate in it during their development. Food-borne infection is an active infection that arises when the food contaminated with pathogenic bacteria is consumed by humans (Addis & Sisay, 2015).

Viruses are microscopic non-living entities in nature and can only reproduce in living cells. They cannot, therefore, survive for extended periods of time outside the host.

Foodborne illnesses have been linked to over 100 different types of enteric viruses; the most prevalent viruses that cause foodborne illnesses are Noroviruses and Hepatitis A (Koopmans & Duizer, 2004).

Hepatitis A virus particles are resilient organisms that can spread through direct or indirect human contact, contaminated food, water, and environmental surfaces (Mbithi, et al., 1992). Hepatitis A outbreaks in 2003 caused nearly 1,000 cases of sickness in several states as well as three fatalities. Green onions imported from four farms in Mexico, where hepatitis A is widespread, were connected to outbreaks; as a result, the FDA prohibited imports from these farms (Wheeler et al., 2005).

Most cases of acute viral gastroenteritis, including an estimated 5.4 million foodborne diseases incidents in the US each year, are caused by Norovirus. Noroviruses are single-stranded RNA viruses that are nonenveloped. The virus may survive in temperatures ranging from below freezing to 60°C (Scallan et al., 2011). It can also persist on foods and surfaces in the environment, which accelerates its spread, especially through secondary transmission to family members or food handlers (Glass et al., 2009). Norovirus outbreaks are common throughout the European Union, and it can be challenging to determine whether epidemics that appear to be unrelated to one another are related. For instance, over the course of three months, six outbreaks connected to frozen raspberries were examined individually. The contaminated raspberries were initially thought to not be from the same batch and came from a single Serbian producer. Molecular investigations revealed that the raspberries responsible for six different outbreaks were genuinely infected from the same source. Furthermore, alterations were made to Danish legislation requiring professional catering facilities to heat-treat frozen raspberries (Müller et al., 2015).

Parasites are unicellular microorganisms with a well-organized nucleus but a permeable cell wall. Compared to bacteria, they are larger. They only multiply in

hosts, just as viruses, not in food. They could spread from animals to animals, from humans to animals, or from humans to humans. A number of parasites have come to attention as important foodborne pathogens. The tissues and organs of ill humans and animals are home to these organisms (Dorny et al., 2009). *Toxoplasma gondii*, *Trichinella spiralis*, and *Cyclospora cayetanensis* are the three most prevalent foodborne parasites. 41 cases of congenital toxoplasmosis and 156 confirmed cases of trichinellosis were reported in the European Union in 2015. The countries with the greatest reporting rates were Lithuania, Romania, and Bulgaria respectively (EFSA, 2016).

Foodborne illnesses are most frequently caused by bacteria, which come in a wide range of forms, characteristics, and kinds. Certain potentially risky bacteria, such as *Clostridium botulinum*, *C. perfringens*, *Bacillus subtilis*, and *Bacillus cereus*, are extremely heat-resistant due to their ability to generate spores (Brown, 2000). Certain bacteria, like *Clostridium botulinum* and *Staphylococcus aureus*, can produce toxins that are resistant to heat (Rose et al., 1988). Most infections develop best in temperatures between 20 °C and 45 °C, making them mesophilic. Nonetheless, some foodborne pathogens, often known as psychrotrophs, can grow in refrigerators or at temperatures below 10 °C. Examples of these pathogens are *Yersinia enterocolitica* and *Listeria monocytogenes* (Azizoglu & Kathariou, 2010). According to the U.S. Food and Drug Administration (FDA), it is estimated that each year roughly 48 million Americans get sick from foodborne diseases, leading to approximately 128,000 hospitalizations and 3,000 deaths (FDA, 2022).

Even though there are numerous bacteria that can lead to foodborne illnesses and intoxications, *Salmonella* species, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *Campylobacter* species, *Clostridium* species, *Escherichia coli* O157:H7, *Shigella* species, *Yersinia enterocolitica*, *Vibrio* species, and *Brucella* species are the most significant and prevalent ones (Tao et al., 2020).

### 2.1.1 *Escherichia coli*

*Escherichia coli* is a rod-shaped, gram-negative bacteria that does not form spores. It could be mobile or immobile; certain rods have flagella, while others do not have. *Escherichia coli* is a facultative anaerobe that ferments simple carbohydrates like glucose to produce lactic, formic, and acetic acid. While growth can happen at pH values as low as 4.3 and as high as 9 to 10, the ideal pH range for growth of *Escherichia coli* is 6.0 to 8.0 (Jang et al., 2017).

The majority of *Escherichia coli* are non-pathogenic and are found in both human and animal intestines, where they support intestinal health. On the other hand, consuming food contaminated with specific strains of *Escherichia coli* can result in mild to severe gastrointestinal diseases (Kaper et al., 2004).

When food or water that has been contaminated by the feces of humans or animals is consumed, pathogenic *Escherichia coli* can cause disease in human beings. Animal products are frequently contaminated when they are processed and slaughtered. Irrigation water can get contaminated when agricultural crops are fertilized with feces from cattle or other animals. *Escherichia coli* can multiply in meats, vegetables and other foods and live in the environment for extended periods of time (Allocati et al., 2013).

Considering the pathogenic mechanism as a guide, six well-defined families of pathogenic *Escherichia coli* have been identified: Enterotoxigenic *Escherichia coli* (ETEC), Enteropathogenic *Escherichia coli* (EPEC), Enteroinvasive *Escherichia coli* (EIEC), Diffusely adherent *Escherichia coli* (DAEC), Enteroaggregative *Escherichia coli* (EAaggEC), and Enterohemorrhagic *Escherichia coli* (EHEC, also known as Shiga toxin-producing *Escherichia coli* (STEC)) (Kaper et al., 2004).

The different pathotypes of *Escherichia coli* are clonal groups that are distinguished by shared O (lipopolysaccharide) and H (flagellar) antigens that define serogroups (which include only O antigen) or serotypes (which contain both O and H antigens).

Enterotoxigenic *Escherichia coli* (ETEC) is the source of watery diarrhea, which can vary in severity from a minor self-limiting illness to a serious purging illness. The organism is the primary cause of diarrhea in visitors to underdeveloped nations and is a major contributor to diarrhea in children in the developing world. Enterotoxins, which are produced by ETEC when it colonizes the mucosa on the surface of the small intestine, are the source of intestinal secretions (Kaper et al., 2004).

The pathotype of *Escherichia coli* that was initially identified was enteropathogenic. Even though significant outbreaks of child diarrhea linked to EPEC have mostly disappeared from developed countries, EPEC is still a significant contributor to often fatal infant diarrhea in economically disadvantaged countries. Diarrhea is caused by EPEC bacteria adhering to the intestinal mucosa of infants and animals. The serotypes O55:H7 are the cause of EPEC, which is a rare event that can arise from contaminated food (Pakbin et al., 2021).

Enteroinvasive *Escherichia coli* (EIEC) are biologically, chemically, and pathogenically similar to *Shigella* species. *Shigella* and *E. coli* are taxonomically identical at the species level; however, some key biochemical investigations separate EIEC from *Shigella*. EIEC are facultative intracellular pathogenic bacteria that enter the body through the consuming contaminated food or water which were contaminated with feces. It results in an imbalance in the gut and uses the large intestine's epithelial cells' intracellular environment as a place to reside. This produces moderate watery diarrhea, anorexia, and fever. There are 21 serotypes in Enteroinvasive *Escherichia coli* (Govindarajan et al., 2020).

Diffusely adherent *Escherichia coli* (DAEC) is an organism that can cause diarrhea, especially in children. It is distinguished by its unique way of adhering to epithelial cells. These bacteria are known for their persistent and diffuse adhesion to the intestinal cell surface, which is made possible by certain adhesins like Afa/Dr adhesins. Diarrhea cases across a range of demographic groups have been linked to DAEC strains, indicating a widespread effect on gastrointestinal health. The creation of vaccines as well as customized treatments is made more difficult by the genetic variety among DAEC strains, which points to several evolutionary pathways (Pakbin et al., 2021).

In developing nations especially, enteroaggregative *Escherichia coli* (EAEC) is an emerging pathogenic group that is known to cause diarrhea in all age groups, both acute and persistent. EAEC can be identified using the HEp-2 cell adhesion assay, which is typified by its "stacked brick" aggregative adherence pattern to epithelial cells. Typical EAEC strains are characterized by the presence of the global virulence gene regulator AggR regulon, which controls the production of several virulence components. EAEC infections are frequently associated with diarrhea that lasts longer than 14 days, which has a major negative influence on the growth and health of children (Govindarajan et al., 2020).

A subset of pathogenic *Escherichia coli* strains known as Enterohemorrhagic *Escherichia coli* (EHEC) or Shiga toxin-producing *Escherichia coli* (STEC) is distinguished by its capacity to cause severe foodborne illness (Griffin & Tauxe, 1991). EHEC infections frequently cause bloody diarrhea, which can develop into hemolytic uremic syndrome (HUS), a potentially fatal condition that is more common in young people and the elderly (Nguyen & Sperandio, 2012). EHEC's pathogenicity is mostly related to the production of Shiga toxins. EHEC is usually spread by eating contaminated food, especially raw milk, fresh produce, and undercooked ground beef (Muniesa et al., 2006).

Two dangerous strains of the bacteria *Escherichia coli*, known as *Escherichia coli* O157:H7 and *Escherichia coli* O104:H4, are known to cause serious foodborne infections. It is commonly known that *E. coli* O157:H7 can result in hemolytic uremic syndrome (HUS), hemorrhagic colitis, and severe diarrhea, especially in young children and the elderly (Griffin & Tauxe, 1991). Shiga toxins, which are principally responsible for intestinal lining destruction and can result in systemic problems such renal failure, are produced by this strain (Rangel et al., 2005).

Due to its prevalence in undercooked beef and other contaminated foods, *E. coli* O157:H7 was initially recognized as a serious pathogen during an outbreak from contaminated hamburgers in 1982 (Lim, Yoon, & Hovde, 2010). Conversely, *E. coli* O104:H4 came to the attention of the world after a significant outbreak in Germany in 2011 that was linked to fenugreek sprouts. Between May and September 2011, the outbreak resulted in about 3,000 cases, with a median age of 46 years, and 18 deaths among the patients. Unlike O157:H7, O104:H4 is characterized by the production of Shiga toxin along with an enteroaggregative adherence pattern, causing prolonged diarrhea and HUS (Frank et al., 2011).

The epidemiology of *E. coli* O157:H7 indicates that ruminants, particularly cattle, are a key reservoir for this infection and are mostly associated with it. *E. coli* O157:H7 primarily infects humans when they consume contaminated food or drink or come into direct contact with the feces of infected animals (Ferens & Hovde, 2011). However, the O104:H4 strain complicates preventative methods as it is more frequently associated with contaminated plant food products than animal reservoirs. Antimicrobial resistance of *E. coli* is an emerging and significant public health problem. Besides, *E. coli* climbed from fourth place in 2017 to second place in 2024 in the “Bacterial Priority Pathogens List” made by World Health Organization (WHO) (WHO, 2024). In addition, WHO has stated that the reported resistance rate for third generation cephalosporin-resistant *E. coli* in 76 countries is 42%, which is a major concern (WHO, 2023).

## **Antibiotic Resistance**

Antibiotics are compounds used to prevent and treat bacterial infections, and they have revolutionized medical practice since they were developed, saving countless lives. Alexander Fleming discovered penicillin, the first genuine antibiotic, in 1928, representing an important era in medical history. Fleming discovered that *Penicillium* mold prevented the growth of bacterial colonies, which led to the invention of penicillin as an antibiotic (Walsh, 2003). This unintentional finding started in the age of antibiotics and supported the additional study into chemicals that could kill or restrict the growth of bacteria. Based on the sort of the target and how they kill or stop bacterial development, antibiotics are divided into various categories. Beta-lactams are the most common, followed by macrolides, fluoroquinolones, tetracyclines, and aminoglycosides. Every antibiotic class has a unique mode of action and target.

Penicillin, cephalosporins, carbapenems, and monobactams are all examples of beta-lactam antibiotics. All members of this antibiotic family have the same chemical structure. They have the beta-lactam ring and demonstrate their antibacterial activity by interfering with the formation of bacterial cell wall. Beta-lactams bind to and inhibit various enzymes known as penicillin-binding proteins (PBPs). PBPs belong to a group of enzymes referred to as transpeptidases. These enzymes are essential in the cross-linking of peptidoglycan, a major component of the bacterial cell wall. Blocking PBPs weakens the cell walls, leading bacteria to burst due to osmotic pressure (Walsh, 2003).

Macrolides are another important class of antibiotics, which includes erythromycin, azithromycin, and clarithromycin. These group of antibiotics work by attaching to the bacterial ribosome's 50S subunit, inhibiting the translocation step in protein synthesis. As a result, macrolides effectively inhibit bacterial growth by stopping them from generating the proteins required for growth and replication. They are

especially efficient for treating respiratory and soft tissue infections, and they are frequently recommended especially for people who are allergic to penicillin (Arsic et al., 2018).

Fluoroquinolones, including ciprofloxacin, levofloxacin, and norfloxacin, are another group of antibiotics that inhibit bacterial DNA replication and cell division. They accomplish this by blocking the activity of the enzymes DNA gyrase and topoisomerase IV, which are required for bacterial DNA replication. Fluoroquinolones bind to these enzymes and cause significant mistakes in DNA replication, resulting in bacterial mortality. This class is distinguished by its broad-spectrum activity, which makes it useful against a wide range of bacterial infections, including those of the urinary tract, respiratory system, and certain types of gastroenteritis (Jacoby, 2005).

Tetracyclines, which include doxycycline, minocycline, and tetracycline, are broad-spectrum antibiotics that block bacterial protein synthesis by interacting with the 30S ribosomal subunit. This binding blocks the attachment of tRNA to mRNA-ribosome complex during the translation process, which is an important process in protein synthesis. The bacterial cells are essentially prevented from making the proteins required for their survival and growth by the interference with this mechanism. Tetracyclines are often used to treat infections such as acne, chlamydia, and Lyme disease because they are efficient against a wide range of bacterial organisms (Chopra & Roberts, 2001).

Aminoglycosides, which include gentamicin, tobramycin, and amikacin, are powerful antibiotics that attach to the 30S subunit of bacterial ribosome. This interaction causes mRNA misreading, which results in the synthesis of defective proteins that disrupt numerous cellular activities, eventually leading to cell death. Aminoglycosides are particularly effective against aerobic gram-negative bacteria,

but they should be used with caution because of potential adverse effects such as nephrotoxicity and ototoxicity (Mingeot-Leclercq & Tulkens, 1999).

The term “antibiotic resistance” describes the ability of bacteria to survive, live and multiply in the presence of antibiotics which are designed to destroy and kill them. This phenomenon poses a huge public health concern worldwide because it lowers the efficacy of antibiotics which causes difficulty in treating common infections. Antibiotic resistance is typically caused by genetic changes in bacteria. These genetic changes give bacteria the ability to overcome the antibiotics by preventing it from reaching its target or modify the target site of the antibiotics. These alterations can occur because of bacterial genome mutations or horizontal gene transfer which allows bacteria to acquire resistance genes from other antibiotic resistance bacteria. The misuse and overuse of antibiotics in human medicine and agriculture have accelerated the spread of antibiotic-resistant bacteria (Lee Ventola, 2015).

One of the key reasons of antibiotic resistance is inappropriate antibiotic usage, such as treating viral infections with inadequate antibiotics or failing to complete a specified antibiotic dose. Antibiotics are frequently used at subtherapeutic levels in agriculture to stimulate growth and prevent illnesses in healthy animals which contributes significantly to antibiotic resistance. According to the report of Food and Drug Administration (FDA) 62% of antibiotics prescribed for use in food-producing animals in the US in 2015 were also prescribed for human consumption. Furthermore, nearly 70% of all medically significant antibiotics sold in the United States are for use in animals (FDA, 2016). Also, The Organization for Economic Co-operation and Development (OECD) reports that in the USA and the EU, more than 75% of yearly antibiotic use is related to agriculture (OECD, 2016). These approaches create selective pressure that promotes the emergence of resistant bacteria. Furthermore, resistant bacteria can be transmitted to humans by the food chain, direct contact with animals or environmental pathways such as contaminated

water. In addition, antibiotic resistance in medical facilities has been worsened by the widespread usage of the antibiotics.

The consequences of antibiotic resistance are significant. Increased mortality, longer hospital stays, and higher medical expenses are the results of antibiotic resistance. Resistant bacteria infections can be challenging to cure and need the use of different, more costly, more harmful and less effective treatments. For example, the commonly encountered resistant bacterium methicillin resistant *Staphylococcus aureus* (MRSA) result in infections that are expensive and much more difficult to cure than non-resistant infections. According to estimates from The Center for Disease Control and Prevention there are over 2.8 million antibiotic resistant infections in the US alone each year, which lead to over 35000 fatalities (Craig, 2019).

Antimicrobial resistance (AMR) is a huge concern to world health, particularly because it reduces the effectiveness of therapies for foodborne infections. Foodborne pathogens with multidrug resistance (MDR) are of particular concern since they inhibit the treatment of infections acquired through contaminated food products. Recent research has emphasized the widespread prevalence of MDR among foodborne pathogenic bacteria. An investigation conducted in the United States, for instance, on beef sellers revealed a notable prevalence of bacteria resistant to antibiotics, many of which were also resistant to several drugs (Tate et al., 2021). According to research, *Campylobacter*, *Salmonella*, and *Enterococcus* were found in beef gathered from marketplaces in nine states at rates of 0%, 0.6%, and 53.5% respectively. All *Salmonella* isolates showed resistance to at least one antimicrobial agent, as did 73.6% of *Enterococcus* isolates. Individual and multidrug resistance levels were significantly greater in *Enterococcus* from retail beef. Comparably, studies conducted in northeastern Ohio on farms revealed a notable spread of resistant bacteria. As a result of the isolation, the prevalence of *Campylobacter* was reported to be 8%, the prevalence of *Listeria monocytogenes* was 7.9%, and the prevalence of *Salmonella* was 1.5%. Although their prevalence was not very high,

multidrug resistance was observed in 77%, 100% and 57.3% of *Salmonella*, *L. monocytogenes* and *Campylobacter* isolates, respectively (Hailu et al., 2021).

Surveillance in Northwestern Germany revealed a high frequency of MDR pathogens such as *Salmonella spp.*, which were isolated more frequently than other pathogens in food chains (Klees et al., 2020). Bangladesh demonstrates a similar trend for common foodborne pathogens. Rafiq et al. found that 75% of *Salmonella*, 95% of *Staphylococcus*, and 100% of *Streptococcus* isolates from various sources were multidrug resistant (Rafiq et al., 2022). The prevalence of MDR pathogens in foods has a direct impact on food safety and public health. Studies like the one in Dhaka, where pathogenic bacteria in chicken markets exhibited serious AMR patterns, highlight the problem's global scope (Parvin et al., 2022). MDR was reported in 100% of *Salmonella spp.* and 97.2% of *S. aureus*.

### **2.1.2 Antibiotic Resistance of *E. coli***

Antibiotic resistance in *Escherichia coli* (*E. coli*) is an increasing global problem, especially given its frequency in food isolates and the implications for public health. Antibiotic resistance in *E. coli* strains provides substantial issues in the treatment of infections and the control of bacterial spread. Multidrug-resistant (MDR) strains are becoming increasingly widespread.

The frequency of antibiotic resistance in *E. coli* varies greatly by geography and source. Research conducted in community settings, such that conducted by Nji et al. has demonstrated that commensal *E. coli* bacteria have a high incidence of antibiotic resistance, suggesting a significant reservoir of resistance among healthy human populations (Nji et al., 2021).

*E. coli* resistance patterns vary by region, according to research. For example, Chen et al. found that water sources (river and stream) in Hangzhou City could serve as

antibiotic resistance reservoirs. *E. coli* isolates were evaluated for susceptibility to 18 antibiotics. Tetracycline resistance was seen in most of the isolates, with ampicillin, piperacillin, trimethoprim/sulfamethoxazole, and chloramphenicol following closely behind. The antibiotic resistance rates of *E. coli* isolates from two different water sources were similar (Z. Chen et al., 2017).

MDR strains of *E. coli* are especially dangerous since their resistance to several antibiotic classes makes treatment choices more difficult. Bryce et al. found a significant frequency of MDR *E. coli* among pediatric urinary tract infections, which was linked to the routine use of antibiotics in primary care. The results of their comprehensive analysis, which included research from OECD nations, showed that the combined prevalence of resistance to ampicillin, trimethoprim, co-amoxiclav, and ciprofloxacin was 53.4%, 23.6%, 8.2%, and 2.1%, respectively; the lowest incidence was observed for nitrofurantoin, at 1.3% (Bryce et al., 2016).

Antimicrobial resistance was shown to be highly prevalent in *E. coli* O157:H7/NH, which was isolated from the feces of ruminant animals, according to research done in 2012 by Rahimi. Out of the 25 identified strains of *Escherichia coli* O157:H7/NH, 24 showed resistance to one or more antibiotic agents (Ebrahim Rahimi, 2012).

Seker and Kus (2019) examined *E. coli* 157 strains isolated from the feces of adult ruminants in Turkey and discovered a large fraction resistant to conventional antibiotics. From 417 fecal samples, 16 strains of *E. coli* O157 were isolated. The 16 *E. coli* bacteria that were identified in this investigation showed high resistance rates to amoxicillin/clavulanic acid, trimethoprim/sulfamethoxazole, tetracycline, neomycin and ampicillin as 56.2%, 62.5%, 68.7%, 68.7%, 68.7% respectively (Seker & Kus, 2019).

Rectal swap samples were obtained from 133 diarrheal calves in a different investigation carried out in Erzurum, Turkey. From all the examples *E. coli* was isolated, that is, a total of 133 *E. coli* was isolated. It was observed that 78.9% of the

isolates were resistant to oxytetracycline, 69.2% to trimethoprim-sulfamethoxazole, 60.9% to neomycin and 58.6% to erythromycin. In 71.4% of the isolates, multiple resistance was identified at the same time (Cengiz & Adigüzel, 2020).

Food is among the sources from which MDR *E. coli* is frequently isolated. Kumar et al. discovered that seafood from India contains many antibiotic-resistant strains of *E. coli*, potentially serving as a large source of resistance genes. Using fourteen different antibiotics – including ampicillin, cephalothin, and ciprofloxacin- the antibiotic resistance of 116 microorganisms obtained from seafood was examined. It is observed that seven strains have resistant to at least five antibiotics (Kumar et al., 2005).

Baz studied the molecular characterization and resistance characteristics of *E. coli* bacteria from soft cheese, ice cream, and yogurt in Mansoura city. A total of 130 samples of fresh soft cheese, ice cream, and yoghurt were collected at random from markets and small vendors in various areas of Egypt and tested for the presence of cytotoxigenic *Escherichia coli*. The findings demonstrated that 3% of all samples tested had toxic *E. coli*, with frequencies of 10%, 2%, and 0% in fresh soft cheese, small-scale ice cream, and traditional yoghurt, respectively. However, all these isolates were resistant to streptomycin (Baz, 2019).

A study conducted by Leverstein-van Hall et al. in the Netherlands in 2011 revealed that Dutch patients and retail chickens carried the same resistance gene. 98 samples were taken from retail chickens and MDR *E. coli* was isolated from 92 of these samples. Similarly, in the study conducted by Petternel et al. in Austria, 100 minced meat samples were collected. MDR *E. coli* was isolated from 20 of these samples (Leverstein-van Hall et al., 2011).

Another study in Saudi Arabia collected 540 beef samples to assess the prevalence of *E. coli* and to further identify the isolated *E. coli*. The isolation yielded 120 *E. coli*

isolates, five of which were the *E. coli* O157:H7 strain. Within the same investigation, 180 *E. coli* isolates from patients admitted to the hospital with food poisoning and/or diarrhea were gathered from several Saudi polyclinics. While all strains isolated from food samples and patients were completely resistant to penicillin, amoxicillin-clavulanic acid, and erythromycin, the strains were resistant to gentamicin and ampicillin at 83% and 75% respectively (Hemeg, 2018).

In a different investigation, the incidence of *E. coli* and the isolates' resistance to antibiotics were investigated in the Mexican Honeydew melon farming system. 445 samples from irrigation water, harvested melons, packing workers' hands, boxes, and discarded melons were taken for this purpose. There were 32 strains of *E. coli* found. Examining the resistance profile of *E. coli* strains, it was shown that 94% of the isolates were resistant to ampicillin, 97% to meropenem, and all of the isolates were resistant to ertapenem. Apart from these high levels of resistance, 47% of the strains were identified as multidrug resistant (Enciso-Martínez et al., 2022).

In another study conducted in Turkey to see the prevalence of *E. coli* in meat and dairy products, 10 samples of beef, chicken, white cheese and cheddar cheese were taken from markets in Tekirdağ every month. A total of 480 samples were studied in the study. As a result of the analysis, 4 *E. coli* O157 were isolated. The isolated *E. coli* was found in beef, but could not be isolated from chicken meat, white cheese and kosher cheese samples. When the antibiotic resistance profiles were examined, it was seen that all *E. coli* isolates were resistant to ampicillin, trimethoprim and meropenem. 75% of the isolates were resistant to ciprofloxacin, streptomycin, sulphamethoxazole and tetracycline. As a result of the study, it was interpreted as pleasing that the prevalence of *E. coli* was not high in meat and dairy products, while the high level of resistance profiles of these isolates was said to pose a significant threat to public health (Atabey et al., 2021).

As explained and mentioned in the above studies, the antimicrobial resistance of *E. coli* poses a major threat to public health since it makes the treatment of infections more difficult and increases cost of treatment. Therefore, it is of great importance to develop new treatment methods and to find alternative antimicrobial strategies.

## 2.2 Bacterial Cell Wall

One of the most important structures in bacteria is the cell wall, which is mainly made up of peptidoglycan, a mesh-like substance that gives the cell its form and structural stability. It is a robust structure that wraps the cell membrane, determining its shape and protecting it from inner turgor pressure and its external environment. The wall performs several vital tasks, including protecting the organism physically, mediating interactions between the bacteria and its surroundings, and aiding in maintaining of the organism's shape. This structure is essential for bacterial survival because it guards against osmotic lysis and mechanical stress. It is generally present in all bacteria, with the exception of those in the *Mycoplasma* genus, which do not have cell walls (Egan et al., 2017).

As a reflection of their many ecological niches and evolutionary pathways, bacteria can differ greatly in the composition and design of their cell walls (Chapot-Chartier & Kulakauskas, 2014). This structure is most noticeable in Gram-positive bacteria, whose thick cell walls are made up of many layers of teichoic acid and peptidoglycan. On the other hand, Gram-negative bacteria have an outer membrane made of lipopolysaccharides and a thinner coating of peptidoglycan, which both contribute to their pathogenicity and capacity to escape immune responses (Chapot-Chartier & Kulakauskas, 2014).

The peptidoglycan layer of the cell wall is a dynamic structure that is constantly changing and is essential for cell division and proliferation. By breaking links within the peptidoglycan network, enzymes like autolysins facilitate cell wall extension and the addition of new peptidoglycan subunits, which is a major function of their

activity in cell wall metabolism. In order to maintain the structural integrity of the cell during growth and division, this process is strictly controlled in time with the cell cycle. The cell wall serves as a regulatory barrier in addition to its structural purpose, regulating the movement of molecules into and out of the cell and providing defense against external pressures (Wicken, 1985).

Gram-positive bacteria have thick peptidoglycan layers on their cell walls. However, gram-negative bacteria have thin peptidoglycan cell walls, but they also have an outer membrane that gram-positive bacteria lack (Chapot-Chartier & Kulakauskas, 2014).

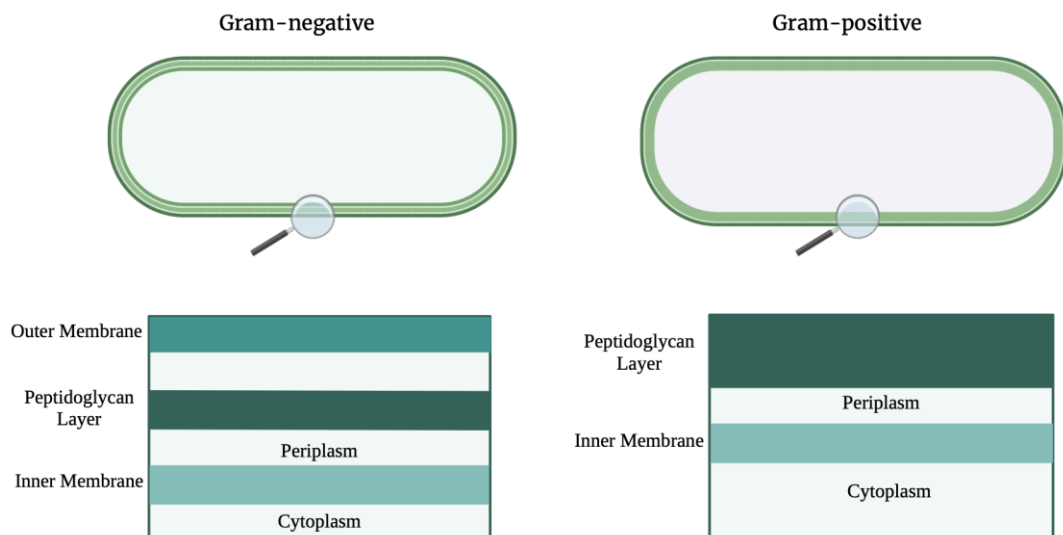


Figure 2.1 Cell wall structure of gram-negative and gram-positive bacteria. This figure was generated using Biorender (<https://app.biorender.com>). Adapted from Egan et al. (2017).

### 2.2.1 Gram-Negative Cell Wall Structure

Gram-negative cell walls are robust enough to tolerate high pH and temperatures, as well as a turgor pressure of roughly 3 atm. Furthermore, the gram-negative cell wall

possesses sufficient elasticity to increase its normal surface area by many times (Beveridge, 1999).

Gram-negative bacteria's cell walls have a common structural framework that they strictly adhere to. The outer membrane of gram-negative bacteria is located on top of a thin layer of peptidoglycan. A dense gel-like matrix called periplasm exists between the outer and inner membranes. The gram-negative envelope is made up of the inner membrane (cytoplasmic membrane) and the cell wall (outer membrane, peptidoglycan layer, and periplasm) (Beveridge, 1999).

Similar to the cytoplasmic membrane, the inner layer of the two-layered outer membrane is composed of phospholipids. The layer that faces the outside environment is different from the inner membrane in that it lacks unsaturated fatty acids and has a structure that is rich in lipopolysaccharides (LPS). Porins are transmembrane proteins found in the outer membrane. Low molecular weight components can enter cells through the hydrophilic pores or water-filled channels that are created by porins. Particular substances can only pass through certain porins, however, all low molecular weight (600–700 Dalton) molecules can pass through porins that are non-specific (Silhavy et al., 2010).

The periplasmic space is between the cytoplasmic membrane and the outer membrane. The ability of the outer membrane to retain enzymes outside of the cytoplasmic membrane is a crucial function. The periplasmic space is where these enzymes are found. Hydrolytic enzymes, which carry out the first breakdown of nutrients, binding proteins, which facilitate the transfer of substances, and chemoreceptors, which trigger an organism's movement in response to a chemical stimulus, are all found in the periplasm (Beveridge, 1999).

