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PHAGE THERAPY AGAINST CONTAMINATION OF PATHOGENIC *ESCHERICHIA COLI* STRAINS (O104:H4, O157:H7, AND O26) DURING GARDEN CRESS (*LEPIDIUM SATIVUM* LINN.) VEGETATION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

İREM İSKENDER

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

AUGUST 2022

Approval of the thesis:

PHAGE THERAPY AGAINST CONTAMINATION OF PATHOGENIC *ESCHERICHIA COLI* STRAINS (O104:H4, O157:H7, AND O26) DURING GARDEN CRESS (*LEPIDIUM SATIVUM* LINN.) VEGETATION

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ABSTRACT

PHAGE THERAPY AGAINST CONTAMINATION OF PATHOGENIC *ESCHERICHIA COLI* STRAINS (O104:H4, O157:H7, AND O26) DURING GARDEN CRESS (*LEPIDIUM SATIVUM* LINN.) VEGETATION

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August 2022, 117 pages

The number of outbreaks caused by foodborne pathogens due to contaminated fresh produces has increased worldwide. *Escherichia coli* is one of the most causative foodborne pathogens. The major transmission way of foodborne pathogens to fresh produce is irrigation water. Once foodborne pathogens are transmitted, the elimination of pathogens from the fresh produce is harder. The most commonly used disinfected agents are chlorine-derived compounds for fresh produce. Yet, usage of them has been related to increasing chemical risk. Thus, the new prevention method for foodborne pathogens that could be applied to the field should be searched. This study aims to investigate an alternative prevention method for the pathogenic *E. coli* strains; O157:H7, O104:H4, and O26 on freshly consumed garden cresses via irrigation water during vegetation. Firstly, cresses were contaminated with these strains with either 10^5 CFU/mL and/or 10^8 CFU/mL via irrigation water. Six groups were designed regarding *E. coli* contamination and phage treatment days. Next, single and cocktail phage therapies against each strain with the multiplicity of infection (MOI) = -1 or 100 was applied according to the irrigation schedule. On the

30th day, leaves were collected to count total and biofilm-forming *E. coli* and compared with their control groups. Furthermore, *E. coli* adherence on the leaf surface was observed with Scanning Electron Microscopy (SEM). Three different phage therapies, single and cocktail phage with two different MOI values, against *E. coli* O104:H4 were applied. Single phage therapy against *E. coli* O104:H4 with MOI = 100 resulted in ~0,47 log and ~0,33 log reductions in total and biofilm-forming *E. coli*, respectively. Single phage therapy against *E. coli* O104:H4 with MOI = -1 led to ~2,60 log and ~2,45 log reductions in total and biofilm-forming *E. coli* count, respectively. Phage cocktail therapy against *E. coli* O104:H4 with MOI = 100 caused ~1,06 logs and ~0,93 log reductions in total and biofilm-forming *E. coli* counts, respectively. When phage cocktail therapy against *E. coli* O157:H7 with MOI = 100 was applied, ~1,94 log reduction in total *E. coli* and ~2,35 log reduction in biofilm-forming *E. coli* count were observed. Single phage therapy against *E. coli* O26 with MOI = 100 was applied and total and biofilm-forming *E. coli* counts were reduced by ~1,83 logs, and ~1,90 logs, respectively. Accordingly, the results of this study show that phage therapy against pathogenic *E. coli* strains might be a promising application as a biocontrol agent during irrigation on freshly consumed leafy greens on the field. To eradicate *E. coli* contamination during vegetation by phage therapy, further studies should be conducted.

Keywords: *Escherichia coli*, foodborne pathogen, bacteriophage, phage therapy, leafy green

ÖZ

BAHÇE TERESİ (*LEPIDIUM SATIVUM* LINN.) VEJETASYONU SIRASINDAKİ PATOJEN *ESCHERICHIA COLI* SUŞLARININ (O104:H4, O157:H7 VE O26) KONTAMİNASYONUNA KARŞI FAJ UYGULAMASI

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Ağustos 2022, 117 sayfa

Gıda kaynaklı patojenlerin kontamine ettiği taze tüketilen gıdaların sebebiyet verdiği salgınlar dünya çapında artış göstermektedir. Bu tip salgınlara, en çok yol açan patojenlerden biri *Escherichia coli*'dir. Gıda kaynaklı patojenlerin taze tüketilen gıdalara bulaşmasının en temel yollarından biri sulama suyudur ve bulaştıktan sonra elimine edilmesi daha zordur. Eliminasyon sırasında en yaygın olarak kullanılan dezenfektanlar klor bazlı kimyasal maddelerdir. Ancak, bu tip maddelerin kullanımı insan sağlığı için giderek kimyasal risk teşkil etmektedir. Bu nedenle, gıda kaynaklı patojenlerden korununabilmek için sahada uygulanabilecek yeni bir yöntem araştırılmalıdır. Bu çalışma, taze tüketilen bahçe terelerinin vejetasyonu sırasında sulama suyu ile patojenik *E. coli* suşları (O104:H4, O157:H7 ve O26) için alternatif bir korunma yöntemi araştırmayı amaçlamıştır. İlk olarak, tereler sulama suyu ile bu suşlarla 10^5 CFU/mL ve/veya 10^8 CFU/mL bakteri yükleriyle kontamine edildi. *E. coli* kontaminasyonu ve faj uygulama günleri farklı olan altı grup oluşturuldu. Sonrasında, planlanan sulama takvimine göre her bir suşa karşı enfeksiyon çokluğu (MOI) = -1 veya 100 olan tekli ve kokteyl faj uygulaması yapıldı. 30. günde

yapraklar toplanarak toplam ve biyofilm oluşturan *E. coli* sayımı yapıldı ve kontrol grupları ile karşılaştırıldı. Ayrıca, Taramalı Elektron Mikroskobu ile yaprak yüzeyindeki *E. coli* tutunması gözlemlendi. *E. coli* O104:H4'e karşı iki farklı MOI değerine sahip tekli ve kokteyl olmak üzere üç farklı faj uygulaması yapıldı. *E. coli* O104:H4'e karşı MOI = 100 olan tekli faj uygulaması toplam ve biyofilm oluşturan *E. coli* sayısında sırasıyla ~0,47 log ve ~0,33 log azalma görüldü. *E. coli* O104:H4'e karşı MOI = -1 olan tekli faj uygulaması, toplam ve biyofilm oluşturan *E. coli* sayısında sırasıyla ~2,60 log ve ~2,45 log azalmaya yol açtı. *E. coli* O104:H4'e karşı MOI = 100 olan koktely faj uygulaması, toplam ve biyofilm oluşturan *E. coli* sayılarında sırasıyla ~1,06 log ve ~0,93 log azalmaya neden oldu. *E. coli* O157:H7'ye karşı MOI = 100 olan kokteyl faj uygulandığında, toplam *E. coli*'de ~1,94 log azalma ve biyofilm oluşturan *E. coli*'de ~2,35 log azalma gözlendi. *E. coli* O26'ya karşı MOI = 100 olan tekli faj uygulaması yapıldı ve toplam ve biyofilm oluşturan *E. coli* sayıları sırasıyla ~1,83 log ve ~1,90 log azladı. Buna göre, bu çalışmanın sonuçları, patojenik *E. coli* suşlarına karşı faj uygulamasının, sahada taze tüketilen yeşil yapraklılar üzerinde sulama sırasında biyokontrol ajanı olarak umut verici bir uygulama olabileceğini göstermektedir. Faj uygulaması ile vejetasyon sırasında *E. coli* kontaminasyonunu tamamen elimine etmek için çalışmalar sürdürülmelidir.

Anahtar Kelimeler: *Escherichia coli,* gıda kaynaklı patojen, bakteriyofaj, faj uygulaması, yeşil yapraklı gıdalar

To My Beloved Parents

ACKNOWLEDGMENTS

Firstly, I would like to state my respect and gratitude to my supervisor Assoc. Prof. Dr. Yeşim Soyer for her endless support, vision, and guidance. She always believes in me even though the times I did not believe in myself. She encourages me and pushes me to become better. She is my role model since I took her class, and I am fortunate enough to be her graduate student. I will always be grateful for her.

Special thanks to my co-supervisor Prof. Dr. Sertaç Önde for his guidance in this research and his positivity. I knew he was a wonderful lecturer, but I learned that he is also a wonderful co-supervisor.

I am deeply grateful to Mustafa Güzel. It was a great opportunity to work with such an amazing person. He always helped me when I was in trouble. I hope I can be at least half of the mentor that he is. He is not only a mentor and a lab mate but an actual mate to me.

I would like to express my love and gratitude to my lab friends Aysu Deniz, Utku Uysal, Ayşenur Yücefaydalı, Diala Konyalı, Segâh Yetişkin, Deniz Tunalıer, Filiz Yeniköylü, Arda Tuhanioğlu, Şahin Namlı, Berfin Özışık, and Hilal Samut. You are the best lab mates that anyone wants. I could not do the harvests without your help.

I owe a special thanks to Ahmet Can Özgören for his emotional, transportational, and computational help. He always believes in me and supports me.

I would like to thank to The Scientific and Technological Council of Turkey (TUBITAK) for funding this research. This study is part of both TUBITAK 118 O 673 and TUBITAK 220 O 073 projects.

I would like to thank my uncle for his lifelong guidance and support. When I was a child, I wished that I would be successful like him and he was and always will be my role model. I am grateful that you are my uncle and always by my side with your support and wisdom.

Last but not least, I am eternally grateful to my parents Ayfer – Mehmet İskender. I cannot find the right word to thank them but thanks for always supporting me, being there for me, believing in me, and wishing the best for me. You are the best parents that anyone wishes for. I love you so much.

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CFU	Colony Forming Unit
GRAS	Generally Recognizes as Safe
HACCP	Hazard Analysis and Critical Control Point
MOI	Multiplicity of Infection
MPN	Most Probable Number
OD	Optical Density
PFU	Plaque Forming Unit
Rpm	Revolutions Per Minute
RTES	Ready-To-Eat Salad
Stx	Shiga toxin
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

The current trend is fresh produce consumption because of health concerns around the world. People would like to eat more nutritious food to live healthier. Being fed nutritious food is important since one of the risk factors for mortality is the low consumption of fresh produce, globally. Moreover, World Health Organization (WHO) states that a diet rich in fresh produce has been linked to avoiding some chronic diseases such as obesity, cardiovascular disease, diabetes, and hypertension (WHO, 2003).

As the fresh produce consumption rate increase throughout the world, the risk of foodborne illnesses and outbreaks due to contaminated fresh produce is increasing too (McDaniel & Jadeja, 2019). WHO indicates that almost 1 in 10 people in the world get sick and 420000 people die after the consumption of contaminated food every year. Moreover, the main foodborne pathogens that cause foodborne diseases by consumption of fresh produces are Enterohaemorrhagic *E. coli* with fresh produce, *Listeria* with ready-to-eat foods, *Vibrio cholera* with vegetables, Norovirus with fresh produce, and *Entamoeba* with fresh produce (WHO, 2020). Also, globally, more than 300 million foodborne diseases and nearly 200000 deaths are occurring each year because of *E. coli* infection (CDC, 2019b). The risk of getting foodborne illnesses is mainly in consequence of the fresh produce's raw consumption. When eating up raw foods, there is no further elimination step for foodborne pathogens such as pasteurization or heat treatment. For foodborne illnesses to be listed as an outbreak, there should be two or more cases resulting in the consumption of the same food product. Moreover, the main foodborne pathogens that cause outbreaks are *E.*

coli, *Salmonella*, *L. monocytogenes*, *Campylobacter*, and Norovirus (McDaniel & Jadeja, 2019).

Among pathogenic *E. coli* strains, enterohemorrhagic *Escherichia coli* (EHEC) induces illnesses in humans like diarrhea, bloody diarrhea, and hemolytic uremic syndrome, HUS, and this subgroup is Shiga toxin-producing *Escherichia coli* (STEC) (Mellmann et al., 2011). The main cause of *E. coli* infections is STEC and it can be grouped as STEC O157 and non-O157 STEC. STEC O157 outbreaks associated with green leafy vegetables were first discovered in 1995 and non-STEC in 2010 in the United States, whereas, green leafy vegetables were associated with STEC 157 outbreaks first in 2012 in Canada (Marshall et al., 2020). Furthermore, enteroaggregative *Escherichia coli* (EAEC) is the cause of persistent diarrhea in developing and developed countries and there are several outbreaks due to this strain worldwide (Kaper et al., 2004). The emergence of the new Shiga toxin-producing EAEC which is *E. coli* O104:H4 stood out after the German outbreak caused by contaminated sprouts in 2011. *E. coli* O104:H4 was different from the ones that were known as STEC although it has a gene for the production of Shiga toxin that is special for EHEC, it is more like the enteroaggregative *E. coli* (EAEC). The outbreak was spread to other neighboring countries. At the end of the outbreak, there were more than 3100 acute gastroenteritis cases, 850 HUS cases, and 53 death in total due to multi-drug resistant strain of *E. coli* O104:H4. This was the largest foodborne outbreak in Germany in 60 years (EFSA, 2012). Moreover, large outbreaks of infant diarrhea caused by enteropathogenic *E. coli* (EPEC) have mostly disappeared from developed countries, however, it is still an important foodborne agent in developing countries for fatal infant diarrhea (Kaper et al., 2004).

One of the major sources of foodborne illnesses from fresh produce is leafy greens. In the reported outbreaks between 1998 to 2016, leafy greens were associated with 10 % to 40 % of fresh produce-related ones (Johnson, 2019). There were nine multistate outbreaks associated with the leafy green between 2019 to 2021 and *E. coli* O157 is the main foodborne pathogen that contaminated the leafy greens during these outbreaks (CDC, 2021). The contamination risk of fresh leafy green vegetables

could come from different sources such as animals, poorly composted manure, affinity to urban areas, and untreated irrigation water (Mercanoglu Taban & Halkman, 2011). Water is a contamination source both pre- and post-harvest, significantly. In the aerenchyma and roots of vegetables, contaminated irrigation water is one of the main sources of enteric bacteria (Martínez-Vaz et al., 2014). As an example, in the study conducted by Mitra et al., water contaminated with *E. coli* O157:H7 was sprinkled on the spinach where *E. coli* O157:H7 was internalized in the plant. The pathogen lived in phylloplane for 14 days and increased its titer and colonization area (Mitra et al., 2009). For fresh produce, water sterilization is commonly done with chlorine-derived compounds because it is not expensive and decreases the number of pathogens present in the water. On the other hand, their usage has been associated with rising chemical risk for the health of humans due to the disinfection byproducts accumulation (Gadelha et al., 2019). Additionally, chlorine usage in organic production is forbidden as a result of health and environmental risks. Chlorine dioxide, also a disinfectant, has a more antimicrobial effect at neutral pH than chlorine, but it is not effective in fresh produce within the permitted levels. Furthermore, organic acids can be an alternative to water sterilization for fresh produce and their usage is easy, however, they could affect the sensory quality and a long contact time is needed which is not convenient for the food industry. Peroxyacetic acid can be used as a sanitizer with no by-products and is not corrosive but it has a little antimicrobial effect within the permitted levels and its usage is not allowed in organic production (Ölmez & Kretzschmar, 2009).

Disinfectant usage against bacteria is one of the roughest stresses they could encounter, however, if bacterial tolerance increases, the adaptive resistance to bacterial virulence and antibiotics could be enhanced. In this way, an increase in disinfectant tolerance in bacteria becomes a key factor for food safety (Sun et al., 2019). Moreover, the overuse and misuse of antimicrobials in humans, animals, and agriculture lead to a gradual increase in antibiotic resistance. As a result, the bacteria which can be treated with antibiotics now either need the last line of antibiotics or are untreatable. *E. coli, Klebsiella pneumoniae, Acinetobacter baumannii,* and

Pseudomonas aeruginosa strains are reported as Multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) worldwide. The emergence of antibiotic resistance is acknowledged as one of the most important public health problems and has significant mortality rates as MDR bacterial infections are high. Also, *E. coli* is the most common Gram-negative pathogen among resistant bacteria (Paitan, 2018). *E. coli* represents a major resistance gene reservoir which might be a reason for treatment failures. During the last decades, the number of resistance genes has increased in *E. coli* isolates and most of the resistance genes were gained by horizontal gene transfer. *E. coli* can gain these resistance genes from other bacteria but also have the ability to pass on its resistance genes to other bacteria (Poirel et al., 2018). Thereby, a need for a new method for the treatment of fresh produce against foodborne pathogens has emerged.

For an alternative prevention method, bacteriophages which are natural bacteria hunters could be used. Bacteriophages do not show risks to human health, unlike chemical compounds. They have minimal effect on humans' normal microflora due to their host specificity. In addition, as biocontrol agents, bacteriophages have plenty of advantages. For example, bacterial resistance to phages is limited by phages' narrow host range, phages' dose is self-controlled and they can clear some biofilms (Loc-Carrillo & Abedon, 2011).

Bacteriophages have a huge potential for preventing foodborne pathogens in food production in both pre- and post-harvest steps. Through the farm-to-fork continuum, phages can be used at any step (Endersen et al., 2014). Additionally, up to today, phage therapy might be the most eco-friendly treatment for antimicrobial prevention. There were several studies conducted on phage therapy against foodborne pathogens like *E. coli*, *L. monocytogenes*, *Salmonella supp.*, *Shigella spp.* and *Campylobacter jejuni* show great results for prevention (Moye et al., 2018). Moreover, there are studies of phage therapy against *E. coli* on fresh produces. In one of these studies, phage therapy against *E. coli* O157:H7 was applied to fresh-cut lettuce and they reported a significant reduction number of pathogens (Ferguson et al., 2013). In another study conducted by Viazis et al., it was reported that a significant reduction

of *E. coli* O157:H7 when phage therapy was applied to organic baby spinach and baby romaine lettuce that was purchased at a supermarket (Viazis et al., 2011).

In the literature, phage therapy against foodborne pathogens has been applied to the end product of leafy green vegetables. On the other hand, there is no study on phage therapy against foodborne pathogens on leafy green vegetables during their vegetative phase.