One characteristic that differentiates between the bacterial cell wall is peptidoglycan. Peptidoglycan was first found to be the target of ground-breaking beta-lactam

antibiotics, but because it may also hold the key to discovering novel antibiotic targets, it has attracted a lot of attention. A polymer known as peptidoglycan is made up of small peptide chains that are covalently cross-linked to one another by linear glycan chains. Glycan chains are extended disaccharide polymers that are connected by  $\beta$ -1,4 glycosidic linkages and comprise N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues. MurNAc's lactoyl residues are covalently bonded to peptides, which typically consist of two to five amino acid residues. The pentapeptide chain in the majority of Gram-negative bacteria, such as *E. coli*, is made up of D-alanine (D-ala),  $\gamma$ -D-glutamate (D-glu), meso-diaminopimelic acid (mDAP), and L-alanine (L-ala). An isopeptide linkage binds D-ala to D-glu and meso-diaminopimelic acid (mDAP). Since it can create cross-links with other peptide chains, the dibasic amino acid meso-diaminopimelic acid is essential to the peptide's structure. Peptides in gram-negative bacteria are immediately cross-linked to one another by means of two meso-diaminopimelic acid residues or D-ala and meso-diaminopimelic acid of nearby peptides from neighboring glycan strands. Peptidoglycan has a distinctive meshlike structure that is caused by cross-linking (Vollmer et al., 2008).

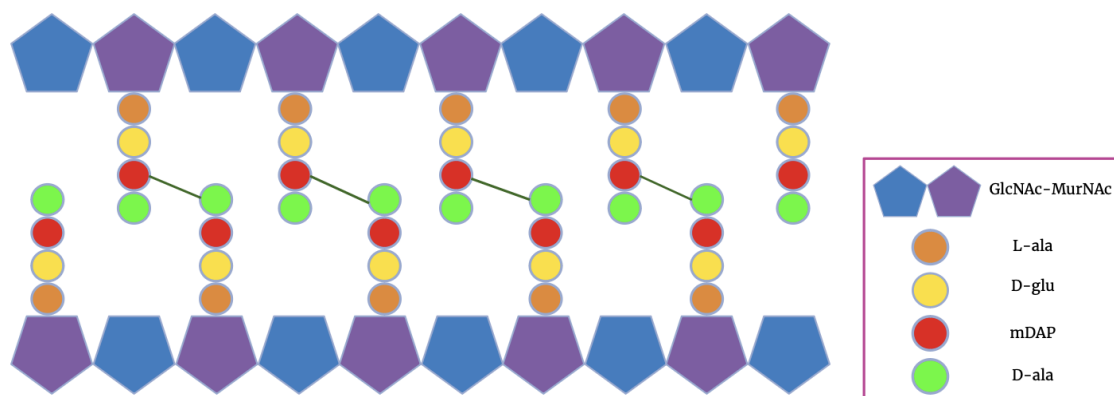


Figure 2.2 Structure of the peptidoglycan. This figure was generated using Biorender (<https://app.biorender.com>). Adapted from Garde et al. (2021).

### 2.3 Bacteriophages

Bacteriophages, also known as phages, are viruses that can infect only bacteria. Phages are mandatory intracellular parasites that do not have their own metabolic activities.

The lysis of the bacteria has been observed by microbiologist throughout history. In 1896, British microbiologist Ernest H. Hankin discovered that the *Vibrio cholera* could be killed by the Jumna and Ganges rivers in India (Summers, 1993). Russian microbiologist Nikolai Gamaleya discovered in 1898 that an infectious “ferment” was responsible for lysing the *Bacillus anthracis*. Frederick William Twort, a British pathologist who led the London-based Brown Institute noticed that staphylococci colonies often became transparent and then died. He observed that the agent responsible for this is an infectious strain that could pass through filters and be killed by heat treatment. Twort stated that this might be caused by enzyme capable of evolve, an amoeba, an ultramicroscopic virus or living protoplasm (Duckworth, 1976). Félix Hubert d'Herelle isolated *Enterobacter aerogenes*, the causative agent of locust illness in Yucatan. He saw that *Enterobacter aerogenes* cultures developed what are known as “plaques” or holes. Afterwards, he looked into a bacillary dysentery outbreak among military personnel in Paris in 1915. He pointed out that a filterable plaque-forming agent kills *Shigella* cultures, and the agent usually reveals itself during the healing phase. In 1917, this discovery was published. d'Herelle immediately realized that he had identified a new type of virus, and he employed it to treat bacterial diseases. He created the plaque test and coined the term “bacteriophage”. Bacteriophage term was derived from the “bacteria” and the Greek word “phagein” meaning “eat”. He founded numerous phage centers and promoted and carried out phage therapy in numerous nations (Duckworth, 1976). Together with G. Eliava, he founded the Tbilisi Institute in 1934, which is still in operation today (Deresinski, 2009). d'Herelle noticed elevated phage titers in stool samples from dysentery patients not long after they were discovered. Scientists were almost

instantly inspired by this discovery to investigate the potential of viruses of bacteria for medical usage. However, the discovery of penicillin by Alexander Fleming in 1928 marked the beginning of the era of antibiotics against pathogens, and as a result, the employment of bacteriophages as antimicrobial agent became less significant.

Bacteriophages, which have common characteristics of viruses, contain DNA or RNA as genetic material, and their structure generally consists of proteins or lipoproteins. Phages are unable to produce any genetic information since they lack their own metabolic systems. Since phages do not have ribosomes, they cannot produce their proteins and cannot show their metabolic activities unless they infect the host cell. Like other viruses, they can only proliferate once they infect the host cell (Forde & Hill, 2018).

Bacteriophages are not capable of infecting mammalian cells; they can only specifically infect bacterial cells. Head, color, tail and tail extensions are the structural components of bacteriophages. Double stranded or single stranded genomic material (DNA or RNA) is found in a structure consisting of protein or lipoprotein called capsid. Demonstration of bacteriophage structure was provided in Figure 2.3. Phages, which consist of approximately 60% of their weight from proteins and 40% from nucleic acids, have different morphological characteristics and different protein structures and like every living thing, their antigenic properties depend on these proteins (Salmond & Fineran, 2015).

At the International Microbiology Congress held in Moscow in 1966, an internationally organized attempt to bring an order for the classification of viruses took place for the first time. Later, a committee called the International Committee on Taxonomy of Viruses (ICTV) was established with the task of developing a single, universal, taxonomic scheme for all viruses that infect animals, plants, fungi, bacteria and archaea. Previously, phages were divided morphologically into two main categories: tailed (head & tail) and PFP (polyhedral, filamentous, or

pleomorphic) phages (Mazaheri & Fard, 2016). Phages were subsequently classified into four basic morphological groups namely, tailed (order *Caudovirales*), polyhedral (*Microviridae*), filamentous (*Inoviridae*) and pleomorphic (*Plasmaviridae*). The majority of the phages (96%) have tails morphologically and others are PFP. More than half of tailed phages had long, flexible and non-contractile tails (*Siphoviridae*), while some had long, non-flexible, contractile tails (*Myoviridae*), followed by families with long, non-flexible, non-contractile tails (*Podoviridae*) (Ackermann, 2009). However, advances in molecular biology and genomic sequencing technologies have ushered in a new era in phage classification. Today, ICTV adopts a genomic based approach that classifies phages according to their genomic material rather than their morphology. Genomic classification of phages allows the identification of phages that share common genetic sequences but differ significantly in morphology. In a recent release, ICTV made a drastic change to phage classification: ICTV abolished the order *Caudovirales*, which was replaced by the class *Caudoviricetes* to group all tailed bacterial and archaeal viruses, as well as the families *Siphoviridae*, *Myoviridae*, *Podoviridae*. ICTV also recognized the importance of morphological (non-taxonomic) terms such as “*podovirus*”, “*myovirus*”, or “*siphovirus*”, noting that these terms could be used to reflect distinguishing features, but these terms do not have any official taxonomic meaning after 2022 approval vote (Turner et al., 2023). This genomic based taxonomic classification led to one class, seven orders, thirty-one families, two hundred fourteen genera, eight hundred fifty-eight species (Walker et al., 2022).

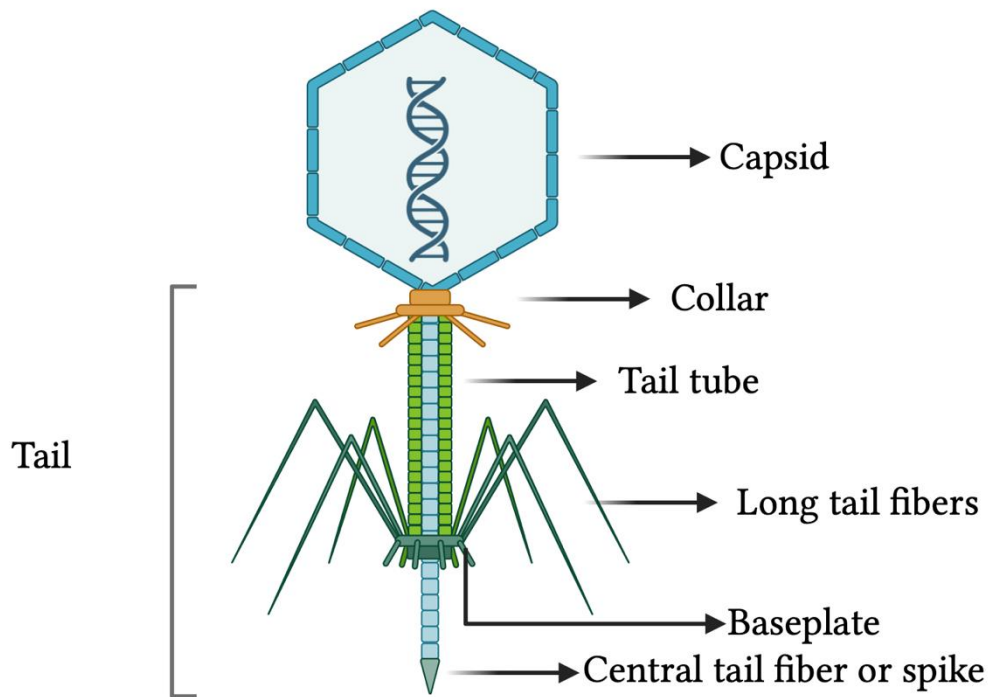


Figure 2.3. Structure of bacteriophage. This figure was generated using Biorender (<https://app.biorender.com>).

The life of bacteriophages basically continues by carrying their genome from one susceptible host to another bacterial cell (infection cycle) and ensuring the formation of new generations of phages. Although the efficiency and timing of the infection cycle of phage depends on the metabolic state of the host, phages follow certain steps during infection. The first step is adsorption (Sabour & Griffiths, 2010). Infection begins with the binding of specialized adsorption structures, such as tail extensions, to specific surface proteins on the host bacteria. When phages bind to gram-negative bacteria, almost all the proteins, oligosaccharides and liposaccharides found in the outer membrane can be used by phages (Clokier et al., 2011). On the other hand, when phages bind to gram-positive bacteria, the receptor-recognizing domains of phage proteins are limited to single regions on polypeptide chains. Once phages adhere irreversibly to their hosts, the next step is DNA transfer (penetration). Phage nucleic acid is densely packed within the capsid to create a high internal pressure of

approximately 50 atm, which creates a driving force for DNA injection into the cell. After binding of phage to the host, the phage genome must pass through the outer membrane, peptidoglycan layer and inner membrane to reach the transcription and translation metabolism of its host. To pass these barriers, the peptidoglycan-degrading enzyme usually carried at the end of the phage tail is used, and then the DNA is quickly drawn into the cell, using either metabolic energy or membrane potential (Giri, 2021). Phage DNA rapidly circularizes using sticky ends or terminal repeats to protect itself from exonucleases and restriction enzymes within the bacterial cell. The next step is DNA replication and particle formation. The phage genome completes its replication, translation and transformation in the host's metabolic system and replicates the phage structural proteins and DNA. Before the DNA is packaged inside the phage head, the phage structure is formed, then the phage genome is transferred to the capsid and finally the tail is attached to the phage head, thus creating the phage structure. The last step of the infection cycle is phage release. Newly formed phages leave the cell to find their new hosts. For this purpose, bacteriophages have two types of strategies. Filamentous phages expel their progeny through the cell wall without bursting the host cell. In the most common strategy, phages use lysine enzymes. Lysins, a muralytic enzyme encoded by phages, degrade the cell's peptidoglycan layer to the point that it can no longer withstand the internal osmotic pressure, thus the cell bursts open and phage particles are released (Sabour & Griffiths, 2010).

If there are more phage particles compared to the number of bacteria (high multiplicity of infection, MOI) the number of pores formed in the bacterial cell wall becomes so high that the cell cannot maintain turgor pressure and the cell loses its integrity (Figure 2.4). This mechanism of phage-mediated cell lysis is called "lysis from without" (Anany et al., 2015). In that case, bacterial cell loses its integrity, but phage replication or new phage particle formation does not occur. However, if the MOI is moderate enough to allow phage particles to infect the host cell, two infection

scenarios can develop depending on both host and virus characteristics and conditions.

### Lysis from without

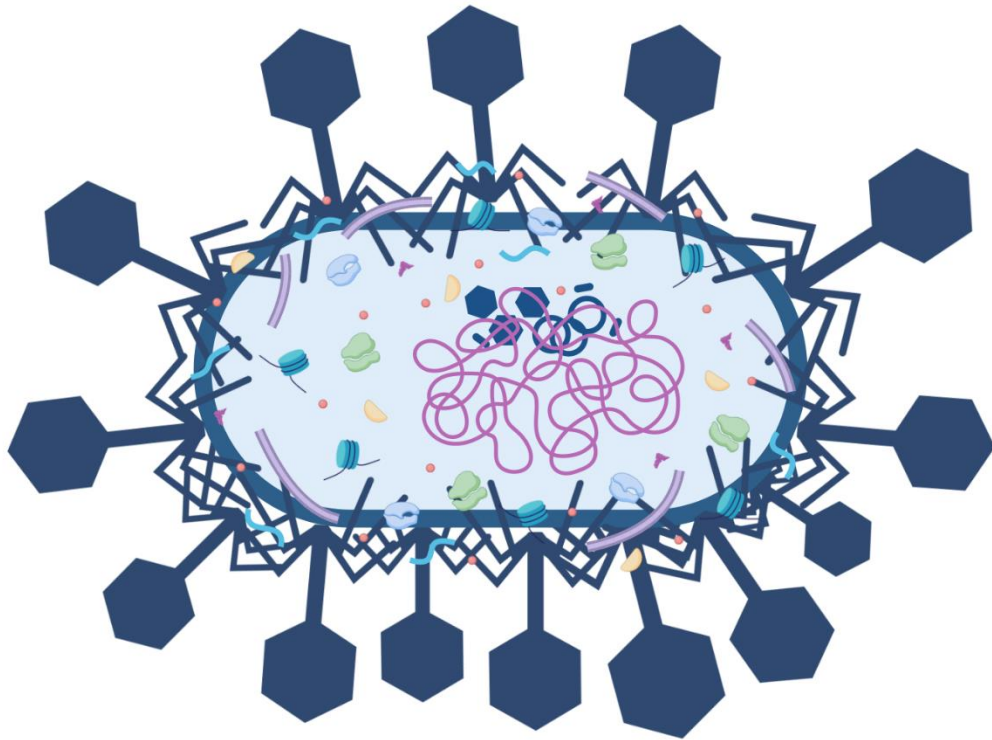


Figure 2.4. Lysis from without mechanism. This figure was generated using Biorender (<https://app.biorender.com>).

The life cycles of phages are basically divided into two main pathways: the lytic cycle and the lysogenic cycle. Both cycles involve processes in which the phage interacts with a host bacterium and replicates, but each produces different results. Phages that follow the lytic cycle are called virulent phages, while phages that can follow both the lytic and the lysogenic cycle are called temperate phages. Virulent phages infect the cell, inject their own DNA, and take over the cell for their own replication. It terminates the variety of biosynthetic processes of the cell and replaces them with processes aimed at the synthesis of phage proteins. Newly formed phages accumulate within the infected cell. Afterwards, the cell is lysed down enzymatically

(lysine) from the inside and the newly formed phage particles are released into the environment. This cycle is called lytic cycle. On the other hand, temperate bacteriophages inject their DNA/RNA into the bacterial cell, then the phage genome is physically inserted into the host genome via a specific enzyme called integrase. Thus, prophage is formed as can be seen in Figure 2.5. When phage DNA integrates into host DNA, the host cell can gain new or additional properties upon expression of genes, known as lysogenic transformation (Sabour & Griffiths, 2010). For example, prophages play an important role in transporting genes that produce shiga toxin (Nanda et al., 2015) The host bacteria can continue to divide and carry prophages indefinitely without lysing until the lytic cycle is triggered by external factors such as ultraviolet light, presence of antibiotics, etc.

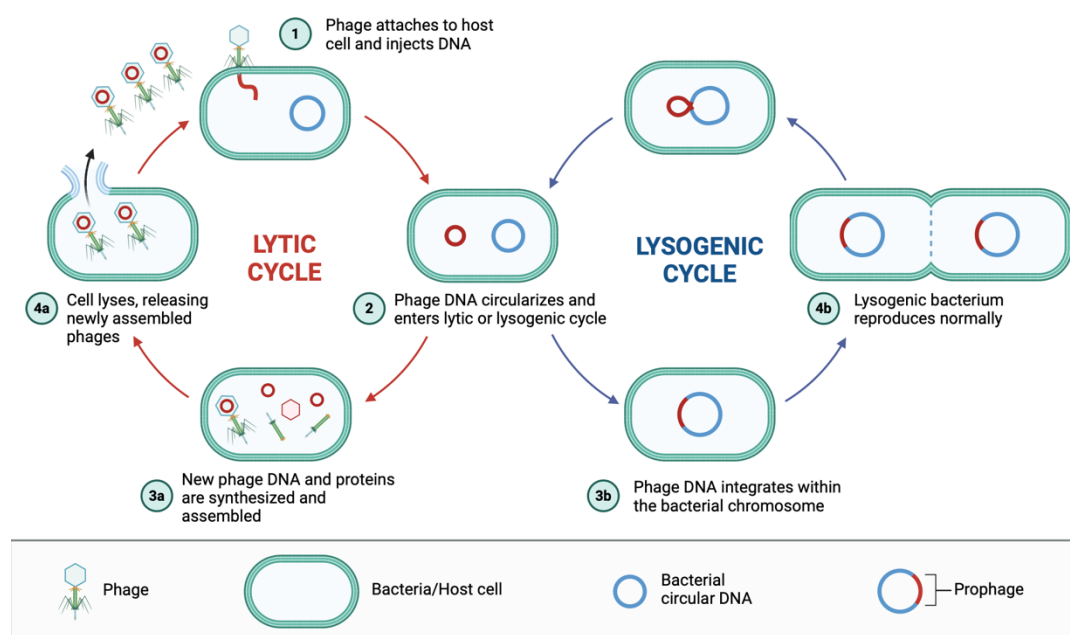


Figure 2.5. Life cycles of bacteriophages. This figure was generated using Biorender (<https://app.biorender.com>).

Phages are becoming popular as biocontrol agent in food industry because of their specificity, efficacy, and safety. One of the primary advantages of utilizing phages as biocontrol agents in food is their ability to target and infect specific bacteria. This specific target mechanism of phages, unlike broad-spectrum antimicrobials,

guarantees that beneficial microorganisms in the food or in the gut microbiome are not negatively impacted. Another advantage of phages as biocontrol agent in food industry is that phages do not change the odor, taste, color or texture of the food, making certain that the sensory properties of food product stay the same (Endersen & Coffey, 2020). Furthermore, phages limit themselves, which means they multiply only when target bacteria are present, and they become functionless when their target bacteria are not found in the environment. This property diminishes the risk of environmental accumulation thus they are an environmentally friendly option for biocontrol agent in food industry. Besides, phages cannot infect eukaryotic cells thus they are safe for humans and animals. The U.S. Food and Drug Administration (FDA) has given an approval for several phage products for their usage in foods, underscoring their safety (Huang et al., 2022). Also, phages do not leave a potentially harmful residues in food matrix which makes them an attractive option for biocontrol agent in food industry without disrupt the food quality. Phages remain active throughout a wide pH and temperature range, making them useful for a variety of food processing and storage conditions. Thus, they can be used in the various stages of food processing which reduces the risk of foodborne illnesses and outbreaks (Anany et al., 2015).

Due to these above-mentioned advantageous properties, experiments of different phages on different food surfaces against various foodborne pathogens have been presented in the literature. Bueno et al. showed that two lytic bacteriophages, vB\_SauS-phi-IPLA35 and vBi\_SauS-phi-IPLA88, were effective against *Staphylococcus aureus* in both fresh and hard-type cheese. Pasteurized milk was first contaminated with  $10^6$  CFU/mL *S. aureus*, then  $10^6$  PFU/mL phage cocktail was applied, and it was seen that the *S. aureus* concentration decreased by 3.83 log CFU/mL after 3 hours and after 6 hours the number of viable *S. aureus* cells was below the detection limit (Bueno et al., 2012).

Additionally, Tabla and colleagues evaluated the synergistic effects of these phages with high hydrostatic pressure (HHP) on pasteurized milk under 48-hour incubation conditions at 25°C. Pasteurized milk was contaminated with two different initial concentrations of *S. aureus* ( $10^6$  CFU/mL and  $10^4$  CFU/mL). It was observed that phage application with 400 MPa HHP was capable of reducing *S. aureus* contamination below the detection limit (10 CFU/mL) at both different initial concentrations (Tabla et al., 2012).

These trials are not limited to milk or *S. aureus*. Zhang and colleagues investigated the inhibition effect of *Shigella* phages against the foodborne pathogen *Shigella spp.* on ready-to-eat seasoned chicken meat. Chicken samples were contaminated either with only one species or with a cocktail of species (*S. flexneri 2a*, *S. dysenteriae*, and *S. sonnei*), and the samples were incubated at 4 °C for 72 hours. It was reported that the number of hosts decreased by 2 log CFU/g after 48 hours, and the host could not be detected after 72 hours (Zhang et al., 2013).

Similarly, Leverentz et al. proved that when phage cocktail was applied to melon pieces contaminated with *Listeria monocytogenes* strain LCDC, there was a 6.8 log decrease in the number of viable cells at the end of 7 days at 10°C (Leverentz et al., 2004). In another study, the inhibitory effect of lytic FO1-E2 phage against *Salmonella* was tested in ready-to-eat foods. Turkey deli meat, chocolate milk, hot dog and seafood were contaminated with  $10^3$  CFU/g *Salmonella*, then  $10^8$  PFU/g phage was applied and incubated at 8°C and 15°C for 6 days. When the samples at 8°C were examined, it was seen that there were no living cells left in the samples after phage application. However, in samples at 15°C, the number of *S. Typhimurium* decreased by 3 log CFU/g in hotdogs and seafood, and by 5 log CFU/g in turkey deli meat and chocolate milk (Guenther et al., 2012).

Besides direct applications, phages have been integrated into various innovative applications in the food industry, particularly in food packaging. In a study published

in 2017, Radford et al. created xanthan coatings on poly(lactic acid) films using bacteriophages that dramatically reduced the growth of *Listeria monocytogenes*. According to the study, these coatings demonstrated the potential of active packaging methods to successfully manage foodborne pathogens, with reductions of up to 6.31 log CFU/g at 4°C (Radford et al., 2017).

Phages have been studied for their efficacy in controlling bacterial contamination on stainless steel surfaces, which are common in food processing environments. Ganegama Arachchi et al. (2013) investigated to evaluate the efficiency of three bacteriophages (LiMN4L, LiMN4p, and LiMN17) individually and in combination for lysing strains of *Listeria monocytogenes* on stainless steel. Within 75 minutes on stainless steel surfaces, the phage cocktail reduced *L. monocytogenes* to undetectable levels (Ganegama Arachchi et al., 2013).

In addition, the efficiency of phage P100 in reducing *Listeria monocytogenes* biofilms on stainless steel surfaces in moist conditions at room temperature was investigated by Montañez-Izquierdo et al. (2012). Higher concentrations of phage treatments (7 and 8 log PFU/ml) considerably decreased *L. monocytogenes* biofilms, with a mean reduction of 5.29 log CFU/cm<sup>2</sup> after 48 hours, according to the study. This illustrates how phages can be used in food processing facilities to supplement hygienic practices (Montañez-Izquierdo et al., 2012).

### **2.3.1.1 Bacteriophages against *E. coli***

Our understanding of microbiology, cell biology, biochemical processes, genetics and evolution has greatly benefited from the use of *E. coli*, one of the most researched model microorganisms. Bacteriophages that infect *E. coli* and its strains (coliphage) are also considered as model microorganisms. These include T-even phages (T2, T4 and T6) along with T-odd phages ( T1, T3 and T7) as well as lambda phage and M13 phage.

T4 and T4-related phages are a class of viruses that share the most important characteristics. Phage T4 is one of the most well investigated phages that demonstrates the *Myoviridae* morphotype. It has a large icosahedral head and a contractile tail which aids the injection of its DNA into its host (Yap & Rossmann, 2014). T4 is a lytic phage whose lytic cycle is highly effective and T4 has been widely utilized in molecular biology to examine replication and transcription processes (Yap & Rossmann, 2014). T4 and T4 related bacteriophages is found in the microbiota of humans and animals. These phages are the result of a long-lasting coevolution with *E. coli* in gastrointestinal tract (GIT). In addition, these phages have evolved to survive and proliferate in GIT ecological system which includes a variety of host bacteria. The abundance of these bacteriophages in many ecological locations including wastewater makes it easier to isolate novel bacteriophages for further usage such as phage therapy. These phages do not contain virulence genes in their genomes. For the reasons mentioned above, these phages are among the best candidates for phage applications and phage therapies against pathogenic *E. coli* strains (Nikulin et al., 2023).

T7 is another lytic phage that is smaller than T4, having a less complex structure and shorter genome. T7 phage is a member of *Podoviridae* morphotype. It has been reported that the whole genome of T7 bacteriophage encodes approximately 50 proteins and consist of approximately 40 kb of double stranded DNA packed in the icosahedron shape of phage capsid. On top of that, T7 phages are utilized as a model system for viral research and RNA polymerase of T7 phage is frequently used in molecular biology due to its extreme selectivity for its own promoters which allows for the high-level expression of recombinant proteins in *E. coli* (Yu et al., 2022).

Phage lambda is other important virus that infect *E. coli*. Esther Lederberg (1951) in the University of Wisconsin identified phage lambda by chance during ultraviolet irradiation of *Escherichia coli* strain K-12 in the laboratory. Phage lambda is notable

for its capacity to alternate between lytic and lysogenic cycles. It uses a complicated regulatory circuit which includes the CI repressor to maintain lysogeny and the Cro protein to transition to the lytic cycle under particular conditions. These particular conditions include damage to DNA by UV light or chemicals, cell starvation, or environmental stresses such as sudden and extreme temperature changes. The genome of phage lambda is approximately 48.5 kb long which integrates into the *E. coli* chromosome allowing the virus to replicate with host and carry along during cell division. Lambda phage has been widely used in molecular biology, particularly for the development of cloning and expression systems. Lambda vectors are employed for DNA cloning due to their capacity to package recombinant DNA and infect *E. coli* effectively (Casjens & Hendrix, 2015).

Bacteriophage M13 is a filamentous bacteriophage that infects *Escherichia coli* using the F pilus as its receptor. Unlike lytic phages, M13 does not kill its host; instead, it integrates its DNA into the host in a form known as a replicative form (RF), which replicates the phage DNA without disrupting the host's normal cellular processes (Rakonjac et al., 2011). The M13 phage's lifecycle is conducive to continuous production of phage particles without lysing the bacterial cells, allowing for sustained production of phage particles if the host cells are alive. Structurally, M13 is composed of a circular single-stranded DNA (ssDNA) genome approximately 6.4 kb long. M13 phage has a wide range of uses, particularly in biotechnology and nanotechnology. One of the most well-known applications of M13 phage is phage display technology, which involves genetically fusing peptides or proteins to one of the phage coat proteins and displaying them on the phage surface. This approach has become a valuable tool in bioengineering and medicine for identifying new ligands for proteins, peptides, and antibody fragments with high affinity for specific molecular targets (Fadaie et al., 2023).

In addition to the *E. coli* phages mentioned above, which have been used as model microorganisms in various fields of biology, various *E. coli* phages used for biocontrol purposes against pathogens are also available in the literature. A notable

study isolated the DW-EC bacteriophage, demonstrating its effectiveness against Enterotoxigenic *E. coli* in chicken meat, fish meat, tomatoes, lettuce, and cucumber. (Dewanggana et al., 2022). In experiments on various foods, this phage was found to reduce pathogen levels by up to 87% after 6 days of incubation at refrigerator temperature.

Zhou et al. (2022) created phage vB\_EcoM\_SQ17, which was used to treat both Enterohemorrhagic *E. coli* O157:H7 and Enterotoxigenic *E. coli*. Phage SQ17 was investigated for its capacity to kill EHEC O157:H7 in skim and whole UHT milk at 4°C or 25°C for 7 or 4 days, using viable bacterial cell counts. After treating the milk with phage SQ17 at 4°C, the contamination level of EHEC O157:H7 strain EO157-1 stayed below the detection limit (<10 CFU/ml) To test the bactericidal efficacy of phage SQ17 in solid foods, raw beef and lettuce were infected with *E. coli* O157:H7. The viable cell counts were considerably decreased by 2.35 log CFU/piece after 12 hours at 4°C and by 0.70 log CFU/piece after 4 hours at 25°C when the raw beef was treated with phage SQ17. Similarly, adding phage SQ17 to fresh lettuce dramatically reduced viable cell counts by 2.23 log CFU/piece after 12 hours at 4°C and 3.83 log CFU/piece after 12 hours at 25°C (Zhou et al., 2022).

In addition to biocontrol purposes, bacteriophages can also be used for detection purposes. The use of *Escherichia coli* phages in food safety, notably for detecting foodborne pathogens, has improved dramatically, with multiple biotechnological techniques used to assure quick and specific identification of contamination. Phage-based detection approaches, such as those based on modified bacteriophages including reporter genes, enable the viewing of phage-bacteria interactions via measurable signals such as luminescence or color change, providing a direct indicator of pathogen presence (J. Chen et al., 2017). For example, lyophilized phages have been employed for colorimetric detection in foods, providing a practical strategy (Singh et al., 2013). Furthermore, the use of immunomagnetic separation in conjunction with phage tests has accelerated the detection procedure, making it

possible to quickly isolate and identify targeted pathogens from complicated food samples. In order to identify *E. coli* O157:H7 in beef, this method consists of gathering bacteria on magnetic beads and then infecting the host cell with phages that are designed to produce signals that can be detected if the infection is effective (Favrin et al., 2003).

#### **2.4 Bacteriophage-encoded Lysins**

At the end of their replication cycle, bacteriophages produce lysins known as "phage-derived lysins," which lyse their host bacterial cell and facilitate the release of newly generated phage particles (Young, 1992). These enzymes have received interest due to their potential to act as novel antimicrobials, particularly against multidrug resistant bacteria. They degrade the peptidoglycan layer of bacterial cell walls, resulting in cell lysis (Young, 1992). Unlike broad-spectrum antibiotics, lysins have a narrow host range and are selective to certain bacterial species or even serotypes, lowering the possibility of disrupting beneficial microbiota (Abdelrahman et al., 2021).

Furthermore, bacteria can become resistant to phages. However, phage lysins are special in comparison to other phages and antibiotics since resistance is extremely rare (Shah et al., 2023). This is because lysin can attach to and cleave highly conserved sites within the cell wall (Fischetti, 2005). Lysins are perceived as more "natural" than real phages, therefore using them instead of phages may result in fewer regulatory difficulties and higher market acceptance (Maciejewska et al., 2018). Lysins can be administered to food surfaces in a variety of ways, including sprays and washes, without the requirement for bacterial hosts (Khan et al., 2023).

### **2.4.1 Structure and Mechanism of Bacteriophage-encoded Lysins**

Generally, lysins consist of two discrete functional domains: the cell wall-binding domain (CBD) and the enzymatic active domain (EAD) (Lai et al., 2020). The EAD is in charge of the enzyme's catalytic action, resulting in peptidoglycan bond breakdown. On the contrary, the CBD guarantees the enzyme's specificity by attaching to specific substrates inside the bacterial cell wall, which can vary greatly throughout bacterial species (Schmelcher et al., 2012). Gram-positive and Gram-negative bacteria have different cell wall compositions, which is reflected in the structural differences between lysins those targeting Gram-positive bacteria and those targeting Gram-negative bacteria.