Elimination of foodborne pathogens in fresh produce is crucial. Outbreak risks caused by contaminated irrigation water will be decreased by eliminating possible foodborne pathogens before harvest and during plantation. Consequently, the purpose of this study is to investigate the impact of phage therapy against pathogenic *E. coli* subgroups which are O157:H7, O104:H4, and O26 during garden cresses' vegetative stage with irrigation water at cresses optimum growth temperature for a solution to reduce the risk of foodborne disease. In this study, the phage therapy was applied against each *E. coli* strain for six different groups which have different irrigation schedules. In addition, the effect of phage therapy against *E. coli* O104:H4 with a single phage solution and phage cocktail solution was compared. Also, the comparison between phage therapy against *E. coli* O104:H4 with a single phage solution with MOI = 100 and MOI = -1 was done. The total and biofilm-forming *E. coli* count results were analyzed by comparing their control groups. To visualize *E. coli* attachment on the cress's leaf surface, SEM analyzes were done. This data would be helpful to increase the phage therapy against foodborne pathogens during the vegetation stage of food plants in the field by irrigation water.

CHAPTER 2

LITERATURE REVIEW

2.1 Foodborne Pathogens

Food consumption and human disease were associated with each other as early as 460 B.C. and this was reported by Hippocrates that there was a strong connection between human disease and consumption of food (Hutt, 1984). It is identified that there are more than 250 foodborne diseases (CDC, 2020b). Foodborne disease, called food poisoning colloquially, is generally the result of the consumption of contaminated food. This contamination could be caused by bacteria and/or their toxins, viruses, or parasites. Foodborne diseases occur when a pathogen (e.g., bacteria, viruses, parasites) in the food is ingested and establishes itself in the human host, or when the pathogen establishes itself in a food product that produces toxins and is again ingested by the host (Bintsis, 2017). The contamination of food could occur at any stage of the farm-to-fork continuum and result from various sources such as unsafe food processes, storage, and environmental contamination that includes water, soil, or air pollution (WHO, n.d.). Moreover, the foodborne disease is usually classified into two groups which are foodborne infection and foodborne intoxication. The time from ingestion to occur of symptoms is longer in foodborne infections than in foodborne intoxication because the period of incubation is involved in foodborne infections (Bintsis, 2017).

The most common foodborne disease symptoms are vomiting, diarrhea, nausea, and stomach cramps but these can be different among different diseases. Although symptoms could be mild in some cases, they could be severe and even life-threatening in other cases. The people who are in the risk groups are more likely to

develop a foodborne disease when they get it. This group includes young children, older adults, immunocompromised people, and pregnant women (CDC, 2020b).

According to WHO, nearly 1 in 10 people in the world, almost 600 million, get sick after the consumption of contaminated food and 420000 people die every year. 40 % of foodborne disease cases occur in children under 5 years, with 125000 deaths per year. Moreover, 550 million people get sick and 230000 people die from diarrheal diseases which is the most occurring disease caused by contaminated food consumption, every year. Almost 110 billion US$ is lost for productivity and medical expenses due to unsafe food in low- and middle-income countries every year (WHO, 2020). Also, CDC reported that nearly 1 in 6 Americans get a foodborne disease, 128000 are hospitalized and 3000 people die from foodborne diseases (CDC, 2020b).

The major pathogens that cause foodborne diseases and their most associated foods are *Salmonella* with eggs, poultry, and other animal origin products, Enterohaemorrhagic *E. coli* with unpasteurized milk, undercooked meat, and fresh produce, *Campylobacter* with raw milk and raw or undercooked poultry, *Listeria* with unpasteurized dairy products and ready-to-eat foods, *Vibrio cholera* with rice, vegetables, and several types of seafood, Norovirus with fresh produce and shellfish, Hepatitis A with raw or undercooked seafood, *Entamoeba* with fresh produce, and prions with bovine products containing brain tissue (WHO, 2020).

In Turkey, foodborne disease is the second rank in poisoning cases at emergency services after drug poisoning. Between 2016 and 2020, there were total of 5842 foodborne disease cases which are shown in Table 2.1. According to this data, the highest case number and number of affected people were in 2019 (Başaran, 2021).

Table 2.1 Number of Foodborne Diseases and Affected People between 2016 - 2020 in Turkey (Başaran, 2021)

Year	*Estimated number of foodborne diseases*	*Estimated number of affected people*
2016	1224	4361
2017	931	3403
2018	1276	5401
2019	1452	9976
2020	959	4055

2.1.1 *Escherichia coli*

Escherichia coli, *E. coli*, is a Gram-negative, non-sporulating, rod-shaped, facultative anaerobe bacteria. It was first identified by Theodor Escherich in 1885 (Lim et al., 2010). Its natural habitat is warm-blooded animals and reptiles' intestines and faces. The gut microbiota consists of more than 500 bacteria species 10^{10} - 10^{11} cells/g of large-intestinal content and *E. coli* is found there. *E. coli* is the predominant aerobic microorganism in the gastrointestinal (GI) tract even though anaerobic bacteria in the bowel are outnumbered (Tenaillon et al., 2010). *E. coli* colonizes the GI tract of human infants therein a few hours after birth. Generally, *E. coli* and its host, human, co-exist in a good health where they mutually benefit each other for decades. Commensal *E. coli* strains seldom cause disease except in immunocompromised hosts or if there is a breach in normal GI barriers. Although most *E. coli* strains are harmless, even good for the human host, some strains can be pathogenic with their virulence factors which can cause diseases in healthy humans. In general, there are three clinical syndromes due to the infection of pathogenic *E. coli*. The first is enteric/diarrheal disease, the second is urinary tract infections (UTIs) and the last is sepsis/meningitis (Kaper et al., 2004).

Pathogenic *E. coli* are categorized into 6 pathotypes that are related to diarrhea and are referred to as diarrheagenic *E. coli*. These pathotypes are enterohemorrhagic *E. coli* (EHEC) also referred to as Shiga toxin-producing *E. coli* (STEC) or Verocytotoxin-producing *E. coli* (VTEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (CDC, 2014a). EHEC, ETEC, and EPEC can cause disease by using mostly the same virulence factors which are present in strains of humans in animals, too. Various *E. coli* pathotypes are characterized via shared O lipopolysaccharide, LPS, and H which are flagellar antigens that identified serogroups, O antigen only, or serotypes, O and H antigens. Pathogenic *E. coli* strains mostly remain extracellular, however, EIEC is an intracellular pathogen, truly, that is the ability to invade and replicate within epithelial cells and macrophages. Moreover, pathogenic *E. coli* strains have specific factors for adherence that allow colonizing the sites that *E. coli* does not inhabit normally like the small intestine (Kaper et al., 2004).

Among the pathotypes, EHEC has the ability to produce Shiga toxins (Stxs), cause hemorrhagic colitis, and hemolytic uremic syndrome (HUS), a life-threatening disease, in humans. Several serotypes of EHEC are related to human diseases like O91:H21, O157:NM, O11:H8, and O157:H7 (Lim et al., 2010). According to CDC, 265000 EHEC infections are seen annually in the United States (CDC, 2014a).

The first described *E. coli* pathotype was EPEC. It was isolated from the children with diarrhea during an infant diarrhea outbreak in the United Kingdom in 1945. Still in developing countries, EPEC is an important cause of fatal infant diarrhea (Kaper et al., 2004). EPEC is known to be non-invasive and delivers its proteins directly to host cells for subverting several host cells' functions which lead to disease (Ochoa & Contreras, 2011). Moreover, besides humans, EPEC could infect animals like cattle, dogs, rabbits, and cats. The dose of infection in healthy adults is estimated to be 10^8 CFU (Yang et al., 2017).

EAEC is a cause of frequently persistent diarrhea in both developing and developed countries. Also, it has been known to cause several outbreaks, worldwide. When EAEC infects the host, intestinal mucosa colonization is comprised and EAEC begins to segregate enterotoxins and cytotoxins. Studies showed that EAEC induces mild but important mucosal damage (Kaper et al., 2004).

ETEC infection is a major reason for travelers' diarrhea and an important cause of diarrhea, especially among children in developing, lower-income countries (CDC, 2014b). When a host gets an ETEC infection, it colonizes the small bowel mucosal surface and produces enterotoxins that cause intestinal secretion (Kaper et al., 2004).

EIEC is genetically, pathogenically, and biochemically closely related to *Shigella* spp. Most of the EIEC infections cause watery diarrhea which is a variation from other *E. coli* infections, however, it might be a reason for invasive inflammatory colitis and sometimes dysentery. When a host gets infected by EIEC, pathogenesis begins with penetration of epithelial cells, then endocytic vacuole lysis, intracellular multiplication, movement through the cytoplasm, directionally, and extension into epithelial cells adjacent (Kaper et al., 2004).

DAEC is identified by a characteristic, adherence to HEp-2 cell monolayers' diffuse pattern. It has been associated with reason for diarrhea, especially in children under 12 months of age. When a host gets a DAEC infection, bacteria induce a cytopathic effect. This effect is characterized by a long cellular extension development that wraps the adherent bacteria (Kaper et al., 2004). Recent studies show that although the number of DAEC strains isolated from patients' feces is high, there has not been any pathogenicity identified in adults (Javadi et al., 2020).

2.1.1.1 *Escherichia coli* O157:H7

Escherichia coli O157:H7 is the most important EHEC serotype that causes severe diseases in humans worldwide. Also, this serotype is the most often isolated EHEC serotype from sick people in the United States, the United Kingdom, and Japan. *E.*

coli O157:H7 was first identified as a human pathogen in 1982 when it was associated with bloody diarrhea outbreaks in Oregon and Michigan, USA, and it was also related to HUS cases in 1983 (Lim et al., 2010). 1982 outbreak was caused by undercooked ground beef consumption in a fast-food restaurant chain (Muniesa et al., 2006). Globally, *E. coli* O157:H7 is accountable for 20 % of foodborne outbreaks (Getaneh et al., 2021). Also, it is estimated that *E. coli* O157:H7 cause 73000 diseases, 2200 hospitalizations, and 60 deaths in the United States, annually (Mead et al., 1999). CDC reported that after the peak in 1999, infections of *E. coli* O157:H7 are decreasing. Nonetheless, serious outbreaks and sporadic cases continue to occur. This infection is one of the main public health concerns in Europe, North America, and around the world. *E. coli* O157:H7 infections have much higher rates of hospitalization and fatality than other enteric pathogens like *Salmonella* or *Campylobacter spp.* although the total infection cases are lower in *E. coli* O157:H7. Furthermore, *E. coli* O157:H7 is the main cause of HUS in the United States (Lim et al., 2010).

E. coli O157:H7's name comes from its somatic (O) antigen 157 and flagella (H) antigen 7. Moreover, the major reservoir of *E. coli* O157:H7 is cattle, and it is usually asymptomatic when carrying O157:H7. Other animals such as sheep, goats, turkeys, and pigs can also have *E. coli* O157:H7 in their feces. The major infection route for *E. coli* O157:H7 is foodborne transmission. This transmission is generally linked to ground beef, unpasteurized milk, salami, beef jerky, lettuce, radish sprouts, apple cider, and fresh spinach. The biggest *E. coli* O157:H7 outbreak was in 1996 caused by contaminated radish sprouts where 7966 individuals got infected in Japan (Lim et al., 2010). From 1982 to 2002, CDC reported 350 *E. coli* O157:H7 outbreaks and the route of transmission of these outbreaks are foodborne (52 %), unknown (21 %), person-to-person interactions (14 %), waterborne (9 %), and contact with the animal (3 %) (Rangel et al., 2005). The reason why transmission routes are various can be the very low dose of *E. coli* O157:H7 infection (1-10^2 CFU) (Muniesa et al., 2006). Moreover, *E. coli* O157:H7 has a long survival time in manure-treated soil which is a year and 21 months in raw manure (Lim et al., 2010).

The major virulence factors of *E. coli* O157:H7 Shiga toxins, LEE (locus of enterocyte effacement) pathogenicity island, and plasmid O157 (pO157). When *E. coli* O157:H7 infection occurs, Shiga toxins are produced inside the colon and travel to the kidney via the bloodstream which results in renal inflammation. EHEC like other enteropathogenic bacteria, insert its receptor to adhere to the intestinal epithelium in the host. During this process, they create attaching effacing (A/E) lesions in the intestine and cause diarrhea. A/E lesions are encoded within the LEE island. Furthermore, *E. coli* O157:H7 carry large plasmids named pO157 which encode proteins about bacterial pathogenesis such as defense mechanisms against oxidative stress (Muniesa et al., 2006).

2.1.1.2 *Escherichia coli* O104:H4

Escherichia coli O104:H4 is a rare isolated pathogenic serotype of *E. coli* and the cause of the Germany outbreak in 2011. Genome sequence analysis procures from some isolates of outbreak indicates that the *E. coli* O104:H4 strain is an EAEC that has attained the Shiga toxin genes, conceivably via transduction of bacteriophage. Assembly of genome ratifies that *stx*2 prophage gene clusters in two copies, nonetheless, the *E. coli* O104:H4 strain has also an additional set of virulence and antibiotic-resistance factors (Garcia-Angulo et al., 2013). Moreover, optical mapping of an isolate from a German outbreak and four other outbreaks demonstrated identity with an EAEC strain isolated from an HIV-positive adult who suffered from persistent diarrhea living in Central Africa in the late 1990s, however, this African strain lacked *stx*2 prophage (Muniesa et al., 2012).

On May 8, 2011, the largest outbreak of STEC cases occurred in Germany. 3842 reported cases including 2987 gastroenteritis cases with 18 deaths, and 855 HUS cases with 35 deaths. The outbreak peaked on May 22 and finished on July 4 (Muniesa et al., 2012). The featured outcomes of this outbreak are the prevalent involvement of adult women and commonly severe neurological complications like epileptic seizures. Nearly 100 cases with 39 HUS cases reasoned by the outbreak

strain have been reported in other European countries (Bielaszewska et al., 2011). Epidemiological evidence shows that outbreaks were related to a seed of fenugreek shipment that arrived in Germany from Egypt in 2009. The seeds were plated around Germany including the farm where grew the contaminated sprouts. *E. coli* O104:H4 has been seldom related to human cases before and has never been reported in animals. Analysis indicated that fenugreek sprouts were the infection vehicle in the outbreak (Garcia-Angulo et al., 2013). The epidemiological analysis showed that the infection was initially foodborne, but human-to-human transmission also plays a role. Transmission by human-to-human was suggested for this outbreak in France and The Netherlands considering the delayed onset compared with the 7 to 9 days of incubation time for *E. coli* O104:H4 infections (Muniesa et al., 2012).

The epidemic strain characterization indicated that it has the *stx*2 genes, however, it lacks the pathogenicity island of LEE, unlike the EHEC. The high level of HUS development related to *E. coli* O104:H4 is not because of the toxin activity differences with *E. coli* O157:H7 but could be explained by this strain's enteroaggregative adherence phenotype leading the bacteria to colonize the intestinal mucosa efficiently, rising the tissue exposure in the host to the toxin (Garcia-Angulo et al., 2013).

2.1.1.3 Enteropathogenic *Escherichia coli* O26

The studies showed that one of the most common diarrheagenic *E. coli* is EPEC in China and is found in many food sources (Shen et al., 2022). According to WHO, EPEC includes 12 O serogroups which are O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 (WHO, 1987). Strains of EPEC could be divided into two groups which are typical (tEPEC) and atypical (aEPEC). Both groups have a LEE pathogenicity island and could cause A/E lesions. Only tEPEC has the adherence factor plasmid (EAF) of EPEC, whereas only aEPEC has heat-stable enterotoxin 1 of enteroaggregative *E. coli* (Sekse et al., 2011). Moreover, EPEC O26 is defined as aEPEC (Trabulsi et al., 2002).

Escherichia coli O26 belonging O26 serogroup includes both EPEC and STEC strains. STEC O26 is a major non-O157 serogroup related to hemorrhagic colitis and HUS while EPEC O26 is related to less-severe enteritis. *E. coli* O26 associated with human diseases generally expresses the H antigen, which is H11, or they are a nonmotile cause of lack expression of the H antigen. Nonetheless, the molecular analysis indicated that nonmotile *E. coli* O26 belongs to the clonal complex of H11 (Sekse et al., 2011). The studies showed that when the presence of OI-122, a highly virulent factor of EHEC strains, compared between STEC O26 and aEPEC O26, most of them contained OI-122 which indicates that STEC O26 and aEPEC O26 are closely related. In fact, in another study in Germany, it is shown that most of the aEPEC strains related to bloody diarrhea are likely EHEC which had lost phages that encodes Stx during the infection (Hernandes et al., 2009).

Even though the epidemiological relation of aEPEC with diarrhea is controversial, it is high maintenance worldwide and some strains' involvement in diarrheal outbreaks promotes the idea that some strains of aEPEC are diarrheagenic (Hernandes et al., 2009). Most aEPEC serotypes are isolated from different species of animals. Also, the relation between EPEC O26 and calves is well-known (Trabulsi et al., 2002). Additionally, foods such as vegetables, raw meats, and pasteurized milk are known to be an aEPEC vehicle to human infections (Affonso Scaletsky, 2019). Epidemiological studies showed that aEPEC identification is increasing in both developing and developed countries. While some studies indicate a relation between aEPEC with acute childhood diarrhea, others show that there is a relation between aEPEC and persistent diarrhea (Hernandes et al., 2009).

2.2 Foodborne Outbreaks

A foodborne outbreak is identified as if two or more people experience similar symptoms due to the consumption of a common food (CDC, 2015). The surveillance systems aim to monitor the foodborne outbreaks occurring worldwide, each year and this is imperative. For example, the Centers for Disease Control and Prevention

(CDC) operates in the U.S. since 1966, and the European Centre for Disease Prevention and Control (ECDC) operates in the European Union (Schirone & Visciano, 2021).

In the EFSA-ECDC annual reports, it is reported that the first four foodborne diseases in humans are campylobacteriosis, salmonellosis, Shiga toxin-producing *E. coli*, and yersiniosis during the 2015-2019 period. In contrast, the most severe diseases are listeriosis and the West Nile virus infection with the highest fatality and hospitalization rates (Schirone & Visciano, 2021).

According to the National Outbreak Reporting System (NORS), there is a total of 23105 foodborne outbreaks, 441559 foodborne illnesses, 19955 hospitalizations, and 462 deaths from 1971 to 2020 in the U.S. (CDC, 2022c). The 27 European Union (EU) member countries reported that in 2019, there were 5175 foodborne outbreaks, 49463 foodborne illnesses, 3859 hospitalizations, and 60 deaths. The number of people who died because of a foodborne pathogen increased as regards the previous year (Sarno et al., 2021).

The list in Table 2.2, represents the selected foodborne outbreaks worldwide, and it is lined according to the number of people infected from that foodborne outbreak.