The structure of lysins those targeting Gram-positive bacteria is modular. A protein that is modular is made up of discrete structural units, or domains, each of which has a unique purpose. The modular structure of these proteins allows for a great degree of versatility and specificity in their functions. As a result, endolysins that specifically target Gram-positive bacteria frequently have a straightforward structure with distinct CBD and EAD (Bai et al., 2016). Endolysins can readily reach the thick peptidoglycan layer in Gram-positive bacteria since there is no outer membrane and the peptidoglycan layer is exposed. Since there is no barrier for lysins those targeting Gram-positive bacteria, they can bind to and breakdown peptidoglycan with efficiency, which makes them quite powerful against these kinds of bacteria.

The outer membrane of Gram-negative bacteria presents an extra challenge for lysins targeting Gram-negative bacteria, as it serves as a considerable barrier to many antibacterial agents. Since lysins those targeting Gram-negative bacteria do not require CBD to attach to the cell residue following host cell lysis in order to reduce premature lysis, the OM can serve as an effective defense against free lysin attacks against Gram-negative bacteria. As a result, lysins those targeting Gram-negative

bacteria typically have a globular structure and one EAD to cause cell wall disruption (Ajuebor et al., 2016).

The EAD catalyzes the breakdown of the PG by reflecting an endolysin's lytic action. Depending on the particular bond of the PG that the EAD attacks, endolysins can be divided into at least five distinct groups (Schmelcher et al., 2012).

The first group is N-acetyl- $\beta$ -D-muramidases, also known as lysozymes. By adding water to the bond, lysozymes cleave the  $\beta$ -1,4-glycosidic link between N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) which is the one of the glycosidic linkages in the sugar strand of peptidoglycan layer thus it causes hydrolysis of peptidoglycan layer.

The second group consists of lytic transglycosylases. Unlike lysozymes, lytic transglycosylases function without the need for water. Lytic transglycosylases also target the  $\beta$ -1,4-glycosidic bond between MurNAc and GlcNAc. By breaking the bond and quickly reestablishing it to a neighboring hydroxyl group in the same molecule, these enzymes carry out an intramolecular transglycosylation. An 1,6-anhydro bond forms in the MurNAc residue as a result of this reaction.

The third group includes N-acetyl- $\beta$ -D-glucosaminidases. The other glycosidic bond in the sugar strand, N-acetylglucosaminyl- $\beta$ -1,4-N-acetylmuramine, is broken by N-acetyl- $\beta$ -D-glucosaminidases.

Amidases represent the final group. They break down the amide link that holds the peptide and sugar chains together. The endopeptidases are the final group. They split inside the peptides that comprise the PG units' connecting stem. Cleavage of bonds can take place between stem peptides or inside an interpeptide bridge.

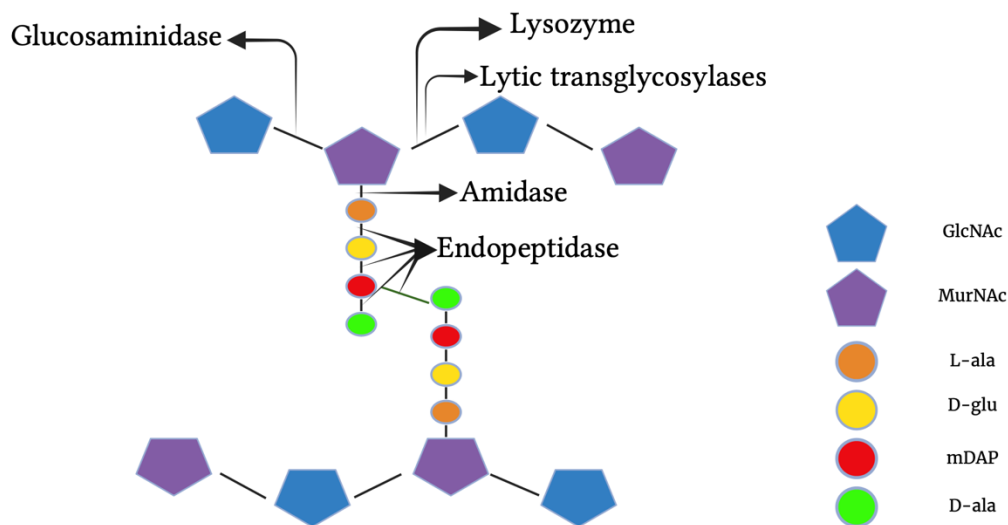


Figure 2.6. Demonstration of lysin cleavage sites with the peptidoglycan structure. This figure was generated using Biorender (<https://app.biorender.com>). Adapted from Ajuebor et al. (2016).

The enzyme nomenclature used by the International Union of Biochemistry and Molecular Biology (IUBMB) classifies all lysins as hydrolases, with the exception of lytic transglycosylases. In addition, IUBMB classifies both muramidases and glucosaminidases as glycosidases (IUBMB, 2024). Enzymes that cut within the PG's stem peptide and those that target bonds within the interpeptide bridge are two other categories into which endopeptidases can be subdivided.

#### 2.4.2 Application of Bacteriophage-encoded Lysins

There are studies in the literature on the lysine enzymes of phages targeting different hosts. For example, Obeso et al. investigated the effect of lysin LysH5 on contaminated milk. It was observed that the concentration of *S. aureus* was reduced by 8 log(CFU/mL). In addition, it is stated that the LysH5 was also affect the *S. epidermidis* not just *S. aureus*. Also, the synergistic effect between nisin and LysH5 was pointed out in dairy products against *S. aureus* (Obeso et al., 2008). In another

investigation, lysin LysSA11 was applied to ham meat previously contaminated with multi-drug resistant *S. aureus* (MRSA). It was reported that the bacterial contamination concentration was decreased by 3 log(CFU/mL) within the first 15 minutes at 4 °C and it was also stated that the LysSA11 can be used as decontaminant when cleaning steel knives (Chang et al., 2017). Furthermore, according to a study conducted by Son et al., it was stated that lysin LysB4EAD and LysSA11 reduced the *S.aures* and *B.cereus* concentrations below the detectable limit within 2 hours in boiled rice (Son et al., 2020).

Studies investigating the effects of lysine against food pathogens are not limited to *S. aureus*. For instance, lysin LysZ5 was studied against the *L. monocytogenes* in soya milk. It was observed that the concentration of *L. monocytogenes* in soya milk was reduced by 4 logs(CFU/mL) within the first 3 hours at 4 °C (Zhang et al., 2012). In another study, conducted by Ibarra-Sanchez et al. lysin PlyP100 was reduced the load of *L. monocytogenes* in Queso Fresco cheese by approximately 4.5 log(CFU/mL) within 7 days of storage at 4 °C (Ibarra-Sánchez et al., 2018). Also, lysin PlyP100 in combination with nisin completely eliminated the contamination of *L. monocytogenes* in half of the cheeses (Ibarra-Sánchez et al., 2018). Moreover, lysin Ply500 was conjugated to silica nanoparticles and the synergistic effect of silica conjugated lysin was investigated on lettuce. It 4 log reduction on iceberg lettuce was observed (Solanki et al., 2013).

Liu et al. investigated the effect of LysWL59 and LysWL60 on *Salmonella* Typhimurium on lettuce. Lettuce was treated with the 2.5mM LysWL59 and 0.5mM EDTA and 93% reduction was observed within one hour. Also, it is noted that the LysWL59 and LysWL60 have a broad host range against Gram negative bacteria pretreated with chloroform (Liu et al., 2019). In another study, conducted by Han et al. lysin LyS15S6 showed lytic activity against *E. coli*, *Shigella*, *P.aeruginosa*, *A. baumannii*, *Klebsiella*. It was noted that the LyS15S6 has a high enzymatic activity and thermostability and LyS15S6 reduced the *Salmonella* load by approximately 3

log(CFU/mL) within the 2 hours of incubation time at 8 °C of incubation temperature (Han et al., 2019).

There are also well investigated lysins against *E. coli*. As an example, the effects of lysin PlyEc2 were tested on Romaine lettuce. PlyEc2 eliminated the 99.7% of *E. coli* O157:H7 on Romaine lettuce. Also, the taste and the texture of the treated Romaine lettuce remained the same (Xu et al., 2021). In another study the lysin EndoT5 was investigated. It was pointed out that the EndoT5 was showed a lytic reduction between 4-5 log when permeabilizing agents were also added. In addition, synergistic effect of EndoT5 with polymyxin B, chlorhexidine and poly-l-lysine was studied and 5 log reduction, 5 log reduction and 4 log reduction was observed respectively. Also, EndoT5 is thermostable lysin which means after incubating, EndoT5 at 90°C for 30 minutes, the 65% of initial lytic activity retained (Shavrina et al., 2016).

Another well investigated lysin against *E.coli* is lysin SPN9CC. According to the Lim et al., SPN9CC has ability to lyse 23 Gram-negative strains tested within the 5 minutes. It is also stated that using SPN9CC with outer membrane permeabilizers can increase the activity of lysin by 4 logs. Besides, SPN9CC is stable between 24°C and 65°C, however the maximum lytic activity was observed at 50°C (Lim et al., 2014).

## **2.5 Whole Genome Sequencing**

The "command and control molecule" of cells, DNA contains all of the genetic information of living things. DNA expresses the information (genes) contained within it in the form of amino acids through RNAs. Figuring out the nucleotide sequences in DNA, or reading the DNA, is the process of sequencing it. Ever since Franklin, Watson, and Crick (1953) determined the structure of DNA, researchers have been studying the process of sequencing genomes. Since determining the

human genome's sequence is the first step toward treating genetic illnesses, this task has taken on great significance.

The first polynucleotide sequence has been identified twelve years after Watson and Crick revealed the double helix structure of DNA. This nucleotide sequence, which has 77 bases, is the yeast alanine tRNA (Holley et al., 1965). Bacteriophage MS2, whose genome is 3569 nucleotides long, has been sequenced in 1976 using a similar technique (Fiers et al., 1976). Two different methods for sequencing DNA were developed in 1977: the Maxam-Gilbert method and the Sanger method (Maxam & Gilbert, 1977) (Sanger et al., 1977).

### **2.5.1 First Generation Sequencing**

The core concept of the Maxam-Gilbert technique is the chemical break down of terminally tagged DNA molecules from the nucleobases (adenine, cytosine, guanine, and thymine) in DNA. To put this technique in brief, restriction enzymes are used to cut the DNA that must be sequenced, resulting in different-sized double-stranded DNA. The radioactive material  $^{32}\text{P}$  is used to mark the 3' ends of these DNA segments. To create a single-stranded structure, the tagged DNA fragments are denatured using a variety of techniques (such as heat, chemicals, etc.). Four tubes are equally filled with the suspension that contains these DNA fragments that have been denatured. Each sample is mixed with a chemical that has the ability to degrade one of the bases. For instance, guanine is bound using dimethyl sulfate. The DNA fragments are separated in an electrical environment from largest to smallest size using agarose gel electrophoresis, which is applied to each of these tubes independently. An X-ray sensitive film is placed on this gel, and the film has an effect on radioactivity ( $^{32}\text{P}$ ). The regions impacted by radioactive phosphorous show up as black spots or bands at the end of the film development process (autoradiography). The base sequence of the DNA from the 5' end to the 3' end is revealed when autoradiographic bands are read from bottom to top (Maxam &

Gilbert, 1977). However, the Sanger method has become more preferred by scientists because Maxam-Gilbert method uses toxic chemicals and is a slower method than Sanger.

One of the most significant advances in DNA sequencing technology is the Sanger method. Chemical analogs of deoxyribonucleotides (dNTPs) are used in the chain termination procedure. These analogues are called dideoxynucleotides. Dideoxynucleotides (ddNTPs) are unable to bind to the 5' phosphate of the subsequent dNTP because they lack the 3' hydroxyl group required for DNA chain extension. Random chain terminations and the formation of a DNA chain in every possible combination occur when ddNTPs are combined with conventional dNTPs at a certain concentration and employed in the DNA extension procedure. In gel electrophoresis, four parallel reactions are carried out for each type of ddNTP. Considering that the smaller piece will go a greater distance on the gel, the nucleotide sequence can be determined (Sanger et al., 1977). The Sanger technique dominated the market and served as the gold standard for about 30 years after it was mechanized and initially made available for use by Applied Biosystems in 1986. The read length of first-generation DNA sequencing equipment is just a little bit less than one kilobase (kb). Longer sections were sequenced using the "shotgun sequencing" approach. The process involves first dividing the region to be sequenced into pieces and then reassembling it once the sequencing is complete. The human genome project was finished in three years with the use of the shotgun sequencing technique (Venter et al., 2001).

### **2.5.2 Second Generation Sequencing**

In the past fifteen years, new sequencing methods have quickly become accessible for use in research. The first emerging second-generation sequencing technology allows for the determination of nucleotides based on the light intensity produced by pyrophosphate, which is released into the environment as a byproduct of enzymatic

reactions that add nucleotides to the DNA chain during DNA synthesis. This technique, known as pyrosequencing, relies on the idea of sequencing by synthesis because it necessitates the direct action of DNA polymerase (Andarnelundin, 1985). Due to its ability to parallelize sequencing operations and dramatically increase the amount of DNA that could be sequenced at once, this technique marked a turning point in the history of sequencing technology. Pyrosequencing technology was first used by the 454 machine to introduce second generation sequencing technologies, which were later expanded upon by Solexa which was later acquired by Illumina (Voelkerding et al., 2009).

Following this sequencing method, Applied Biosystem developed SOLID, a third option. This method operates on the concept of sequencing by ligation rather than sequencing by synthesis. Instead of DNA polymerase, the DNA ligase enzyme is utilized to ligate fluorescently tagged oligonucleotides. Although SOLID technology cannot compete with Illumina machines' reading duration and coverage characteristics, it is nonetheless cost-effective per base (Voelkerding et al., 2009). The Ion Torrent platform is the final notable second-generation sequencing platform. Unlike other approaches, Ion Torrent technology does not employ fluorescence or luminescence. This method sequences DNA by calculating the pH shift caused by hydrogen ion release during the DNA synthesis process (Rothberg et al., 2011).

### **2.5.3 Third Generation Sequencing**

Simple differences between third-generation sequencing technology and earlier generations include single-molecule sequencing and real-time sequencing. In contrast to prior technologies, third-generation sequencing does not require DNA amplification (Schadt et al., 2010). One of the most prominent third-generation sequencing technologies is Single Molecule Real Time, which is accessible on Pacific Biosciences' PacBio devices. Real-time single molecule sequencing method monitors DNA polymerase activity in real time within nanostructure gaps known as

Zero-mode waveguides (ZMWs). PacBio machines are extremely useful in de novo genome sequencing since they can generate reads longer than 10 kb (Rhoads & Au, 2015).

Nanopore sequencing is the most promising third-generation sequencing technology. Nanopore technology works on the foundation of monitoring the ionic current as molecules flow through nano-sized pores glued to the membrane. By monitoring the changing ionic current, the molecule passing through the pore can be determined. Each DNA base (dNTPs) has a unique influence on the ion current, making identification possible. When appropriate sized pores are employed, sequencing can be accomplished by measuring the change, as each nucleotide generates a separate change in ionic current. Despite its high error rate, nanopore technology is critical because it enables longer readings at a lower cost and faster rate than earlier technologies (Laver et al., 2015).

In comparison to second-generation sequencing methods, third-generation sequencing technologies require less beginning material. Third-generation sequencing technologies can produce longer and faster results. Another distinction between these two sequencing systems is that second-generation sequencing methods require cDNA sequencing for RNA sequencing, whereas some third-generation sequencing methods allow for direct RNA sequencing. When compared to the resulting data, both generations are complex due to the enormous data amount. While read lengths are the primary challenges in processing data collected through second-generation sequencing technology, reading errors are the primary challenges in third-generation sequencing (Schadt et al., 2010).

Table 2.1 Comparison of sequencing technologies



	Illumina (HiSeq400)	Oxford Nanopore (MinION)
Read length	Up to 150 bp	Up to 4 Mbp
Number of reads	1-25M	7-12M
Error rate	<1%	5-15%
Processing time	4-56 hr	~6 hr
Advantages	Highly Accurate	Long reads Portable Device



## CHAPTER 3

### MATERIALS & METHODS

#### 3.1 Materials

##### 3.1.1 Chemicals

In this study, the kits and chemicals used were carefully chosen and of analytical grade. Detailed information on the preparation of culture mediums, and chemicals, as well as a list of the kits and chemicals employed and their manufacturers, were provided in Appendices A and B.

##### 3.1.2 Bacterial Isolate and Bacteriophage

The bacterial strain employed in this study, Enteroaggregative *E. coli* (EAEC) serotype O104:H4, was acquired from the General Directorate of Public Health in Türkiye. This isolate, identified by the METU ID MET A1-200, exhibited resistance to Tetracycline, Nalidixic acid, Trimethoprim-Sulfamethoxazole, Streptomycin, and Ampicillin. The bacteriophage, MET P1-301, was isolated from a cattle fecal sample coming from Adıyaman on 02.10.2020 during a previous study conducted at the METU Food Safety Laboratory. All of the bacterial strains used in this study were detailed in the Table 3.1

Table 3.1 Bacterial strains used in this study

<b>METU ID</b>	<b>Genus</b>	<b>Species</b>	<b>Serotype</b>	<b>Pathogroup</b>	<b>Source</b>
A1-200	<i>Escherichia</i>	<i>coli</i>	O104:H4	EAEC	Public Health Agency of Turkey
A1-179	<i>Escherichia</i>	<i>coli</i>	O157:H7	EHEC	National Food Reference Laboratory
A1-90	<i>Escherichia</i>	<i>coli</i>	O26	EPEC	Water- Public Health Agency of Turkey
K1-42	<i>Escherichia</i>	<i>coli</i>	ND	ETEC	Food
A2-251	<i>Salmonella</i>	<i>enterica</i>	Typhimurium	NA	National Food Reference Laboratory

\*ND: Not Detected, NA: Not Applicable

## 3.2 Methods

### 3.2.1 Bacteriophage DNA Isolation

Fresh phage solution was used for bacteriophage DNA isolation. To prepare fresh phage solution, 100 µL was taken from the phage stock (MET P1-301), 500 µL was taken from host (MET A1-200), which was inoculated the day before and incubated at 37 °C for 18 hours, and kept in Eppendorf tube for 15 minutes to achieve phage adsorption to host cell and it was transferred to 50 mL Luria Bertani (LB) broth and was incubated in shaking incubator at 37 °C for 18 hours at 150 rpm. After incubation, it was transferred to 50 mL falcon tube, and centrifuged at 4000 rpm for

30 minutes. The supernatant was filtered through 0.22  $\mu\text{m}$  filter to obtain phage solution. Initially, 100  $\mu\text{L}$  from phage solution was taken and serial dilutions were made in an Eppendorf tube containing 900  $\mu\text{L}$  0.9% NaCl solution. From this serial dilution 100  $\mu\text{L}$  was taken along with 100  $\mu\text{L}$  from the host that was incubated 18 hours before the experiment and added to soft LB agar, vortexed, and poured over the surface of LB agar-containing Petri dishes and spread evenly. It was incubated at 37°C for 18 hours, and the counted plaques were multiplied by the dilution factors to find the titer of the phage solution in CFU/mL. After ensuring the phage titer was higher than  $10^8$  PFU/mL, a phage DNA isolation kit (Norgen, Canada) was used for phage DNA isolation, and the procedure of the kit was followed. It was incubated at 37°C for 18 hours, and the counted plaques were multiplied by the dilution factors to find the titer of the phage solution in CFU/mL.

After ensuring the phage titer was higher than  $10^8$  PFU/mL, a phage DNA isolation kit (Norgen, Canada) was used for phage DNA isolation, and the procedure of the kit was followed. Since the phage was enriched with its host, 10  $\mu\text{L}$  DNase I was added to the falcon to avoid contamination from the host genome and incubated at room temperature for 15 minutes. Then, to stop the DNase I activity, it was incubated at 75°C for 5 minutes. Subsequently, 500  $\mu\text{L}$  of Lysis Buffer B included in the kit was added and vortexed vigorously for 10 seconds. Proteinase K (20mg/mL) was used to increase DNA yield. 4  $\mu\text{L}$  of Proteinase K was added and incubated at 55°C for 15 minutes, followed by 15 minutes at 65°C, and the falcon tube was mixed by inverting every 5 minutes during this incubation. 320  $\mu\text{L}$  of isopropanol was added and the lysate was thus prepared. In order to isolate DNA from this lysate, silica membrane spin columns included in the kit were used. Up to 650  $\mu\text{L}$  of lysate can be added in spin columns. For this reason, 650  $\mu\text{L}$  of lysate was added, centrifuged at 8000 rpm for 1 minute, the liquid passing through the silica membrane into the collection tube was drained, the column was reassembled, and this process was continued until all the lysate in the falcon tube passed through the silica membrane. Afterwards, the column washing process was performed to purify the DNA attached

to the column containing silica membrane from other contaminants such as proteins and salts attached to the column, and for this, 400  $\mu\text{L}$  of Wash Solution A from the kit was added to the column and centrifuged at 8000 rpm for 1 minute. The liquid that passed down the membrane into the collection tube was drained, the column was reassembled, and this process was repeated 3 times. Finally, it was centrifuged at 14000 rpm for 2 minutes to dry the column, and the liquid passing through the silica membrane into the collection tube was drained. The final step is to elute the DNA attached to the column containing silica membrane. For this, the collection tube at the bottom of the column was removed and the column was transferred into a clean Eppendorf tube, and 75  $\mu\text{L}$  of the Elution Buffer included in the kit was added to the column. Afterwards, the column was centrifuged at 8000 rpm for 1 minute and the DNA sample was obtained.

Qubit fluorometer and Qubit high sensitivity DNA kit were used to determine the concentration of the obtained DNA. The device was calibrated before determining the DNA concentration. Standards number 1 and 2, included in the high sensitivity DNA kit, were used for calibration. 10  $\mu\text{L}$  of these standards were transferred into Qubit tubes and 190  $\mu\text{L}$  of the Qubit working solution included in the kit was added into it. These standards were vortexed vigorously for 5 seconds and placed into the device after incubating for 2 minutes, and the device created the calibration curve with these standards. Afterwards, 10  $\mu\text{L}$  of the DNA sample was transferred into Qubit tubes and 190  $\mu\text{L}$  of Qubit working solution was added into it. After the sample was vortexed and incubated at room temperature for 2 minutes, it was placed into the device and concentration data in  $\text{ng}/\mu\text{L}$  was obtained.

### **3.2.2 Whole Genome Sequencing of Bacteriophage**

The whole genome of the phage was sequenced using the Oxford Nanopore MinION Mk1b device. Bacteriophage DNA was isolated one day before and kept at  $-20^{\circ}\text{C}$ . Before sequencing, MinKNOW software was installed to collect raw data from the

device and convert them into readings, and the software and the number of pores of the flow cell were checked. If the number of pores of the flow cell is above 700, it is considered to be usable for genome sequencing. Rapid Barcoding Sequencing Kit (SQK-RBK004) was used for sequencing and library preparation. Briefly, 7.5  $\mu\text{L}$  of phage DNA was mixed with 2.5  $\mu\text{L}$  of the barcode included in the kit. Barcoded sample was incubated for 1 minute at 30°C and then at 80°C for 1 minute and were placed on ice and allowed to cool. Increased yield can be achieved by cleaning and concentrating the DNA using AMPure XP beads. For this, AMPure XP beads were collected in an Eppendorf tube and added in equal volume to the barcoded sample. It was incubated in a rotating mixer at room temperature for 5 minutes and then placed on a magnetic rack. As a result of waiting in the magnetic rack, AMPure XP beads concentrated on the magnetic side and the resulting supernatant was withdrawn with a pipette. Afterwards, the beads were washed with 200  $\mu\text{L}$  of freshly prepared 70% ethanol without disturbing the pellet, and the ethanol was withdrawn with the help of a pipette without disturbing the pellet, and this step was repeated once again. The Eppendorf tube was removed from the magnetic rack and the pellet was resuspended in 10  $\mu\text{L}$  of 10 mM Tris-HCl pH 7.5–8.0 and 50 mM NaCl. It was incubated for 2 minutes at room temperature. Afterwards, the beads were placed on a magnetic rack for at least 1 minute until the supernatant was clear and colorless. 10  $\mu\text{L}$  of supernatant was transferred to a clean 1.5 mL Eppendorf tube and 1  $\mu\text{L}$  of fast adapter was added and incubated for 5 minutes at room temperature. Thus, the sequencing library was created. The created library was loaded into the flow cell and sequencing was started. Sequencing has been started through the MinKNOW application, and the amount of data acquired can be immediately checked through the application. Sequencing was continued until at least 100x coverage of the phage genome was obtained.

To compare the results and obtain more accurate readings, Illumina sequencing technology was also used. Illumina sequencing was done commercially by BM Labosis (Ankara, Türkiye).

### **3.2.3 Bioinformatic Analysis of Bacteriophage Genome**

During sequencing, the Oxford Nanopore system takes a certain number of data and saves multiple FASTQ files to prevent data loss. For this reason, they all needed to be merged before analysis, and all files belonging to the phage were merged using the "merge" code in the terminal and one FASTQ file was obtained for the phage genome. For bioinformatics analyses, firstly, the read quality was checked with FASTQC software. For bioinformatics analyses, software recommended by Shen and Millard was used (Shen & Millard, 2021). The European server of the Galaxy web platform (usegalaxy.eu) was used to perform bioinformatics analyses. After cleaning the adapters with the PoreChop program using default settings, the reading quality was checked once again with FASTQC. The assembly of the readings was done with FLYE software and the contigs were visualized with the Bandage program. Contigs were checked for assembly errors. For this purpose, the sequence was mapped to the raw reads using the Map with BWA-MEM tool. The resulting BAM file was confirmed and checked by the Pilon program. The closest relatives of the phages were determined using the Basic Local Alignment Search Tool (BLASTn). Phage genome was annotated using BV-BRC (BACTERIAL AND VIRAL BIOINFORMATICS RESOURCE CENTER). Virulence genes and antibiotic resistance genes were checked using PhageLeads (<https://phageleads.dk/>). tRNAs in the phage genome were found using tRNAscan-SE (<http://trna.ucsc.edu/tRNAscan-SE/>). The molecular taxonomy of phages was determined based on BLASTn results. Phages were considered to be the same genus when they were compared to reference phages in the NCBI database and shared high nucleotide similarity (>95%) and protein similarities (90%) (Turner et al., 2021)

### **3.2.4 Bioinformatic Analysis of Lysin**

The lysine gene region obtained after annotation was used for further analysis. Within the scope of further analysis, physical and chemical properties of endolysin,

such as molecular weight, isoelectric point, instability index, were analyzed with the help of the ProtParam (Wilkins et al., 2005) tool. Through Interpro (Paysan-Lafosse et al., 2023), protein families, protein domains and homologous superfamilies of lysin gene regions were determined. BLASTP was used to find previously identified proteins whose protein sequence was similar to this lysine. I-Tasser (Yang & Zhang, 2015) was used to find the secondary structure, solvent accessibility, and ligand binding site of lysine. Both I-Tasser and Swiss-Model (Waterhouse et al., 2024) online tools were used to determine the tertiary structure of lysin.

### 3.2.5 Primer Design and PCR Amplification of Lysin Gene Region

In order to amplify the insert DNA with restriction enzymes, restriction enzymes were added to both the start and end of the target area when designing primers. *SacI* (5'-GAGCTC-3') and *BamHI* (5'-GGATCC-3') were the restriction enzymes employed, and their selection was based on the enzymes present in multiple cloning site of the pET-28a (+) vector. In order to guarantee effective DNA cleavage by the restriction enzyme for both the forward and reverse primers, a leader sequence was incorporated into the primer design. *BamHI* was added to the forward primer, whereas *SacI* was added to the reverse primer. The forward primer was designated with the ATG start codon, whereas the reverse primer was designated with the TAG stop codon. The correspondingly designed forward and reverse primers are provided below.

**Forward (5'→3')**: **ATAGGATCC**ATGCAACTCTCA

**Reverse (5'→3')**: GTATATCCAAAGTAGGAGCTCGAC

The leader sequence is represented by the forward primer's bold initial base sequence. The leader sequence in the reverse primer is the final three bold base sequences. Restrictions enzymes are shown by underlined sequences. The remaining

base sequences are the first 9 nucleotides of the lysin for the forward primer and the last 12 nucleotides of the lysin for the reverse primer.

The Multiple Primer Analysis program was used to verify the proposed primer's GC content, melting temperature ( $T_m$ ), and primer dimer probabilities. Next, it was verified that the designed primers did not bind anywhere else in the bacteriophage's whole genome using the SnapGene tool and Primer BLAST.

Polymerase chain reaction (PCR) was used to amplify the target region with restriction enzymes and a master mix was prepared for this. The reagents and quantities required for the master mix are given in the table below.

Table 3.2 Reagents of Master Mix

<b>Reagent</b>	<b>Volume (<math>\mu</math>L)</b>
ddH <sub>2</sub> O	16.5
Buffer	2.5
MgCl <sub>2</sub>	2
dNTPs	0.5
Forward Primer	1
Reverse Primer	1
<i>Taq</i> DNA Polymerase	0.5
DNA Template	1

Following the preparation of the master mix, the polymerase chain reaction was run under the following parameters.

Table 3.3 Polymerase Chain Reaction Conditions

Temperature (°C)	Time	Number of Cycles
95°C	5 min	1x
95°C	30 sec	
55°C	30 sec	30x
72°C	30 sec	
72°C	5 min	1x

The 1.5% agarose gel with wells were prepared and 5 µl of the PCR product was put into each well. 110 Voltage was applied to the gel for 50 minutes while it was running through an electrophoresis equipment. To observe the PCR result, the gel was immersed in ethidium bromide solution for 5 minutes and in double-distilled water for 40 minutes. The BIO-RAD GelDoc Go Imaging System was then used to visualize the gel.

### 3.2.6 DNA Purification from Gel

QIAquick Gel Extraction Kit (QIAGEN, Germany) was used to purify the lysine region with restriction enzyme amplified by PCR. Previously, with the help of a sterilized scalpel, the positive bands observed in the gel under UV light were cut and placed into Eppendorf tubes. Weighing the Eppendorf both before and after the gel piece was inserted allowed to calculate the weight of the gel. The kit's instructions were followed, and all centrifugation procedures were completed at 13000 rpm in order to purify the lysine region with restriction enzyme amplified by PCR from the gel. Three volumes of the kit's Buffer QC were applied to one volume of gel, assuming that the volume of 100 mg of gel equaled 100 µL. When the gel was completely dissolved in Buffer QC and the mixture turned yellow, 1 gel volume of isopropanol was added. Afterwards, this mixture was transferred into the spin column and centrifuged for 1 minute. The volume of the spin column was 800 µL and this process was repeated until the mixture was completely passed through the

column and the liquid flowing into the collection tube was discarded. 500  $\mu$ L of QC Buffer was added into the column and centrifuged for 1 minute, and the liquid flowing into the collection tube was discarded. To wash the column, 750  $\mu$ L PE Buffer was added and centrifuged for 1 minute. Again, the liquid flowing into the collection tube was discarded and centrifuged for an additional 1 minute to dry the column. The column was transferred to a clean Eppendorf tube and 50  $\mu$ L EB Buffer was added to the column and the column was incubated for 4 minutes then centrifuged for 1 minute to complete lysine region with restriction enzyme elution and purification. Qubit fluorometer and Qubit high sensitivity DNA kit were used to determine the concentration of the obtained lysine region with restriction enzyme amplified by PCR.

### **3.2.7 Plasmid Isolation**

The vector to transfer lysin gene region to *E. coli* BL21 was selected as pET-28(a). The pET-28(a) includes kanamycin resistance as marker, and N-terminal his-tag for easy purification. In addition, it has a strong T7 promoter and allows IPTG induced expression via lac operon. Its genomic map was provided in the figure below.