Table 2.2. Selected Foodborne Outbreaks Worldwide

Outbreak Agent	*Food*	*Country*	*Year*	*Number of People Infected*	*Number of Death*	*Reference*
Salmonella Enteritidis	Ice Cream	The United States	1994	224000	0	(Homas et al., 1996)
Salmonella Typhimurium	Milk	The United States	1985	5295	9	(CDC, 1985a)
E. coli O104:H4	Fenugreek Sprouts	Germany	2011	3842	53	(Muniesa et al., 2012)

Table 2.2 (continued)

Listeria monocytogenes	Processed Meat	South Africa	2017-2018	1060	216	(National Listeria Incident Management Team, 2018)
Salmonella Oranienburg	Onion	The United States	2021	1040	0	(CDC, 2022b)
Salmonella Poona	Cucumber	The United States	2015	907	6	(CDC, 2016a)
Salmonella Typhimurium	Peanut Butter	The United States	2008-2009	714	9	(CDC, 2009b)
Salmonella Typhimurium	Meat	Wales	1989	640	3	(Pennington, 2014)
Hepatitis A	Green Onions	The United States	2003	601	3	(Wheeler et al., 2005)
E. coli O157:H7	Undercooked Hamburgers	The United States	1992-1993	>500	4	(CDC, 1993)
E. coli O157:H7	Meat	Scotland	1996	496	21	(WHO & FAO, 2002)
Norovirus and ETEC	Lettuce	Denmark	2010	260	0	(Ethelberg et al., 2010)
E. coli O157:H7	Romaine Lettuce	The United States	2018	210	5	(CDC, 2018b)
E. coli O157:H7	Spinach	The United States	2006	199	3	(CDC, 2006)

Table 2.2 (continued)

E. coli O157:H7	Meat	Wales	2005	157	1	(Pennington, 2014)
Listeria monocytogenes	Cantaloupe	The United States	2011	147	33	(McCollum et al., 2013)
E. coli O157:H7	Milk	The United Kingdom	1999	114	0	(Pennington, 2014)
Shigella sonnei	Iceberg Lettuce	Norway	1994	110	0	(Kapperud et al., 1995)
Listeria monocytogenes	Cheese	The United States	1985	86	29	(CDC, 1985b)
E. coli O157:H7	Unpasteurized Apple Juice	The United States	1996	70	1	(Cody et al., 1999)
Listeria monocytogenes	Deli Meats	Canada	2008	57	24	(Currie et al., 2015)

2.2.1 Foodborne Outbreaks through Contaminated Leafy Greens

For a healthy diet, vegetables are essential as they contain nutrients that help to protect against stroke, heart disease, and some cancers (CDC, 2021). However, contamination of them is concerning as they are mostly consuming raw which means there is no cooking for killing the present pathogens (Herman et al., 2015) as washing the leafy greens cannot remove all pathogens due to their ability to stick to the leaf surface. Pathogens can contaminate the leafy greens at any point through the farm-to-fork continuum such as through irrigation water, the soil where leafy greens grow, in packing and processing, from food handlers, or in the kitchen (CDC, 2021). Both harvest and post-harvest contamination sources such as water, insects, processing equipment, vehicles of transport, and human handling. Leafy greens could undergo

some processes which result in changes in a physical state like the creation of cut surfaces and crevices which could allow pathogen adherence and protection of some sanitation methods (Turner et al., 2019).

Herman et al. defined leafy green-associated outbreaks as two or more people getting sick from the consumption of a common leafy green food. Also, they defined leafy green food as any vegetable with edible leaves such as cabbage, ferns, chard, green onions, collard greens, lettuce, spinach, turnip greens, leeks, and mustard greens (Herman et al., 2015).

Foodborne outbreaks associated with lettuce, spinach, and parsley have been reported since 2002 (Delaquis et al., 2007). Leafy greens are often linked to foodborne outbreaks, and it is estimated that leafy greens are the most common source of foodborne disease in the U.S. Leafy green outbreaks could result in serious health problems (Herman et al., 2015). Moreover, green leafy foods have ranked the risk as the most important human infection from food of non-animal origin in the European Union (EFSA, 2014). In a study, it is reported that leafy greens were the second most common cause of hospitalizations and the fifth most common cause of death from foodborne disease (Turner et al., 2019). According to CDC, there were 51 foodborne outbreaks associated with leafy greens, mainly lettuce, from 2014 to 2018. Recently, CDC reported that there were 9 multistate leafy green outbreaks in 2019-2021 (CDC, 2021). Table 2.3 shows selected foodborne outbreaks linked to leafy greens worldwide and it is lined considering the number of people infected from that outbreak.

Table 2.3. Selected Foodborne Outbreaks Linked to Leafy Greens Worldwide

Outbreak Agent	*Food*	*Country*	*Year*	*Number of People Infected*	*Number of Death*	*Reference*
E. coli O104:H4	Fenugreek Sprouts	Germany	2011	3842	53	(Muniesa et al., 2012)
Hepatitis A	Green Onions	The United States	2003	601	3	(Wheeler et al., 2005)
Cyclospora cayetanensis	Fresh Express Salad Mix	The United States	2018	511	0	(CDC, 2018c)
Norovirus and ETEC	Lettuce	Denmark	2010	260	0	(Ethelberg et al., 2010)
Cyclospora cayetanensis	Fresh Basil	The United States	2019	241	0	(CDC, 2019c)
Salmonella Saintpaul	Alfalfa Sprouts	The United States	2009	235	0	(CDC, 2009a)
Salmonella Bareilly	Bean Sprouts	The United Kingdom	2010	231	0	(Cleary et al., 2010)
E. coli O157:H7	Romaine Lettuce	The United States	2018	210	5	(CDC, 2018b)
E. coli O157:H7	Spinach	The United States	2006	199	3	(CDC, 2006)
E. coli O157:H7	Romaine Lettuce	The United States	2020	167	0	(CDC, 2020a)
Enteroinvasive *E. coli*	Salad	The United Kingdom	2014	157	0	(Newitt et al., 2016)
Shigella sonnei	Iceberg Lettuce	Norway	1994	110	0	(Kapperud et al., 1995)

Table 2.3 (continued)

Enteroinvasive *E. coli*	Leafy Greens	Sweden	2017	83	0	(Lagerqvist et al., 2020)
E. coli O157:H7	Romaine Lettuce	The United States	2019	62	0	(CDC, 2019a)
E. coli O103	Clover Sprouts	The United States	2020	51	0	(CDC, 2020c)
Salmonella Newport	Alfalfa Sprouts	The United States	2010	44	0	(CDC, 2010)
E. coli O157:H7	Leafy Greens	The United States	2020	40	0	(CDC, 2020d)
E. coli O157:H7	Organic Spinach and Spring Mix Blend	The United States	2012	33	0	(CDC, 2012b)
E. coli O157:H7	Ready-to-eat Salads	The United States	2013	33	0	(CDC, 2013)
E. coli O26	Raw Clover Sprouts	The United States	2012	29	0	(CDC, 2012a)
E. coli O157:H7	Leafy Greens	The United States	2018	25	1	(CDC, 2018a)
Listeria monocytogenes	Pre-Packaged Salads	The United States	2016	19	1	(CDC, 2016b)
E. coli O157:H7	Prepackaged Baby Spinach	The United States	2022	15	0	(CDC, 2022a)
Listeria monocytogenes	Packaged Salads	The United States	2022	10	1	(CDC, 2022d)

In Table 2.4 and Table 2.5, a summary of fresh green vegetable outbreaks and their causative agents in the U.S. and Europe from 2004 to 2012 is shown.

Table 2.4. Summary of Fresh Green Vegetable Outbreaks in the U.S., 2004-2012 (Callejón et al., 2015)

Type of Pathogen	*Salad*	*Leafy*	*Sprouts*
Norovirus	97	62	0
Salmonella spp.	8	8	14
Escherichia coli	10	22	4
Listeria monocytogenes	0	0	2
Campylobacter spp.	4	2	0
Shigella spp.	1	2	0
Staphylococcus	2	0	0
Bacillus spp.	1	0	0
Giardia	0	1	0
Cyclospora spp.	1	1	0
Hepatitis A	0	1	0

Table 2.5. Summary of Fresh Green Vegetable Outbreaks in Europe, 2004-2012 (Callejón et al., 2015)

Type of Pathogen	*Salad*	*Leafy*	*Sprouts*
Norovirus	15	26	0
Salmonella spp.	8	12	11
Escherichia coli	3	0	3
Campylobacter spp.	2	1	0
Shigella spp.	1	0	0
Staphylococcus	0	0	1
Bacillus spp.	2	0	0
Cryptosporidium spp.	1	0	0

Table 2.5 (continued)

Hepatitis A	2	0	2

2.2.1.1 *Escherichia coli* Outbreaks in Leafy Greens

Since the reservoir of *E. coli* is animals, it was not a consideration that fresh produce could be a vector for *E. coli* O157:H7 until the mid-1990s when various outbreaks linked to raw or minimally processed agricultural products revealed that contamination can happen (Delaquis et al., 2007). The major source of *E. coli* O157:H7 foodborne outbreaks is leafy greens and other row crop vegetables (CDC, 2021).

STEC contamination of leafy greens is an important public health problem and was 40 outbreaks in the U.S. and Canada from 2009 to 2018 (Irvin et al., 2021). The study by Turner K. et al. (2019), shows that there was at least one outbreak associated with leafy greens in California, U.S. from 2002 to 2016. Among those outbreaks, *E. coli* was the major foodborne agent (38/40). Moreover, it is reported that *E. coli* was responsible for 48 % of foodborne outbreaks linked to leafy greens, whereas *Salmonella* was 27 % and *L. monocytogenes* was 24 % from 1996 to 2016 in the U.S. (Turner et al., 2019). In all leafy green outbreaks from 1973 to 2012 in the United States, STEC caused almost 2/3 of multistate outbreaks, more than 45 % of hospitalization, and almost half of the deaths (Herman et al., 2015).

2.3 Leafy Greens

Leafy greens have been eaten since prehistoric times. They are a great nutrition source, especially salad greens, spinach, and kale are rich in vitamins A, C, E, and K. Additionally, bok choy, broccoli, and mustard are rich in Vitamin B too. Also, these leafy greens are high in carotenoids and antioxidants and contain a high amount of fiber, magnesium, iron, folate, calcium, and potassium. Moreover, they have a

very little amount of sodium, cholesterol, carbohydrates, and a low glycemic index (Yan, 2016). Leafy greens consumption is increasing internationally resulting in efforts in promoting better nutrition (Mercanoglu Taban & Halkman, 2011). Modifications in eating habits and less time for food preparation could be a solution for the emerging claim for a healthy and not time-consuming diet in industrialized countries. As an example, ready-to-eat leafy green salads (RTESs) consumption has increased in Europe. In particular, there is an average 10 % annual increase in the consumption of RTESs, and the endorsement has been reaching about 600 million Euros in Italy (Arienzo et al., 2020).

Besides the potential benefits of the consumption of leafy greens, the number of outbreaks due to foodborne pathogens linked to the consumption of leafy greens and their RTESs has increased all over the world during the last two decades. Leafy greens that are used in RTESs maintain most of their microflora after minimal processing and this microflora's pathogens have a great potential for threatening food safety (Mercanoglu Taban & Halkman, 2011). The majority of naturally present microorganisms on vegetables and fruits are non-pathogenic epiphytic bacteria. Nonetheless, both of them are vulnerable to pathogen contamination as they are mostly grown in a natural environment (Franz et al., 2009). Microorganisms that live in soil and irrigation water can contaminate plants through roots or wounded and/or cut surfaces where they can get internalized by the coating of the plant which creates a natural biofilm to protect microorganisms from treatments (Arienzo et al., 2020). Studies showed that human pathogens are present in fresh produce and some human pathogens like *E. coli* O157:H7 and *Salmonella* have the ability to colonize the crops when they are contaminated (Franz et al., 2009). Furthermore, RTESs might play a role in propagating the bacteria that carry antibiotic resistance genes (Arienzo et al., 2020).

Leafy greens usually do not contain any preservatives or antimicrobial agents and seldom receive heat treatment before consumption (Kokkinos et al., 2017). Since there is almost no treatment against foodborne pathogens for leafy greens before consumption, there are limitations for acceptable levels of pathogens present in the

food. Concerning the European Regulation (EC) No 1441/2007, the acceptable level of *E. coli*, *Salmonella* spp., and *L. monocytogenes* in RTESs is 100 CFU/g during their shelf-life (The European Community, 2007).

Many outbreaks due to fresh produce consumption have been known or suspected to have originated from contamination from the field indicating irrigation water was the contamination route. Water always has a key role in pathogen transmission via the fecal-oral route (Kokkinos et al., 2017). Recent studies demonstrated that there is strong evidence for irrigation water as a contamination source of fresh produce during primary production. Irrigation water originates from different water sources and among them, it could be identified, from a lower contamination risk to higher, wells, rainwater harvesting, rivers, and reservoirs. The study conducted by Decol, L. T. et al. (2017), confirmed that irrigation water is a very important risk factor as a source of microbial contamination during primary production. Moreover, they analyzed two sources of irrigation water and concluded that there a high counts of *E. coli*, higher than the acceptable microbial limits for irrigation water. In addition, *E. coli* O157:H7 was present in more than 20 % of samples (Decol et al., 2017).

Lepidium sativum Linn. is chosen as a model organism. It is an edible herb and belongs to the family of Brassicaceae (Diwakar et al., 2010). It is commonly known as garden cress (GC). GC is a cool-season, annual and erect herbaceous plant that can grow rapidly up to 50 cm. It needs minimal resources, can grow in semi-arid regions and does not need much fertilizer (Behrouzian et al., 2014). It has both broad and curly leaves, but they taste the same. It could be harvested throughout the year and cut when the sprouts are 5-10 cm in height. Its leaves are consumed raw in salads, but they can also be cooked and used as a garnish (S. Sharma & Agarwal, 2011). It is high in nutritional value (Behrouzian et al., 2014). The major characteristic feature of the Garden cress is that it has the ability to grow in any type of soil and climate condition (Wadhwa et al., 2012).

Lepidium sativum Linn. was used as a food source by the ancient Egyptians and become popular in several parts of Europe and around the world (S. Sharma &

Agarwal, 2011). The Persians used to eat GC even before the bread was invented as Xenophon mentioned in 400 B.C. The origin of *Lepidium sativum* is not known, however, it is estimated that Ethiopia and around countries or even in western Asia could be the place of origin. Nowadays, GC is cultivated around the world (Wadhwa et al., 2012). In short, GC was chosen because it is an example of a freshly consumed green leafy vegetable and its germination time is short.

2.3.1 Attachment of Pathogens to Leafy Greens

Biofilms are complex structures of microorganisms where the bacterial cells are attached to both the surface and each other and are buried in the self-produced matrix of extracellular polymeric substances (Yaron & Römling, 2014). Leafy greens as carriers for pathogenic biofilms become a significant threat to microbial safety as biofilms have a significant resiliency against decontamination techniques that are applied during post-harvest. The mechanism for contamination of leafy greens by the colonization of pathogens generally occurs in stages. First, the bacterial attachment to the phyllosphere or rhizoplane happens. Second, the adaptation of the pathogen to environmental parameters, and last, bacterial survival and multiplication on the parts of the plants occur (Darabă, 2021).

The surface properties of leafy greens are suitable for pathogen attachment and colonization which causes biofilm formation at certain parts of the leaf such as stomata, trichomes' base, junctions of the epidermal cell wall, and grooves in the veins and depressions (Darabă, 2021). An extensive study including 47 plant species belonging to 27 botanical families demonstrated that phyllosphere bacteria were predominantly linked to structures of leaf such as trichomes, stomata, veins, and epidermal cell wall junctions, independent of species (Macarisin et al., 2013). Also, bacterial contamination load is an important factor for attachment to leafy greens. The first step of biofilm formation on leafy greens is bacterial contamination, then colonization. This could happen via various routes such as irrigation water, contaminated soil, seeds, and roots. Colonization also depends on the bacterial ability

to adapt to the new niche. After the colonization happens, the formation of biofilm is initiated (Darabă, 2021). Beattie and Lindow (1999) explained that bacteria on leaves have two strategies which are the "tolerance strategy" which needs the ability to show resistance to exposure to environmental stresses on surface leaves and the "avoidance strategy" in which bacteria search the sites of plants that are protected against those stresses (Beattie & Lindow, 1999). Moreover, after the biofilm is formed, it can protect the attached bacteria against the immune response of plants, endogenous or exogenous antimicrobial compounds, and environmental stressors. In some cases, the bacterial attachment could be reversible, however, if pathogens form exopolymeric material which makes them fix on the leafy greens more strongly, the attachment becomes irreversible and cannot be removed by simply washing (Darabă, 2021).

Recent studies showed that human enteric pathogens like *E. coli* and *Salmonella* can attach and even internalize on plant surfaces (Macarisin et al., 2013). For instance, *E. coli* has an attachment-adhesion system thanks to the production of a variety of pili, fimbriae and non-fimbrial adhesins, and flagella which could create alternative functions for attachment and adhesion (Darabă, 2021). Studies revealed that virulence factors of *E. coli* O157:H7 related to pathogenesis in humans like curli fimbriae were also associated with attachment of bacterial cells and persistence on leaf surfaces (Macarisin et al., 2013).

2.3.2 Prevention Methods against Foodborne Pathogens on Leafy Greens

Effective food safety prevention methods are needed to be applied through the production, processing, and distribution for controlling, reducing, or eliminating microbial hazards on fresh produce. Leafy greens can be grown and harvested under a variety of geographical and climatic conditions and can get contaminated via several sources. The hazard analysis and critical control point (HACCP) which is a formal program is not feasible for the primary production of fresh produce due to the

lack of strict critical control points and the legal obligation of keeping records and documentation (Gil et al., 2015).

Some of the potential human pathogen sources through the production chain are using low-quality irrigation water concerning human enteric pathogen abundance, untreated manure, using unhygienic farm equipment, and washing water. The most used method for removing the microorganisms is washing. Water washing is a good method for the elimination of soil and other foreign materials (Rosberg et al., 2021). Moreover, a study conducted by Uhlig et al. (2017) indicated that almost 1 log CFU/g reduction in total aerobic bacteria count and 0.5 log CFU/g reduction in *Enterobacteriaceae* count in the simulation of household washing procedure. They also suggested that the reduction could be dependent on water flow force which means there is a physical force needed to eliminate bacteria (Uhlig et al., 2017).