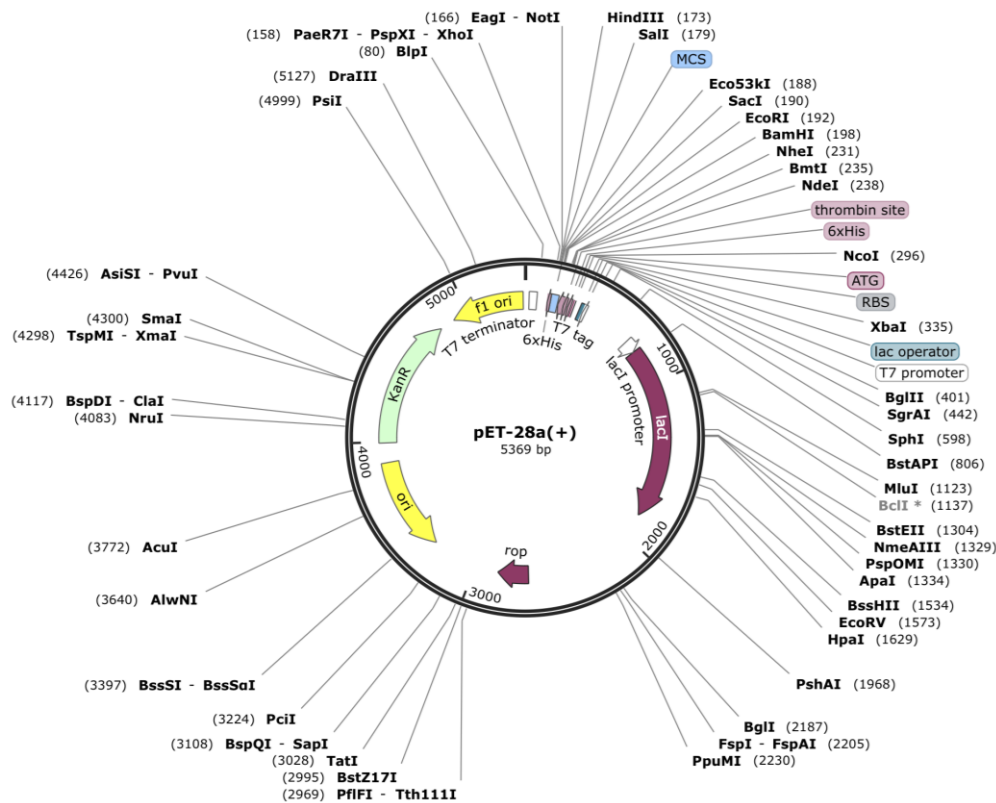


Figure 3.1 Genomic map of pET-28(a) vector. Retrieved from [https://www.snapgene.com/plasmids/pet\\_and\\_duet\\_vectors\\_\(novagen\)/pET-28a\(%\)2B](https://www.snapgene.com/plasmids/pet_and_duet_vectors_(novagen)/pET-28a(%)2B)

WizPrep Plasmid DNA Mini Kit was used for plasmid isolation, the protocol of this kit was followed, and all centrifugation processes were performed at 13000 rpm. The cell containing the pET-28a (+) plasmid was incubated in 10 mL LB broth at 37°C for 18 hours. Cultured bacterial cells were transferred to a 1.5 mL Eppendorf tube, centrifuged for 1 min, and the supernatant was discarded. This process was repeated until the entire culture was utilized. 200 µL of PD1 Buffer was added and the cell pellet was dispersed by pipetting and vortexing. Then, 200 µL PD2 Buffer was added, and the tube was mixed gently by inverting 10 times and the lysate was waited for 2 minutes to be homogeneous. Then, 300 µL PD3 Buffer was added, and the tube was mixed gently by inverting 10 times and centrifuged for 10 minutes. The spin column included in the kit was placed in a clean Eppendorf tube, and the supernatant

obtained after centrifugation was transferred to the column and centrifuged for 1 minute, and after centrifugation, the liquid collected in the Eppendorf tube was discarded. 600  $\mu$ L Wash Buffer was added to the column and centrifuged for 1 minute to wash to column. The liquid flowing into the Eppendorf tube was discarded and centrifuged for an additional 2 minutes to dry the column. The dried column was transferred to a new Eppendorf tube and 50  $\mu$ L Elution Buffer was added and incubated for 10 minutes, then centrifuged for 1 minute and the plasmid was obtained. Qubit fluorometer and Qubit high sensitivity DNA kit were used to determine the concentration of the obtained plasmid.

### **3.2.8 Double Digestion of Plasmid and DNA Purified from Gel**

Restriction enzymes *BamHI* and *SacI* were used to cut the plasmid and insert DNA at specific positions so that these fragments could be easily joined by the DNA ligase enzyme to form recombinant DNA. Restriction enzymes were purchased from New England Biolabs and their recommended protocol was used (New England Biolabs, USA) The double digestion procedure was applied both for the DNA isolated from the gel and for the isolated plasmid. Digestion with restriction enzyme was done sequentially, starting with *SacI* enzyme first. 40  $\mu$ L DNA, 5  $\mu$ L 10X rCutSmart Buffer, 1  $\mu$ L *SacI* and 4  $\mu$ L double-distilled water were combined in an Eppendorf tube and incubated at 37°C for 15 minutes. At the end of the incubation, DNA was purified from the first digestion using a spin column. Afterwards, digestion with *BamHI* was started. 40  $\mu$ L DNA, 5  $\mu$ L 10X NEBuffer r3.1, 1  $\mu$ L *BamHI* and 4  $\mu$ L double-distilled water were combined in an Eppendorf tube and incubated at 37°C for 15 minutes and digestion was completed.

### 3.2.9 Ligation

T4 Ligase (Vazyme Biotech Co. Ltd., China) enzyme was used to ligate the digested plasmid and insert DNA, and the reaction given below was carried out by using the protocol provided by the manufacturer.

Table 3.4 Reagents and Their Amounts for Ligation Reaction

Reagent	Amount
Nuclease free water	10 $\mu$ L
T4 Ligase Buffer	1 $\mu$ L
Insert DNA	0.3 pmol
Plasmid	0.03 pmol
T4 Ligase	1 $\mu$ L

This mixture was mixed by pipetting and incubated at 16°C overnight, and at the end of the incubation, the T4 ligase enzyme was inactivated by keeping it at 65°C for 20 minutes and then the mixture was kept on ice.

### 3.2.10 Preparation of Competent Cell

*Escherichia coli* BL21 strain has been made chemically competent. For this, *Escherichia coli* BL21 strain was inoculated into a 15 mL falcon tube containing 10 mL LB broth and incubated overnight in a shaking incubator at 37°C. The next day, 250  $\mu$ L of *Escherichia coli* BL21 strain was inoculated into an Erlenmeyer containing 50 mL of LB broth and incubated in a shaking incubator at 37°C until the optical density (OD) became 0.5 to 0.6 at 600 nm. After the desired OD was reached, the cells were transferred to a 50 mL falcon tube and centrifuged at 4000 rpm for 10 minutes, the supernatant was discarded and placed on ice. Afterwards, 15 mL of 100mM CaCl<sub>2</sub> solution, previously kept on ice, was added to the cell pellet and the cell pellet was dispersed by pipetting, and this mixture was kept on ice for 20 minutes

and centrifuged at 4000 rpm for 10 minutes, the supernatant was discarded. 4 mL of the 100 mM CaCl<sub>2</sub> + 15% glycerol mixture, previously kept on ice, was added and mixed. This mixture was divided into 200 µL volumes and distributed into 1.5 mL Eppendorf tubes, and competent cells were stored at -80 °C (Li et al., 2010).

### **3.2.11 Transformation**

The competent cell and plasmid with ligated insert DNA were placed on ice, and 50 µL of competent cells were transferred to two separate Eppendorf tubes, one of which was used as a control. 5 µL of the plasmid with ligated insert DNA was added to the non-control Eppendorf tube and incubated sequentially on ice for 20 min, at 42°C for 1 min, and again on ice for 2 min. Afterwards, 950 µL LB broth was added to Eppendorf tubes and incubated for 45 minutes in a shaking incubator at 37°C. At the end of the incubation, 100 µL was taken from Eppendorf tubes and inoculated onto LB agar plates containing kanamycin using the spread plate method, and the Petri dishes were incubated at 37°C for 18 hours. Colony PCR was performed on the colonies observed after incubation (Sambrook & Russel, 2001)

### **3.2.12 Colony PCR**

Colonies observed as a result of transformation were transferred to Eppendorf tubes containing 50 µL LB broth with the help of a sterile loop, and this was used as a DNA template for PCR. The reagents of the PCR master mix and the amounts of these reagents and PCR conditions are the same as those given in section 3.2.5. If a positive band was seen as a result of the PCR, the remaining PCR template in the Eppendorf tube was completely withdrawn with the help of a pipette and added into 5mL LB broth containing kanamycin with the pipette tip and incubated overnight in a shaking incubator at 37°C. The next day, 750 µL of cells were transferred to a cryotube, 750 µl of glycerol was added, it was mixed by inverting the cryotube

several times until the mixture was homogeneous and stored in replicate at -80°C to be used in protein production.

### **3.2.13 Production and Purification of Lysin**

To produce and purify lysine, the procedure of Shen et al. was used with minor modifications. Cells stored at -80°C were revived by transferring them to LB agar containing kanamycin using a sterile loop. As a result of 18 hours of incubation at 37°C, a single colony was selected from LB agar and transferred to LB broth containing 5 mL kanamycin with the help of a sterile loop and incubated for 18 hours at 37°C. At the end of the incubation, all 5 mL of LB broth was transferred into the 50 mL LB broth in the Erlenmeyer containing kanamycin and incubated at 37°C until the OD at 600 nm was 0.5-0.6. When OD<sub>600</sub> was 0.5 to 0.6, Isopropyl β-d-1-thiogalactopyranoside (IPTG) with a final concentration of 0.2mM was added and incubated at 15°C for 24 hours. Then, bacterial pellets were harvested by centrifugation at 9500 rpm at 4°C for 10 min and suspended in 50 mmol/L Tris-HCl buffer (pH 8.0). Then, the supernatant containing soluble lysine was harvested from the disrupted bacterial suspensions by centrifugation at 12200 rpm at 4°C for 20 min. The harvested supernatant was passed through a 0.45 μm filter to prevent contamination ( Shen et al., 2022).

Thermo Scientific HisPur™ Ni-NTA Purification Kit (Rockford, USA) was used to purify dissolved and impure lysine and the kit's protocol was used. All centrifugation steps were performed at 2720 rpm for 2 minutes. 200 μL protein extract and 200 μL Equilibration Buffer were mixed in a sterile Eppendorf tube. Meanwhile, the bottom of the spin column in the kit was removed, placed in an empty Eppendorf tube and centrifuged. 400 μL of Equilibration Buffer was added to the column and subjected to centrifugation. After discarding the flowing liquid in the Eppendorf tube, the initially prepared dissolved protein and buffer mixture was added to the spin column and waited for 30 minutes for the proteins to adhere to the column, and then

centrifuged. After discarding the flowing liquid in the Eppendorf tube, 400  $\mu\text{L}$  Wash Buffer was added and centrifuged. The column washing step was repeated 3 times under the same conditions. Afterwards, 200  $\mu\text{L}$  Elution Buffer was added to the column and centrifuged. The elution step was repeated 3 times, as was the washing step. A new, sterile Eppendorf tube was used for each step. The resulting lysine was stored at  $-20^{\circ}\text{C}$  and Thermo Scientific™ Multiscan Sky Microplate Spectrophotometer  $\mu\text{Drop}$  Plate (Waltham, USA) was utilized to measure absorbance at 280nm and The Beer Lambert Law which was shown in Equation 1 was used to calculate protein concentration. Mass extinction coefficient was predicted by Protparam bioinformatic tool and path length was obtained from manual of the equipment.

$$\text{Concentration } \left(\frac{\text{mg}}{\text{mL}}\right) = \frac{\text{Absorbance}}{\text{path length (cm)} \times \text{mass extinction coefficient}(\text{M}^{-1} \cdot \text{cm}^{-1})} \quad \text{Eqn(1)}$$

### 3.2.14 Antimicrobial Activity of Lysin

Antimicrobial activity of lysin was determined by making minor modifications to the procedure of Guo et al. *E. coli* O104:H4, *E. coli* O157:H7, EPEC, ETEC and *Salmonella* Typhimurium were grown in 50 mL LB broth in an Erlenmeyer at  $37^{\circ}\text{C}$  in a shaking incubator until the exponential phase ( $\text{OD}_{600}=0.4-0.5$ ). Afterwards, the cells were harvested by centrifugation at 9000 rpm for 15 minutes, and then the cells were suspended in Tris-HCl buffer and  $\text{OD}_{600}$  was adjusted to 0.1 (Guo et al., 2017). Different systems were set up to observe antimicrobial activity. 300  $\mu\text{L}$  bacteria were transferred into an Eppendorf tube and used as a bacterial control. 100  $\mu\text{L}$  bacteria and 200  $\mu\text{L}$  lysine were transferred to an Eppendorf tube, and this system was used to observe the effect of lysine on bacteria. In another system, 100  $\mu\text{L}$  bacteria, 100  $\mu\text{L}$  Tris-HCl and 100  $\mu\text{L}$  EDTA were mixed in an Eppendorf tube, and this system was used to observe the effect of Tris-HCl and EDTA on bacteria. To observe the effect of lysine with Tris-HCl and EDTA, a system was created by mixing 100  $\mu\text{L}$  bacteria, 100  $\mu\text{L}$  Tris-HCl, 100  $\mu\text{L}$  EDTA and 100  $\mu\text{L}$  lysine in an Eppendorf tube.

Furthermore, two distinct systems were set up: one with 100  $\mu\text{L}$  of bacteria, 100  $\mu\text{L}$  of Tris-HCl, and 100  $\mu\text{L}$  of lysine, and another with 100  $\mu\text{L}$  of bacteria, 100  $\mu\text{L}$  of EDTA, and 100  $\mu\text{L}$  of lysine in order to investigate the impact of lysine with Tris-HCl and EDTA independently.

After two hours of incubation at 37°C, serial dilutions were prepared, and inoculated onto Brilliant-Green Phenol-Red Lactose Sucrose (BPLS) Agar for *E. coli* strains and Xylose Lysine Deoxycholate (XLD) Agar for *Salmonella* Typhimurium using the spread plate method and incubated at 37°C overnight. Antimicrobial activity was checked by counting colonies the next day.

### **3.2.15 Statistical Analysis**

Each spectrophotometric measurement and inoculation were carried out in triplicate. Minitab was used to evaluate the data using a Tukey comparison, and a P value of less than 0.05 was deemed statistically significant.



## CHAPTER 4

### RESULTS & DISCUSSION

#### 4.1 Bioinformatic Analyses of MET P1-301 genome

The genome of bacteriophage MET P1-301 was sequenced using both Illumina and Oxford Nanopore Technologies. Therefore, genomic feature of phage MET P1-301 was determined by three different ways: using only Illumina reads, using only Oxford Nanopore reads, and performing hybrid assembly with these two reads (Table 4.1).

Table 4.1 Genomic features of MET P1-301 according to both Illumina and Nanopore sequencing technologies.

	<b>Illumina</b>	<b>Nanopore</b>	<b>Hybrid assembly</b>
<b>Genome Length (bp)</b>	89274	89638	89187
<b>GC%</b>	39%	39%	39%
<b>Close Relative</b>	<i>Escherichia coli</i> phage vB_EcoM_Shy	<i>Escherichia coli</i> phage vB_EcoM_Shy	<i>Escherichia coli</i> phage vB_EcoM_Shy
<b>Upper Kingdom</b>	<i>Heunggongvirae</i>	<i>Heunggongvirae</i>	<i>Heunggongvirae</i>
<b>Pyhlum</b>	<i>Uroviricota</i>	<i>Uroviricota</i>	<i>Uroviricota</i>
<b>Class</b>	<i>Caudoviricetes</i>	<i>Caudoviricetes</i>	<i>Caudoviricetes</i>
<b>Genus</b>	<i>Felixonavirus</i>	<i>Felixonavirus</i>	<i>Felixonavirus</i>
<b>tRNA</b>	24	24	24
<b>Virulence Gene</b>	-	-	-

<b>Antimicrobial Gene</b>	-	-	-
<b>Integrase Gene</b>	-	-	-
<b>CDS</b>	154	190	154

Raw reads of both technologies were in .fastq format; however, Nanopore read were single end while Illumina reads are paired end. Nanopore single end reads are sequences created from one end of DNA, which offers longer reads, and ability to identify repetitive regions in the genome. On the other hand, paired end reads of Illumina created from both ends of DNA, which provides accuracy and better error correction due to overlap of the reads and the ability to align them more accurately. Therefore, sequencing technology should be selected accordingly. For instance, if the goal is to identify variant in repetitive regions, Nanopore technology should be preferred (Edge & Bansal, 2019) whereas; if the goal is to figure out small indels and obtain high accuracy (Luo et al., 2019), Illumina should be preferred. However, recent studies have shown that sequencing accuracy has increased, and error rates have decreased with Nanopore's new kits with new chemistry (Sanderson et al., 2023). Furthermore, hybrid assembly combines the strength of Illumina and Nanopore read and results in a more comprehensive and accurate genome assembly. Thus, hybrid assembly is particularly beneficial for producing high-quality genome assemblies that are both accurate and complete (Wick et al., 2017).

Raw reads were analyzed according to the procedure described by Shen & Millard (2021). Firstly, quality control analysis of raw reads was done using FASTQC tool. As expected, quality scores of reads obtained by Illumina (35) were higher than the those of Nanopore (15) since. Then, trimming and subsampling was performed to A. After the phage genome was assembled. The length of the genome of MET P1-301 was 89 kb. The GC% content of the genome is 39%.

Assembled contigs was utilized to find the close relative phage by using BLAST. It was observed that the close relative of MET P1-301 is *Escherichia coli* phage vB\_EcoM\_Shy with an identity of 97%. BLAST Taxonomy result showed that MET P1-301 belongs to the upper kingdom of *Heunggongvirae*, the phylum *Uroviricota*, and the class *Caudoviricetes*. The molecular taxonomy result demonstrated that this phage is tailed phage with a double stranded DNA (dsDNA) (Turner et al., 2023). In addition, MET P1-301 belongs to genus *Felixonavirus*. Genus *Felixonavirus* is known with its ability to infect both genus *Salmonella* and *Escherichia* (Barron-Montenegro et al., 2021). However, it was also reported that the bacteria have begun to develop resistance against phages belonging to the genus *Felixonavirus* as bacteria exposed to phage. For instance, study conducted by Rivera et al. (2019), demonstrated that as host interact with phage, single nucleotide polymorphisms were detected in two genes which are responsible for the lipopolysaccharide polymerization in cell wall.

In order phages to be used in various biocontrol approaches, the genome must not contain virulence genes and/or antibiotic resistance genes. When it was check using PhageLeads, it was observed that the genome does not contain these genes.

The phage genome also may contain integrase gene that facilitate site-specific recombination between the phage genome attachment size and the bacterial genome attachment site (Groth & Calos, 2004). The genome of phage MET P1-301 was also searched against the presence of integrase gene, and it was not found in the genome. Since phage integrase genes accelerate the transmission of antimicrobial resistance genes across bacterial populations, they are important for food safety. Xiong et al. (2019) looked at the presence of class 1 integrase genes in fresh produce, raw meats and aquatic products in southern China. They discovered that these samples had a high frequency of class 1 integrase gene and AMR genes which indicates their function in spread of AMR genes in food chain.

Another feature of phage genomes is that they can contain tRNA and it's a common feature. There are 24 tRNAs in the MET P1-301 genome. This observation is consistent with the concept that phages frequently carry tRNA genes to boost the codon usage of their own genes and improve translation within the host cell. Codon usage is the phenomenon that various codons are employed to encode same amino acid. This selective employment of tRNAs operates to compensate the variations in codon usage between phage and its host. This guarantees the efficient protein synthesis throughout the infection cycle of bacteriophage thereby increasing the fitness of the phage (Bailly-Bechet et al., 2007). Furthermore, tRNAs in the phage genome may assist the integration of phage genome into the host DNA (Fouts, 2006).

The genome was annotated by using BV-BRC. The result demonstrated the fact that coding sequences were very dense in the genome as expected. Since the large gaps often interpreted as inappropriate annotation (Turner et al., 2021). The annotation results of contig sequenced using Illumina revealed 154 coding sequences (CDS), whereas the results of contig sequenced using Nanopore has 190 coding sequences. This difference is expected, and this difference is since Illumina sequencing produce short reads that may not fully resolve complex genomic regions, and this may cause the underestimation of coding sequences. There is no such a study to demonstrate the annotation difference in phage genomes due the sequencing technology. However, a study conducted to compare the Illumina and Nanopore sequencing for determining the human nasal microbiota. It was found that the Nanopore sequencing identified more bacterial genera than Illumina sequencing (Heikema et al., 2020). In another study, for example, the genome of *Gluconobacter oxydans* was sequenced using both technologies and it was concluded that combining Illumina and Nanopore reads provided the best result since they detected the structural variants and coding sequences that were not identified by using Illumina reads alone (Kranz et al., 2017).

## 4.2 Bioinformatic Analyses of LysEc301

According to the annotation of MET P1-301 genome, there is a single open reading frame that encodes phage lysin with a length of 465 bp which encodes 154 amino acids. After the open reading frame of lysin was determined, the lysin protein encoded by MET P1-301 was named with a unique identifier which includes information about the type of phage protein, the genus and the species of the host and phage ID code. Therefore, the name LysEc301 was given to this protein.

The physical and the chemical properties of LysEc301 were analyzed by utilizing ProtParam online tool as can be seen in Table 4.2 below.

Table 4.2 Properties of LysEc301.

<b>Number of amino acids</b>	154
<b>Molecular weight (Da)</b>	17124.81
<b>Theoretical pI</b>	9.36
<b>Instability index</b>	30.78
<b>Aliphatic index</b>	81.75
<b>Grand average of hydropathicity (GRAVY)</b>	-0.332
<b>Mass extinction coefficient</b>	1.164
<b>Domain</b>	Endolysin-autolysin
<b>Superfamily</b>	Lysozyme-like
<b>Family</b>	N-acetyl--D-muramidase

Molecular weight of LysEc301 was 17.1 kDa and theoretical isoelectric point (pI) is 9.36. pI is the term indicate the pH where protein has no net electrical charge thus have minimum solubility in water. Therefore, at pH 9.36 LysEc301 is less efficient. Most foods however do not have such a high pH, they are typically neutral or acidic. It mainly includes lysine and alanine amino acids with a percentage of 11% and 10.4%, respectively. Aspartate and glutamate are proteins that are negatively

charged. The total number of negatively charged amino acids is 17 with 6 aspartate residues and 11 glutamate residues. Histidine, lysine and arginine are proteins that are positively charged. The total number of positively charged amino acids is 24 with 6 arginine residues, 1 histidine residues and 17 lysine residues. Therefore, LysEc301 has a net positive charge. Lysin is typically involved in the breakdown of the cell wall of bacteria. It often requires interaction with negatively charged bacterial cell wall components such as peptidoglycan. The net positive charge of LysEc301 may enhance binding to these negatively charged structures.

The instability index of LysEc301 was computed as 30.78 which shows that protein is stable. The instability index is an estimate that reflects the stability of protein in vitro. The instability index considers the dipeptide composition of protein. The fundamental concept is that certain dipeptides are more commonly found in unstable proteins than in stable ones (Guruprasad et al., 1990). An instability index value less than 40 suggest that the protein is stable, which indicates that LysEc301 is a stable protein. Therefore, LysEc301 is likely to remain robust and functioning in vitro.

In addition, aliphatic index of LysEc301 was computed by ProtParam as 81.75. The aliphatic index is a ratio of aliphatic side chains of protein to its overall volume. Furthermore, according to study conducted by Ikai, proteins with higher aliphatic indices are more stable across a wide range of temperature. An aliphatic index of 85 or higher is generally considered high whereas an index of less than 70 is considered low (Ikai, 1980). Therefore, LysEc301 has a moderate to high stability across a range of temperatures.

Lastly, grand average of hydropathicity (GRAVY) was determined as -0.332. The overall hydrophobic or hydrophilic nature of protein can be assessed by its GRAVY score. It is found by calculating the sum of the hydropathic indices of all amino acids in the protein and dividing the results by the number of amino acids. Hydrophobic amino acids are indicated by positive values while hydrophilic amino acids are

indicated by negative values (Kyte & Doolittle, 1982). In the case of LysEc301, a GRAVY score of -0.332 indicates that the protein is hydrophilic.

BLASTP result showed that LysEc301 has a high sequence similarity (>95%) with the endolysin of *Escherichia* phage REP8. NCBI Conserved Domains data base revealed that LysEc301 has one conserved domain between amino acids 7 and 148 amino acids. LysEc301 belongs to Lysozyme-like superfamily, and it has an endolysin-autolysin domain. Therefore, it was concluded that the LysEc301 cleaves the  $\beta$ -1,4-glycosidic link between N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) and the schematic representation can be seen in Figure 4.1.

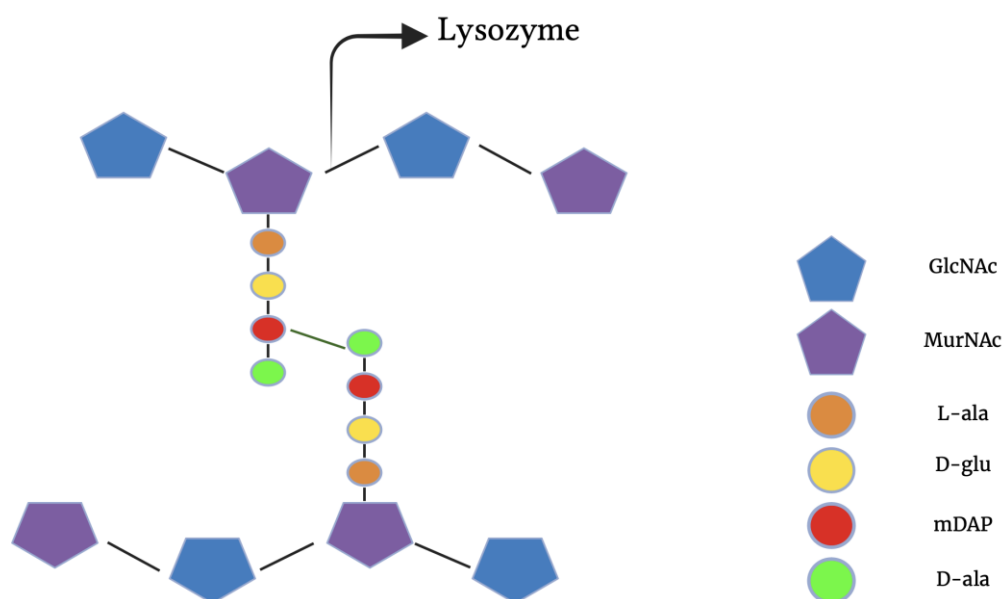


Figure 4.1 Schematic representation of bond that LysEc301 cleaves in peptidoglycan structure.

Lastly, the structure of LysEc301 (Figure 4.2) was determined by using I-Tasser which produced a model with a C-score of 1.08 and TM-score of 0.86. The C-score is a confidence score for estimating the quality of predicted models by I-Tasser. The C-score ranges from -5 to 2 with higher values indicating higher confidence. The

TM-score measures the structural similarity between two protein structures. It ranges between 0 to 1 and values higher than 0.5 indicates a model of correct topology and values closer to 1 signifying high accuracy (Yang & Zhang, 2015). A TM-score of 0.86 indicates a very high degree of accuracy in the predicted structure and suggests that the model closely resembles the actual tertiary structure of the protein. Results showed that the structure of LysEc301 has a structural analogy with an endolysin from *Escherichia coli* O157:H7 phage FAHEc1 with an identity of 0.974.

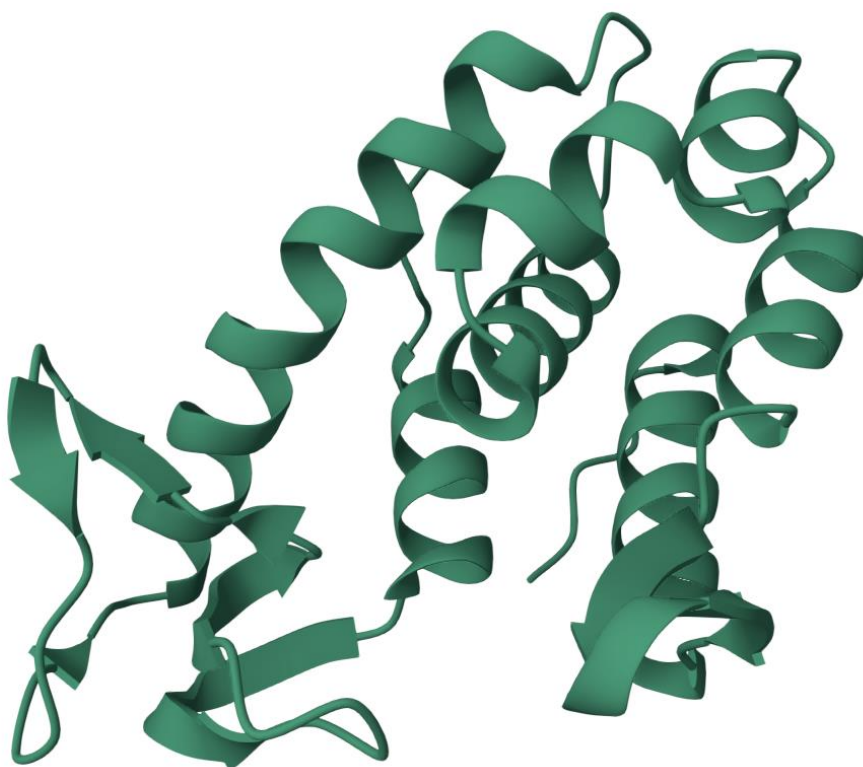


Figure 4.2 The tertiary structure of LysEc301 predicted by I-Tasser

### 4.3 Gene cloning of LysEc301

The lysin gene LysEc301 was cloned to pET-28a(+) plasmid between the *SacI* and *BamHI* restriction sites. The pet28(a) vector is highly used plasmid in protein cloning applications. It is designed for the high-level expression of recombinant proteins in *Escherichia coli*. It has a kanamycin resistance as a selection marker. In addition, its

Multiple Cloning Site (MCS) contains several unique restriction sites including *SacI* and *BamHI* (Novagen, 2005).

Also, pET-28a(+) vector has poly-histidine (6xhis) sequence that is located upstream of MCS which means when a gene is cloned into MCS, his-tag sequence is fused to the N-terminus of expressed protein. The 6xHis residues bind strongly to nickel, which allows efficient purification using metal affinity chromatography (Novagen, 2005).

Another feature of pET-28a(+) is lac operon that controls the expression of genes. IPTG (Isopropyl  $\beta$ -D-1-thiogalactopyranoside) is an analogue of lactose that cannot be metabolized by bacteria. Therefore, it mimics lactose and induces lac operon thus the gene expression (Kosinski et al., 1992).

After the construction of pET-28a(+) plasmid, it was transformed to *E. coli* BL21 cells. *E. coli* BL21 cells was made competent chemically using, which neutralizes the negative charge on cell wall and smooth the path for uptake of plasmid DNA. When the successful transformation is achieved, *E. coli* BL21 cells will gain the kanamycin resistant which is used as marker. The colonies after transformation were observed on LB agar containing kanamycin and the result was demonstrated in Figure 4.3.