There are no practical technologies that ensure the removal of all the pathogens on the product without damaging the quality, however, it is critical to apply a sanitizing wash step to eliminate pathogens in fresh produce processing. In contrast, there is a possibility of pathogen cross-contamination in the washing step due to the reusing and recirculating of water. Thereby, the usage of a sanitizing agent is crucial in wash water to prevent the survival and transfer of pathogens (Luo et al., 2011).

Several sanitizing agents are used in the food industry. Chlorine is the most common sanitizer that is used as chlorinated water in the produce industry to eliminate pathogen contamination. The advantages of using chlorine are low cost and easily available, however, it is hazardous at high levels, react with organic matter, is affected by organic matter, is dependent on pH, and is corrosive. Chlorine dioxide is an alternative option, and it is more tolerant of organic matter and pH, it has higher antimicrobial efficiency than chlorine, less corrosive than chlorine and ozone, but it is not effective at permitted levels on fresh produce, requires on-site generation, explosive, needs final water after application, not allowed to use on organic produce. Peroxyacetic acid (PAA) is another option for an antimicrobial agent which is not affected by temperature change and is not corrosive at permitted levels. Yet, using

PAA in-tank wash is not efficient, so it is not an ideal option for commercial production, it has a low antimicrobial effect on vegetables at permitted levels and is not allowed used on the organic product. Furthermore, ozone, as a disinfected agent, has been approved by FDA in 2011 to use in food directly in both liquid and gaseous phases for treatment, process, and storage of foods. Ozone has high antimicrobial activity, needs short contact time, and no hazardous formation, whereas it requires on-site generation, is toxic when inhaled, corrosive above 4 ppm, not allowed use for organic products. Additionally, essential oils are used as antimicrobial and anti-spoilage agents in food. In contrast, essential oils are not feasible because of productivity and organoleptic reasons, and usage in the food industry is not ideal as high amounts are required and it is costly. Moreover, organic acids are easy to use, have no toxicity, and can be used for organic products but they need a long contact time, are interferes with sensory quality, and not allowed to be used in organic produce. As a natural alternative, bacteriophages can be used in the food industry as antimicrobial agents. They are viruses with the ability to kill specific target bacteria. It is estimated that bacteriophages have the ability to kill up to nearly half of all bacteria produced daily. The problem with using them in the food industry is their specificity since it is impossible to know which bacteria strains will be in the production or processing of food. Furthermore, sodium acid sulfate is a natural food acid and is recognized as safe (GRAS) by FDA as disinfected agent on fresh produce. Because it is acid, it reduces the product's pH but does not change the taste to sour. On the other hand, there are a few studies on how sodium acid sulfate eliminates pathogens (McDaniel & Jadeja, 2019; Ölmez & Kretzschmar, 2009). Studies showed that chlorine and ozone have the best ratio of efficacy to eliminate pathogens and cost efficiency except for the bacteriophages. At permitted levels, chlorine dioxide, peroxyacetic acid, organic acids, and hydrogen peroxide are not efficient in reducing pathogens again except the bacteriophage (Ölmez & Kretzschmar, 2009).

Usage of disinfectants for the elimination of bacteria is one of the major stress conditions that bacteria could encounter. The increase in bacterial tolerance would enhance the adaptive resistance to bacterial virulence and antibiotics. This result in

an increase in bacterial disinfectant tolerance which becomes a key point of food safety (Sun et al., 2019). About 75 years ago, penicillin and other antibiotics were introduced to the world, and this led to great reductions in death from bacterial infections which saved millions of lives. However, shortly after, a resistance emergency has been published. Overuse and misuse of antibiotics in humans, animals, and agriculture cause a gradual increase in antibiotic resistance. This cause untreatable bacteria or needs the last line of antibiotics for treatment that once treatable bacteria. MDR, XDR, and PDR strains of *E. coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumonia* are reported worldwide which becoming a critical global issue. Antibiotic resistance emergency is now acknowledged as one of the most important public health problems. Furthermore, *E. coli* is the most common Gram-negative pathogen among antibiotic-resistant bacteria (Paitan, 2018). *E. coli* might be the reason for treatment failures due to the major resistance genes reservoir. During the last decades, an increase in resistance genes number in *E. coli* has been identified, and many of these genes were gained via horizontal gene transfer. *E. coli* could be a donor and a recipient for the resistance genes, so they can gain resistance genes from other bacteria and also pass on its genes to others. Generally, *E. coli* antibiotic resistance is recognized as one of the most challenging health problems in both humans and animals worldwide (Poirel et al., 2018).

2.4 Bacteriophages

Bacteriophages or bacterial viruses or phages were discovered at the beginning of the 20th century. In fact, they were discovered twice. First, British pathologist Frederick William Twort described the "*Micrococcus*" colonies were glassily transmitted via a transmissible agent in 1915 in London. He had some ideas about how this transmission occurs, and one of the ideas was the agent was viral and found in nature. Second, a French Canadian scientist Félix Hubert d'Hérelle observed the *Shigella* cultures' lysis in broth in 1917 at the Pasteur Institute of Paris. He is the

father of the name of bacteriophage and also invented some methods which are still in use, accepted intracellular virus multiplication, and advertised phage therapy for infectious diseases. After the invention of electron microscopy in 1940, bacteriophages' viral nature was recognized. The International Committee for Taxonomy of Viruses (ICTV) classified bacteriophages into six genera which are T4, φX174, MS2, λ, and PM2 in its first report. New groups were included over time. Most of the phages have dsDNA, however, some small groups have ssDNA, ssRNA, or dsRNA (Ackermann, 2003). Among the well-characterized bacteriophages, the ones that have a single genome are the majority. Nearly 85 % of bacteriophages are dsDNA covered by a protein shell. Thereby, it is suggested that dsDNA phages are the most varied entities on Earth. (Nami et al., 2021). Moreover, bacteriophages usually do not have any envelopes and have only protein and DNA. But few of them have lipid-containing envelopes or just have lipids in their particle wall. Their head-tail structure is unique. Even though a few viruses other than phages have similar structures, they cannot compare with tailed phages (Ackermann, 2003).

Bacteriophages can be present in the soil, seawater, oceanic and terrestrial surfaces, extreme environments, food, animal, and human GI tract (Principi et al., 2019; Litt et al., 2020). They do not have an independent replication system, so they seize the DNA replication and mechanism of protein synthesis of host bacteria. When replication is done, bacteriophages disintegrate the bacterial cell wall's peptidoglycan layer in order to release their progeny phages (Litt et al., 2020). For this reason, phages produce their progeny phages and kill the host, continually (Nami et al., 2021). Because of their high specificity and unique nature, they are highly desirable as antimicrobial agents (Litt et al., 2020). Additionally, they have featured elements of biogeochemical cycles on Earth (Nami et al., 2021).

Bacteriophages regulate the ecosystem by killing, metabolic reprogramming, or transfer of genes. The identified phage number increased from 1468 to 3852 between 2015 to 2020 in the RefSeq database supported by the National Center for Biotechnology Information (NCBI). For the phage classification, two challenges exist. The first one is that known taxa phages are limited. ICTV is in charge of the

official taxonomy of viruses and organizes them in order, family, sub-family, genera, and species. The second one is that most of the phages can share protein homologs although they are in different taxa (Shang et al., 2021).

The most common classification of phages divides them into two groups which are lytic and lysogenic bacteriophages considering their biological cycle. The biological cycle means the invasion of and attachment to bacteria. Once the lytic phage enters the cell, it redirects the bacterial synthetic machinery in order to produce the viral genome and its proteins. Lastly, assembly and packing of new phages occur and the host is lysed as new phages are released. On the other hand, lysogenic phage integrates their genetic material into the host genome. When the host divides, the viral chromosomes are transmitted to daughter cells. Only the lytic phages can be used in treatments of bacterial infections as their ability to kill specific bacteria (Principi et al., 2019).

2.4.1 Bacteriophage Therapy

After the discovery of bacteriophages, their ability to infect and kill specific bacteria allows for the exploration of their potential therapeutic effect against pathogens, known as bacteriophage or phage therapy. The first usage of phages for therapeutic in humans was done in 1919. On the other hand, antibiotic discovery and then an increase in their usage during the 1940s and 1950s, along with a lack of knowledge about phages and some other factors, allow for a decrease in phage clinical use in Western Europe and North America. Nonetheless, phage therapy has been continued to evaluate in the former Soviet Union and a few Eastern European countries. Recently, phage therapy and other phage-associated technologies have been gaining interest in the West. Two main events played a key role in regaining phage interest as antimicrobial agents. The first one is the emergence and widespread distribution of antimicrobial-resistant bacteria which cause the limitation of therapeutic options. The situation is complicated by a few new antimicrobial drugs on the market. The

second one is increasing broad-spectrum damage of antibiotics' ability to affect the microbiome (Vikram et al., 2020).

In theory, every bacteria can be lysed by at least one bacteriophage. Accordingly, bacteriophages are more significantly effective than antibiotics (Principi et al., 2019). The current antibiotic-resistant bacteria threat in Western countries has renewed the interest in phages as biocontrol agents. Even some commercialized products based on bacteriophages are available in the market (García et al., 2008). Bacteriophages' most characteristic feature is their specific killing ability which they can only kill when they recognize the pathogen. Because their activity has a narrow spectrum, bacteriophages have an advantage over antibiotics which is antibiotics have the ability to influence the whole microbiome with the reduction of beneficial bacteria, the emergence of antibiotic-resistant bacteria, and the outgrowth of secondary pathogens. Compared with antibiotics, phages are safer and more tolerated since they can only replicate in the target bacteria (Principi et al., 2019). Moreover, phages might help to overcome several bacterial infections, including multidrug-resistant bacterial infections (Vikram et al., 2020). In addition, phages have been proposed as a green strategy to control biofilm since they provide a specific, natural, non-toxic, and feasible approach to controlling the formation of biofilm (Rogovski et al., 2021).

Although bacteriophage therapy seems to have an advantage over antibiotics and disinfectant agents, the main setback of this method is that there is a need for the determination of host bacteria before the treatment and not enough protocols to test the susceptibility of bacteria *in vitro*. Moreover, when bacterial elimination is not completed via phage therapy, the result could be the re-emergence of pathogens (Rogovski et al., 2021).

2.4.1.1 Application of Bacteriophages in Food Industry

The concept of phage therapy on foods is applying lytic phages with strong lytic efficiency against foodborne pathogens on foods where contamination risk is high. Phages have the ability to lyse target bacteria and eliminate foodborne pathogens so that foods are safer for consumption (Vikram et al., 2020). Phages give the increasing global demand for natural biocontrol agents without the usage of biocides or chemical preservatives. One of the major advantages of the phages is that they cannot persist for a long time without a host in the environment, unlike biocides and antibiotics which are persistent in the soil causing an increase in the risk of bacterial resistance (O'sullivan et al., 2018).

Fighting against foodborne pathogens by phage treatment can be applied at all steps in the 'farm-to-fork' continuum. Phages can prevent or eliminate the colonization and diseases in livestock via phage therapy, decontaminate the carcasses and other raw, fresh produce like fruits and vegetables and disinfect equipment in use and contact surfaces as phage biosanitation and biocontrol, and prolong the shelf-life of manufactured foods like natural preservatives as biopreservation (García et al., 2008). The nature of bacteriophages does not affect the quality of fresh produce, dissimilar from the treatments of chemical disinfection, which makes phages possible and a competitive choice for biocontrol agents. Phages are stable in a wide variety of neutral solutions like phosphate-buffered saline, deionized water, or 0.9 % saline solution after being suspended. Thanks to this characteristic, phages do not affect the flavor, nutritional value, or texture of the foods (López-Cuevas et al., 2021).

There are a limited number of foodborne pathogens related to fruit and vegetable outbreaks. For this reason, phage application preparation has been marked to investigate the phages' biocontrol capacity against these foodborne pathogens (López-Cuevas et al., 2021). Phage therapy is being accepted as a biocontrol agent, increasingly and more effective at specific target foodborne pathogens in several foods shown in Table 2.6 (Vikram et al., 2020).

Table 2.6. Selected studies of phage therapy of foods (Vikram et al., 2020)

Bacteria	*Food*	*Results*
Campylobacter jejuni	Chicken	Single phage therapy on chicken skin reduced ~1 log of bacterial count
E. coli O157:H7	Beef	Phage cocktail therapy on beef eliminated ~78 % of the bacterial count
E. coli O157:H7	Tomato, Broccoli, and Spinach	Phage cocktail therapy on tomato, broccoli, and spinach decreased ~1-3 logs of bacterial counts
E. coli O157:H7	Lettuce and Cantaloupe	Phage cocktail therapy on lettuce and cantaloupe reduced ~1.9 and ~2.5 logs of bacterial counts
E. coli O157:H7	Leafy Green Vegetables	Phage cocktail therapy on leafy greens eliminated ~2-4 logs of bacterial count
E. coli O157:H7	Beef and Lettuce	Phage cocktail therapy on beef and lettuce decreased ~94 % and ~87 % of bacterial counts
Listeria monocytogenes	Melon and Apple Slices	Phage cocktail therapy on melon and apple slices reduced ~2 and ~0.4 logs of bacterial counts
Campylobacter jejuni	Chicken	Single phage therapy on chicken skin reduced ~1 log of bacterial count
E. coli O157:H7	Beef	Phage cocktail therapy on beef eliminated ~78 % of bacterial count
E. coli O157:H7	Tomato, Broccoli, and Spinach	Phage cocktail therapy on tomato, broccoli, and spinach decreased ~1-3 logs of bacterial counts
E. coli O157:H7	Lettuce and Cantaloupe	Phage cocktail therapy on lettuce and cantaloupe reduced ~1.9 and ~2.5 logs of bacterial counts
E. coli O157:H7	Leafy Green Vegetables	Phage cocktail therapy on leafy greens eliminated ~2-4 logs of bacterial count
E. coli O157:H7	Beef and Lettuce	Phage cocktail therapy on beef and lettuce decreased ~94 % and ~87 % of bacterial counts

Table 2.6 (continued)

Listeria monocytogenes	Melon and Apple Slices	Phage cocktail therapy on melon and apple slices reduced ~2 and ~0.4 logs of bacterial counts
Listeria monocytogenes	Cheese	Single phage therapy on ripened red-smear soft cheese decreased ~3.5 logs of bacterial counts
Listeria monocytogenes	Salmon	Single phage therapy on raw salmon fillets eliminated ~1.8-3.5 logs of bacterial counts
Listeria monocytogenes	Cheese	Single phage therapy on queso fresco cheese reduced ~3 logs of bacterial counts
Salmonella spp.	Melon	Phage cocktail therapy on melon slices decreased ~3.5 logs at 5 and 10°C and ~2.5 logs at 20°C of bacterial counts
Salmonella spp.	Egg and Lettuce	Phage cocktail therapy on eggshells and lettuce eliminated ~1 and ~2-4 logs of bacterial counts
Salmonella spp.	Chicken	Phage cocktail therapy on chicken skin reduced ~1 log of bacterial counts
Salmonella Enteritidis	Salmon	Phage cocktail therapy on raw salmon fillets eliminated ~3.2 logs at 18°C of bacterial counts
Salmonella spp.	Chicken	Single phage therapy on chicken breast fillets decreased ~0.9 logs of bacterial counts
Salmonella spp.	Poultry	Single phage therapy on skinless and skin-on poultry products eliminated ~1 log of bacterial counts
Salmonella spp.	Lettuce and Cucumber	Phage cocktail therapy on lettuce and cucumber reduced ~4.7 logs and ~5.8 logs of bacterial counts
Shigella spp.	Chicken	Phage cocktail therapy on spiced chicken decreased ~1-4 logs of bacterial counts
Staphylococcus aureus	Whole Milk	Phage cocktail therapy on whole milk decreased under the detection level of bacterial count

Phage therapy achieves many difficulties of the traditional chemical-based and irradiation-based approaches, as well as many consumer concerns. For instance, traditional approaches for decontamination are broad-spectrum, which means not only killing the intended pathogens but also the food's natural microflora which is often beneficial. However, because of the specificity of phages, phage therapy allows targeted foodborne pathogens to be eliminated in foods while does not alter the natural microflora and preserve the nutritional value of foods (Vikram et al., 2020). Moreover, with phage therapy on food, there are no safety issues since phages are present naturally in high numbers in the environment and also present in many food materials (O'sullivan et al., 2018). Commercial phage therapy as a biocontrol method has several features which make this method consumer-friendly. As an example, many of the commercial phages do not include any additives or preservatives and some of them have Kosher, Halal, and Organic certificates so their usage is suitable for organic produce. Furthermore, phage therapy on foods is also cost-efficient. For instance, the costs of treated food for some biocontrol methods other than phages like irradiation range from 10 to 30 cents per pound, whereas phage therapy costs range from 1 to 4 cents per pound (Vikram et al., 2020).

2.4.1.2 Phage Therapy against *Escherichia coli* on Food

Phages of *Escherichia coli* have been investigated to use in a variety of applications for the improvement of food safety. *E. coli* phages have been useful to reduce the contamination on vegetables, milk, and meat. The best result on *E. coli* reduction with phage therapy to date has been gained with post-harvest applications (O'sullivan et al., 2018).

Phage therapy of STEC, especially *E. coli* O157:H7, is done by using both commercial and non-commercial phages has been reported in several studies. For instance, a single phage that is isolated from sewage, used against STEC on beef slices reduced the bacterial count by ∼2 logs. Moreover, a phage cocktail prepared by using 3 commercial phages applied on ground beef and ∼1.2 logs reduction on *E.*

coli O157:H7 counts was reported. In another study, ∼77 % elimination of *E. coli* O157:H7 counts was reported when a phage cocktail was applied to beef. Furthermore, a single phage isolated from sewage and livestock manure, applied against *E. coli* O157:H7 on baby spinach and cut green peppers, and ∼1-4 logs decreased is noted. It is reported that the effect of a phage cocktail on decreasing *E. coli* O157:H7 counts on fresh-cut lettuce and cantaloupes (Vikram et al., 2020). EcoShield™ (Formerly ECP-100™, Intralytix, Inc.) is a commercial phage formulation to reduce *E. coli* O157:H7 evaluated by Abuladze et al. (2008). They observed 99.5 %, 99 %, and 97 % reductions in samples of broccoli stored at 10°C for 24 hours, 120 hours, and 168 hours, respectively. For tomato slices and spinach samples, similar reductions were observed (Abuladze et al., 2008). In a study conducted by Sharma et al. (2009), EcoShield™ was evaluated to reduce the viable *E. coli* O157:H7 counts on contaminated fresh-cut lettuce and cantaloupe for 1-2 days, and 2, 5, and 7 days storage at 4°C. They observed that phage therapy can decrease ∼2 logs CFU/cm^2 on lettuce, on the other hand, ∼2-3 logs CFU/mL are reduced on cantaloupe (M. Sharma et al., 2009).