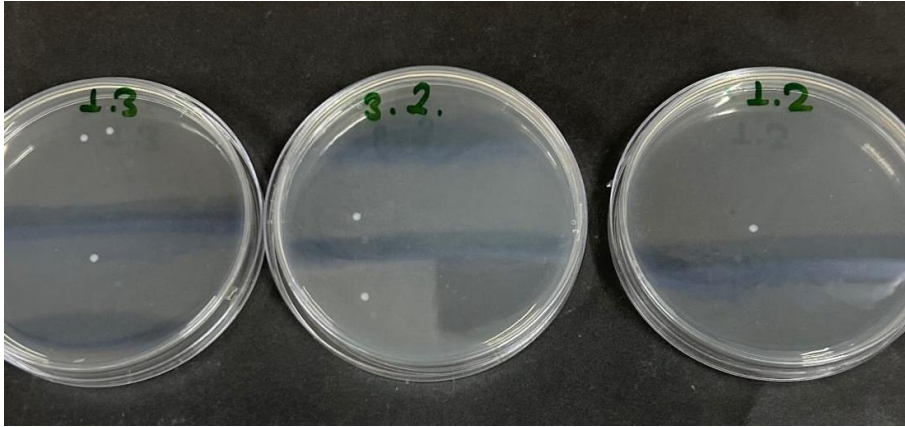


Figure 4.3 Colonies observed after transformation on LB agar containing kanamycin.

To confirm the presence of LysEc301 genes in the colonies observed after transformation, the colony PCR was performed, and results can be seen in Figure 4.4. All the samples give positive result for LysEc301 gene, and they were grown kanamycin containing LB broth and were incubated at 37°C for overnight. Then, it was mixed with 50% (v/v) glycerol in 1.5mL cryotubes and stored in -80 °C stocks of METU Food Safety Laboratory.

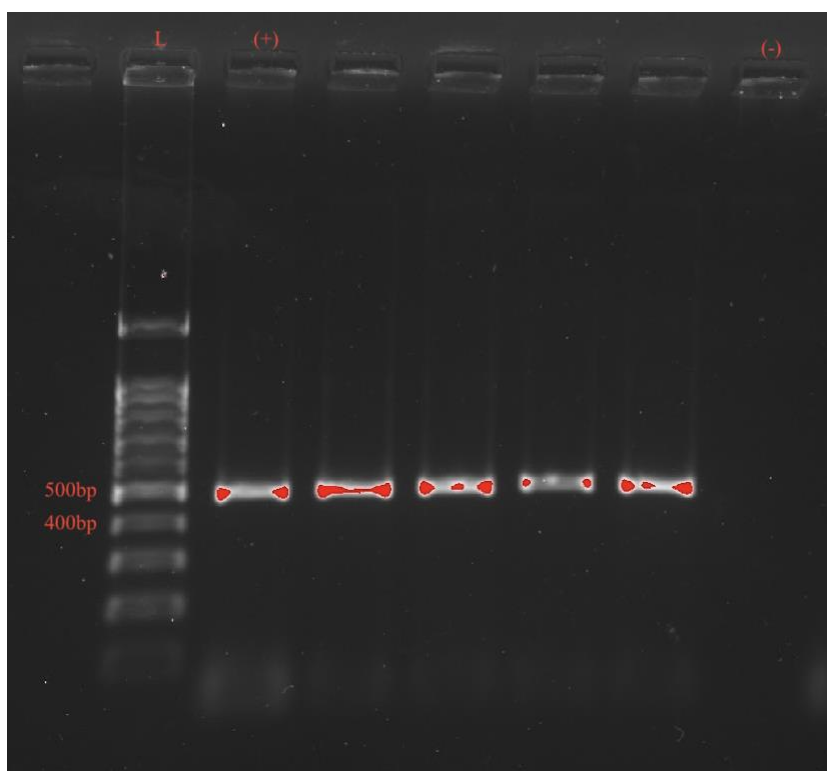


Figure 4.4 Colony PCR gel image for cloned *E. coli* BL21 cells. L represents ladder, (+) represents positive control and (-) represents negative control.

#### 4.4 Expression and Purification of LysEc301

Transformed and cloned *E. coli* BL21 cells were grown in LB broth containing kanamycin for protein production. Protein production is fundamental and highly regulated process which is crucial for life. Shortly, the protein expression process begins with the transcription where the DNA sequence is copied in to messenger RNA (mRNA). Once the mRNA is generated, it is transported to the ribosome which is the protein synthesis machinery of the cell. In the ribosome, translation takes place, during which the ribosome reads mRNA. Each three nucleotide specifies a particular amino acid and that amino acids are brought to the ribosome by transfer RNA (tRNA). The ribosome facilitates the bonding of amino acids into polypeptide chain, which eventually folds into functional protein (G. W. Li & Xie, 2011).

The pET-28a(+) plasmid confers kanamycin resistance to the host cells in this case *E. coli* BL21. Including kanamycin in growth medium ensures that only bacteria containing the plasmid will survive and proliferate. Thus, this maintains the selective pressure necessary to keep the plasmid within the bacterial population. This is crucial for efficient protein production since it ensures that the cell expressing the LysEc301 are the ones that are being propagated (Novagen, 2005).

To induce the protein expression IPTG which as analogue of lactose was used in this study. In the pET vector systems, the gene of interest is placed under the control of T7 promoter which is regulated by the lac operator. The lac operon is a set of genes controlled by the lac repressor (LacI) protein. In the absence of an inducer, LacI binds to the operator sequences thus prevents transcription of downstream genes. When the IPTG added to the culture it binds to the LacI, causes conformational changes that releases LacI from the operator sequence. Unlike lactose, IPTG is not metabolized by *E. coli* which means its concentration remains constant and this provides a stable and sustained induction (Mahoney, 1998).

The concentration of IPTG is important factor to consider. Generally, the concentration of IPTG for inducing protein expression in recombinant DNA technology ranges between 0.1 mM to 1.0 mM (Larentis et al., 2014). Typically, standard starting concentration is 1 mM which is effective for most applications. However, in cases when expressing proteins that are particularly toxic to host cells, lower concentrations such as 0.1 to 0.5 mM are preferred. However, in the previous studies, it was stated that 1.0 mM IPTG had a toxic effect on *E. coli* BL21 cells (Larentis et al., 2014). Besides, in the study conducted by Shen et al., the effect of different IPTG concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM) on the recombinant lysin was tested. They stated that the no significant difference was observed in the yield of recombined lysin when induced with 0.2-1 mM IPTG, however the yield was the higher when protein production was induced with the 0.1 mM IPTG ( Shen et al., 2022). Thus, in this study, 0.2 mM concentration of IPTG was used.

Temperature is also a critical factor in protein production since it affects the growth rate of *E. coli* BL21, the expression level of LysEc301 and the proper folding of the protein. Initially, *E. coli* BL21 was grown at 37°C, its optimal growth temperature to achieve rapid cell division and increase the cell density to an the OD<sub>600</sub> of 0.4-0.5. This optical density makes sures that the cells are in their exponential growth phase, which is characterized by high metabolic activity and is ideal for induction with IPTG. After the induction, the temperature is lowered to 15°C to improve the quality of expressed protein. Since the lower temperatures reduce the rate of protein synthesis, allowing more time for proper folding and reducing the formation of inclusion bodies, which are aggregates of misfolded proteins (Palmer & Wingfield, 2012).

After the production of the recombinant LysEc301(rLysEc301), it was purified using Thermo Scientific HisPur™ Ni-NTA Purification Kit (Rockford, USA) since the protein was tagged with 6xhis residues. Nitrilotriacetic acid resin (NTA resin) is chelating agent that binds to nickel ions tightly and provides stable matrix for affinity purification. The His-tag has a high affinity for these nickel ions. This interaction allows for efficient purification of the target protein from bacterial lysate (Bornhorst & Falke, 2000).

After the production and the purification of the rLysEc301, the concentration of it was determined spectrophotometrically by using Nanodrop. Proteins absorb UV light at 280 nm primarily due to the presence of aromatic amino acids such as tryptophan and tyrosine. These proteins have a conjugated system of double bond. When UV light at 280 nm hits these aromatic rings, it provides the energy needed for electrons to jump from a lower energy level (ground state) to a higher energy level (excited state). This absorption of energy at 280 nm results in a measurable absorbance peak (Brackenridge, 1959) and the Beer Lambert Law relates the absorbance of a solution to its concentration, path length and molar extinction coefficient (Cejnar et al., 1993).

Absorbance value at 280 nm was measured as 0.01, the path length is 1 cm and mass extinction coefficient was predicted by ProtParam as  $1.164 \text{ M}^{-1}\cdot\text{cm}^{-1}$ . Hence, the concentration of rLysEc301 was calculated as  $85.9 \mu\text{g/mL}$  by using The Beer Lambert Law.

Identifying proteins can be achieved through several methods including but not limited to mass spectrometry, Enzyme-Linked Immunosorbent Assay (ELISA), Bradford assay and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Mass spectrometry identify protein weight with high precision (Mann & Pandey, 2001), whereas ELISA quantifies proteins through antigen-antibody interactions (Murdock & Jacob, 1997). The Bradford assay quantifies total protein concentration using Coomassie dye binding (Ku et al., 2013). Finally, SDS-PAGE separates proteins with respect to their molecular weight and allows identification through size comparison (Raynal et al., 2010).

However, activity assay of the protein is also a method to prove the presence of the protein. Activity analysis can be performed if the target protein has been purified since it allows for the assessment of functional properties of protein that indicates its presence and proper folding. Thus, in this study, rLysEc301 was identified by using its lytic activity.

#### **4.5 Lytic Activity Determination**

The lytic activity assay for phage-derived endolysins typically involves measuring the ability of enzyme to lyse the cells. This assay is crucial for evaluating the efficacy of lysins as antibacterial agents. Lytic activity of rLysEc301 was tested against *E. coli* O104:H4, *E. coli* O157:H7, EPEC, ETEC and *Salmonella* Typhimurium. Results are analyzed using one-way ANOVA with Tukey comparison.

In these assays, EDTA and Tris-HCl were used to analyze whether they had a synergistic effect or not. EDTA is a chelating agent that binds divalent metal ions such as  $Mg^{2+}$  and  $Ca^{2+}$ , which are crucial for stability and integrity of bacterial cell wall. By chelating these ions, EDTA destabilizes the cell wall thus making it more susceptible to cell lysis by phage-derived lysins (Powell, 1999). On the other hand, Tris-HCl is a buffering agent that maintains a stable pH environment during the assay. The enzymatic activity of lysins can be highly dependent to pH and Tris-HCl ensures that the pH remains constant and allows endolysin to function efficiently (Bjerneroth et al., 1994).

The lytic activity of rLysEc301 against *E. coli* O104:H4 showed a significant reduction in bacterial load. The combined treatment of rLysEc301 with EDTA or Tris-HCl did not show significant difference with rLysEc301 treatment alone. rLysEc301 alone achieved approximately 3 log reduction of *E. coli* O104:H4 at the end of the 2 hours incubation at 37°C.

Similarly, the same trend was observed against *E. coli* O157:H7 and *Salmonella* Typhimurium. Again, rLysEc301 alone reduced the concentration of *E. coli* O157:H7 by approximately 3 logs, while it reduced the concentration of *Salmonella* Typhimurium by approximately 2.5 logs. Furthermore, EDTA and Tris-HCl did not show neither synergistic nor antagonistic effect with LysEc301.

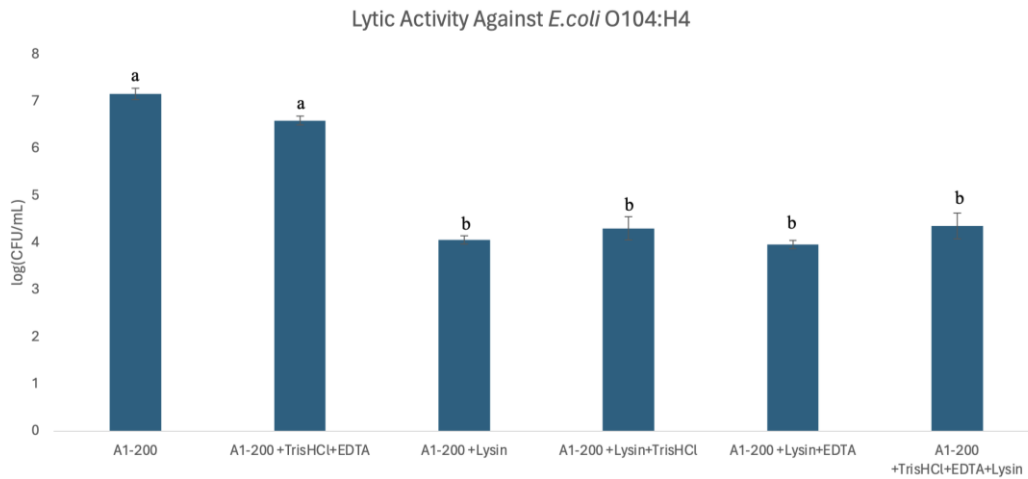


Figure 4.5 Lytic activity of LysEc301. Each column represents different treatment against *E. coli* O104:H4.

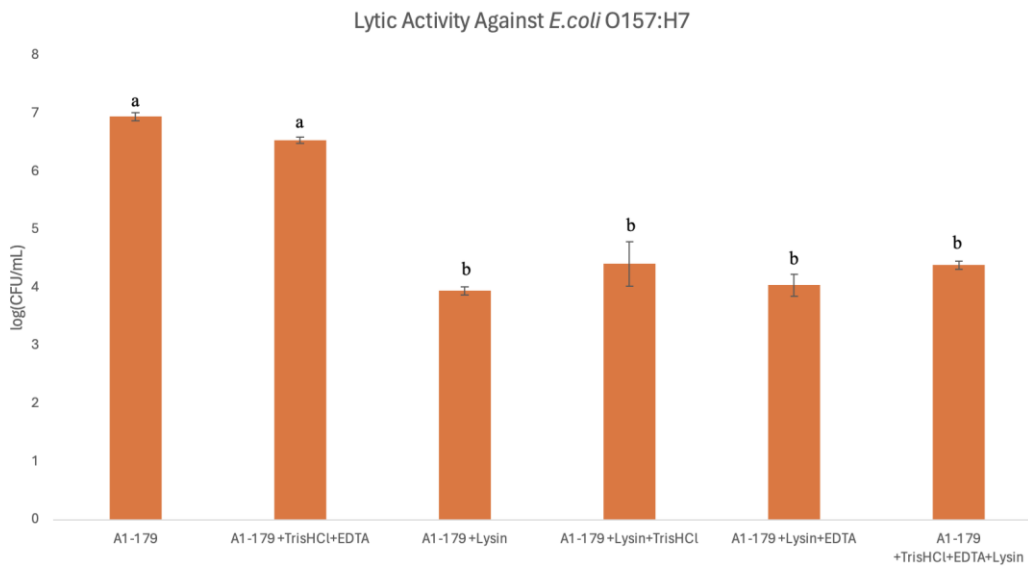


Figure 4.6. Lytic activity of LysEc301. Each column represents different treatment against *E. coli* O157:H7.

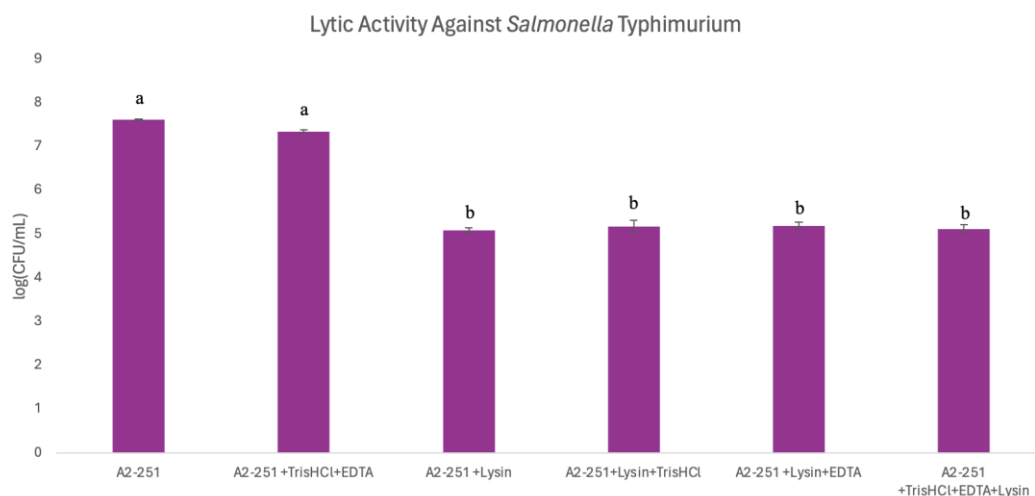


Figure 4.7 Lytic activity of LysEc301. Each column represents different treatment against *Salmonella Typhimurium*.

Moreover, the lytic activity of rLysEc301 against EPEC and ETEC was also demonstrated a significant reduction in the bacterial load. However, the reduction of EPEC and ETEC loads achieved by rLysEc301 alone is around 1.5 log (CFU/mL). On the other hand, combination of rLysEc301 with EDTA showed synergistic effect against both EPEC and ETEC. The combination of rLysEc301 with EDTA reduced EPEC and ETEC concentration approximately 2.5 and 3 log (CFU/mL) respectively.

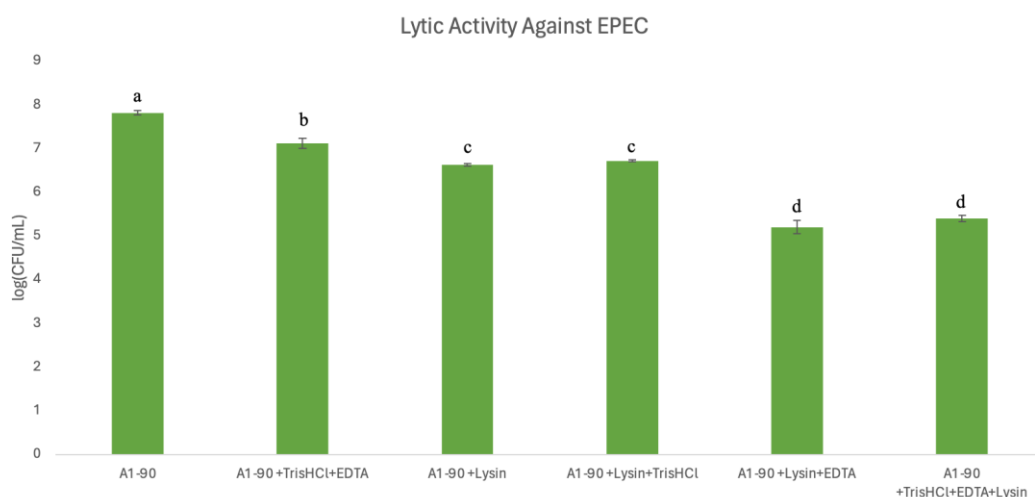


Figure 4.8 Lytic activity of LysEc301. Each column represents different treatment against EPEC.

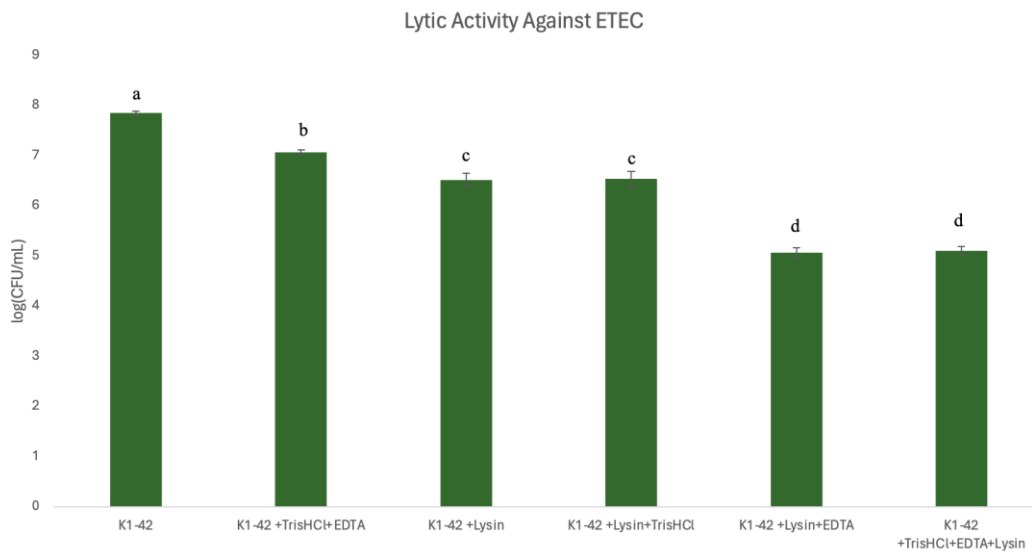


Figure 4.9 Lytic activity of LysEc301. Each column represents different treatment against ETEC.

The rLysEc301 exhibit significant lytic activity against various bacterial strains, yet its efficacy notably enhanced by the presence of EDTA in the case of EPEC and ETEC. This situation can be attributed to the structural and compositional differences in the bacterial cell wall of these strains. It can be stated that EPEC and ETEC could have additional outer membrane barriers such as lipopolysaccharides, which can block the accesses of the rLysEc301 to peptidoglycan layer thus can reduce the activity of rLysEc301. However, EDTA disturbs these barriers thereby, increases the permeability of the cell wall. In contrast, *E. coli* O157:H7, *E. coli* O104:H4, and *Salmonella* Typhimurium could have less robust outer membrane structures which allows rLysEc301 to demonstrate its lytic activity without a need for EDTA. This indicates that the effectiveness of rLysEc301 was influenced by the specific outer membrane composition of target bacteria.

The enhanced lytic activity of rLysEc301 with EDTA against ETEC and EPEC was consistent with the literature also. For instance, the study by Thummeepak et al. (2016) highlighted that the use of EDTA can enhance the antibacterial activity of

phage-derived lysins by providing easier access to peptidoglycan layer of the cell wall. In addition, Yetiskin S., (2023) proved that the EDTA increased the activity of recombinant endolysin which is a member of endopeptidase family against *Salmonella* Kentucky significantly.

In the literature, it was reported that lysin LysECP26, which has also lysozyme-like catalytic domain tried against *E. coli* O157:H7. It was seen that when LysECP26 used with the combination of distilled water, and 5 mM lactate, there was no significant difference in the bacterial reduction compared to control. However, when LysECP26 combined with 5 mM citrate or 5mM EDTA, significant reduction in the bacterial load was observed (Park & Park, 2020). In another study, the efficiency of lysin LysSP1 against *Salmonella* Typhimurium was investigated. It was seen that the LysSP1 alone was ineffective in reducing *Salmonella* Typhimurium cell counts. On the other hand, when it was combined with the 5 mM EDTA, it was observed that the *Salmonella* Typhimurium concentration was reduced by 3.2 log (CFU/mL) (Jiang et al., 2021). However, LysEc301 did not require the EDTA to reduce the load of *E. coli* O104:H4, *E. coli* O157:H7, *Salmonella* Typhimurium and just the 0.5 mM of EDTA was sufficient to reduce the concentration of EPEC and ETEC. These findings highlight the variable effectiveness of lysins depending on the bacterial strain and the presence of additional agents. The intrinsic ability of LysEc301 to reduce the bacterial load without the need for EDTA indicates a potentially broader spectrum of activity. These variations underscore the importance of optimizing lysin formulations for targeted bacterial agent and taking into account the specific interactions between lysins and chelating agents to maximize their efficacy.

Furthermore, the host range analysis for MET P1-301 bacteriophage was performed. The host range analysis determines the spectrum of bacterial strains or species that phage can infect and lyse. As a result, the host range analysis revealed that bacteriophage MET P1-301 cannot infect *E. coli* O157:H7, ETEC and *Salmonella* Typhimurium, whereas it can infect *E. coli* O104:H4 and EPEC. While recombinant

lysin of phage MET P1-301 (rLysEc301) can affect the load of *E. coli* O104:H4, *E. coli* O157:H7, EPEC, ETEC, and *Salmonella* Typhimurium. This indicates that phage-derived lysins can offer a broad spectrum of activity compared to bacteriophages. Lysins can directly degrade the peptidoglycan layer of bacterial cell wall, while phages are affected by the presence of specific receptors required for phage attachment and infection. Moreover, Guzel et al. (2024) verified that even if the bacteriophages that contain same lysin gene, they can differ in the host range analysis. Also, they proved that the most important factor in the bacteriophage infection was the ligand binding site of receptor binding proteins since in their study they examined 2 different bacteriophages with the same lysin gene but different host ranges, and they remarked that the structures of their receptor binding proteins were the same, but the ligand binding regions of these proteins were different.

Besides, lysins act rapidly and can achieve immediate bacterial lysis, unlike bacteriophages that require bacterial replication (Khan et al., 2023). Additionally, lysins do not propagate and release progeny that might contribute horizontal gene transfer which is a concern regarding bacteriophage therapy (Shah et al., 2023). The enhanced stability of lysins under varying temperatures and pH conditions also make them more suitable for diverse applications in food safety (Schmelcher et al., 2012). Also, consumer acceptance of lysins is higher compared to phages as lysins are perceived as safer and more controlled, which reduces concerns about the use of viruses in food products. Therefore, rLysEc301 can be used as antimicrobial agent instead of phages.

## CHAPTER 5

### CONCLUSION

The increasing prevalence of antibiotic-resistant *E. coli* strains cause the investigation of new alternative antimicrobial strategies. This study focused on the bacteriophage MET P1-301 genome and its putative lysin, LysEc301, as potential agent against pathogenic *E. coli* strains. Through whole genome sequencing using Illumina and Oxford Nanopore Technologies, LysEc301 was identified and characterized. It was predicted by bioinformatic analysis that LysEc301 was a stable protein. The lysin gene was successfully cloned, expressed, and purified.

While MET P1-301 bacteriophage cannot infect *Salmonella* Typhimurium, ETEC, and *E. coli* O157:H7, the lysin of MET P1-301, rLysEc301, bacteriophage could significantly reduce the concentration of these stains compared to the control. It was demonstrated that rLysEc301 exhibited significant bactericidal activity against *E. coli* O104:H4, *E. coli* O157:H7, EPEC, ETEC and *Salmonella* Typhimurium. The antimicrobial activity of rLysec301 against a range of pathogens highlights its advantage over narrow-spectrum phages.

Besides the narrower range of host of phages, another drawback of using phages in food applications is the consumer acceptance. While phages are natural and often specific to their hosts, their introduction into food is frequently met with skepticism. Therefore, phage usage in the food industry is limited. However, enzymes are more readily accepted in food industry. Considering its acceptance and effectivity over bacteriophages, it can be concluded that LysEc301 could serve as an effective alternative to traditional antibiotics.

Future study should focus on optimizing expression and purification methods to increase yield and efficacy of rLysEc301. Furthermore, the stability and efficacy of rLysEc301 under various environmental conditions and its potential synergistic effects when combined with other antimicrobial agents should be investigated. Besides, in vivo studies are required to figure out the therapeutic potential and safety of rLysEc301 in real-world applications.

## REFERENCES

- Abdelrahman, F., Easwaran, M., Daramola, O. I., Ragab, S., Lynch, S., Oduselu, T. J., Khan, F. M., Ayobami, A., Adnan, F., Torrents, E., Sanmukh, S., & El-Shibiny, A. (2021). Phage-encoded endolysins. In *Antibiotics* (Vol. 10, Issue 2, pp. 1–31). MDPI AG. <https://doi.org/10.3390/antibiotics10020124>
- Ackermann, H.-W. (2009). Phage Classification and Characterization. In M. R. J. Clokie & A. M. Kropinski (Eds.), *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions* (Vol. 501). Humana Press. <https://doi.org/10.1007/978-1-60327-164-6>
- Addis, M., & Sisay, D. (2015). Citation: Addis M, Sisay D (2015) A Review on Major Food Borne Bacterial Illnesses. *J Trop Dis*, 3(4), 176. <https://doi.org/10.4176/2329-891X.1000176>
- Ajuebor, J., McAuliffe, O., O'Mahony, J., Ross, R. P., Hill, C., & Coffey, A. (2016). Bacteriophage endolysins and their applications. *Science Progress*, 99(2), 183–199. <https://doi.org/10.3184/003685016X14627913637705>
- Allocati, N., Masulli, M., Alexeyev, M. F., & Di Ilio, C. (2013). Escherichia coli in Europe: An overview. In *International Journal of Environmental Research and Public Health* (Vol. 10, Issue 12, pp. 6235–6254). MDPI. <https://doi.org/10.3390/ijerph10126235>
- Anany, H., Brovko, L. Y., El-Arabi, T., & Griffiths, M. W. (2015). Bacteriophages as antimicrobials in food products: History, biology and application. In *Handbook of Natural Antimicrobials for Food Safety and Quality* (pp. 69–87). Elsevier Ltd. <https://doi.org/10.1016/B978-1-78242-034-7.00004-9>
- Andarnelundin~, P. \*. (1985). Enzymatic Method for Continuous Monitoring of Inorganic Pyrophosphate Synthesis?. In *ANALYTICAL BIOCHEMISTRY* (Vol. 151).
- Arsic, B., Barber, J., Čikoš, A., Mladenovic, M., Stankovic, N., & Novak, P. (2018). 16-membered macrolide antibiotics: a review. In *International*

- Journal of Antimicrobial Agents* (Vol. 51, Issue 3, pp. 283–298). Elsevier B.V. <https://doi.org/10.1016/j.ijantimicag.2017.05.020>
- Atabey, C., Kahraman, T., & Koluman, A. (2021). *Prevalence and Antibiotic Resistance of Salmonella sp., E. coli O157, and L. monocytogenes in Meat and Dairy Products*.
- Azizoglu, R., & Kathariou, S. (2010). *Impact of Growth Temperature and Agar Versus Liquid Media on Freeze-Thaw Tolerance of Yersinia enterocolitica*.
- Bai, J., Kim, Y. T., Ryu, S., & Lee, J. H. (2016). Biocontrol and rapid detection of food-borne pathogens using bacteriophages and endolysins. In *Frontiers in Microbiology* (Vol. 7, Issue APR). Frontiers Media S.A. <https://doi.org/10.3389/fmicb.2016.00474>
- Bailly-Bechet, M., Vergassola, M., & Rocha, E. (2007). Causes for the intriguing presence of tRNAs in phages. *Genome Research*, 17(10), 1486–1495. <https://doi.org/10.1101/gr.6649807>
- Barron-Montenegro, R., García, R., Dueñas, F., Rivera, D., Opazo-Capurro, A., Erickson, S., & Moreno-Switt, A. I. (2021). Comparative analysis of Felixonavirus genomes including two new members of the genus that infect *Salmonella infantis*. *Antibiotics*, 10(7). <https://doi.org/10.3390/antibiotics10070806>
- Baz, A. (2019). Prevalence, Molecular Characterization and Antimicrobial Resistance of Vero Toxigenic E.Coli in Fresh Soft Cheese, Ice Cream and Yoghurt in Mansoura City. *Alexandria Journal of Veterinary Sciences*, 62(1), 38. <https://doi.org/10.5455/ajvs.42871>
- Bean, N. H., Griffin, P. M., Goulding, J. S., & Ivey, C. B. (1990). Copyright© International Association of Milk, Food and Environmental Sanitarians Reprinted from. In *CDC Surveillance Summaries. MMWR* (Vol. 53, Issue 8).
- Benklaouz, M. B., Aggad, H., & Benameur, Q. (2020). Resistance to multiple first-line antibiotics among *Escherichia coli* from poultry in Western Algeria. *Veterinary World*, 13(2), 290–295. <https://doi.org/10.14202/vetworld.2020.290-295>