Originally, most of the studies and phage preparations were focused on *E. coli* O157:H7 among the *E. coli* serotypes. On the other hand, because of the increasing rate of non-O157 STEC-related diseases, recent studies have focused on targeting STEC in general. As an example, phage cocktail therapy on ultra-high temperature treated (UHT) milk and raw milk reduced completely the strains of *E. coli* ATCC 25922 and O127:H6 at 4°C and 25°C. In another study, it is reported that a cocktail phage therapy with two phages completely eliminated *E. coli* O5:H in UHT milk at 4°C and 25°C (Vikram et al., 2020). However, up to our knowledge, there is no study for bacteriophage therapy against *E. coli* O26, no study for the application of bacteriophage against *E. coli* O104:H4, and no study for bacteriophage therapy against foodborne pathogens on leafy greens on the field.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Materials

All the substances used in this study were selected carefully. The list of chemicals and materials with their commercial manufacturers is detailed in Appendix B and C.

3.2 *Escherichia coli* isolates

In this study, three different pathogenic *E. coli* strains were used from Middle East Technical University (METU) Food Safety Laboratory, shown in Table 3.1. These *E. coli* strains were used in cress contamination and growth of bacteriophages. The strains used in the study are *E. coli* O157:H7 (MET K1 - 030), *E. coli* O104:H4 (MET A1 - 080), and *E. coli* O26 (MET A1 - 090) represent the most frequently found serotypes in green leafy vegetables. All isolates were stored at -80°C and obtained from METU Food Safety Laboratory in Food Engineering Department. *E. coli* O157:H7 (MET K1 - 030) were taken from Ankara University and both *E. coli* O104:H4 (MET A1 - 080) and *E. coli* O26 (MET A1 - 090) were taken from the Turkish Public Health Institution. For storage, the isolates were given a METU ID Code, frozen with glycerol, and stored at -80°C. Table 3.1 shows the isolated information in detail.

Table 3.1. *E. coli* serotypes used in the study

Isolate ID Code	*Genus*	*Species*	*Serotype*	*Pathogenic Group*	*Year of Isolation*	*Source of Isolation*
MET K1 - 030	*Escherichia*	*coli*	O157:H7	EHEC	2013	Food
MET A1 - 080	*Escherichia*	*coli*	O104:H4	EAEC	2019	Human
MET A1 - 090	*Escherichia*	*coli*	O26	EPEC	2019	Human

3.3 Phage Host Range Determination

To evaluate for phage cocktails, 23 *E. coli* bacteriophage isolates were used from METU Food Safety Laboratory. Spot tests were performed for each phage against target strains. Luria-Bertani (LB) agar Petri plates were divided into 6 equal parts and labeled with phage ID and host bacterial ID. The host bacteria were selected as the same as the bacteria used in contamination, shown in Table 3.1. 100 μL of host bacteria was put into the 4 mL semi-solid LB broth, spilled into the LB agar, and drawn eight to cover everywhere in the agar. It was put aside for 15 min to solidify. When it was solidified, 5 μL of each phage stock was spotted on the bacterial lawn where they were labeled. Then, the plates were set aside again to dry at room temperature. After they were dried, they were carefully placed into the 37°C incubator. The next day, the host range of each phage was determined as to where the phages were given the clear zone for the bacteria (Fong et al., 2017).

3.4 Phage Titer Determination

To determine phage titer, 100 μL of phage stock stored at 4°C was put into a 1.5 mL Eppendorf tube containing 900 μL 0.85 % sterile NaCl solution. Serial dilutions were made up to 10^{-9} and a double-plaque assay was performed for the last 4 dilutions. After the plates were solidified, they were placed in the incubator at 37°C for 24 hours. The next day, plaques were counted, and the total number was reported in plaque-forming units (PFU/mL) (Bonilla et al., 2016).

3.5 Phage Solution Preparation

The bacteriophages used in phage therapy were determined based on their initial titers, killing efficiency in the cocktail, and host ranges. For preparing phage

solution, 100 µL of phage stock and 500 µL of host bacteria for a single phage solution and 33 µL of each phage stock, and 500 µL of host bacteria for phage cocktail solution which included 3 phages were mixed in the 1.5 mL sterile Eppendorf tube, vortexed, and wait for 5 min. The mix was transferred to 50 mL LB broth and put into the shaker incubator at 37°C and 150 rpm, overnight. The next day, LB broth was transferred into the 50 mL sterile Falcon tube and placed in the centrifuge at 4000 rpm at 4°C for 30 min. When it was finished, the supernatant was taken with a sterile syringe and filtered with 0.22 µm filters. The filtered titer was determined as detailed in Part 3.5 and adjusted to 10^7 PFU/mL with sterile 0.85 % NaCl.

3.5.1 Phage Solution Against *E. coli* O104:H4

The single phage and phage cocktail solutions were prepared against *E. coli* O104:H4. For a single phage solution, the phage with METU ID code MET P1 – 303 was selected and the solution was prepared as described in Part 3.5.

For the phage cocktail, several configurations with phage isolates from METU Food Safety Laboratory were tried and the phages who were given the highest titer were chosen. The chosen ones were the phages with METU ID codes MET P1 – 303, MET P1 – 316, and MET P1 – 349, and a phage cocktail solution was made as in Part 3.5. The phages used for phage solution against *E. coli* O104:H4 are shown in detail in Table 3.2.

Table 3.2. Phage Isolates used for Phage Solution against *E. coli* O104:H4

METU ID	*Original Host*	*Initial Titer*	*Source of Isolate*	*City of Isolate*
MET P1 – 303	*E. coli* O104:H4	2 X 10^7 PFU/mL	Cattle Farm / Cow Feces	Adıyaman
MET P1 – 316	*E. coli* O157:H7	1 X 10^9 PFU/mL	Poultry Farm / Chicken Feces	Bolu
MET P1 – 349	*E. coli* O26	6 X 10^7 PFU/mL	Wastewater	Ankara

3.5.2 Phage Solution Against *E. coli* O157:H7

Phage cocktail solution was prepared against *E. coli* O157:H7. Possible phage configurations were tried and determined the configuration which was given the highest titer against *E. coli* O157:H7. The configuration contained the phages with METU ID codes MET P1 – 303, MET P1 – 311, and MET P1 – 322. Detailed information about phages was shown in Table 3.3. The phage cocktail solution was prepared as described in Part 3.5.

Table 3.3. Phage Isolates used for Phage Solution against *E. coli* O157:H7

METU ID	*Original Host*	*Initial Titer*	*Source of Isolate*	*City of Isolate*
MET P1 – 303	*E. coli* O104:H4	2 X 10^7 PFU/mL	Cattle Farm / Cow Feces	Adıyaman
MET P1 – 311	*E. coli* O157:H7	1 X 10^9 PFU/mL	Cattle Farm / Cow Feces	Şanlıurfa
MET P1 – 322	EAgEC	3 X 10^7 PFU/mL	Wastewater	Ankara

3.5.3 Phage Solution Against *E. coli* O26

A single phage solution was prepared against *E. coli* O26. First, the phages with the possibility to affect *E. coli* O26 were tried in different phage cocktail configurations. However, none of the configurations was shown as high a titer as the single phage. This is the reason why a single phage solution for *E. coli* O26 was used in this study. Among the single phage solutions, the highest titer belonged to the solution made with MET P1 – 346, shown in detail in Table 3.4. The single phage solution made with MET P1 – 346 was prepared as depicted in Part 3.5.

Table 3.4. Phage Isolate used for Phage Solution against *E. coli* O26

METU ID	*Original Host*	*Initial Titer*	*Source of Isolate*	*City of Isolate*
MET P1 – 346	*E. coli* O26	3 X 10^{10} PFU/mL	Poultry Farm / Chicken Feces	Şanlıurfa

3.6 Cress Cultivation

Garden cress (*Lepidium sativum* Linn.) was used as a model plant and the seeds were bought commercially. Garden cress seeds were weighed to be 1 g/pot. Then, seeds were sterilized with 12 % bleach solution for 12 min, rinsed three times with distilled sterile water, and filtered with autoclaved filter paper to get rid of excess water. Each pot (10.9 X 20.1 X 4.9 cm) that was autoclaved before received approximately 1 g of seed and planted with commercial peat which was also autoclaved before. For all groups, there were three replicates. Moreover, in each trial, there was a negative control group with three replicates. Next, each pot was irrigated with 60 mL of sterile distilled water and put into the climate chamber. For the first week, the climate chamber was set to 20°C with 65 % humidity with 16 hours daytime (8,000 lux) and 8 hours nighttime to germinate seeds. After the first week, the climate chamber's temperature was reduced to 15 °C where humidity and light settings were the same until the harvest day which is the 30th day. The cresses were irrigated two times a week with sterile water and one time with sterile water, contaminated water, or phage therapy which was schematized in Table 3.5. The cresses were reached almost 6 cm long with a leaf width of 0.5 – 1 cm on the 30th day which is enough growth for garden cress to harvest. For this schedule to work, cresses were planted on Friday.

Table 3.5. Weekly Schedule of Irrigation of Cresses

	Monday	*Wednesday*	*Friday*
1st Week	Irrigation with sterile water	Irrigation with sterile water	Irrigation with sterile water / *E. coli* contaminated water
2nd Week	Irrigation with sterile water	Irrigation with sterile water	Irrigation with sterile water / *E. coli* contaminated water / phage therapy
3rd Week	Irrigation with sterile water	Irrigation with sterile water	Irrigation with sterile water / *E. coli* contaminated water / phage therapy
4th Week	Irrigation with sterile water	Irrigation with sterile water	Irrigation with phage therapy

3.7 Phage Therapy Application

For contamination of cresses, the isolates which are given in Table 3.1 were inoculated on the Brain Heart Infusion (BHI) agar and incubated at 37 °C for 16 hours. Then, one colony was selected and transferred to the 100 mL BHI broth and put into the shaker incubator at 37 °C and 150 rpm for 8 hours. After, cells were decanted to 1.5 mL Eppendorf tubes and centrifuged at 7700 rpm for 15 minutes in the mini centrifuge. The supernatant was pulled without disturbing the pellet. 1 mL sterile 0.85 % NaCl was poured into each pellet and mixed via pipetting. To make sure the bacterial load of the contamination solution, the solution's optical density was measured spectrophotometrically at 600 nm. OD_{600} = 0.1 means the bacterial load is 10^8 CFU/mL (Zhao et al., 2010) and the solution was diluted with 0.85 % NaCl to adjust the bacterial load 10^5 CFU/mL. Next, the solution was transferred to the sprays which were sterilized with 70 % ethanol and rinsed with sterile distilled water before the experiment. The contamination solution was sprayed to the leaves where each pot received 50 mL solution in the biosafety cabinet that was sterilized

with UV for 30 min before the experiment. The cresses were irrigated with contaminated water according to Table 3.6.

Table 3.6. Irrigation Schedule for Each Phage Treatment Group

	Therapy Group 1	*Therapy Group 2*	*Therapy Group 3*	*Therapy Group 4*	*Therapy Group 5*	*Therapy Group 6*
7th Day	Irrigation with *E. coli* contaminated water	Irrigation with *E. coli* contaminated water	Irrigation with *E. coli* contaminated water	Irrigation with distilled sterile water	Irrigation with distilled sterile water	Irrigation with distilled sterile water
14th Day	Irrigation with phage solution	Irrigation with distilled sterile water	Irrigation with distilled sterile water	Irrigation with *E. coli* contaminated water	Irrigation with *E. coli* contaminated water	Irrigation with distilled sterile water
21st Day	Irrigation with phage solution	Irrigation with phage solution	Irrigation with distilled sterile water	Irrigation with phage solution	Irrigation with distilled sterile water	Irrigation with *E. coli* contaminated water
28th Day	Irrigation with phage solution	Irrigation with phage solution	Irrigation with phage solution	Irrigation with phage solution	Irrigation with phage solution	Irrigation with phage solution
30th Day	Harvest and sample collection	Harvest and sample collection	Harvest and sample collection	Harvest and sample collection	Harvest and sample collection	Harvest and sample collection

The phage solutions which were prepared as described in Part 3.5 for phage therapy were transferred into the sprays that were sterilized with 70 % ethanol and rinsed with sterile distilled water before the application. The phage therapy was applied onto the leaves where each pot received 50 mL of phage solution by spraying. This application was done in the biosafety cabinet which was sterilized with UV for 30 min just before the application. The cresses received phage therapy according to Table 3.6. For a control group, cresses were just contaminated, not received any phage treatment considering Table 3.7. Also, for each experiment, there was a control group with 3 replicates that were planted as described in Part 3.6. These

cresses only received distilled sterile water three times a week to make sure there was no other *E. coli* contamination than the ones contaminated in purpose.

Table 3.7. Irrigation Schedule of Control Groups

	Control Group 1	*Control Group 2*	*Control Group 3*
7th Day	Irrigation with *E. coli* contaminated water	Irrigation with distilled sterile water	Irrigation with distilled sterile water
14th Day	Irrigation with distilled sterile water	Irrigation with *E. coli* contaminated water	Irrigation with distilled sterile water
21st Day	Irrigation with distilled sterile water	Irrigation with distilled sterile water	Irrigation with *E. coli* contaminated water
28th Day	Irrigation with distilled sterile water	Irrigation with distilled sterile water	Irrigation with distilled sterile water
30th Day	Harvest and sample collection	Harvest and sample collection	Harvest and sample collection

3.8 Total *E. coli* and Biofilm-forming *E. coli* Enumeration

On the 30th day, the cresses' size was approximately 6 cm long with a leaf width of 0.5-1 cm which was enough growth for harvest. The harvest was carried out by cutting leaves with sterile scissors. Totally 2 grams of sample was collected for both total *E. coli* and biofilm-forming *E. coli* count. Until the homogenization of samples, they were kept at 4°C in sterile bags. The total *E. coli* count and biofilm-forming *E. coli* counts' enumeration was done by the Most Probable Number (MPN) technique with few changes (Luo et al., 2011).

3.8.1 Enumeration for Total *E. coli* Count

To begin with, 2 g of the sample was diluted ten-fold (g/mL) with the buffered peptone water (BPW) and put into the stomacher for 1 min for homogenization. Then, the sample was transferred to the stomacher bag. 1 mL was pulled from the

filter side of the stomacher bag and put in each 1st well of the 12-well plates that were filled with 2.25 mL of sterile BPW except for the 1st well a day before and kept at 4°C until using. Dilutions were made up to 10^{-8} for each row of the well. In total, there were 3 dilutions up to 10^{-8} for each pot for the total *E. coli* count. Next, 12-well plates were put into the incubator at 37°C for 24 h (ISO, 2017). After 24 h, the cells which gave turbidity were transferred to the brilliant-green phenol-red lactose sucrose (BPLS) agar Petri plates by streaking. Plates were incubated at 37°C for 24 h. The next day, yellow colonies were counted and recorded since *E. coli* produces yellow colonies on BPLS agar as it can ferment lactose (Malaka & Indah Prahesti, 2020).

3.8.2 Enumeration for Biofilm-Forming *E. coli* Count

First, 2 gr of the sample was put into the 50 mL Falcon tubes which were filled with 5 mL BPW a day before and kept at 4°C until used and vortexed for 30 seconds to remove planktonic cells from the surface (Rathinasabapathi, 2004). Then, leaves were transferred to sterile bags, diluted ten-fold (g/mL) with BPW, and put into the stomacher for 1 min to homogenize. The rest of the experiment was done exactly detailed in Part 3.8.1.

3.9 SEM Analysis

For Scanning Electron Microscopy (SEM) analysis, one wide and curly leaf was cut from each pot and fixed with high purity (99.7 %) methanol for 30 s (Neinhuis & Edelmann, 1996). Next, the leaves were dipped in each serial ethanol concentration which was 10 %, 30 %, 50 %, 70 %, 90 %, and 96 % for 10 min to dry. For the final step, leaves were dipped in ≥ 99 % acetone for 10 min (Pathan et al., 2010). Field Emission SEM (Quanta 400F, Eindhoven, Holland) was used to analyze the surface of the leaves after coating the samples with gold-palladium with an accelerating

voltage of 1020 kV with 5000 X – 10000 X magnification levels at METU Central Laboratory.

3.10 Statistical Analysis

Both the control groups and therapy groups were planted with 3 replicates in one trial. The difference between the control and therapy groups the means of *E. coli* cell counts in LogMPN were analyzed by ANOVA followed by Tukey's test to determine the difference among MOIs, single phage or phage cocktail treatment, and with control and therapy groups for both total *E. coli* count and biofilm-forming *E. coli* count for each strain. Data were analyzed by RStudio software. The p-value where $p < 0.05$ was considered significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Host Range of Bacteriophages

All 23 *E. coli* phages of METU Food Safety Laboratory stocks were spotted against *E. coli* O104:H4, *E. coli* O157:H7, *E. coli* O26 which are the main strains used in the study, and against Enteroaggregative *E. coli* (EAgEC) (MET K1 – 052) in order to determine the host range of phages. In addition, phages were spotted against some *Salmonella enterica* subspecies such as Enteriditis, Hadar, Anatum, and Infantis since *E. coli* and *Salmonella* have high similarity levels like 76.3 - 100 % within the housekeeping genes which indicates they are closely related species (Hu et al., 2010). Phages used in phage therapy solutions for this study were specified according to their host range, initial titer, and efficiency of killing the pathogens. The results of the host range of all 23 *E. coli* phages are listed in Table 4.1.

Table 4.1. Host Range Results of Bacteriophages

	E. coli O104:H4	*E. coli O157:H7*	*E. coli O26*	*EAgEC*	*S.* Enteriditis	*S.* Infantis	*S.* Hadar	*S.* Anatum
MET P1-303	+	+	+	+	-	-	-	T
MET P1-304	+	T	T	-	-	-	-	T
MET P1-307	+	+	-	T	-	-	-	-
MET P1-311	T	+	T	-	-	-	-	-
MET P1-313	T	T	-	-	-	-	-	-

Table 4.1 (continued)

MET P1-316	+	+	-	+	-	-	-	-
MET P1-319	+	-	-	+	-	-	-	-
MET P1-322	+	-	+	+	-	-	-	T
MET P1-325	T	+	+	T	-	-	-	-
MET P1-328	T	T	+	-	-	-	-	-
MET P1-331	-	-	-	-	-	-	-	-
MET P1-334	-	-	-	-	-	-	-	-
MET P1-337	+	-	-	+	-	-	-	-
MET P1-340	+	-	-	+	-	-	-	-
MET P1-343	-	-	+	-	-	-	-	-
MET P1-346	+	+	+	-	-	-	-	-
MET P1-349	+	+	+	T	-	-	-	-
MET P1-352	+	T	-	T	-	-	-	-
MET P1-355	+	+	+	-	-	-	-	-
MET P1-358	+	T	+	T	-	-	-	-
MET P1-361	T	+	T	-	-	-	-	-
MET P1-364	T	+	+	T	-	-	-	T

Table 4.1 (continued)

MET P1-367	T	+	T	T	-	T	-	T

*: +: Positive, - : Negative, T : Turbid

As it can be seen from Table 4.1, all phages that are used in the study have the ability to kill at least three different strains.

4.2 SEM Images

SEM analysis was done in order to visualize the difference in biofilm structures on the leaf surface between the control and therapy groups. The selected leaves were wide, curly, and in the center where each leaf received the phage therapy within the required amount each time.

Pathogens like *E. coli* have the ability to penetrate the stoma and junctions of the cut sides of the leaves in order to be protected from disinfectants. To examine the details about 1-5 nm in size such as stoma in the leaf of leafy greens, SEM has been used in order to obtain high-resolution images. In one study, the entry of internalization of *E. coli* is through the cavity of the stoma in the lettuce and the formation of bacterial biofilm around the stoma was observed by SEM images. It was concluded that contamination sites of pathogens in leafy greens are localized in the stomata, mainly (Gomes et al., 2009). Likewise, the formation of bacterial biofilms in and around the stoma was observed in almost all SEM images of this study. Nonetheless, the classical rod-shaped *E. coli* cells were not observed but this result was expected as phages lysed the *E. coli* cells. Based on the SEM images, it could be confirmed that *E. coli* were lysed, and enumeration is consistent with the images.

4.2.1 Negative Control Groups

Negative control group samples were tested against the *E. coli* presence. For each trial, the results were negative which means there was no host bacteria contamination in the control groups. The expectation for the negative control groups was no bacterial presence, and this was confirmed with both enumeration results and SEM images for this study. The leaf surface and stomas on negative control were clear which is shown in Fig. 4.1.

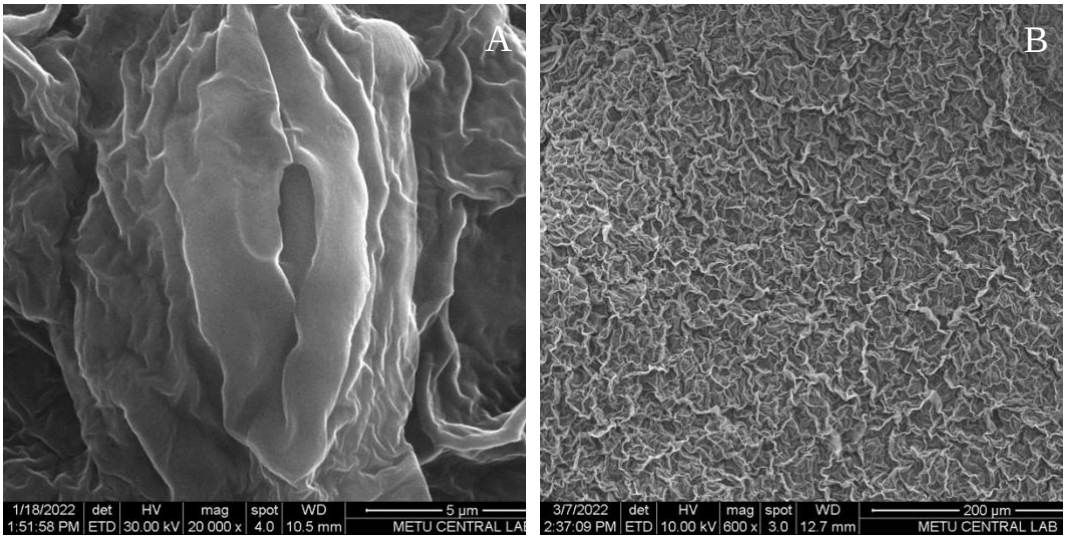


Figure 4.1. SEM images of negative control groups. A closed image of semi-open stoma (A) and a cross-sectional area of leaf surface (B).

4.3 Phage Therapy Against *E. coli* O104:H4

Application of phage therapy against *E. coli* O104:H4 was done in three different phage solutions in this study. The first solution was made with a single phage and its MOI was adjusted to 100. The second solution was made with the same single phage, but this time its MOI was adjusted to -1 and for the last solution, the phage cocktail was made with MOI equal to 100. All 6 therapy groups for each trial were irrigated based on Table 3.6. Each group was planted as 3 replicates and results for *E. coli* counts were calculated as the average of the replicates for each group. On the 30th

day, cresses were harvested to collect samples for total and biofilm-forming *E. coli* counts. Results were compared with their control groups. The control groups of single phage and phage cocktail therapies with MOI = 100 were the same since their *E. coli* O104:H4 contamination load was 10^5 CFU/mL. In addition, single phage therapy with MOI = 100 and MOI = -1 were compared to see the effectiveness of MOI, and single phage therapy and phage cocktail therapy were compared to determine which therapy is better for eliminating bacterial load when their MOI is the same. Moreover, ANOVA and Tukey's test were done to results in order to specify the difference between control and therapy groups.

4.3.1 Single Phage Therapy with MOI =100

For the single phage therapy, METU ID with MET P1 – 303 phage was used with 10^7 PFU/mL and *E. coli* O104:H4 with 10^5 CFU/mL was used to contaminate the cresses for both therapy and control groups. In the 1st group, the total *E. coli* count was reduced by 0,2 logs with 8576,30 MPN/g, and the biofilm-forming *E. coli* count was decreased by 0,13 logs with 1584,89 MPN/g. There were 0,33 log reductions in total *E. coli* with 6309,57 MPN/g and 0,2 log reductions in biofilm-forming *E. coli* count with 1359,25 MPN/g in the 2nd group. In the 3rd group, 0,07 log reductions in total *E. coli* with 11660,04 MPN/g and 0,13 log reductions in biofilm-forming *E. coli* counts with 1584,89 MPN/g were observed. Total and biofilm-forming *E. coli* counts were reduced by 1 log with 5411,27 MPN/g and 0,53 logs with 2154,26 MPN/g, respectively in the 4th group. In the 5th group, total *E. coli* was reduced by 0,6 logs with 13592,50 MPN/g, and biofilm-forming *E. coli* count was decreased by 0,53 logs with 2154,26 MPN/g. In the 6th group, there were 0,67 log reductions in total *E. coli* with 11660,04 MPN/g and 0,47 log reductions in biofilm-forming *E. coli* count with 1847,99 MPN/g. The results were shown in Table 4.2. The total *E. coli* results were visualized in Fig. 4.2 and the biofilm-forming *E. coli* results in Fig. 4.3.

As regards Commission Regulation (EC) No 2073/2005, *E. coli* O104:H4 limitation for the bacterial load on sprouts is zero (*Commission Regulation (EC) No 2073/2005*

on Microbiological Criteria for Foodstuffs, 2020). However, we could not eliminate the total bacterial load down to zero with a single phage therapy with MOI =100. The lowest total bacterial load for this therapy was log3,73 MPN/g which is 5411,27 MPN/g.

It is much harder to eliminate bacterial biofilms with chemical prevention methods than free-flowing cells of the same bacterial species because they are more tolerant to disinfectants. STEC biofilms are specifically important biohazards in food. Prevention of the formation of biofilms, inactivation, or removal of biofilms is essential in order to improve food safety (Wang et al., 2012). In this study, the biofilm-forming *E. coli* O104:H4 counts have been reduced significantly in all groups of each trial. Although single phage therapy with MOI = 100 could not eliminate the biofilm-forming *E. coli* count down to zero, the best result of this single phage therapy was log3,13 MPN/g which is 1359,25 MPN/g.

The SEM images for control groups that are contaminated with 10^5 CFU/mL of *E. coli* O104:H4 are in Fig. 4.4 and for single therapy against *E. coli* O104:H4 with MOI = 100 are in Fig. 4.5.

Table 4.2 Total and Biofilm-Forming *E. coli* Counts of Single Phage Therapy Against *E. Coli* O104:H4 when MOI = 100

	Total E. coli Count				*Biofilm-Forming E. coli Count*			
	Contaminated Cresses		*Phage Treated Cresses*		*Contaminated Cresses*		*Phage Treated Cresses*	
Groups	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*
1st group	4,13	13592,52	3,93	8576,30	3,33	2154,26	3,20	1584,89
2nd group	4,13	13592,52	3,80	6309,57	3,33	2154,26	3,13	1359,25
3rd group	4,13	13592,52	4,07	11660,04	3,33	2154,26	3,20	1584,89
4th group	4,73	54112,79	3,73	5411,27	3,87	7356,98	3,33	2154,26
5th group	4,73	54112,79	4,13	13592,50	3,87	7356,98	3,33	2154,26
6th group	4,73	54112,79	4,07	11660,04	3,73	5411,27	3,27	1847,99

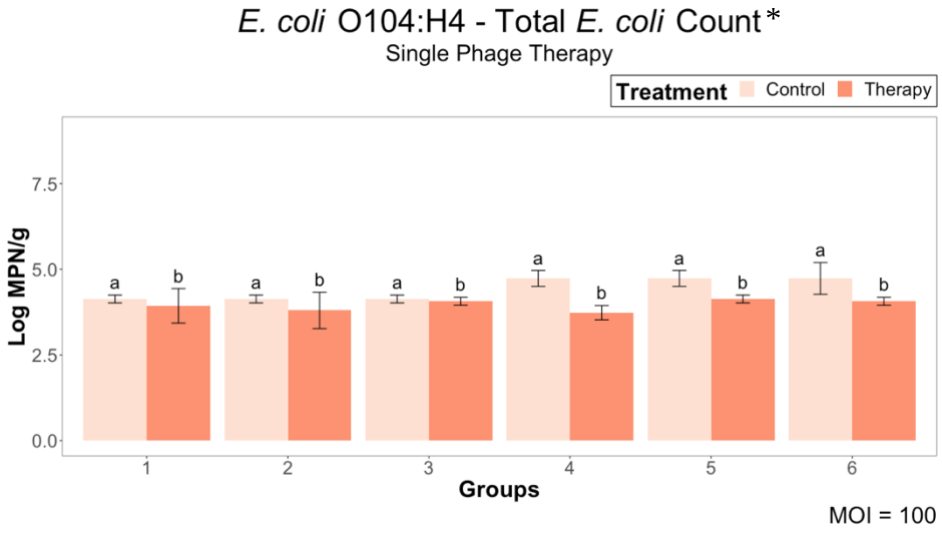


Figure 4.2. Total *E. coli* Count of Contaminated (Control) and Phage Treated (Therapy) Cresses of Single Phage Therapy against *E. coli* O104:H4 with MOI = 100

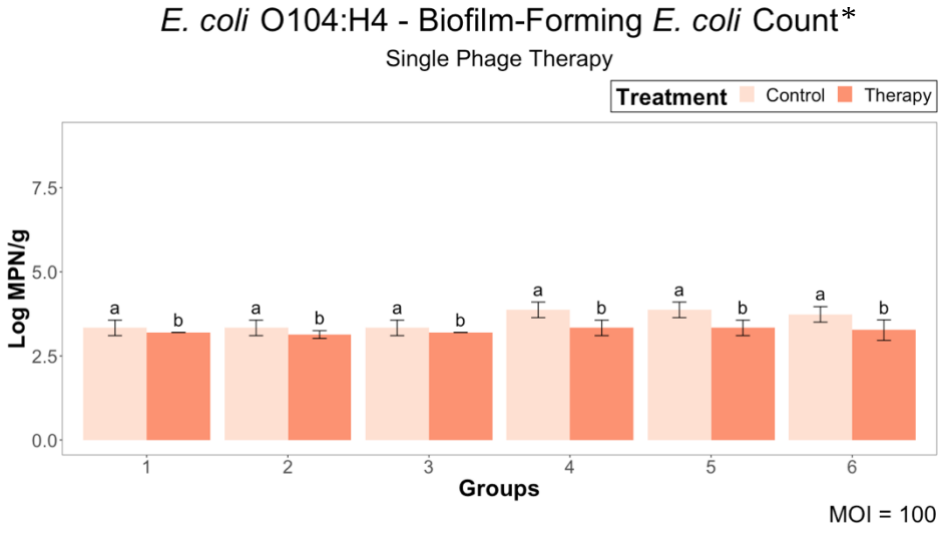


Figure 4.3. Biofilm-Forming *E. coli* Count of Contaminated (Control) and Phage Treated (Therapy) Cresses of Single Phage Therapy against *E. coli* O104:H4 with MOI = 100

* Results were shown as a mean of 3 replicates ± standard deviation. Values with different letters represent the statistical difference between therapy and control groups. $P < 0.05$ considered as statistically different.

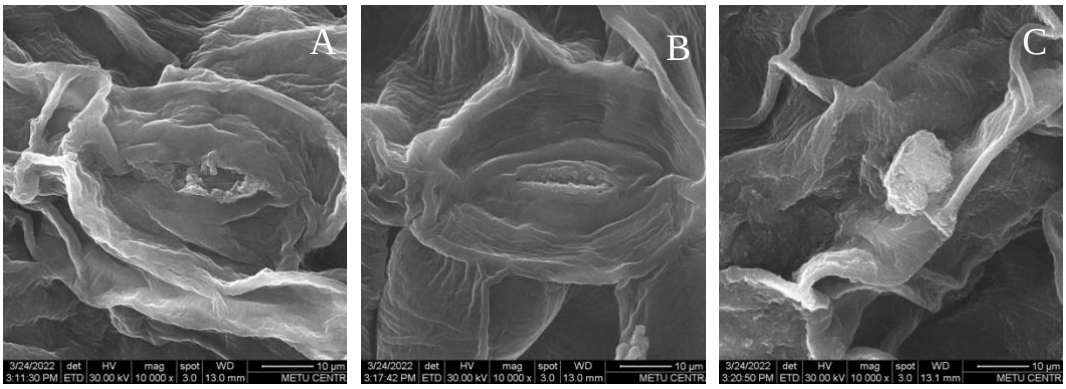
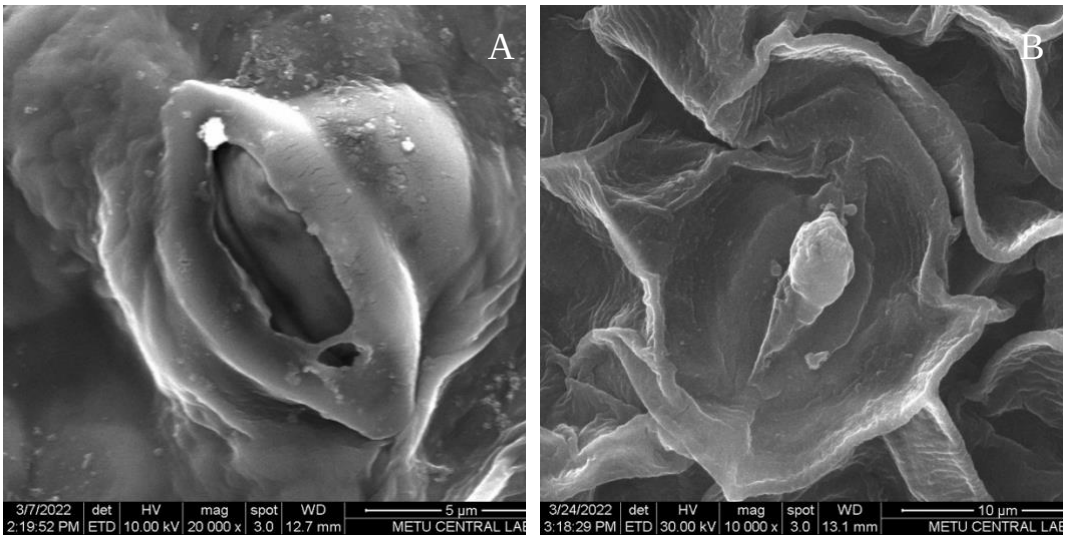


Figure 4.4. SEM images of control groups of single phage and phage cocktail therapies against *E. coli* O104:H4 with MOI = 100. Bacterial growth in the stoma for 1st group (A), bacterial growth in the stoma for 2nd group (B), and bacterial biofilm on the surface of the leaf in 3rd group (C).