- Beveridge, T. J. (1999). Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles. In *JOURNAL OF BACTERIOLOGY* (Vol. 181, Issue 16).
- Bjerneroth, G., Sammeli, O., Li, Y. C., & Wiklund, L. (1994). Effects of alkaline buffers on cytoplasmic pH in lymphocytes. *Critical Care Medicine*, 22(10), 1550–1556.
- Bornhorst, J. A., & Falke, J. J. (2000). *Purification of Proteins Using Polyhistidine Affinity Tags*.
- Brackenridge. (1959). *THE TYROSINE AND TRYPTOPHAN CONTENT OF BLOOD SERUM IN MALIGNANT DISEASE*.
- Brown, K. L. (2000). *Control of bacterial spores*. <http://bmb.oxfordjournals.org/>
- Bryce, A., Hay, A. D., Lane, I. F., Thornton, H. V., Wootton, M., & Costelloe, C. (2016). Global prevalence of antibiotic resistance in paediatric urinary tract infections caused by *Escherichia coli* and association with routine use of antibiotics in primary care: Systematic review and meta-analysis. In *BMJ (Online)* (Vol. 352). BMJ Publishing Group. <https://doi.org/10.1136/bmj.i939>
- Bueno, E., García, P., Martínez, B., & Rodríguez, A. (2012). Phage inactivation of *Staphylococcus aureus* in fresh and hard-type cheeses. *International Journal of Food Microbiology*, 158(1), 23–27.  
<https://doi.org/10.1016/j.ijfoodmicro.2012.06.012>
- Casjens, S. R., & Hendrix, R. W. (2015). Bacteriophage lambda: Early pioneer and still relevant. In *Virology* (Vols. 479–480, pp. 310–330). Academic Press Inc.  
<https://doi.org/10.1016/j.virol.2015.02.010>
- Cejnar, M., Kobler, H., & Hunyor, S. N. (1993). *Quantitative photoplethysmography: Lambert-Beer law or inverse function incorporating light scatter*.
- Cengiz, S., & Adigüzel, M. C. (2020). Determination of virulence factors and antimicrobial resistance of *e. Coli* isolated from calf diarrhea, part of eastern Turkey. *Ankara Universitesi Veteriner Fakultesi Dergisi*, 67(4), 365–371.  
<https://doi.org/10.33988/auvfd.640990>

- Chang, Y., Kim, M., & Ryu, S. (2017). Characterization of a novel endolysin LysSA11 and its utility as a potent biocontrol agent against *Staphylococcus aureus* on food and utensils. *Food Microbiology*, *68*, 112–120.  
<https://doi.org/10.1016/j.fm.2017.07.004>
- Chapot-Chartier, M. P., & Kulakauskas, S. (2014). Cell wall structure and function in lactic acid bacteria. *Microbial Cell Factories*, *13*.  
<https://doi.org/10.1186/1475-2859-13-S1-S9>
- Chen, J., Picard, R. A., Wang, D., & Nugen, S. R. (2017). Lyophilized Engineered Phages for *Escherichia coli* Detection in Food Matrices. *ACS Sensors*, *2*(11), 1573–1577. <https://doi.org/10.1021/acssensors.7b00561>
- Chen, Z., Yu, D., He, S., Ye, H., Zhang, L., Wen, Y., Zhang, W., Shu, L., & Chen, S. (2017). Prevalence of antibiotic-resistant *Escherichia coli* in drinking water sources in Hangzhou City. *Frontiers in Microbiology*, *8*(JUN).  
<https://doi.org/10.3389/fmicb.2017.01133>
- Chopra, I., & Roberts, M. (2001). Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews*, *65*(2), 232–260.  
<https://doi.org/10.1128/mnbr.65.2.232-260.2001>
- Clifford, K., Desai, D., da Costa, C. P., Meyer, H., Klohe, K., Winkler, A., Rahman, T., Islam, T., & Zaman, M. H. (2018). Antimicrobial resistance in livestock and poor quality veterinary medicines. *Bulletin of the World Health Organization*, *96*(9), 662–664. <https://doi.org/10.2471/BLT.18.209585>
- Clokier, M. R. J., Millard, A. D., Letarov, A. V., & Heaphy, S. (2011). Phages in nature. *Bacteriophage*.
- Craig, M. (2019). *CDC's Antibiotic Resistance Threats Report, 2019 EXTENDED SPECTRUM  $\beta$ -LACTAMASE (ESBL)-PRODUCING ENTEROBACTERIACEAE*.
- Deresinski, S. (2009). Bacteriophage therapy: Exploiting smaller fleas. In *Clinical Infectious Diseases* (Vol. 48, Issue 8, pp. 1096–1101).  
<https://doi.org/10.1086/597405>

- Dewanggana, M. N., Evangeline, C., Ketty, M. D., Waturangi, D. E., Yogiara, & Magdalena, S. (2022). Isolation, characterization, molecular analysis and application of bacteriophage DW-EC to control Enterotoxigenic *Escherichia coli* on various foods. *Scientific Reports*, *12*(1).  
<https://doi.org/10.1038/s41598-021-04534-8>
- Dorny, P., Praet, N., Deckers, N., & Gabriel, S. (2009). Emerging food-borne parasites. *Veterinary Parasitology*, *163*(3), 196–206.  
<https://doi.org/10.1016/j.vetpar.2009.05.026>
- Duckworth, D. H. (1976). “Who Discovered Bacteriophage?” In *BACTEREzOLOGICAL REviEws* (Vol. 40, Issue 4).  
<https://journals.asm.org/journal/br>
- Ebrahim Rahimi. (2012). Antimicrobial resistance of *Escherichia coli* O157:H7/NM isolated from faeces of ruminant animals in Iran. *Journal of Cell and Animal Biology*, *6*(6). <https://doi.org/10.5897/jcab11.082>
- Edge, P., & Bansal, V. (2019). Longshot enables accurate variant calling in diploid genomes from single-molecule long read sequencing. *Nature Communications*, *10*(1). <https://doi.org/10.1038/s41467-019-12493-y>
- EFSA. (2016). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA Journal*, *14*(12). <https://doi.org/10.2903/J.EFSA.2016.4634>
- EFSA. (2022). The European Union One Health 2021 Zoonoses Report. *EFSA Journal*, *20*(12). <https://doi.org/10.2903/j.efsa.2022.7666>
- Egan, A. J. F., Cleverley, R. M., Peters, K., Lewis, R. J., & Vollmer, W. (2017). Regulation of bacterial cell wall growth. In *FEBS Journal* (Vol. 284, Issue 6, pp. 851–867). Blackwell Publishing Ltd. <https://doi.org/10.1111/febs.13959>
- Enciso-Martínez, Y., Barrios-Villa, E., Sepúlveda-Moreno, C. O., Ballesteros-Monrreal, M. G., Valencia-Rivera, D. E., González-Aguilar, G. A., Martínez-Téllez, M. A., & Ayala-Zavala, J. F. (2022). Prevalence of Antibiotic-Resistant *E. coli* Strains in a Local Farm and Packing Facilities of Honeydew

- Melon in Hermosillo, Sonora, Mexico. *Antibiotics*, 11(12).  
<https://doi.org/10.3390/antibiotics11121789>
- Endersen, L., & Coffey, A. (2020). The use of bacteriophages for food safety. In *Current Opinion in Food Science* (Vol. 36, pp. 1–8). Elsevier Ltd.  
<https://doi.org/10.1016/j.cofs.2020.10.006>
- Ermenlieva, N., Tsankova, G., Naydenova, D., Todorova, T. T., Tsankova, D., & Georgieva, E. (2018). Foodborne Bacteria: Potential Bioterrorism Agents. In *Food Safety - Some Global Trends*. InTech.  
<https://doi.org/10.5772/intechopen.75965>
- Fadaie, M., Dianat-Moghadam, H., Ghafouri, E., Naderi, S., Darvishali, M. H., Ghovvati, M., Khanahmad, H., Boshtam, M., & Makvandi, P. (2023). Unraveling the potential of M13 phages in biomedicine: Advancing drug nanodelivery and gene therapy. In *Environmental Research* (Vol. 238). Academic Press Inc. <https://doi.org/10.1016/j.envres.2023.117132>
- Favrin, S. J., Jassim, S. A., & Griffiths, M. W. (2003). Application of a novel immunomagnetic separation-bacteriophage assay for the detection of *Salmonella enteritidis* and *Escherichia coli* O157:H7 in food. *International Journal of Food Microbiology*, 85(1–2), 63–71.  
[https://doi.org/10.1016/S0168-1605\(02\)00483-X](https://doi.org/10.1016/S0168-1605(02)00483-X)
- FDA. (2016). *2015 Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals*.
- FDA. (2022). *Foodborne Illness-Causing Organisms in the U.S.*  
<http://www.fda.gov/educationresourcelibrary>
- FDA. (2023). *Foodborne Pathogens*. <https://www.fda.gov/food/outbreaks-foodborne-illness/foodborne-pathogens>
- Ferens, W. A., & Hovde, C. J. (2011). *Escherichia coli O157:H7: Animal Reservoir and Sources of Human Infection*.
- Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Iserentant, D., Merregaert, J., Min Jou, W., Molemans, F., Raeymaekers, A., Van den Berghe, A., Volckaert, G., & Ysebaert, M. (1976). Complete nucleotide sequence of

- bacteriophage MS2 RNA: primary and secondary structure of the replicase gene. *Nature*, 260(5551), 500–507. <https://doi.org/10.1038/260500a0>
- Fischetti, V. A. (2005). Bacteriophage lytic enzymes: Novel anti-infectives. In *Trends in Microbiology* (Vol. 13, Issue 10, pp. 491–496). <https://doi.org/10.1016/j.tim.2005.08.007>
- Forde, A., & Hill, C. (2018). Phages of life – the path to pharma. In *British Journal of Pharmacology* (Vol. 175, Issue 3, pp. 412–418). John Wiley and Sons Inc. <https://doi.org/10.1111/bph.14106>
- Fouts, D. E. (2006). Phage\_Finder: Automated identification and classification of prophage regions in complete bacterial genome sequences. *Nucleic Acids Research*, 34(20), 5839–5851. <https://doi.org/10.1093/nar/gkl732>
- Frank, C., Werber, D., Cramer, J. P., Askar, M., Faber, M., an der Heiden, M., Bernard, H., Fruth, A., Prager, R., Spode, A., Wadl, M., Zoufaly, A., Jordan, S., Kemper, M. J., Follin, P., Müller, L., King, L. A., Rosner, B., Buchholz, U., ... Krause, G. (2011). Epidemic Profile of Shiga-Toxin–Producing *Escherichia coli* O104:H4 Outbreak in Germany . *New England Journal of Medicine*, 365(19), 1771–1780. <https://doi.org/10.1056/nejmoa1106483>
- Ganegama Arachchi, G. J., Cridge, A. G., Dias-Wanigasekera, B. M., Cruz, C. D., McIntyre, L., Liu, R., Flint, S. H., & Mutukumira, A. N. (2013). Effectiveness of phages in the decontamination of *Listeria monocytogenes* adhered to clean stainless steel, stainless steel coated with fish protein, and as a biofilm. *Journal of Industrial Microbiology and Biotechnology*, 40(10), 1105–1116. <https://doi.org/10.1007/s10295-013-1313-3>
- Garde, S., Chodiseti, P. K., & Reddy, M. (2021). Peptidoglycan: Structure, Synthesis, and Regulation. *EcoSal Plus*, 9(2). <https://doi.org/10.1128/ecosalplus.ESP-0010-2020>
- Giri, N. (2021). Bacteriophage Structure, Classification, Assembly and Phage Therapy. *Biosciences Biotechnology Research Asia*, 18(2), 239–250. <https://doi.org/10.13005/bbra/2911>

- Glass, R. I., Parashar, U. D., & Estes, M. K. (2009). Norovirus Gastroenteritis. *New England Journal of Medicine*, 361(18), 1776–1785.  
<https://doi.org/10.1056/nejmra0804575>
- Golkar, Z., Bagasra, O., & Gene Pace, D. (2014). Bacteriophage therapy: A potential solution for the antibiotic resistance crisis. In *Journal of Infection in Developing Countries* (Vol. 8, Issue 2, pp. 129–136).  
<https://doi.org/10.3855/jidc.3573>
- Górski, A., Miedzybrodzki, R., & Borysowski, J. (2019). Phage therapy: A practical approach. In *Phage Therapy: A Practical Approach*. Springer International Publishing. <https://doi.org/10.1007/978-3-030-26736-0>
- Govindarajan, D. K., Viswalingam, N., Meganathan, Y., & Kandaswamy, K. (2020). Adherence patterns of Escherichia coli in the intestine and its role in pathogenesis. In *Medicine in Microecology* (Vol. 5). Elsevier B.V.  
<https://doi.org/10.1016/j.medmic.2020.100025>
- Griffin, P. M., & Tauxe, R. V. (1991). *The Epidemiology of Infections Caused by Escherichia coli O157:H7, Other Enterohemorrhagic E. coli, and the Associated Hemolytic Uremic Syndrome*.
- Groth, A. C., & Calos, M. P. (2004). Phage integrases: Biology and applications. In *Journal of Molecular Biology* (Vol. 335, Issue 3, pp. 667–678). Academic Press. <https://doi.org/10.1016/j.jmb.2003.09.082>
- Guenther, S., Herzig, O., Fieseler, L., Klumpp, J., & Loessner, M. J. (2012). Biocontrol of Salmonella Typhimurium in RTE foods with the virulent bacteriophage FO1-E2. *International Journal of Food Microbiology*, 154(1–2), 66–72. <https://doi.org/10.1016/j.ijfoodmicro.2011.12.023>
- Guo, M., Feng, C., Ren, J., Zhuang, X., Zhang, Y., Zhu, Y., Dong, K., He, P., Guo, X., & Qin, J. (2017). A novel antimicrobial endolysin, LysPA26, against Pseudomonas aeruginosa. *Frontiers in Microbiology*, 8(FEB).  
<https://doi.org/10.3389/fmicb.2017.00293>
- Guruprasad, K., Reddy, B. V. B., & Pandit, M. W. (1990). Correlation between stability of a protein and its dipeptide composition: a novel approach for

- predicting in vivo stability of a protein from its primary sequence. In *Protein Engineering* (Vol. 4, Issue 2). <http://peds.oxfordjournals.org/>
- Guzel, M., Yucefaydali, A., Yetiskin, S., Deniz, A., Yaşar Tel, O., Akçelik, M., & Soyer, Y. (2024). Genomic analysis of *Salmonella* bacteriophages revealed multiple endolysin ORFs and importance of ligand binding site of receptor binding protein. *FEMS Microbiology Ecology*.  
<https://doi.org/10.1093/femsec/fiae079>
- Hailu, W., Helmy, Y. A., Carney-Knisely, G., Kauffman, M., Fraga, D., & Rajashekara, G. (2021). Prevalence and Antimicrobial Resistance Profiles of Foodborne Pathogens Isolated from Dairy Cattle and Poultry Manure Amended Farms in Northeastern Ohio, the United States. *Antibiotics*, *10*(12).  
<https://doi.org/10.3390/antibiotics10121450>
- Han, H., Li, X., Zhang, T., Wang, X., Zou, J., Zhang, C., Tang, H., Zou, Y., Cheng, B., & Wang, R. (2019). Bioinformatic analyses of a potential *Salmonella*-virus-FelixO1 biocontrol phage BPS15S6 and the characterisation and anti-Enterobacteriaceae-pathogen activity of its endolysin LyS15S6. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *112*(11), 1577–1592. <https://doi.org/10.1007/s10482-019-01283-7>
- Heikema, A. P., Horst-Kreft, D., Boers, S. A., Jansen, R., Hiltemann, S. D., de Koning, W., Kraaij, R., de Ridder, M. A. J., van Houten, C. B., Bont, L. J., Stubbs, A. P., & Hays, J. P. (2020). Comparison of illumina versus nanopore 16s rRNA gene sequencing of the human nasal microbiota. *Genes*, *11*(9), 1–17. <https://doi.org/10.3390/genes11091105>
- Hemeg, H. A. (2018). Molecular characterization of antibiotic resistant *Escherichia coli* isolates recovered from food samples and outpatient Clinics, KSA. *Saudi Journal of Biological Sciences*, *25*(5), 928–931.  
<https://doi.org/10.1016/j.sjbs.2018.01.016>
- Holley, R. W., Apgar, J., Everett, G. A., Madison, J. T., Marquisee, M., Merrill, S. H., Penswick, J. R., & Zamir, A. (1965). Structure of a Ribonucleic Acid.

- Science*, 147(3664), 1462–1465.  
<https://doi.org/10.1126/science.147.3664.1462>
- Huang, Y., Wang, W., Zhang, Z., Gu, Y., Huang, A., Wang, J., & Hao, H. (2022). Phage Products for Fighting Antimicrobial Resistance. In *Microorganisms* (Vol. 10, Issue 7). MDPI. <https://doi.org/10.3390/microorganisms10071324>
- Ibarra-Sánchez, L. A., Van Tassell, M. L., & Miller, M. J. (2018). Antimicrobial behavior of phage endolysin PlyP100 and its synergy with nisin to control *Listeria monocytogenes* in Queso Fresco. *Food Microbiology*, 72, 128–134. <https://doi.org/10.1016/j.fm.2017.11.013>
- Ikai, A. (1980). Thermostability and Aliphatic Index of Globular Proteins. In *Biochem* (Vol. 88).
- IUBMB. (2024). *International Union of Biochemistry and Molecular Biology*.
- Jacoby, G. A. (2005). *Mechanisms of Resistance to Quinolones*.  
[https://academic.oup.com/cid/article/41/Supplement\\_2/S120/307501](https://academic.oup.com/cid/article/41/Supplement_2/S120/307501)
- Jang, J., Hur, H. G., Sadowsky, M. J., Byappanahalli, M. N., Yan, T., & Ishii, S. (2017). Environmental *Escherichia coli*: ecology and public health implications—a review. In *Journal of Applied Microbiology* (Vol. 123, Issue 3, pp. 570–581). <https://doi.org/10.1111/jam.13468>
- Jiang, Y., Xu, D., Wang, L., Qu, M., Li, F., Tan, Z., & Yao, L. (2021). *Characterization of a broad-spectrum endolysin LysSP1 encoded by a Salmonella bacteriophage*. <https://doi.org/10.1007/s00253-021-11366-z/Published>
- Kaper, J. B., Nataro, J. P., & Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. In *Nature Reviews Microbiology* (Vol. 2, Issue 2, pp. 123–140). <https://doi.org/10.1038/nrmicro818>
- Khan, F. M., Chen, J. H., Zhang, R., & Liu, B. (2023). A comprehensive review of the applications of bacteriophage-derived endolysins for foodborne bacterial pathogens and food safety: recent advances, challenges, and future perspective. In *Frontiers in Microbiology* (Vol. 14). Frontiers Media SA. <https://doi.org/10.3389/fmicb.2023.1259210>

- Klees, S., Effelsberg, N., Stührenberg, B., Mellmann, A., Schwarz, S., & Köck, R. (2020). Prevalence and epidemiology of multidrug-resistant pathogens in the food chain and the urban environment in northwestern Germany. *Antibiotics*, 9(10), 1–15. <https://doi.org/10.3390/antibiotics9100708>
- Koopmans, M., & Duizer, E. (2004). Foodborne viruses: An emerging problem. *International Journal of Food Microbiology*, 90(1), 23–41. [https://doi.org/10.1016/S0168-1605\(03\)00169-7](https://doi.org/10.1016/S0168-1605(03)00169-7)
- Kosinski, M. J., Rinas, U., & Bailey, J. E. (1992). Isopropyl- $\beta$ -D-thiogalactopyranoside influences the metabolism of *Escherichia coli*. In *Appl Microbiol Biotechnol* (Vol. 36).
- Kranz, A., Vogel, A., Degner, U., Kiefler, I., Bott, M., Usadel, B., & Polen, T. (2017). High precision genome sequencing of engineered *Gluconobacter oxydans* 621H by combining long nanopore and short accurate Illumina reads. *Journal of Biotechnology*, 258, 197–205. <https://doi.org/10.1016/j.jbiotec.2017.04.016>
- Ku, H. K., Lim, H. M., Oh, K. H., Yang, H. J., Jeong, J. S., & Kim, S. K. (2013). Interpretation of protein quantitation using the Bradford assay: Comparison with two calculation models. *Analytical Biochemistry*, 434(1), 178–180. <https://doi.org/10.1016/j.ab.2012.10.045>
- Kumar, H. S., Parvathi, A., Karunasagar, I., & Karunasagar, I. (2005). Prevalence and antibiotic resistance of *Escherichia coli* in tropical seafood. *World Journal of Microbiology and Biotechnology*, 21(5), 619–623. <https://doi.org/10.1007/s11274-004-3555-8>
- Kyte, J., & Doolittle, R. F. (1982). A Simple Method for Displaying the Hydrophobic Character of a Protein. In *J. Mol. Biol* (Vol. 157).
- Lai, W. C. B., Chen, X., Ho, M. K. Y., Xia, J., & Leung, S. S. Y. (2020). Bacteriophage-derived endolysins to target gram-negative bacteria. In *International Journal of Pharmaceutics* (Vol. 589). Elsevier B.V. <https://doi.org/10.1016/j.ijpharm.2020.119833>

- Larentis, A. L., Nicolau, J. F. M. Q., Esteves, G. D. S., Vareschini, D. T., De Almeida, F. V. R., Dos Reis, M. G., Galler, R., & Medeiros, M. A. (2014). Evaluation of pre-induction temperature, cell growth at induction and IPTG concentration on the expression of a leptospiral protein in *E. coli* using shaking flasks and microbioreactor. *BMC Research Notes*, *7*(1).  
<https://doi.org/10.1186/1756-0500-7-671>
- Laver, T., Harrison, J., O'Neill, P. A., Moore, K., Farbos, A., Paszkiewicz, K., & Studholme, D. J. (2015). Assessing the performance of the Oxford Nanopore Technologies MinION. *Biomolecular Detection and Quantification*, *3*, 1–8.  
<https://doi.org/10.1016/j.bdq.2015.02.001>
- Lee Ventola, C. (2015). *The Antibiotic Resistance Crisis Part 1: Causes and Threats* (Vol. 40, Issue 4).
- Leverentz, B., Conway, W. S., Janisiewicz, W., & Camp, M. J. (2004). Optimizing Concentration and Timing of a Phage Spray Application To Reduce *Listeria monocytogenes* on Honeydew Melon Tissue. In *Journal of Food Protection* (Vol. 67, Issue 8). <http://www.safetyalerts.com/>
- Leverstein-van Hall, M. A., Dierikx, C. M., Cohen Stuart, J., Voets, G. M., van den Munckhof, M. P., van Essen-Zandbergen, A., Platteel, T., Fluit, A. C., van de Sande-Bruinsma, N., Scharinga, J., Bonten, M. J. M., & Mevius, D. J. (2011). Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clinical Microbiology and Infection*, *17*(6), 873–880.  
<https://doi.org/10.1111/j.1469-0691.2011.03497.x>
- Li, G. W., & Xie, X. S. (2011). Central dogma at the single-molecule level in living cells. In *Nature* (Vol. 475, Issue 7356, pp. 308–315).  
<https://doi.org/10.1038/nature10315>
- Li, X., Sui, X., Zhang, Y., Sun, Y., Zhao, Y., Zhai, Y., & Wang, Q. (2010). An improved calcium chloride method preparation and transformation of competent cells. *African Journal of Biotechnology*, *9*(50), 8549–8554.  
<https://doi.org/10.5897/AJB10.105>

- Lim, J. A., Shin, H., Heu, S., & Ryu, S. (2014). Exogenous lytic activity of SPN9CC endolysin against gram-negative Bacteria. *Journal of Microbiology and Biotechnology*, 24(6), 803–811. <https://doi.org/10.4014/jmb.1403.03035>
- Liu, A., Wang, Y., Cai, X., Jiang, S., Cai, X., Shen, L., Liu, Y., Han, G., Chen, S., Wang, J., Wu, W., Li, C., Liu, S., & Wang, X. (2019). Characterization of endolysins from bacteriophage LPST10 and evaluation of their potential for controlling *Salmonella Typhimurium* on lettuce. *LWT*, 114. <https://doi.org/10.1016/j.lwt.2019.108372>
- Lorenzo, J. M., Munekata, P. E., Dominguez, R., Pateiro, M., Saraiva, J. A., & Franco, D. (2018). Main groups of microorganisms of relevance for food safety and stability: General aspects and overall description. In *Innovative technologies for food preservation: Inactivation of spoilage and pathogenic microorganisms* (pp. 53–107). Elsevier. <https://doi.org/10.1016/B978-0-12-811031-7.00003-0>
- Luo, R., Sedlazeck, F. J., Lam, T. W., & Schatz, M. C. (2019). A multi-task convolutional deep neural network for variant calling in single molecule sequencing. *Nature Communications*, 10(1). <https://doi.org/10.1038/s41467-019-09025-z>
- Maciejewska, B., Olszak, T., & Drulis-Kawa, Z. (2018). Applications of bacteriophages versus phage enzymes to combat and cure bacterial infections: an ambitious and also a realistic application? In *Applied Microbiology and Biotechnology* (Vol. 102, Issue 6, pp. 2563–2581). Springer Verlag. <https://doi.org/10.1007/s00253-018-8811-1>
- Mahoney, R. R. (1998). Galactosyl-oligosaccharide formation during lactose hydrolysis: a review. In *Food Chemistry* (Vol. 63, Issue 2).
- Mann M, & Pandey A. (2001). *Use of mass spectrometry-derived data to annotate nucleotide and protein sequence databases.*
- Martinović, T., Andjelković, U., Gajdošik, M. Š., Rešetar, D., & Josić, D. (2016). Foodborne pathogens and their toxins. *Journal of Proteomics*, 147, 226–235. <https://doi.org/10.1016/j.jprot.2016.04.029>

- Matsuzaki, S., Rashel, M., Uchiyama, J., Sakurai, S., Ujihara, T., Kuroda, M., Ikeuchi, M., Tani, T., Fujieda, M., Wakiguchi, H., & Imai, S. (2005). Bacteriophage therapy: A revitalized therapy against bacterial infectious diseases. In *Journal of Infection and Chemotherapy* (Vol. 11, Issue 5, pp. 211–219). Springer Japan. <https://doi.org/10.1007/s10156-005-0408-9>
- Maxam, A. M., & Gilbert, W. (1977). A new method for sequencing DNA (DNA chemistry/dimethyl sulfate cleavage/hydrazine/piperidine). In *Biochemistry* (Vol. 74, Issue 2).
- Mazaheri, R., & Fard, N. (2016). A Short Introduction to Bacteriophages. *Trends in Peptide and Protein Sciences*.
- Mbithi, J. N., Springthorpe, V. S., Boulet, J. R., & Sattar, S. A. (1992). Survival of Hepatitis A Virus on Human Hands and Its Transfer on Contact with Animate and Inanimate Surfaces. In *JOURNAL OF CLINICAL MICROBIOLOGY* (Vol. 30, Issue 4). <https://journals.asm.org/journal/jcm>
- Mingeot-Leclercq, M.-P., & Tulkens, P. M. (1999). Aminoglycosides: Nephrotoxicity. In *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY* (Vol. 43, Issue 5).
- Montañez-Izquierdo, V. Y., Salas-Vázquez, D. I., & Rodríguez-Jerez, J. J. (2012). Use of epifluorescence microscopy to assess the effectiveness of phage P100 in controlling *Listeria monocytogenes* biofilms on stainless steel surfaces. *Food Control*, 23(2), 470–477. <https://doi.org/10.1016/j.foodcont.2011.08.016>
- Mora, A., Herrera, A., López, C., Dahbi, G., Mamani, R., Pita, J. M., Alonso, M. P., Llovo, J., Bernárdez, M. I., Blanco, J. E., Blanco, M., & Blanco, J. (2011). Characteristics of the Shiga-toxin-producing enteroaggregative *Escherichia coli* O104:H4 German outbreak strain and of STEC strains isolated in Spain. *International Microbiology*, 14(3), 121–141. <https://doi.org/10.2436/20.1501.01.142>
- Müller, L., Schultz, A. C., Fonager, J., Jensen, T., Lisby, M., Hindsdal, K., Krusell, L., Eshøj, A., Møller, L. T., Porsbo, L. J., Böttiger, B. E., Kuhn, K., Engberg, J., & Ethelberg, S. (2015). Separate norovirus outbreaks linked to one source

- of imported frozen raspberries by molecular analysis, Denmark, 2010-2011. *Epidemiology and Infection*, 143(11), 2299–2307.  
<https://doi.org/10.1017/S0950268814003409>
- Murdock, P. J., & Jacob, D. (1997). A simple monoclonal antibody based ELISA for free protein S. Comparison with PEG precipitation. In *Clin. Lab. Haem* (Vol. 19).
- Nanda, A. M., Thormann, K., & Frunzke, J. (2015). Impact of spontaneous prophage induction on the fitness of bacterial populations and host-microbe interactions. In *Journal of Bacteriology* (Vol. 197, Issue 3, pp. 410–419). American Society for Microbiology. <https://doi.org/10.1128/JB.02230-14>
- Nelson, D. C., Schmelcher, M., Rodriguez-Rubio, L., Klumpp, J., Pritchard, D. G., Dong, S., & Donovan, D. M. (2012). Endolysins as Antimicrobials. In *Advances in Virus Research* (Vol. 83, pp. 299–365). Academic Press Inc. <https://doi.org/10.1016/B978-0-12-394438-2.00007-4>
- Nikulin, N., Nikulina, A., Zimin, A., & Aminov, R. (2023). Phages for treatment of Escherichia coli infections. In *Progress in Molecular Biology and Translational Science* (Vol. 200, pp. 171–206). Elsevier B.V. <https://doi.org/10.1016/bs.pmbts.2023.03.011>
- Nji, E., Kazibwe, J., Hambridge, T., Joko, C. A., Larbi, A. A., Dampthey, L. A. O., Nkansa-Gyamfi, N. A., Stålsby Lundborg, C., & Lien, L. T. Q. (2021). High prevalence of antibiotic resistance in commensal Escherichia coli from healthy human sources in community settings. *Scientific Reports*, 11(1). <https://doi.org/10.1038/s41598-021-82693-4>
- Novagen. (2005). *pET System Manual 11th Edition*. [www.novagen.com](http://www.novagen.com)
- Obeso, J. M., Martínez, B., Rodríguez, A., & García, P. (2008). Lytic activity of the recombinant staphylococcal bacteriophage ΦH5 endolysin active against Staphylococcus aureus in milk. *International Journal of Food Microbiology*, 128(2), 212–218. <https://doi.org/10.1016/j.ijfoodmicro.2008.08.010>
- OECD. (2016). *Antimicrobial Resistance Policy Insight*. [www.oecd.org/health/antimicrobial-resistance.htm](http://www.oecd.org/health/antimicrobial-resistance.htm)

- O'Neill, J. (2014). *Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations*. [https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations\\_1.pdf](https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf)
- Pakbin, B., Brück, W. M., & Rossen, J. W. A. (2021). Virulence factors of enteric pathogenic *Escherichia coli*: A review. In *International Journal of Molecular Sciences* (Vol. 22, Issue 18). MDPI. <https://doi.org/10.3390/ijms22189922>
- Palmer, I., & Wingfield, P. T. (2012). Preparation and extraction of insoluble (Inclusion-body) proteins from *Escherichia coli*. *Current Protocols in Protein Science*, 1(SUPPL.70). <https://doi.org/10.1002/0471140864.ps0603s70>
- Pappas, G., Kiriaze, I. J., & Falagas, M. E. (2008). Insights into infectious disease in the era of Hippocrates. In *International Journal of Infectious Diseases* (Vol. 12, Issue 4, pp. 347–350). <https://doi.org/10.1016/j.ijid.2007.11.003>
- Park, D. W., & Park, J. H. (2020). Characterization of endolysin LysECP26 derived from rV5-Like Phage vB\_EcoMECP26 for inactivation of *Escherichia coli* O157:H7. *Journal of Microbiology and Biotechnology*, 30(10), 1552–1558. <https://doi.org/10.4014/JMB.2005.05030>
- Parvin, M. S., Ali, M. Y., Mandal, A. K., Talukder, S., & Islam, M. T. (2022). Sink survey to investigate multidrug resistance pattern of common foodborne bacteria from wholesale chicken markets in Dhaka city of Bangladesh. *Scientific Reports*, 12(1). <https://doi.org/10.1038/s41598-022-14883-7>
- Pastagia, M., Schuch, R., Fischetti, V. A., & Huang, D. B. (2013). Lysins: The arrival of pathogen-directed anti-infectives. In *Journal of Medical Microbiology* (Vol. 62, Issue PART10, pp. 1506–1516). <https://doi.org/10.1099/jmm.0.061028-0>
- Paysan-Lafosse, T., Blum, M., Chuguransky, S., Grego, T., Pinto, B. L., Salazar, G. A., Bileschi, M. L., Bork, P., Bridge, A., Colwell, L., Gough, J., Haft, D. H., Letunić, I., Marchler-Bauer, A., Mi, H., Natale, D. A., Orengo, C. A., Pandurangan, A. P., Rivoire, C., ... Bateman, A. (2023). InterPro in 2022.