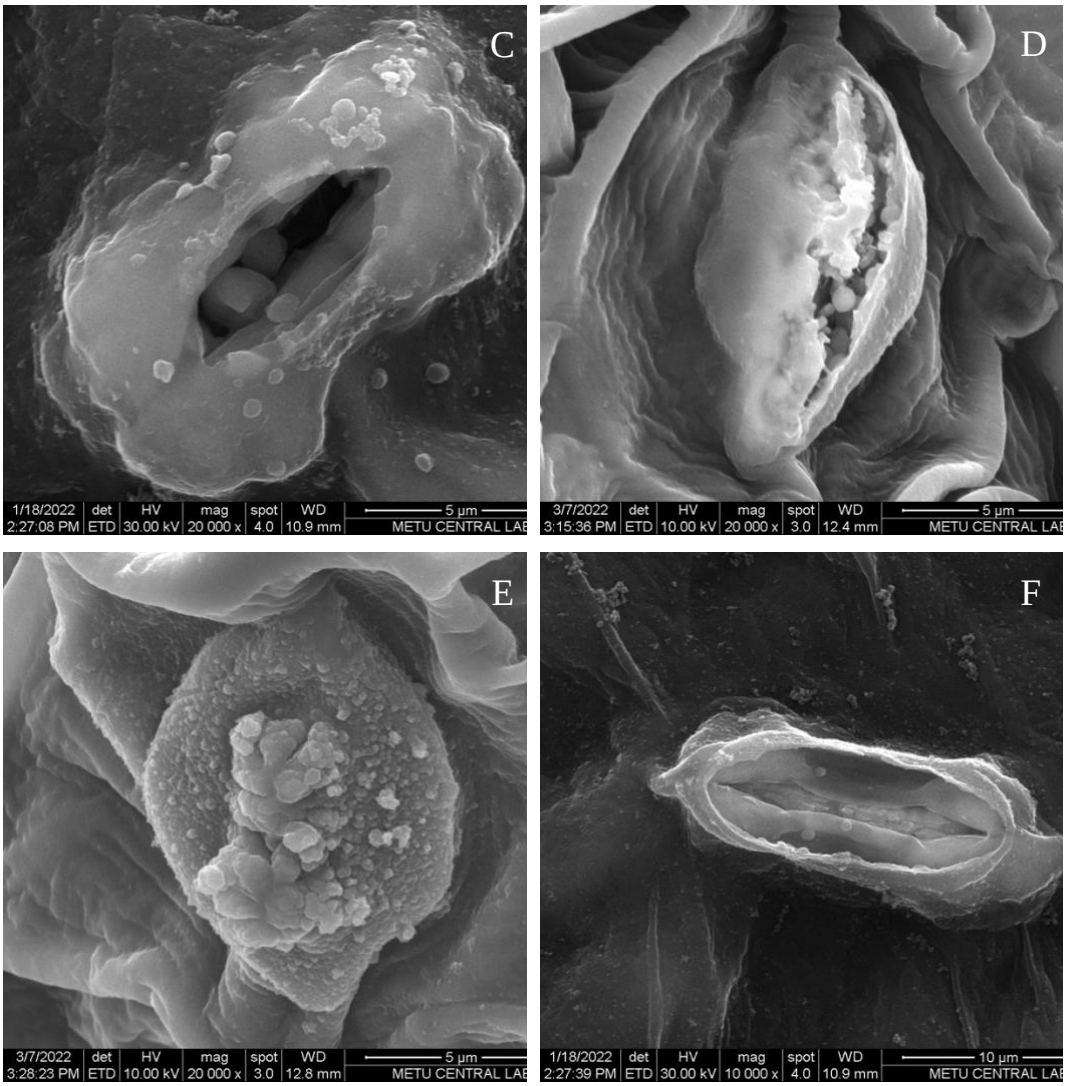


Figure 4.5. SEM images of single phage treated groups against *E. coli* O104:H4 with MOI = 100. Bacterial biofilm on the surface of the leaf in the 1st group (A), bacterial biofilm in the stoma, bacterial growth on the surface of the leaf in the 2nd group (B), bacterial colonies on the stoma's guard cells and the surface of the leaf in the 3rd group (C), bacterial growth in the stoma in 4th group (D), bacterial growth in and above the stoma and on the surface of the leaf in the 5th group (E), and bacterial growth in and above the stoma and surface of the leaf in the 6th group (F).

4.3.2 Single Phage Therapy with MOI = -1

Single phage therapy with MOI = -1 was done with the same phage, MET P1 – 303, and titer (10^7 PFU/mL) as single phage therapy with MOI = 100. However, the contamination of cresses for both therapy and control groups were done with *E. coli* O104:H4 with 10^8 CFU/mL. In the 1st group, 1,87 logs were reduced in total *E. coli* with 251188,64 MPN/g, and 2,27 logs were reduced in biofilm-forming *E. coli* count with 15848,93 MPN/g. In the 2nd group, total *E. coli* was reduced by 2,87 logs with 31622,77 MPN/g and biofilm-forming *E. coli* count was decreased by 2,73 logs with 5411,27 MPN/g. There were 2,73 logs reduction in total *E. coli* with 34142,86 MPN/g and 3,13 logs reduction in biofilm-forming *E. coli* count with 2154,26 MPN/g in the 3rd group. In the 4th group, 2,50 log reduction in total *E. coli* with 630957,34 MPN/g and 1,87 logs reduction in biofilm-forming *E. coli* count with 100000 MPN/g were observed. In the 5th group, total *E. coli* was 3,50 logs reduced by 63095,73 MPN/g and biofilm-forming *E. coli* was 2,80 logs reduced by 11660,03 MPN/g. In the 6th group, total and biofilm-forming *E. coli* counts were decreased by 2,17 logs with 464194,51 MPN/g and 1,9333 logs with 46419,45 MPN/g, respectively. The results were shown in Table 4.3. The total *E. coli* results were visualized in Fig. 4.6 and the biofilm-forming *E. coli* results in Fig. 4.7.

Although there were more log reductions in single phage therapy with MOI = -1 than with MOI = 100, the final bacterial loads are higher and again, we could not eliminate total and biofilm-forming *E. coli* O104:H4 counts down to zero. The lowest total *E. coli* O104:H4 count was log4,40 MPN/g which is 25118,86 MPN/g and the lowest biofilm-forming *E. coli* count was log3,33 MPN/g which is 2154,26 MPN/g.

The SEM images of control groups for single phage with MOI = -1 which are contaminated with 10^8 CFU/mL are in Fig. 4.8 and therapy groups of single phage with MOI = -1 are in Fig. 4.9.

Table 4.3 Total and Biofilm-Forming *E. coli* Counts of Single Phage Therapy Against *E. coli* O104:H4 when MOI = -1

	Total E. coli Count				*Biofilm-Forming E. coli Count*			
	Contaminated Cresses		*Phage Treated Cresses*		*Contaminated Cresses*		*Phage Treated Cresses*	
Groups	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*
1st group	7,27	18479916,31	5,40	2511888,64	6,47	2928869,35	4,20	15848,93
2nd group	7,27	18479916,31	4,40	251188,86	6,47	2928869,35	3,73	5411,27
3rd group	7,27	18479916,31	4,53	34142,86	6,47	2928869,35	3,33	2154,26
4th group	8,30	199526231,50	5,80	630957,34	6,87	7356987,19	5,00	100000
5th group	8,30	199526231,50	4,80	63095,73	6,87	7356987,19	4,07	11660,03
6th group	7,83	68123978,00	5,67	464194,51	6,60	3981071,70	4,67	46419,45

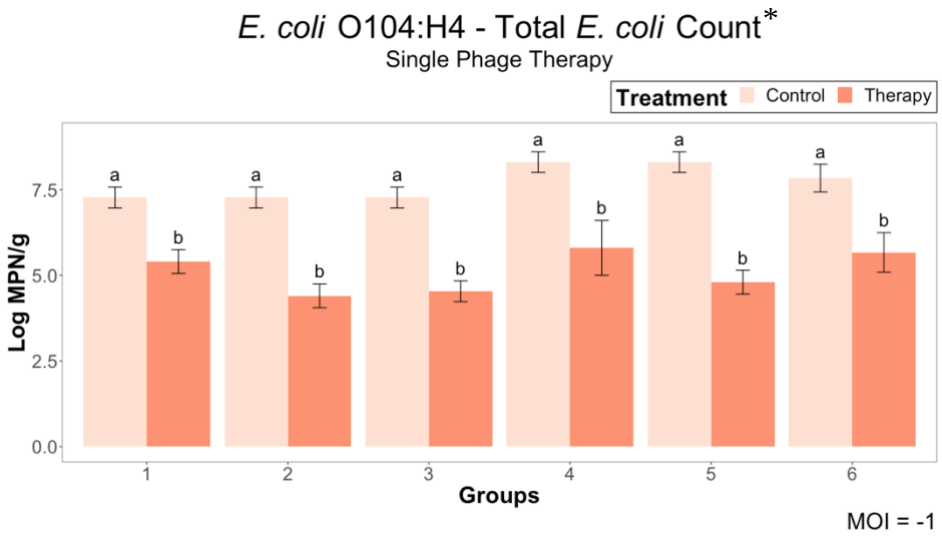


Figure 4.6. Total *E. coli* Count of Contaminated (Control) and Phage Treated (Therapy) Cresses of Single Phage Therapy against *E. coli* O104:H4 with MOI = -1

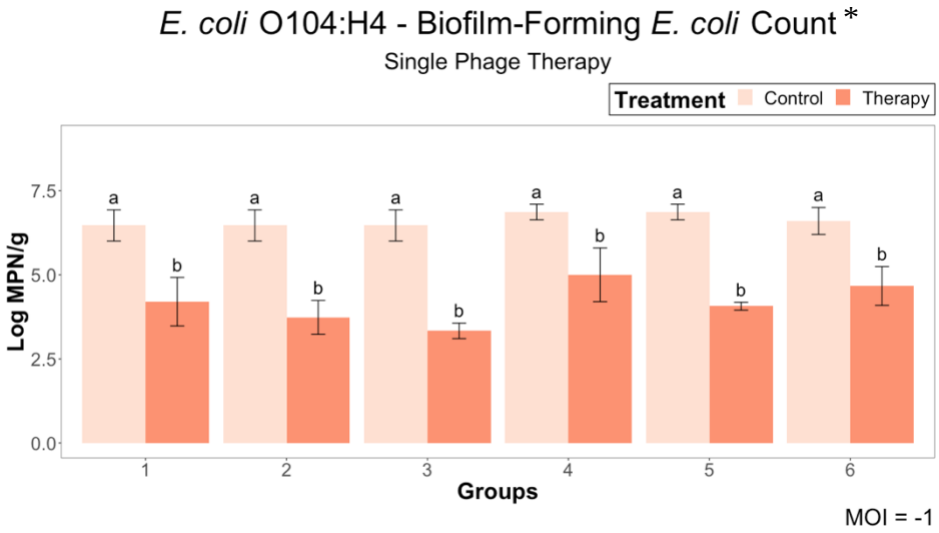


Figure 4.7. Biofilm-Forming *E. coli* Count of Contaminated (Control) and Phage Treated (Therapy) Cresses of Single Phage Therapy against *E. Coli* O104:H4 with MOI = -1

* Results were shown as a mean of 3 replicates ± standard deviation. Values with different letters represent the statistical difference between therapy and control groups. $P < 0.05$ considered as statistically different.

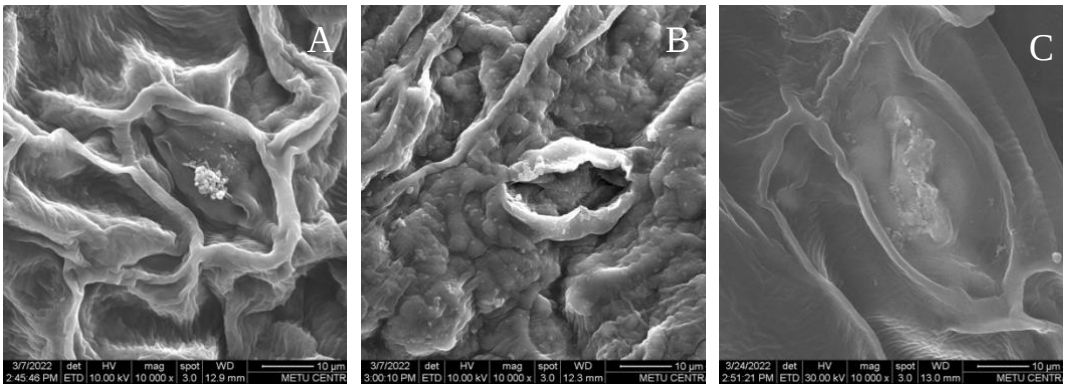


Figure 4.8. SEM images of control groups of single phage therapy with MOI = -1. Bacterial biofilm in the stoma and bacterial growth above the stoma in 1st group (A), bacterial growth above the stoma and on the surface of the leaf in 2nd group (B), and bacterial biofilm in the stoma and bacterial growth on the surface of the leaf in 3rd group (C).

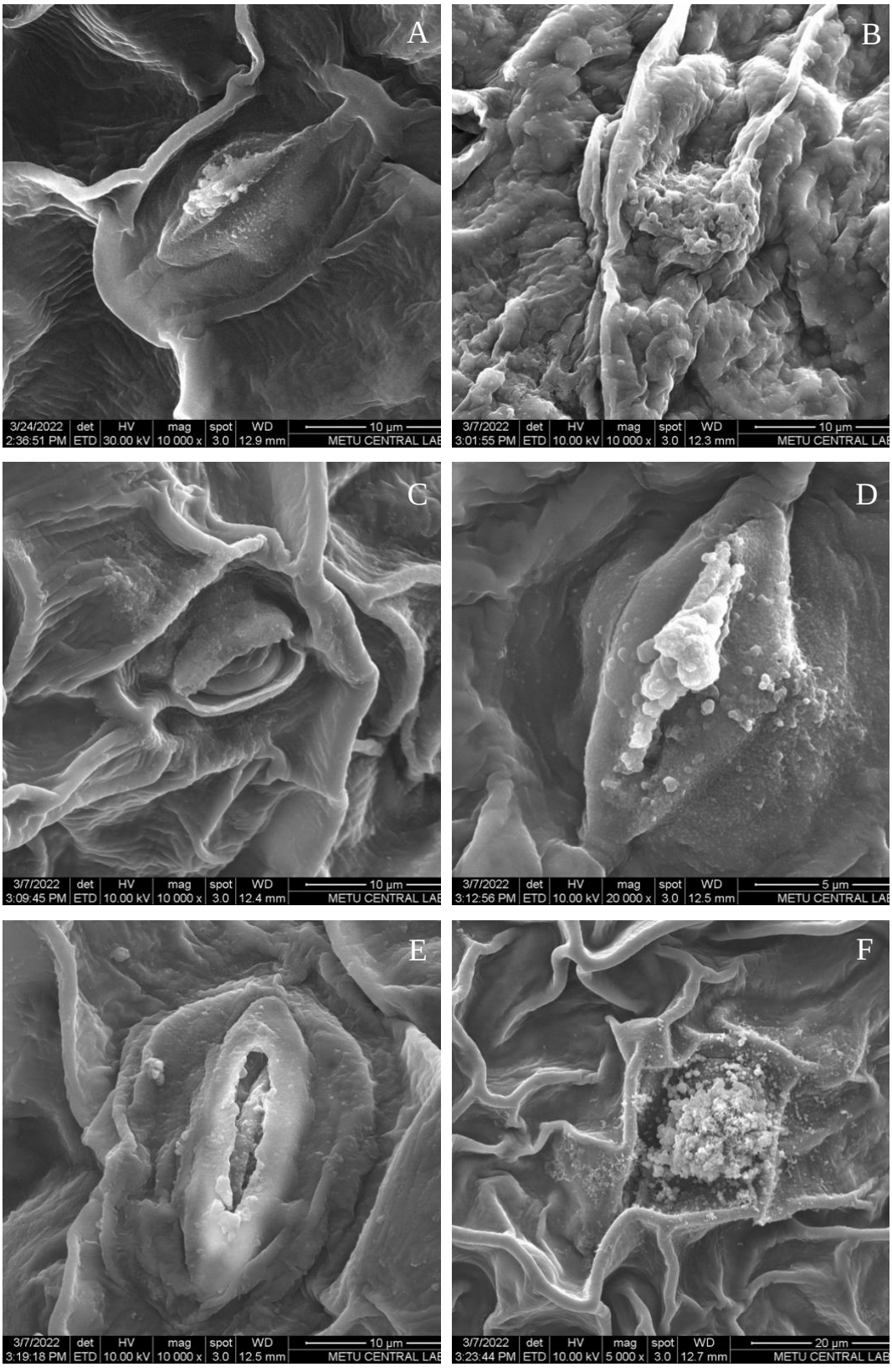

A
3/24/2022 | det | HV | mag | spot | WD
2:36:51 PM | ETD | 30.00 kV | 10 000 x | 3.0 | 12.9 mm
10 µm
METU CENTRAL LAB
B
3/7/2022 | det | HV | mag | spot | WD
3:01:55 PM | ETD | 10.00 kV | 10 000 x | 3.0 | 12.3 mm
10 µm
METU CENTRAL LAB
C
3/7/2022 | det | HV | mag | spot | WD
3:09:45 PM | ETD | 10.00 kV | 10 000 x | 3.0 | 12.4 mm
10 µm
METU CENTRAL LAB
D
3/7/2022 | det | HV | mag | spot | WD
3:12:56 PM | ETD | 10.00 kV | 20 000 x | 3.0 | 12.5 mm
5 µm
METU CENTRAL LAB
E
3/7/2022 | det | HV | mag | spot | WD
3:19:18 PM | ETD | 10.00 kV | 10 000 x | 3.0 | 12.5 mm
10 µm
METU CENTRAL LAB
F
3/7/2022 | det | HV | mag | spot | WD
3:23:44 PM | ETD | 10.00 kV | 5 000 x | 3.0 | 12.7 mm
20 µm
METU CENTRAL LAB

Figure 4.9. SEM images of single phage treated groups against *E. coli* O104:H4 with MOI = -1. Bacterial biofilm in the stoma and bacterial growth above the stoma in the 1st group (A), bacterial biofilm and bacterial colonies on the surface of the leaf in the 2nd group (B), bacterial biofilm on the guard cells of the stoma and the surface of the leaf in the 3rd group (C), bacterial growth and biofilm in the stoma and its guard cells in the 4th group (D), bacterial growth and biofilm in and above the stoma and on the surface of the leaf in the 5th group (E), and bacterial growth and biofilm in and above the stoma and on the surface of the leaf in the 6th group (F).

4.3.3 Phage Cocktail Therapy with MOI = 100

For the phage cocktail therapy, 3 different phages (MET P1 – 303, MET P1 – 316, and MET P1 – 349) were used with 10^7 PFU/mL, and *E. coli* O104:H4 with 10^5 CFU/mL was used to contaminate the cresses for both therapy and control groups. In the 1st group, the total *E. coli* count was reduced by 0,93 logs with 1584,89 MPN/g, and the biofilm-forming *E. coli* count was decreased by 1,0667 logs with 184,79 MPN/g. There was 1 log reduction in total *E. coli* with 1359,25 MPN/g and 0,70 log reduction in biofilm-forming *E. coli* count with 429,83 MPN/g in the 2nd group. In the 3rd group, the total and biofilm-forming *E. coli* counts were reduced by 0,7333 logs with 2511,88 MPN/g and 0,70 logs with 429,83 MPN/g, respectively. In the 4th group, 1,23 logs were reduced in total *E. coli* with 3162,27 MPN/g, and 0,80 logs were reduced in biofilm-forming *E. coli* with 1166,00 MPN/g counts. In the 5th group, total *E. coli* was reduced by 1,60 logs with 1359,25 MPN/g and biofilm-forming *E. coli* count was decreased by 1,23 logs with 429,83 MPN/g. There were 0,87 logs and 1,1 logs reduced in total with 7356,98 MPN/g and biofilm-forming *E. coli* with 429,83 MPN/g counts, respectively, in the 6th group. The results were shown in Table 4.4. The total *E. coli* results were visualized in Fig. 4.10 and the biofilm-forming *E. coli* results in Fig. 4.11.