*Nucleic Acids Research*, 51(D1), D418–D427.

<https://doi.org/10.1093/nar/gkac993>

Powell, J. (1999). Urinary excretion of essential metals following intravenous calcium disodium edetate: an estimate of free zinc and zinc status in man.

*Journal of Inorganic Biochemistry*, 75(3), 159–165.

[https://doi.org/10.1016/S0162-0134\(99\)00054-9](https://doi.org/10.1016/S0162-0134(99)00054-9)

Radford, D., Guild, B., Strange, P., Ahmed, R., Lim, L. T., & Balamurugan, S. (2017). Characterization of antimicrobial properties of Salmonella phage Felix O1 and Listeria phage A511 embedded in xanthan coatings on Poly(lactic acid) films. *Food Microbiology*, 66, 117–128.

<https://doi.org/10.1016/j.fm.2017.04.015>

Rafiq, K., Islam, M. R., Siddiky, N. A., Samad, M. A., Chowdhury, S., Hossain, K. M. M., Rume, F. I., Hossain, M. K., Mahbub-E-Elahi, A. T. M., Ali, M. Z., Rahman, M., Amin, M. R., Masuduzzaman, M., Ahmed, S., Ara Rumi, N., & Hossain, M. T. (2022). Antimicrobial Resistance Profile of Common Foodborne Pathogens Recovered from Livestock and Poultry in Bangladesh.

*Antibiotics*, 11(11). <https://doi.org/10.3390/antibiotics11111551>

Rakonjac, J., Bennett, N. J., Spagnuolo, J., Gagic, D., & Russel, M. (2011).

*Filamentous Bacteriophage: Biology, Phage Display and Nanotechnology Applications*. <http://www.cimb.org>

Ravindran, R., & Jaiswal, A. K. (2019). Wholesomeness and safety aspects of irradiated foods. In *Food Chemistry* (Vol. 285, pp. 363–368). Elsevier Ltd.

<https://doi.org/10.1016/j.foodchem.2019.02.002>

Raynal, B., Lenormand, P., Baron, B., Hoos, S., & England, P. (2010). Quality assessment and optimization of purified protein samples: Why and how?

*Microbial Cell Factories*, 13(1). <https://doi.org/10.1186/s12934-014-0180-6>

Rhoads, A., & Au, K. F. (2015). PacBio Sequencing and Its Applications. In *Genomics, Proteomics and Bioinformatics* (Vol. 13, Issue 5, pp. 278–289).

Beijing Genomics Institute. <https://doi.org/10.1016/j.gpb.2015.08.002>

- Rivera, D., Hudson, L. K., Denes, T. G., Hamilton-West, C., Pezoa, D., & Moreno-Switt, A. I. (2019). Two phages of the genera *felixunavirus* subjected to 12 hour challenge on salmonella infantis showed distinct genotypic and phenotypic changes. *Viruses*, *11*(7). <https://doi.org/10.3390/v11070586>
- Rodríguez-Rubio, L., Gutiérrez, D., Donovan, D. M., Martínez, B., Rodríguez, A., & García, P. (2016). Phage lytic proteins: Biotechnological applications beyond clinical antimicrobials. In *Critical Reviews in Biotechnology* (Vol. 36, Issue 3, pp. 542–552). Taylor and Francis Ltd. <https://doi.org/10.3109/07388551.2014.993587>
- Rose, S. A., Modis, N. K., Bailey, N. E., Rose, S. A., Modi, N. K., Tranter, H. S., Stringer, M. F. &, & Hambleton, P. 1988. (1988). Studies on the irradiation of toxins of *Clostridium botulinum* and *Staphylococcus aureus* Studies on the irradiation of toxins of *Clostridium botu-h u m* and *Staphylococcus aureus* The effects of irradiation of *Clostridium botulinum* neurotoxin type A (BNTA) and staphylococcal enterotoxin. In *Journal of Applied Bacteriology* (Vol. 65).
- Rothberg, J. M., Hinz, W., Rearick, T. M., Schultz, J., Mileski, W., Davey, M., Leamon, J. H., Johnson, K., Milgrew, M. J., Edwards, M., Hoon, J., Simons, J. F., Marran, D., Myers, J. W., Davidson, J. F., Branting, A., Nobile, J. R., Puc, B. P., Light, D., ... Bustillo, J. (2011). An integrated semiconductor device enabling non-optical genome sequencing. *Nature*, *475*(7356), 348–352. <https://doi.org/10.1038/nature10242>
- Sabour, P. M., & Griffiths, Mansel. (2010). *Bacteriophages in the control of food- and waterborne pathogens*.
- Salmond, G. P. C., & Fineran, P. C. (2015). A century of the phage: Past, present and future. In *Nature Reviews Microbiology* (Vol. 13, Issue 12, pp. 777–786). Nature Publishing Group. <https://doi.org/10.1038/nrmicro3564>
- Sambrook, J., & Russel, D. W. (2001). *Molecular Cloning A Laboratory Manual*. Volume 1, 2, & 3 (in one file). *3rd Edition, Vol. 1, Cold Spring Harbor Laboratory Press, New York*.

- San Martín, M. F., Barbosa-Cánovas, G. V., & Swanson, B. G. (2002). Food processing by high hydrostatic pressure. *Critical Reviews in Food Science and Nutrition*, 42(6), 627–645. <https://doi.org/10.1080/20024091054274>
- Sanderson, N. D., Kapel, N., Rodger, G., Webster, H., Lipworth, S., Street, T. L., Peto, T., Crook, D., & Stoesser, N. (2023). Comparison of R9.4.1/Kit10 and R10/Kit12 Oxford Nanopore flowcells and chemistries in bacterial genome reconstruction. *Microbial Genomics*, 9(1). <https://doi.org/10.1099/mgen.0.000910>
- Sanger, F., Nicklen, S., & Coulson, A. R. (1977). *DNA sequencing with chain-terminating inhibitors (DNA polymerase/nucleotide sequences/bacteriophage 4X174)* (Vol. 74, Issue 12).
- Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States-Major pathogens. *Emerging Infectious Diseases*, 17(1), 7–15. <https://doi.org/10.3201/eid1701.P11101>
- Schadt, E. E., Turner, S., & Kasarskis, A. (2010). A window into third-generation sequencing. *Human Molecular Genetics*, 19(R2). <https://doi.org/10.1093/hmg/ddq416>
- Schmelcher, M., Donovan, D. M., & Loessner, M. J. (2012). Bacteriophage endolysins as novel antimicrobials. In *Future Microbiology* (Vol. 7, Issue 10, pp. 1147–1171). <https://doi.org/10.2217/fmb.12.97>
- Seker, E., & Kus, F. S. (2019). The prevalence, virulence factors and antibiotic resistance of escherichia coli O157 in feces of adult ruminants slaughtered in three provinces of Turkey. *Veterinarski Arhiv*, 89(1), 107–121. <https://doi.org/10.24099/vet.arhiv.0074>
- Shah, S., Das, R., Chavan, B., Bajpai, U., Hanif, S., & Ahmed, S. (2023). Beyond antibiotics: phage-encoded lysins against Gram-negative pathogens. In *Frontiers in Microbiology* (Vol. 14). Frontiers Media SA. <https://doi.org/10.3389/fmicb.2023.1170418>

- Shavrina, M. S., Zimin, A. A., Molochkov, N. V., Chernyshov, S. V., Machulin, A. V., & Mikoulinskaia, G. V. (2016). In vitro study of the antibacterial effect of the bacteriophage T5 thermostable endolysin on *Escherichia coli* cells. *Journal of Applied Microbiology*, *121*(5), 1282–1290. <https://doi.org/10.1111/jam.13251>
- Shen, A., & Millard, A. (2021). Phage Genome Annotation: Where to Begin and End. *PHAGE: Therapy, Applications, and Research*, *2*(4), 183–193. <https://doi.org/10.1089/phage.2021.0015>
- Shen, K. S., Shu, M., Tang, M. X., Yang, W. Y., Wang, S. C., Zhong, C., & Wu, G. P. (2022). Molecular cloning, expression and characterization of a bacteriophage JN01 endolysin and its antibacterial activity against *E. coli* O157:H7. *LWT*, *165*. <https://doi.org/10.1016/j.lwt.2022.113705>
- Shim, S. M., Seo, S. H., Lee, Y., Moon, G. I., Kim, M. S., & Park, J. H. (2011). Consumers' knowledge and safety perceptions of food additives: Evaluation on the effectiveness of transmitting information on preservatives. *Food Control*, *22*(7), 1054–1060. <https://doi.org/10.1016/j.foodcont.2011.01.001>
- Silhavy, T. J., Kahne, D., & Walker, S. (2010). The bacterial cell envelope. In *Cold Spring Harbor perspectives in biology* (Vol. 2, Issue 5). <https://doi.org/10.1101/cshperspect.a000414>
- Singh, A., Poshtiban, S., & Evoy, S. (2013). Recent advances in bacteriophage based biosensors for food-borne pathogen detection. In *Sensors (Switzerland)* (Vol. 13, Issue 2, pp. 1763–1786). <https://doi.org/10.3390/s130201763>
- Solanki, K., Grover, N., Downs, P., Paskaleva, E. E., Mehta, K. K., Lee, L., Schadler, L. S., Kane, R. S., & Dordick, J. S. (2013). Enzyme-based listericidal nanocomposites. *Scientific Reports*, *3*. <https://doi.org/10.1038/srep01584>
- Son, B., Kong, M., Cha, Y., Bai, J., & Ryu, S. (2020). Simultaneous control of *Staphylococcus aureus* and *Bacillus cereus* using a hybrid endolysin lysb4ead-lyssa11. *Antibiotics*, *9*(12), 1–11. <https://doi.org/10.3390/antibiotics9120906>

- Summers, W. C. (1993). *Cholera and Plague in India: The Bacteriophage Inquiry of 1927-1936* Downloaded from. <http://jhmas.oxfordjournals.org/>
- Tabla, R., Martínez, B., Rebollo, J. E., González, J., Ramírez, M. R., Roa, I., Rodríguez, A., & García, P. (2012). Bacteriophage performance against *Staphylococcus aureus* in milk is improved by high hydrostatic pressure treatments. *International Journal of Food Microbiology*, 156(3), 209–213. <https://doi.org/10.1016/j.ijfoodmicro.2012.03.023>
- Tao, J., Liu, W., Ding, W., Han, R., Shen, Q., Xia, Y., Zhang, Y., & Sun, W. (2020). A multiplex PCR assay with a common primer for the detection of eleven foodborne pathogens. *Journal of Food Science*, 85(3), 744–754. <https://doi.org/10.1111/1750-3841.15033>
- Tate, H., Li, C., Nyirabahizi, E., Tyson, G. H., Zhao, S., Rice-Trujillo, C., Jones, S. B., Ayers, S., M'Ikanatha, N. M., Hanna, S., Ruesch, L., Cavanaugh, M. E., Laksanalamai, P., Mingle, L., Matzinger, S. R., & McDermott, P. F. (2021). A National Antimicrobial Resistance Monitoring System survey of antimicrobial-resistant foodborne bacteria isolated from retail veal in the United States. *Acta Medica Portuguesa*, 84(10), 1749–1759. <https://doi.org/10.4315/JFP-21-005>
- Thummeepak, R., Kittit, T., Kunthalert, D., & Sitthisak, S. (2016). Enhanced antibacterial activity of *Acinetobacter baumannii* bacteriophage ØABP-01 endolysin (LysABP-01) in combination with colistin. *Frontiers in Microbiology*, 7(SEP). <https://doi.org/10.3389/fmicb.2016.01402>
- Turner, D., Adriaenssens, E. M., Tolstoy, I., & Kropinski, A. M. (2021). Phage Annotation Guide: Guidelines for Assembly and High-Quality Annotation. *PHAGE: Therapy, Applications, and Research*, 2(4), 170–182. <https://doi.org/10.1089/phage.2021.0013>
- Turner, D., Shkoporov, A. N., Lood, C., Millard, A. D., Dutilh, B. E., Alfenas-Zerbini, P., van Zyl, L. J., Aziz, R. K., Oksanen, H. M., Poranen, M. M., Kropinski, A. M., Barylski, J., Brister, J. R., Chanisvili, N., Edwards, R. A., Enault, F., Gillis, A., Knezevic, P., Krupovic, M., ... Adriaenssens, E. M.

- (2023). Abolishment of morphology-based taxa and change to binomial species names: 2022 taxonomy update of the ICTV bacterial viruses subcommittee. *Archives of Virology*, 168(2). <https://doi.org/10.1007/s00705-022-05694-2>
- Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., ... Zhu, X. (2001). *The Sequence of the Human Genome Downloaded from* (Vol. 291). <http://science.sciencemag.org/>
- Voelkerding, K. V., Dames, S. A., & Durtschi, J. D. (2009). Next-generation sequencing: from basic research to diagnostics. In *Clinical Chemistry* (Vol. 55, Issue 4, pp. 641–658). <https://doi.org/10.1373/clinchem.2008.112789>
- Vollmer, W., Blanot, D., & De Pedro, M. A. (2008). Peptidoglycan structure and architecture. In *FEMS Microbiology Reviews* (Vol. 32, Issue 2, pp. 149–167). <https://doi.org/10.1111/j.1574-6976.2007.00094.x>
- Walker, P. J., Siddell, S. G., Lefkowitz, E. J., Mushegian, A. R., Adriaenssens, E. M., Alfenas-Zerbini, P., Dempsey, D. M., Dutilh, B. E., García, M. L., Curtis Hendrickson, R., Junglen, S., Krupovic, M., Kuhn, J. H., Lambert, A. J., Łobocka, M., Oksanen, H. M., Orton, R. J., Robertson, D. L., Rubino, L., ... Zerbini, F. M. (2022). Recent changes to virus taxonomy ratified by the International Committee on Taxonomy of Viruses (2022). *Archives of Virology*, 167(11), 2429–2440. <https://doi.org/10.1007/s00705-022-05516-5>
- Walsh, Christopher. (2003). *Antibiotics : actions, origins, resistance*. ASM Press.
- Waterhouse, A. M., Studer, G., Robin, X., Bienert, S., Tauriello, G., & Schwede, T. (2024). The structure assessment web server: for proteins, complexes and more. *Nucleic Acids Research*. <https://doi.org/10.1093/nar/gkae270>
- Wheeler, C., Vogt, T. M., Armstrong, G. L., Vaughan, G., Weltman, A., Nainan, O. V., Dato, V., Xia, G., Waller, K., Amon, J., Lee, T. M., Highbaugh-Battle, A., Hembree, C., Evenson, S., Ruta, M. A., Williams, I. T., Fiore, A. E., &

- Bell, B. P. (2005). *An Outbreak of Hepatitis A Associated with Green Onions*.  
www.nejm.org
- WHO. (2023). *Antimicrobial resistance*. <https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance>
- WHO. (2024). *Bacterial Priority Pathogens List, 2024*.  
<https://iris.who.int/bitstream/handle/10665/376776/9789240093461-eng.pdf?sequence=1>
- Wick, R. R., Judd, L. M., Gorrie, C. L., & Holt, K. E. (2017). Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Computational Biology*, 13(6). <https://doi.org/10.1371/journal.pcbi.1005595>
- Wicken, A. J. (1985). *Bacterial Cell Walls and Surfaces*.
- Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J.-C., Williams, K. L., Appel, R. D., & Hochstrasser, D. F. (2005). The ExPASy Server 531 53 531 Protein Identification and Analysis Tools in the ExPASy Server. In *From: Methods in Molecular Biology* (Vol. 112, Issue 2). <http://www.expasy.hcuge.ch/>
- Xiong, L., Sun, Y., Shi, L., & Yan, H. (2019). Characterization of antimicrobial resistance genes and class 1 integrase gene in raw meat and aquatic product, fresh vegetable and fruit, and swine manure in southern China. *Food Control*, 104, 240–246. <https://doi.org/10.1016/j.foodcont.2019.05.004>
- Xu, S., Campisi, E., Li, J., & Fischetti, V. A. (2021). Decontamination of *Escherichia coli* O157:H7 on fresh Romaine lettuce using a novel bacteriophage lysin. *International Journal of Food Microbiology*, 341. <https://doi.org/10.1016/j.ijfoodmicro.2021.109068>
- Yang, J., & Zhang, Y. (2015). I-TASSER server: New development for protein structure and function predictions. *Nucleic Acids Research*, 43(W1), W174–W181. <https://doi.org/10.1093/nar/gkv342>
- Yap, M. L., & Rossmann, M. G. (2014). Structure and function of bacteriophage T4. In *Future Microbiology* (Vol. 9, Issue 12, pp. 1319–1337). Future Medicine Ltd. <https://doi.org/10.2217/fmb.14.91>

- Yetiskin, S. (2023). *PRODUCTION AND PURIFICATION OF ENDOLYSINS FROM SALMONELLA BACTERIOPHAGES*. Middle East Technical University.
- Young, R. Y. (1992). Bacteriophage Lysis: Mechanism and Regulation. In *MICROBIOLOGICAL REVIEWS* (Vol. 56, Issue 3).
- Yu, T., Sun, Z., Cao, X., Pang, Q., & Deng, H. (2022). Recent trends in T7 phage application in diagnosis and treatment of various diseases. In *International Immunopharmacology* (Vol. 110). Elsevier B.V.  
<https://doi.org/10.1016/j.intimp.2022.109071>
- Zhang, H., Bao, H., Billington, C., Hudson, J. A., & Wang, R. (2012). Isolation and lytic activity of the Listeria bacteriophage endolysin LysZ5 against Listeria monocytogenes in soya milk. *Food Microbiology*, 31(1), 133–136.  
<https://doi.org/10.1016/j.fm.2012.01.005>
- Zhang, H., Wang, R., & Bao, H. (2013). Phage inactivation of foodborne shigella on ready-to-eat spiced chicken. *Poultry Science*, 92(1), 211–217.  
<https://doi.org/10.3382/ps.2011-02037>
- Zhou, Y., Wan, Q., Bao, H., Guo, Y., Zhu, S., Zhang, H., Pang, M., & Wang, R. (2022). Application of a novel lytic phage vB\_EcoM\_SQ17 for the biocontrol of Enterohemorrhagic Escherichia coli O157:H7 and Enterotoxigenic E. coli in food matrices. *Frontiers in Microbiology*, 13.  
<https://doi.org/10.3389/fmicb.2022.929005>

## APPENDICES

### A. CHEMICALS AND KITS

Table A.1 The list of chemicals and kits with their producers.

<b>Chemicals</b>	<b>Producers</b>
American Bacteriological Agar	Condalab (Madrid, Spain)
Luria Bertani (LB) Broth	Condalab (Madrid, Spain)
Brain Heart Infusion (BHI) Broth	Merck (Darmstadt, Germany)
Sodium chloride	Merck (Darmstadt, Germany)
Brilliant-green phenol-red lactose sucrose Agar	Merck (Darmstadt, Germany)
EDTA disodium salt dihydrate	Applichem (Darmstadt, Germany)
Isopropyl- $\beta$ -D-Thiogalactopyranoside	Bioshop (Burlington, Canada)
Tris HCl	Merck (Darmstadt, Germany)
Sterile Nuclease Free Water	Bioshop (Burlington, Canada)
Proteinase K	Roche
Ethanol	Isolab (Eschau, Germany)
BamHI	NEB (Ipswich, USA)
SacI	NEB (Ipswich, USA)
Bacteriophage DNA Isolation Kit	NORGEN (Thorold, Canada)
WizPrep™ Plasmid DNA Mini Kit	Wizbio (South Korea)
QIAquick® Gel Extraction Kit	QIAGEN (Hilden, Germany)
HisPur™ Ni-NTA Purification Kit	Thermo Scientific (Rockford, USA)

## **B. PREPARATION OF MEDIA**

### **1. Preparation of LB Broth**

25 grams of LB broth was dissolved in 1 Liter distilled water by continuous heating and stirring. Then, it was autoclaved at 121°C for 15 minutes.

### **2. Preparation of LB Agar**

25 grams of LB broth and 6 grams of Agar Bacteriological was dissolved in 1 Liter distilled water by continuous heating and stirring. Then it was autoclaved at 121°C for 15 minutes. Upon cooling, it was poured into Petri Dishes.

### **3. Preparation of Soft LB Agar**

25 grams of LB broth and 15 grams of Agar Bacteriological was dissolved in 1 Liter distilled water by continuous heating and stirring. Then, it was distributed to glass tubes as 4 mL. After, it was autoclaved at 121°C for 15 minutes.

### **4. Preparation of BHI Broth**

37 grams of BHI broth was dissolved in 1 Liter distilled water by continuous heating and stirring. After, it was distributed to glass tubes as 10 mL. Then, it was autoclaved at 121°C for 15 minutes.

### **5. Preparation of 50mM Tris-HCl**

7.9 grams of Tris-HCl in 1 Liter distilled water by continuous heating and stirring. Then, it was autoclaved at 121°C for 15 minutes.

### **6. Preparation of 0.5 mM EDTA**

0.186 grams of EDTA in 1 Liter distilled water by continuous heating and stirring. Then, it was autoclaved at 121°C for 15 minutes.

### **7. Preparation of BPLS Agar**

51.5 grams of BPLS Agar was dissolved in 1 Liter distilled water by continuous heating and stirring. After boiling was observed, it was placed into 50°C water bath. Upon cooling, it was poured into Petri Dishes.

### **8. Preparation of 0.9% NaCl Solution**

9 grams of NaCl was dissolved in 1 Liter distilled water by continuous heating and stirring. Then, it was autoclaved at 121°C for 15 minutes.

### **9. Preparation of 0.1 M Stock Solution IPTG**

0.238 grams of IPTG was dissolved in 10 mL water to prepare 0.1M stock solution



**C. Properties of primer used.**

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	<b>Sequence</b>	<b>T<sub>m</sub>(°C)</b>	<b>GC%</b>	<b>Number of nucleotides</b>	<b>Self/Cross Dimer</b>
<b>Forward</b>	ATAGGATCCATGCAACTCTCA	61.6	42.9	21	No/No
<b>Reverse</b>	GTATATCCAAAGTAGGAGCTCGAC	61.3	45.8	24	No/No

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#### D. Annotation of MET P1-301 bacteriophage genome

Table D. 1 Annotation of genome using only Illumina reads

<b>Locus Tag</b>	<b>Length (bp)</b>	<b>AA Length</b>	<b>Product</b>
MET_P1_301_I_1	384	127	Phage protein
MET_P1_301_I_2	213	70	Phage protein
MET_P1_301_I_3	300	99	Phage protein
MET_P1_301_I_4	360	119	Phage protein
MET_P1_301_I_5	516	171	Phage protein
MET_P1_301_I_6	261	86	Phage protein
MET_P1_301_I_7	546	181	Dihydrofolate reductase (EC 1.5.1.3)
MET_P1_301_I_8	900	299	Thymidylate synthase (EC 2.1.1.45)
MET_P1_301_I_9	372	123	Phage protein
MET_P1_301_I_10	195	64	Phage protein
MET_P1_301_I_11	2361	786	Phage tail fiber
MET_P1_301_I_12	1176	391	Phage tail fiber
MET_P1_301_I_13	303	100	Phage protein
MET_P1_301_I_14	855	284	Phage protein
MET_P1_301_I_15	1470	489	Phage protein
MET_P1_301_I_16	420	139	Phage protein
MET_P1_301_I_17	624	207	Phage baseplate
MET_P1_301_I_18	978	325	Phage protein
MET_P1_301_I_19	342	113	Phage protein
MET_P1_301_I_20	798	265	Phage protein
MET_P1_301_I_21	2229	742	Phage tail tape measure
MET_P1_301_I_22	240	79	Phage protein
MET_P1_301_I_23	399	132	Phage protein

MET_P1_301_I_24	447	148	Phage protein
MET_P1_301_I_25	1353	450	Phage protein
MET_P1_301_I_26	600	199	Phage protein
MET_P1_301_I_27	402	133	Phage protein
MET_P1_301_I_28	483	160	Phage protein
MET_P1_301_I_29	450	149	Phage protein
MET_P1_301_I_30	1107	368	Phage protein
MET_P1_301_I_31	378	125	Phage protein
MET_P1_301_I_32	1347	448	Phage protein
MET_P1_301_I_33	333	110	Phage protein
MET_P1_301_I_34	501	166	Phage protein
MET_P1_301_I_35	1467	488	Phage protein
MET_P1_301_I_36	1602	533	Phage terminase, large subunit
MET_P1_301_I_37	201	66	Phage protein
MET_P1_301_I_38	177	58	hypothetical protein
MET_P1_301_I_39	735	244	Phage protein
MET_P1_301_I_40	558	185	Phage protein
MET_P1_301_I_41	171	56	Phage protein
MET_P1_301_I_42	198	65	Phage protein
MET_P1_301_I_43	111	36	hypothetical protein
MET_P1_301_I_44	141	46	Phage protein
MET_P1_301_I_45	93	30	Phage protein
MET_P1_301_I_46	486	161	Phage protein
MET_P1_301_I_47	108	35	Phage protein
MET_P1_301_I_48	102	33	hypothetical protein
MET_P1_301_I_49	111	36	hypothetical protein
MET_P1_301_I_50	336	111	Phage protein
MET_P1_301_I_51	90	29	hypothetical protein
MET_P1_301_I_52	1416	471	Phage protein

MET_P1_301_I_53	372	123	Phage protein
MET_P1_301_I_54	129	42	hypothetical protein
MET_P1_301_I_55	234	77	Phage protein
MET_P1_301_I_56	591	196	Phage protein
MET_P1_301_I_57	372	123	Phage protein
MET_P1_301_I_58	1386	461	Phage protein
MET_P1_301_I_59	465	154	Phage lysin (EC 3.2.1.17)
MET_P1_301_I_60	399	132	Phage protein
MET_P1_301_I_61	390	129	Phage protein
MET_P1_301_I_62	294	97	Phage protein
MET_P1_301_I_63	345	114	Phage protein
MET_P1_301_I_64	582	193	Phage protein
MET_P1_301_I_65	546	181	Phage protein
MET_P1_301_I_66	219	72	Phage protein
MET_P1_301_I_67	504	167	Phage protein
MET_P1_301_I_68	96	31	hypothetical protein
MET_P1_301_I_69	225	74	Phage protein
MET_P1_301_I_70	429	142	Phage protein
MET_P1_301_I_71	465	154	Phage protein
MET_P1_301_I_72	549	182	Phage protein
MET_P1_301_I_73	102	33	hypothetical protein
MET_P1_301_I_74	96	31	hypothetical protein
MET_P1_301_I_75	90	29	hypothetical protein
MET_P1_301_I_76	90	29	hypothetical protein
MET_P1_301_I_77	249	82	Phage protein
MET_P1_301_I_78	264	87	Phage protein
MET_P1_301_I_79	531	176	Phage protein
MET_P1_301_I_80	93	30	hypothetical protein
MET_P1_301_I_81	342	113	Phage protein

MET_P1_301_I_82	243	80	Phage protein
MET_P1_301_I_83	564	187	Phage protein
MET_P1_301_I_84	204	67	Phage protein
MET_P1_301_I_85	405	134	Phage protein
MET_P1_301_I_86	273	90	Phage protein
MET_P1_301_I_87	159	52	hypothetical protein
MET_P1_301_I_88	333	110	Phage protein
MET_P1_301_I_89	297	98	Phage protein
MET_P1_301_I_90	513	170	Phage protein
MET_P1_301_I_91	291	96	Phage protein
MET_P1_301_I_92	381	126	Phage protein
MET_P1_301_I_93	207	68	Phage protein
MET_P1_301_I_94	174	57	hypothetical protein
MET_P1_301_I_95	201	66	Phage protein
MET_P1_301_I_96	786	261	Phage protein
MET_P1_301_I_97	201	66	Phage protein
MET_P1_301_I_98	234	77	Phage protein
MET_P1_301_I_99	327	108	Phage protein
MET_P1_301_I_100	270	89	Phage protein
MET_P1_301_I_101	135	44	hypothetical protein
MET_P1_301_I_102	348	115	Phage protein
MET_P1_301_I_103	465	154	Phage protein
MET_P1_301_I_104	696	231	Phage protein
MET_P1_301_I_105	549	182	Phage protein
MET_P1_301_I_106	1110	369	Phage rIIB lysis inhibitor
MET_P1_301_I_107	2367	788	Phage rIIA lysis inhibitor
MET_P1_301_I_108	177	58	Phage protein
MET_P1_301_I_109	336	111	Phage protein

MET_P1_301_I_110	1782	593	Nicotinamide phosphoribosyltransferase (EC 2.4.2.12)
MET_P1_301_I_111	882	293	Phage ribose-phosphate pyrophosphokinase
MET_P1_301_I_112	279	92	Phage protein
MET_P1_301_I_113	516	171	Phage protein
MET_P1_301_I_114	321	106	Phage protein
MET_P1_301_I_115	267	88	Phage protein
MET_P1_301_I_116	321	106	Phage protein
MET_P1_301_I_117	486	161	Ribonucleotide reductase of class III (anaerobic), activating protein (EC 1.97.1.4)
MET_P1_301_I_118	300	99	Phage protein
MET_P1_301_I_119	396	131	Phage protein
MET_P1_301_I_120	201	66	Phage protein
MET_P1_301_I_121	165	54	Phage protein
MET_P1_301_I_122	2145	714	Ribonucleotide reductase of class III (anaerobic), large subunit (EC 1.17.4.2)
MET_P1_301_I_123	207	68	Phage protein
MET_P1_301_I_124	243	80	Phage protein
MET_P1_301_I_125	1074	357	Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1)
MET_P1_301_I_126	342	113	Phage protein
MET_P1_301_I_127	2235	744	Ribonucleotide reductase of class Ia (aerobic), alpha subunit (EC 1.17.4.1)
MET_P1_301_I_128	333	110	Phage protein

MET_P1_301_I_129	324	107	Phage protein
MET_P1_301_I_130	756	251	Phage protein
MET_P1_301_I_131	249	82	Phage protein
MET_P1_301_I_132	498	165	Phage protein
MET_P1_301_I_133	1041	346	putative exodeoxyribonuclease
MET_P1_301_I_134	858	285	Phage protein
MET_P1_301_I_135	150	49	Phage protein
MET_P1_301_I_136	282	93	Phage protein
MET_P1_301_I_137	1986	661	Phage DNA primase/helicase
MET_P1_301_I_138	201	66	Phage protein
MET_P1_301_I_139	744	247	putative deoxynucleotide monophosphate kinase
MET_P1_301_I_140	813	270	Phage minor tail protein
MET_P1_301_I_141	423	140	Phage protein
MET_P1_301_I_142	2730	909	DNA polymerase (EC 2.7.7.7), phage-associated
MET_P1_301_I_143	210	69	Phage protein
MET_P1_301_I_144	219	72	Phage protein
MET_P1_301_I_145	147	48	hypothetical protein
MET_P1_301_I_146	225	74	Phage protein
MET_P1_301_I_147	258	85	Phage protein
MET_P1_301_I_148	168	55	Phage protein
MET_P1_301_I_149	219	72	Phage protein
MET_P1_301_I_150	1101	366	DNA ligase, phage-associated
MET_P1_301_I_151	189	62	hypothetical protein
MET_P1_301_I_152	138	45	hypothetical protein
MET_P1_301_I_153	588	195	Phage protein
MET_P1_301_I_154	441	146	Phage protein

Table D. 2 Annotation of genome using only Nanopore reads

<b>Locus Tag</b>	<b>Length (bp)</b>	<b>AA Length</b>	<b>Product</b>
MET_P1_301_N_1	90	29	hypothetical protein
MET_P1_301_N_2	465	154	Phage protein
MET_P1_301_N_3	249	82	Phage protein
MET_P1_301_N_4	696	231	Phage protein
MET_P1_301_N_5	402	133	Phage protein
MET_P1_301_N_6	1110	369	Phage rIIB lysis inhibitor
MET_P1_301_N_7	1386	461	Phage rIIA lysis inhibitor
MET_P1_301_N_8	285	94	Phage protein
MET_P1_301_N_9	927	308	Phage rIIA lysis inhibitor
MET_P1_301_N_10	177	58	Phage protein
MET_P1_301_N_11	306	101	Phage protein
MET_P1_301_N_12	1515	504	Nicotinamide phosphoribosyltransferase (EC 2.4.2.12)
MET_P1_301_N_13	531	176	Phage protein
MET_P1_301_N_14	261	86	Nicotinamide phosphoribosyltransferase (EC 2.4.2.12)
MET_P1_301_N_15	321	106	ribose-phosphate pyrophosphokinase family protein
MET_P1_301_N_16	489	162	Phage ribose-phosphate pyrophosphokinase
MET_P1_301_N_17	279	92	Phage protein
MET_P1_301_N_18	516	171	Phage protein

MET_P1_301_N_19	321	106	Phage protein
MET_P1_301_N_20	267	88	Phage protein
MET_P1_301_N_21	321	106	Phage protein
MET_P1_301_N_22	486	161	Ribonucleotide reductase of class III (anaerobic), activating protein (EC 1.97.1.4)
MET_P1_301_N_23	300	99	Phage protein
MET_P1_301_N_24	396	131	Phage protein
MET_P1_301_N_25	201	66	Phage protein
MET_P1_301_N_26	129	42	hypothetical protein
MET_P1_301_N_27	1998	665	Ribonucleotide reductase of class III (anaerobic), large subunit (EC 1.17.4.2)
MET_P1_301_N_28	93	30	hypothetical protein
MET_P1_301_N_29	204	67	Phage protein
MET_P1_301_N_30	117	38	hypothetical protein
MET_P1_301_N_31	207	68	Phage protein
MET_P1_301_N_32	243	80	Phage protein
MET_P1_301_N_33	348	115	Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1)
MET_P1_301_N_34	594	197	Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1)
MET_P1_301_N_35	159	52	hypothetical protein
MET_P1_301_N_36	342	113	Phage protein
MET_P1_301_N_37	2235	744	Ribonucleotide reductase of class Ia (aerobic), alpha subunit (EC 1.17.4.1)
MET_P1_301_N_38	138	45	Phage protein
MET_P1_301_N_39	333	110	Phage protein

MET_P1_301_N_40	243	80	Phage protein
MET_P1_301_N_41	324	107	Phage protein
MET_P1_301_N_42	645	214	Phage protein
MET_P1_301_N_43	93	30	hypothetical protein
MET_P1_301_N_44	249	82	Phage protein
MET_P1_301_N_45	114	37	hypothetical protein
MET_P1_301_N_46	282	93	Phage protein
MET_P1_301_N_47	99	32	hypothetical protein
MET_P1_301_N_48	936	311	putative exodeoxyribonuclease
MET_P1_301_N_49	558	185	Phage protein
MET_P1_301_N_50	858	285	Phage protein
MET_P1_301_N_51	93	30	hypothetical protein
MET_P1_301_N_52	159	52	Phage protein
MET_P1_301_N_53	108	35	Phage protein
MET_P1_301_N_54	1986	661	Phage DNA primase/helicase
MET_P1_301_N_55	201	66	Phage protein
MET_P1_301_N_56	240	79	putative deoxynucleotide monophosphate kinase
MET_P1_301_N_57	444	147	putative deoxynucleotide monophosphate kinase
MET_P1_301_N_58	549	182	Phage minor tail protein
MET_P1_301_N_59	291	96	Phage minor tail protein
MET_P1_301_N_60	423	140	Phage protein
MET_P1_301_N_61	90	29	hypothetical protein
MET_P1_301_N_62	1359	452	DNA polymerase (EC 2.7.7.7), phage-associated
MET_P1_301_N_63	204	67	Phage protein
MET_P1_301_N_64	1011	336	DNA polymerase (EC 2.7.7.7), phage-associated

MET_P1_301_N_65	288	95	DNA polymerase (EC 2.7.7.7), phage-associated
MET_P1_301_N_66	210	69	Phage protein
MET_P1_301_N_67	219	72	Phage protein
MET_P1_301_N_68	147	48	hypothetical protein
MET_P1_301_N_69	225	74	Phage protein
MET_P1_301_N_70	258	85	Phage protein
MET_P1_301_N_71	135	44	hypothetical protein
MET_P1_301_N_72	219	72	Phage protein
MET_P1_301_N_73	150	49	Phage protein
MET_P1_301_N_74	1101	366	DNA ligase, phage-associated
MET_P1_301_N_75	195	64	hypothetical protein
MET_P1_301_N_76	138	45	hypothetical protein
MET_P1_301_N_77	186	61	Phage protein
MET_P1_301_N_78	588	195	Phage protein
MET_P1_301_N_79	441	146	Phage protein
MET_P1_301_N_80	384	127	Phage protein
MET_P1_301_N_81	213	70	Phage protein
MET_P1_301_N_82	300	99	Phage protein
MET_P1_301_N_83	360	119	Phage protein
MET_P1_301_N_84	453	150	Phage protein
MET_P1_301_N_85	243	80	Phage protein
MET_P1_301_N_86	261	86	Phage protein
MET_P1_301_N_87	546	181	Dihydrofolate reductase (EC 1.5.1.3)
MET_P1_301_N_88	600	199	Thymidylate synthase (EC 2.1.1.45)
MET_P1_301_N_89	246	81	Thymidylate synthase (EC 2.1.1.45)
MET_P1_301_N_90	372	123	Phage protein
MET_P1_301_N_91	114	37	Phage protein
MET_P1_301_N_92	159	52	hypothetical protein

MET_P1_301_N_93	2361	786	Phage tail fiber
MET_P1_301_N_94	1176	391	Phage tail fiber
MET_P1_301_N_95	333	110	Phage protein
MET_P1_301_N_96	303	100	Phage protein
MET_P1_301_N_97	855	284	Phage protein
MET_P1_301_N_98	1470	489	Phage protein
MET_P1_301_N_99	420	139	Phage protein
MET_P1_301_N_100	216	71	Phage protein
MET_P1_301_N_101	624	207	Phage baseplate
MET_P1_301_N_102	132	43	Phage protein
MET_P1_301_N_103	831	276	Phage protein
MET_P1_301_N_104	342	113	Phage protein
MET_P1_301_N_105	93	30	hypothetical protein
MET_P1_301_N_106	555	184	Phage protein
MET_P1_301_N_107	321	106	Phage tail tape measure
MET_P1_301_N_108	513	170	Phage protein
MET_P1_301_N_109	1125	374	Phage tail tape measure
MET_P1_301_N_110	483	160	Phage tail tape measure
MET_P1_301_N_111	237	78	Phage tail tape measure
MET_P1_301_N_112	639	212	Phage protein
MET_P1_301_N_113	411	136	Phage protein
MET_P1_301_N_114	1353	450	Phage protein
MET_P1_301_N_115	600	199	Phage protein
MET_P1_301_N_116	402	133	Phage protein
MET_P1_301_N_117	435	144	Phage protein
MET_P1_301_N_118	264	87	Phage protein
MET_P1_301_N_119	291	96	Phage protein
MET_P1_301_N_120	180	59	Phage protein
MET_P1_301_N_121	969	322	Phage protein

MET_P1_301_N_122	378	125	Phage protein
MET_P1_301_N_123	552	183	Phage protein
MET_P1_301_N_124	696	231	Phage protein
MET_P1_301_N_125	333	110	Phage protein
MET_P1_301_N_126	432	143	Phage protein
MET_P1_301_N_127	381	126	Phage protein
MET_P1_301_N_128	855	284	Phage protein
MET_P1_301_N_129	99	32	hypothetical protein
MET_P1_301_N_130	435	144	Phage protein
MET_P1_301_N_131	399	132	Phage terminase, large subunit
MET_P1_301_N_132	1158	385	Phage terminase, large subunit
MET_P1_301_N_133	201	66	Phage protein
MET_P1_301_N_134	105	34	Phage protein
MET_P1_301_N_135	438	145	Phage protein
MET_P1_301_N_136	183	60	hypothetical protein
MET_P1_301_N_137	207	68	Phage protein
MET_P1_301_N_138	558	185	Phage protein
MET_P1_301_N_139	171	56	Phage protein
MET_P1_301_N_140	207	68	Phage protein
MET_P1_301_N_141	111	36	hypothetical protein
MET_P1_301_N_142	141	46	Phage protein
MET_P1_301_N_143	105	34	hypothetical protein
MET_P1_301_N_144	159	52	Phage protein
MET_P1_301_N_145	264	87	Phage protein
MET_P1_301_N_146	108	35	Phage protein
MET_P1_301_N_147	96	31	hypothetical protein
MET_P1_301_N_148	102	33	hypothetical protein
MET_P1_301_N_149	111	36	hypothetical protein
MET_P1_301_N_150	336	111	Phage protein

MET_P1_301_N_151	153	50	Phage protein
MET_P1_301_N_152	615	204	Phage protein
MET_P1_301_N_153	735	244	Phage protein
MET_P1_301_N_154	231	76	Phage protein
MET_P1_301_N_155	96	31	Phage protein
MET_P1_301_N_156	102	33	hypothetical protein
MET_P1_301_N_157	786	261	Phage protein
MET_P1_301_N_158	234	77	Phage protein
MET_P1_301_N_159	600	199	Phage protein
MET_P1_301_N_160	372	123	Phage protein
MET_P1_301_N_161	528	175	Phage protein
MET_P1_301_N_162	921	306	Phage protein
MET_P1_301_N_163	465	154	Phage lysin (EC 3.2.1.17)
MET_P1_301_N_164	399	132	Phage protein
MET_P1_301_N_165	390	129	Phage protein
MET_P1_301_N_166	294	97	Phage protein
MET_P1_301_N_167	96	31	Phage protein
MET_P1_301_N_168	249	82	Phage protein
MET_P1_301_N_169	321	106	Phage protein
MET_P1_301_N_170	264	87	Phage protein
MET_P1_301_N_171	537	178	Phage protein
MET_P1_301_N_172	219	72	Phage protein
MET_P1_301_N_173	504	167	Phage protein
MET_P1_301_N_174	96	31	hypothetical protein
MET_P1_301_N_175	225	74	Phage protein
MET_P1_301_N_176	243	80	hypothetical protein
MET_P1_301_N_177	108	35	Phage protein
MET_P1_301_N_178	435	144	Phage protein
MET_P1_301_N_179	549	182	Phage protein

MET_P1_301_N_180	201	66	Phage protein
MET_P1_301_N_181	114	37	hypothetical protein
MET_P1_301_N_182	102	33	hypothetical protein
MET_P1_301_N_183	183	60	hypothetical protein
MET_P1_301_N_184	90	29	hypothetical protein
MET_P1_301_N_185	90	29	hypothetical protein
MET_P1_301_N_186	234	77	Phage protein
MET_P1_301_N_187	285	94	Phage protein
MET_P1_301_N_188	270	89	Phage protein
MET_P1_301_N_189	135	44	hypothetical protein
MET_P1_301_N_190	348	115	Phage protein

Table D. 3 Annotation of genome using both Nanopore and Illumina (hybrid) reads

<b>Locus Tag</b>	<b>Length (bp)</b>	<b>AA Length</b>	<b>Product</b>
MET_P1_301_H_1	1602	533	Phage terminase, large subunit
MET_P1_301_H_2	447	148	Phage protein
MET_P1_301_H_3	399	132	Phage protein
MET_P1_301_H_4	240	79	Phage protein
MET_P1_301_H_5	2229	742	Phage tail tape measure
MET_P1_301_H_6	798	265	Phage protein
MET_P1_301_H_7	342	113	Phage protein
MET_P1_301_H_8	978	325	Phage protein
MET_P1_301_H_9	624	207	Phage baseplate
MET_P1_301_H_10	1467	488	Phage protein
MET_P1_301_H_11	420	139	Phage protein
MET_P1_301_H_12	1470	489	Phage protein
MET_P1_301_H_13	855	284	Phage protein

MET_P1_301_H_14	303	100	Phage protein
MET_P1_301_H_15	1176	391	Phage tail fiber
MET_P1_301_H_16	2361	786	Phage tail fiber
MET_P1_301_H_17	195	64	Phage protein
MET_P1_301_H_18	372	123	Phage protein
MET_P1_301_H_19	900	299	Thymidylate synthase (EC 2.1.1.45)
MET_P1_301_H_20	546	181	Dihydrofolate reductase (EC 1.5.1.3)
MET_P1_301_H_21	261	86	Phage protein
MET_P1_301_H_22	516	171	Phage protein
MET_P1_301_H_23	360	119	Phage protein
MET_P1_301_H_24	300	99	Phage protein
MET_P1_301_H_25	213	70	Phage protein
MET_P1_301_H_26	384	127	Phage protein
MET_P1_301_H_27	441	146	Phage protein
MET_P1_301_H_28	588	195	Phage protein
MET_P1_301_H_29	138	45	hypothetical protein
MET_P1_301_H_30	189	62	hypothetical protein
MET_P1_301_H_31	1101	366	DNA ligase, phage-associated
MET_P1_301_H_32	219	72	Phage protein
MET_P1_301_H_33	168	55	Phage protein
MET_P1_301_H_34	258	85	Phage protein
MET_P1_301_H_35	225	74	Phage protein
MET_P1_301_H_36	501	166	Phage protein
MET_P1_301_H_37	147	48	hypothetical protein
MET_P1_301_H_38	219	72	Phage protein
MET_P1_301_H_39	210	69	Phage protein
MET_P1_301_H_40	2730	909	DNA polymerase (EC 2.7.7.7), phage-associated
MET_P1_301_H_41	423	140	Phage protein

MET_P1_301_H_42	813	270	Phage minor tail protein
MET_P1_301_H_43	744	247	putative deoxynucleotide monophosphate kinase
MET_P1_301_H_44	333	110	Phage protein
MET_P1_301_H_45	201	66	Phage protein
MET_P1_301_H_46	1986	661	Phage DNA primase/helicase
MET_P1_301_H_47	282	93	Phage protein
MET_P1_301_H_48	150	49	Phage protein
MET_P1_301_H_49	858	285	Phage protein
MET_P1_301_H_50	1347	448	Phage protein
MET_P1_301_H_51	1041	346	putative exodeoxyribonuclease
MET_P1_301_H_52	498	165	Phage protein
MET_P1_301_H_53	249	82	Phage protein
MET_P1_301_H_54	756	251	Phage protein
MET_P1_301_H_55	324	107	Phage protein
MET_P1_301_H_56	333	110	Phage protein
MET_P1_301_H_57	2235	744	Ribonucleotide reductase of class Ia (aerobic), alpha subunit (EC 1.17.4.1)
MET_P1_301_H_58	342	113	Phage protein
MET_P1_301_H_59	1074	357	Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1)
MET_P1_301_H_60	243	80	Phage protein
MET_P1_301_H_61	207	68	Phage protein
MET_P1_301_H_62	2145	714	Ribonucleotide reductase of class III (anaerobic), large subunit (EC 1.17.4.2)
MET_P1_301_H_63	165	54	Phage protein
MET_P1_301_H_64	201	66	Phage protein

MET_P1_301_H_65	396	131	Phage protein
MET_P1_301_H_66	300	99	Phage protein
MET_P1_301_H_67	486	161	Ribonucleotide reductase of class III (anaerobic), activating protein (EC 1.97.1.4)
MET_P1_301_H_68	321	106	Phage protein
MET_P1_301_H_69	267	88	Phage protein
MET_P1_301_H_70	321	106	Phage protein
MET_P1_301_H_71	516	171	Phage protein
MET_P1_301_H_72	279	92	Phage protein
MET_P1_301_H_73	882	293	Phage ribose-phosphate pyrophosphokinase
MET_P1_301_H_74	378	125	Phage protein
MET_P1_301_H_75	1782	593	Nicotinamide phosphoribosyltransferase (EC 2.4.2.12)
MET_P1_301_H_76	336	111	Phage protein
MET_P1_301_H_77	177	58	Phage protein
MET_P1_301_H_78	2367	788	Phage rIIA lysis inhibitor
MET_P1_301_H_79	1107	368	Phage protein
MET_P1_301_H_80	1110	369	Phage rIIB lysis inhibitor
MET_P1_301_H_81	549	182	Phage protein
MET_P1_301_H_82	696	231	Phage protein
MET_P1_301_H_83	465	154	Phage protein
MET_P1_301_H_84	348	115	Phage protein
MET_P1_301_H_85	135	44	hypothetical protein
MET_P1_301_H_86	270	89	Phage protein
MET_P1_301_H_87	327	108	Phage protein
MET_P1_301_H_88	234	77	Phage protein

MET_P1_301_H_89	201	66	Phage protein
MET_P1_301_H_90	786	261	Phage protein
MET_P1_301_H_91	201	66	Phage protein
MET_P1_301_H_92	174	57	hypothetical protein
MET_P1_301_H_93	207	68	Phage protein
MET_P1_301_H_94	381	126	Phage protein
MET_P1_301_H_95	291	96	Phage protein
MET_P1_301_H_96	513	170	Phage protein
MET_P1_301_H_97	297	98	Phage protein
MET_P1_301_H_98	333	110	Phage protein
MET_P1_301_H_99	159	52	hypothetical protein
MET_P1_301_H_100	273	90	Phage protein
MET_P1_301_H_101	405	134	Phage protein
MET_P1_301_H_102	204	67	Phage protein
MET_P1_301_H_103	564	187	Phage protein
MET_P1_301_H_104	450	149	Phage protein
MET_P1_301_H_105	243	80	Phage protein
MET_P1_301_H_106	342	113	Phage protein
MET_P1_301_H_107	93	30	hypothetical protein
MET_P1_301_H_108	531	176	Phage protein
MET_P1_301_H_109	264	87	Phage protein
MET_P1_301_H_110	249	82	Phage protein
MET_P1_301_H_111	90	29	hypothetical protein
MET_P1_301_H_112	90	29	hypothetical protein
MET_P1_301_H_113	96	31	hypothetical protein
MET_P1_301_H_114	102	33	hypothetical protein
MET_P1_301_H_115	549	182	Phage protein
MET_P1_301_H_116	483	160	Phage protein
MET_P1_301_H_117	465	154	Phage protein

MET_P1_301_H_118	429	142	Phage protein
MET_P1_301_H_119	225	74	Phage protein
MET_P1_301_H_120	96	31	hypothetical protein
MET_P1_301_H_121	504	167	Phage protein
MET_P1_301_H_122	219	72	Phage protein
MET_P1_301_H_123	546	181	Phage protein
MET_P1_301_H_124	582	193	Phage protein
MET_P1_301_H_125	345	114	Phage protein
MET_P1_301_H_126	294	97	Phage protein
MET_P1_301_H_127	390	129	Phage protein
MET_P1_301_H_128	399	132	Phage protein
MET_P1_301_H_129	402	133	Phage protein
MET_P1_301_H_130	465	154	Phage lysin (EC 3.2.1.17)
MET_P1_301_H_131	1386	461	Phage protein
MET_P1_301_H_132	372	123	Phage protein
MET_P1_301_H_133	591	196	Phage protein
MET_P1_301_H_134	234	77	Phage protein
MET_P1_301_H_135	129	42	hypothetical protein
MET_P1_301_H_136	372	123	Phage protein
MET_P1_301_H_137	600	199	Phage protein
MET_P1_301_H_138	1416	471	Phage protein
MET_P1_301_H_139	90	29	hypothetical protein
MET_P1_301_H_140	336	111	Phage protein
MET_P1_301_H_141	111	36	hypothetical protein
MET_P1_301_H_142	102	33	hypothetical protein
MET_P1_301_H_143	108	35	Phage protein
MET_P1_301_H_144	486	161	Phage protein
MET_P1_301_H_145	93	30	Phage protein
MET_P1_301_H_146	141	46	Phage protein

MET_P1_301_H_147	111	36	hypothetical protein
MET_P1_301_H_148	198	65	Phage protein
MET_P1_301_H_149	171	56	Phage protein
MET_P1_301_H_150	558	185	Phage protein
MET_P1_301_H_151	1353	450	Phage protein
MET_P1_301_H_152	735	244	Phage protein
MET_P1_301_H_153	177	58	hypothetical protein
MET_P1_301_H_154	201	66	Phage protein

## **E. Sequence of LysEc301**

### **E.2. Nucleotide Sequence**

atgcaactctcaagaaaaggtttagaagctattaagttcttgaaggctgaagctagaggcttacgaagattctgctgga  
atccaacaattgggtatgggactatccgtattgctggtaagcctgtaagatgggtatgaagattaccgctgaacaagct  
gaacagtatctctcgcagatgtgaaaagttcggtgcagcagtgaaacaagccatcaaggtccaacttctcagaacga  
gttcgatgcacttgaagtgaacatacaacattggcatcacagccatgcaggactctacattatcaagcgtcacaatgc  
tgtaataaggtaggtgtgcagaagctatgcagtggtggaacaaggttacagtcaaagtaagaaggctcacttcaaac  
ggcctgaaaaacagacgtagaatggaagctgacatttatcttgacagtgtatatccaaagtaa

### **E.2. Amino acid sequence**

MQLSRKGLEAIKFFEGLKLEAYEDSAGIPTIGYGTIRIAGKPKMGMKITAE  
QAEQYLLADVEKFVAAVNKAIKVPTSQNEFDALVSETYNIGITAMQDSTFI  
KRHNAGNKVGCAEAMQWWNKVTVKGKKVTSNGLKNRRRMEADIYLDV

## F. ANOVA Results

Table F.1 ANOVA results of lytic activity against A1-200

### Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

*Equal variances were assumed for the analysis.*

### Factor Information

Factor	Levels	Values
Systems	6	A1-200, A1-200 +Lysin, A1-200 +Lysin+EDTA, A1-200 +Lysin+TrisHCl, A1-200 +TrisHCl+EDTA, A1-200 +TrisHCl+EDTA+Lysin

### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Systems	5	30.0987	6.01975	138.33	0.000
Error	12	0.5222	0.04352		
Total	17	30.6210			

### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.208610	98.29%	97.58%	96.16%

### Means

Systems	N	Mean	StDev	95% CI
A1-200	3	7.1590	0.1512	(6.8966, 7.4215)
A1-200 +Lysin	3	4.0587	0.1017	(3.7963, 4.3211)
A1-200 +Lysin+EDTA	3	3.9622	0.1035	(3.6997, 4.2246)
A1-200 +Lysin+TrisHCl	3	4.301	0.301	(4.039, 4.563)
A1-200 +TrisHCl+EDTA	3	6.5927	0.1112	(6.3303, 6.8551)
A1-200 +TrisHCl+EDTA+Lysin	3	4.357	0.338	(4.094, 4.619)

*Pooled StDev = 0.208610*

### Tukey Pairwise Comparisons

#### Grouping Information Using the Tukey Method and 95% Confidence

Systems	N	Mean	Grouping
A1-200	3	7.1590	A
A1-200 +TrisHCl+EDTA	3	6.5927	A
A1-200 +TrisHCl+EDTA+Lysin	3	4.357	B
A1-200 +Lysin+TrisHCl	3	4.301	B
A1-200 +Lysin	3	4.0587	B
A1-200 +Lysin+EDTA	3	3.9622	B

*Means that do not share a letter are significantly different.*

Table F.2 ANOVA results of lytic activity against A1-179

**Method**

Null hypothesis      All means are equal  
 Alternative hypothesis   Not all means are equal  
 Significance level       $\alpha = 0.05$

*Equal variances were assumed for the analysis.*

**Factor Information**

Factor	Levels	Values
Systems	6	A1-179, A1-179 +Lysin, A1-179 +Lysin+EDTA, A1-179 +Lysin+TrisHCl, A1-179 +TrisHCl+EDTA, A1-179 +TrisHCl+EDTA+Lysin

**Analysis of Variance**

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Systems	5	26.6393	5.32785	106.32	0.000
Error	12	0.6014	0.05011		
Total	17	27.2406			

**Model Summary**

S	R-sq	R-sq(adj)	R-sq(pred)
0.223860	97.79%	96.87%	95.03%

**Means**

Systems	N	Mean	StDev	95% CI
A1-179	3	6.9484	0.0894	(6.6668, 7.2300)
A1-179 +Lysin	3	3.9484	0.0894	(3.6668, 4.2300)
A1-179 +Lysin+EDTA	3	4.049	0.232	(3.767, 4.330)
A1-179 +Lysin+TrisHCl	3	4.415	0.468	(4.133, 4.696)
A1-179 +TrisHCl+EDTA	3	6.5411	0.0625	(6.2595, 6.8227)
A1-179 +TrisHCl+EDTA+Lysin	3	4.3920	0.0882	(4.1104, 4.6736)

*Pooled StDev = 0.223860*

**Tukey Pairwise Comparisons**

**Grouping Information Using the Tukey Method and 95% Confidence**

Systems	N	Mean	Grouping
A1-179	3	6.9484	A
A1-179 +TrisHCl+EDTA	3	6.5411	A
A1-179 +Lysin+TrisHCl	3	4.415	B
A1-179 +TrisHCl+EDTA+Lysin	3	4.3920	B
A1-179 +Lysin+EDTA	3	4.049	B
A1-179 +Lysin	3	3.9484	B

*Means that do not share a letter are significantly different.*

Table F.3 ANOVA results of lytic activity against A1-90

**Method**

Null hypothesis All means are equal  
 Alternative hypothesis Not all means are equal  
 Significance level  $\alpha = 0.05$

*Equal variances were assumed for the analysis.*

**Factor Information**

Factor	Levels	Values
Systems	6	A1-90, A1-90 +Lysin, A1-90 +Lysin+EDTA, A1-90 +Lysin+TrisHCl, A1-90 +TrisHCl+EDTA, A1-90 +TrisHCl+EDTA+Lysin

**Analysis of Variance**

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Systems	5	15.2387	3.04774	269.67	0.000
Error	12	0.1356	0.01130		
Total	17	15.3743			

**Model Summary**

S	R-sq	R-sq(adj)	R-sq(pred)
0.106309	99.12%	98.75%	98.02%

**Means**

Systems	N	Mean	StDev	95% CI
A1-90	3	7.8245	0.0684	(7.6908, 7.9583)
A1-90 +Lysin	3	6.6360	0.0317	(6.5023, 6.7698)
A1-90 +Lysin+EDTA	3	5.208	0.180	(5.074, 5.341)
A1-90 +Lysin+TrisHCl	3	6.7265	0.0258	(6.5928, 6.8602)
A1-90 +TrisHCl+EDTA	3	7.1193	0.1436	(6.9856, 7.2530)
A1-90 +TrisHCl+EDTA+Lysin	3	5.4032	0.0914	(5.2694, 5.5369)

*Pooled StDev = 0.106309*

**Tukey Pairwise Comparisons**

**Grouping Information Using the Tukey Method and 95% Confidence**

Systems	N	Mean	Grouping
A1-90	3	7.8245	A
A1-90 +TrisHCl+EDTA	3	7.1193	B
A1-90 +Lysin+TrisHCl	3	6.7265	C
A1-90 +Lysin	3	6.6360	C
A1-90 +TrisHCl+EDTA+Lysin	3	5.4032	D
A1-90 +Lysin+EDTA	3	5.208	D

*Means that do not share a letter are significantly different.*

Table F.4 ANOVA results of lytic activity against K1-42

### Method

Null hypothesis All means are equal  
 Alternative hypothesis Not all means are equal  
 Significance level  $\alpha = 0.05$

*Equal variances were assumed for the analysis.*

### Factor Information

Factor	Levels	Values
Systems	6	K1-42, K1-42 +Lysin, K1-42 +Lysin+EDTA, K1-42 +Lysin+TrisHCl, K1-42 +TrisHCl+EDTA, K1-42 +TrisHCl+EDTA+Lysin

### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Systems	5	18.0150	3.60300	234.02	0.000
Error	12	0.1848	0.01540		
Total	17	18.1998			

### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.124080	98.98%	98.56%	97.72%

### Means

Systems	N	Mean	StDev	95% CI
K1-42	3	7.8492	0.0520	(7.6931, 8.0053)
K1-42 +Lysin	3	6.5112	0.1631	(6.3551, 6.6673)
K1-42 +Lysin+EDTA	3	5.0680	0.1178	(4.9120, 5.2241)
K1-42 +Lysin+TrisHCl	3	6.534	0.192	(6.378, 6.690)
K1-42 +TrisHCl+EDTA	3	7.0644	0.0584	(6.9083, 7.2205)
K1-42 +TrisHCl+EDTA+Lysin	3	5.1074	0.0942	(4.9513, 5.2635)

*Pooled StDev = 0.124080*

### Tukey Pairwise Comparisons

#### Grouping Information Using the Tukey Method and 95% Confidence

Systems	N	Mean	Grouping
K1-42	3	7.8492	A
K1-42 +TrisHCl+EDTA	3	7.0644	B
K1-42 +Lysin+TrisHCl	3	6.534	C
K1-42 +Lysin	3	6.5112	C
K1-42 +TrisHCl+EDTA+Lysin	3	5.1074	D
K1-42 +Lysin+EDTA	3	5.0680	D

*Means that do not share a letter are significantly different.*

Table F.5 ANOVA results of lytic activity against A2-251

**Method**

Null hypothesis            All means are equal  
 Alternative hypothesis   Not all means are equal  
 Significance level         $\alpha = 0.05$

*Equal variances were assumed for the analysis.*

**Factor Information**

Factor	Levels	Values
Systems	6	A2-251, A2-251 +Lysin, A2-251 +Lysin+EDTA, A2-251 +Lysin+TrisHCl, A2-251 +TrisHCl+EDTA, A2-251 +TrisHCl+EDTA+Lysin

**Analysis of Variance**

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Systems	5	21.9806	4.39613	449.79	0.000
Error	12	0.1173	0.00977		
Total	17	22.0979			

**Model Summary**

S	R-sq	R-sq(adj)	R-sq(pred)
0.0988620	99.47%	99.25%	98.81%

**Means**

Systems	N	Mean	StDev	95% CI
A2-251	3	7.61959	0.01602	(7.49523, 7.74395)
A2-251 +Lysin	3	5.0851	0.0882	(4.9607, 5.2095)
A2-251 +Lysin+EDTA	3	5.1970	0.0953	(5.0727, 5.3214)
A2-251 +Lysin+TrisHCl	3	5.1833	0.1607	(5.0590, 5.3077)
A2-251 +TrisHCl+EDTA	3	7.3500	0.0458	(7.2256, 7.4744)
A2-251 +TrisHCl+EDTA+Lysin	3	5.1255	0.1166	(5.0012, 5.2499)

*Pooled StDev = 0.0988620*

**Tukey Pairwise Comparisons**

**Grouping Information Using the Tukey Method and 95% Confidence**

Systems	N	Mean	Grouping
A2-251	3	7.61959	A
A2-251 +TrisHCl+EDTA	3	7.3500	A
A2-251 +Lysin+EDTA	3	5.1970	B
A2-251 +Lysin+TrisHCl	3	5.1833	B
A2-251 +TrisHCl+EDTA+Lysin	3	5.1255	B
A2-251 +Lysin	3	5.0851	B

*Means that do not share a letter are significantly different.*