For the phage therapy against *E. coli* O104:H4, the lowest total and biofilm-forming *E. coli* counts were obtained in phage cocktail therapy but still, the elimination could not down to zero. The lowest total bacterial load was log3,13 MPN/g which is 1359,25 MPN/g and the lowest biofilm-forming *E. coli* count was log2,27 MPN/g which is 184,79 MPN/g. To enhance the effectiveness of phage therapy, additional substances might be used with phages such as essential oils; for example, thymol, carvacrol, *trans*-cinnamaldehyde, and eugenol (Moon et al., 2020).

The SEM images for control groups that are contaminated with 10^5 CFU/mL of *E. coli* O104:H4 are in Fig. 4.4 and for phage cocktail with MOI = 100 SEM images are in Fig. 4.12.

Table 4.4 Total and Biofilm-Forming *E. coli* Counts of Phage Cocktail Therapy Against *E. coli* O104:H4 when MOI = 100

	Total E. coli Count				*Biofilm-Forming E. coli Count*			
	Contaminated Cresses		*Phage Treated Cresses*		*Contaminated Cresses*		*Phage Treated Cresses*	
Groups	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*
1st group	4,13	13592,52	3,20	1584,89	3,33	2154,26	2,27	184,79
2nd group	4,13	13592,52	3,13	1359,25	3,33	2154,26	2,63	429,83
3rd group	4,13	13592,52	3,40	2511,88	3,33	2154,26	2,63	429,83
4th group	4,73	54112,79	3,50	3162,27	3,87	7356,98	3,07	1166,00
5th group	4,73	54112,79	3,13	1359,25	3,87	7356,98	2,63	429,83
6th group	4,73	54112,79	3,87	7356,98	3,73	5411,27	2,63	429,83

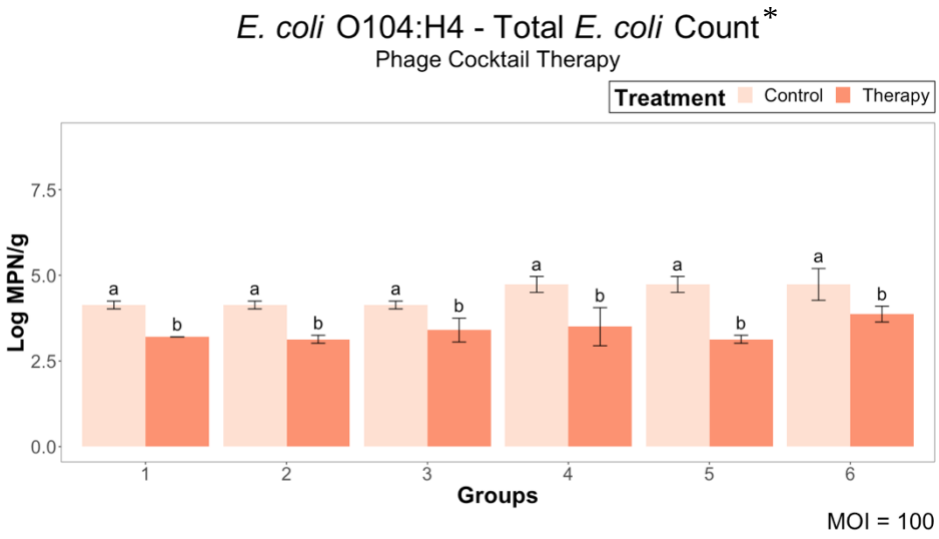


Figure 4.10. Total *E. coli* Count of Contaminated (Control) and Phage Treated (Therapy) Cresses of Phage Cocktail Therapy against *E. Coli* O104:H4 with MOI = 100

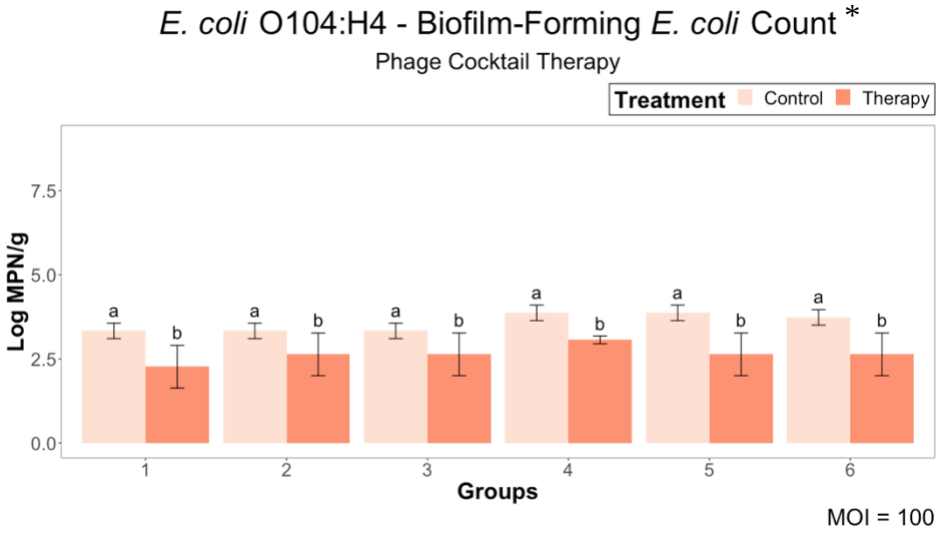


Figure 4.11. Biofilm-Forming *E. coli* Count of Contaminated (Control) and Phage Treated (Therapy) Cresses of Phage Cocktail Therapy against *E. Coli* O104:H4 with MOI = 100

* Results were shown as a mean of 3 replicates ± standard deviation. Values with different letters represent the statistical difference between therapy and control groups. $P < 0.05$ considered as statistically different.

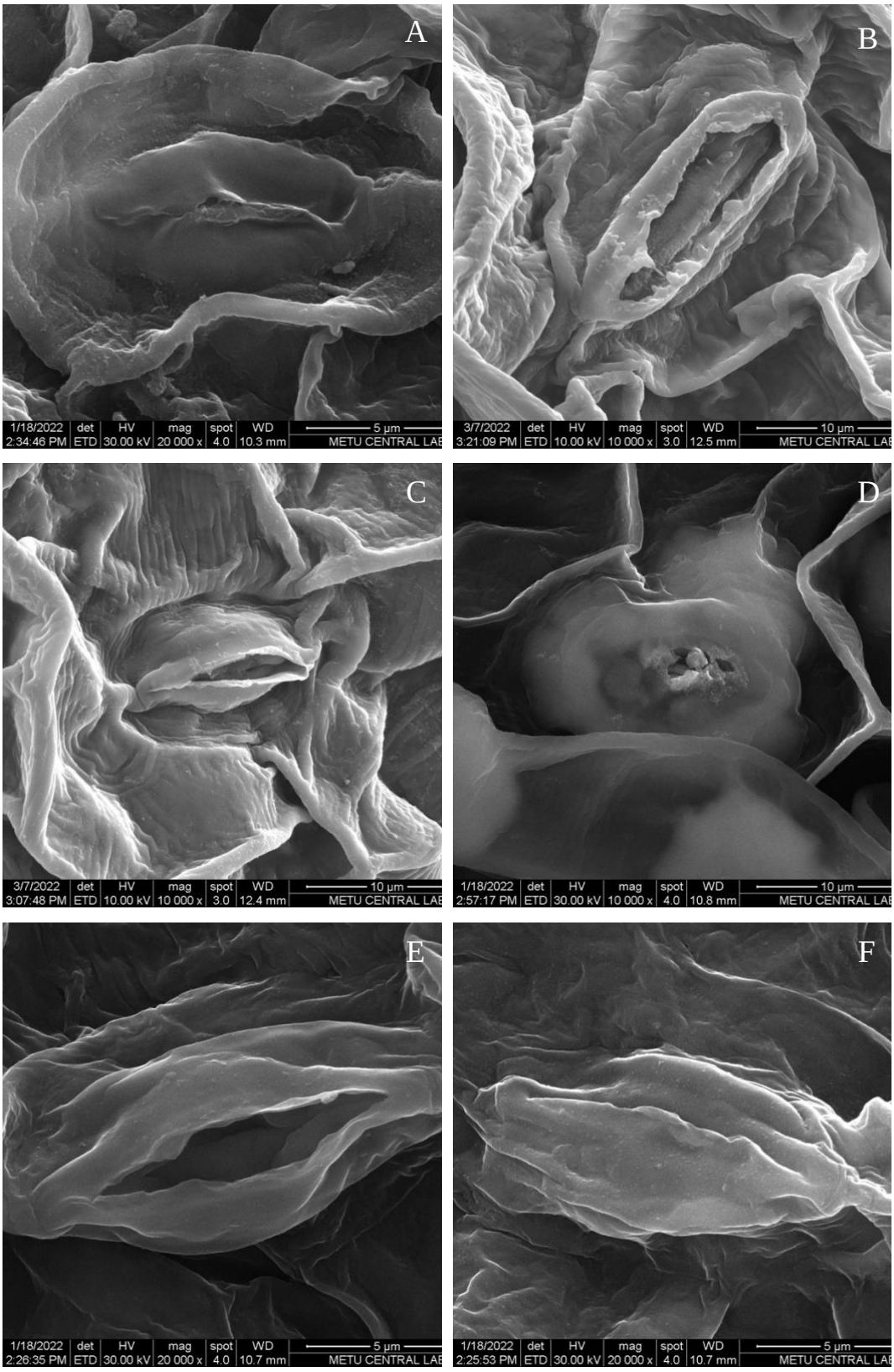

A
1/18/2022 2:34:46 PM | det ETD | HV 30.00 kV | mag 20 000 x | spot 4.0 | WD 10.3 mm | 5 µm | METU CENTRAL LAB
B
3/7/2022 3:21:09 PM | det ETD | HV 10.00 kV | mag 10 000 x | spot 3.0 | WD 12.5 mm | 10 µm | METU CENTRAL LAB
C
3/7/2022 3:07:48 PM | det ETD | HV 10.00 kV | mag 10 000 x | spot 3.0 | WD 12.4 mm | 10 µm | METU CENTRAL LAB
D
1/18/2022 2:57:17 PM | det ETD | HV 30.00 kV | mag 10 000 x | spot 4.0 | WD 10.8 mm | 10 µm | METU CENTRAL LAB
E
1/18/2022 2:26:35 PM | det ETD | HV 30.00 kV | mag 20 000 x | spot 4.0 | WD 10.7 mm | 5 µm | METU CENTRAL LAB
F
1/18/2022 2:25:53 PM | det ETD | HV 30.00 kV | mag 20 000 x | spot 4.0 | WD 10.7 mm | 5 µm | METU CENTRAL LAB

Figure 4.12. SEM images of phage cocktail treated groups against *E. coli* O104:H4 with MOI = 100. Bacterial biofilm on the surface of the leaf in the 1st group (A), bacterial colonies in and above the stoma in the 2nd group (B), bacterial biofilm above the stoma and surface of the leaf in the 3rd group (C), bacterial biofilm on the leaf surface in 4th group (D), little colonies of bacteria above the stoma in the 5th group (E), and bacterial colonies above the closed stoma in the 6th group (F).

4.3.4 Comparison of Single Phage Therapy with Different MOI Values

In this study, METU ID code MET P1 – 303 phage with titer 10^7 PFU/mL was used in both single phage therapies against *E. coli* O104:H4 but their MOI values were different. In MOI = 100 bacterial load was 10^5 CFU/mL and in MOI = -1 bacterial load was 10^8 CFU/mL. Results were compared between these two therapies. In the 1st group, total *E. coli* was 1,47 logs and biofilm-forming *E. coli* was 1 log less in MOI = 100 phage therapy. In the 2nd group, total and biofilm-forming *E. coli* counts were less by 0,6 logs in MOI = 100 phage therapy. Total *E. coli* was 0,47 logs and biofilm-forming *E. coli* count was 0,13 logs less in MOI = 100 phage therapy in the 3rd group. In the 4th group, total and biofilm-forming *E. coli* counts were 2,07 logs and 1,67 logs less in MOI = 100 phage therapy, respectively. In MOI = 100 phage therapy, total *E. coli* was 0,67 logs less and biofilm-forming *E. coli* count was 0,73 logs less in the 5th group. In the 6th group, total of *E. coli* was 1,60 logs and biofilm-forming *E. coli* 1,40 logs less in MOI = 100 phage therapy. The total *E. coli* results were visualized in Fig. 4.13 and the biofilm-forming *E. coli* results in Fig. 4.14.

When bacteriophages with high MOI values are applied, they are greatly effective in bacteria-killing by the process named ‘lysis from without’ (López-Cuevas et al., 2021). It is emphasized that the efficiency of killing the bacteria of phages is dependent on MOI in several studies (Bigwood et al., 2008; Huang et al., 2018; Mangieri et al., 2020). The results of this study fit this phenomenon. Both total and biofilm-forming *E. coli* counts were less in single phage therapy with MOI = 100 in all groups when counts were compared to single phage therapy with MOI = -1.

In the experimental studies of phage therapy applications on fresh produce, the bacterial loads are generally higher in order to determine phages' reduction effect, however, natural pathogen density is usually lower in fresh produce (López-Cuevas et al., 2021). In this study, it is observed that when the initial bacterial load was lower, 10^5 CFU/mL, there is more reduction in both total and biofilm-forming *E. coli* counts in the single phage therapy against *E. coli* O104:H4 with the same titer, 10^7 PFU/mL, than single phage therapy with 10^8 CFU/mL of initial bacterial load. These results could be associated with the natural load of pathogens in the fresh produce.

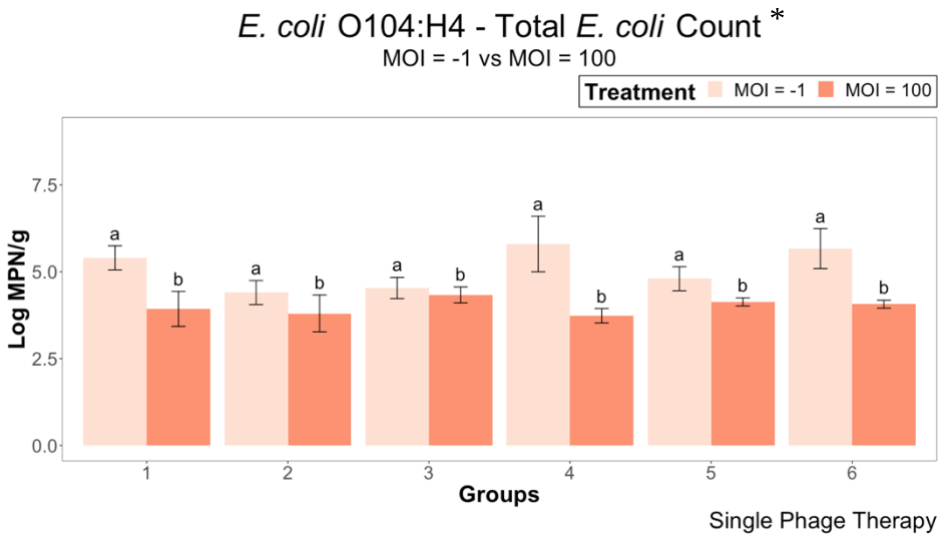


Figure 4.13. Comparison of Total *E. coli* Count on Phage Treated Cresses of Single Phage Therapy against *E. Coli* O104:H4 with MOI = 100 or MOI = -1

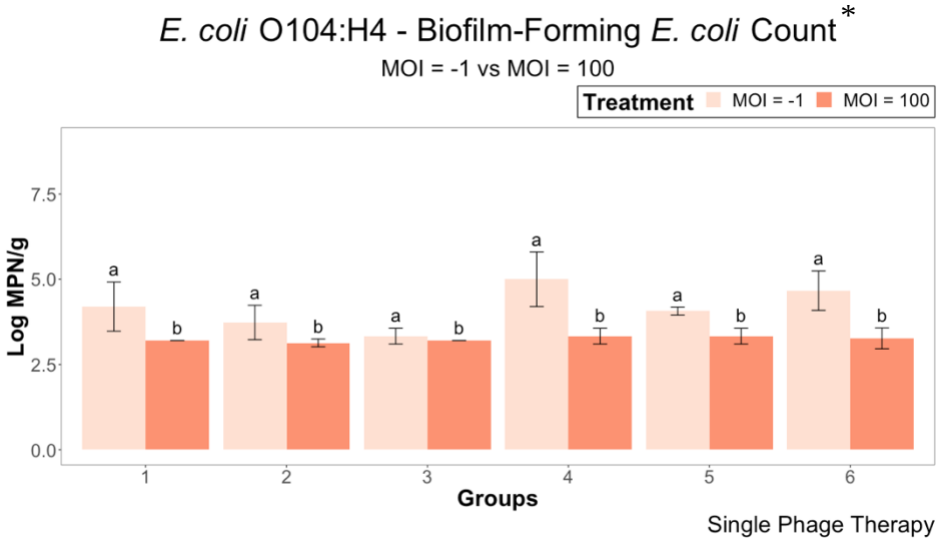


Figure 4.14. Comparison of Biofilm-Forming *E. coli* Count on Phage Treated Cresses of Single Phage Therapy against *E. coli* O104:H4 with MOI = 100 or MOI = -1

* Results were shown as a mean of 3 replicates ± standard deviation. Values with different letters represent the statistical difference between therapy and control groups. $P < 0.05$ considered as statistically different.

4.3.5 Comparison of Phage Cocktail Therapy and Single Phage Therapy

METU ID codes MET P1 – 303, MET P1 – 316, and MET P1 – 349 were used for phage cocktail therapy, and MET P1 – 303 was used for single phage therapy with both MOI = 100. Results were compared between these two therapies. In the 1st group, total *E. coli* was 0,73 logs less and biofilm-forming *E. coli* was 0,93 logs less in phage cocktail therapy. In the 2nd group, 0,67 logs less and 0,50 logs less in total and biofilm-forming *E. coli* counts, respectively in phage cocktail therapy. The total *E. coli* was 0,67 logs and biofilm-forming *E. coli* 0,57 logs less in phage cocktail therapy in the 3rd group. Although there was not much change in results for the 4th group, total *E. coli* was 0,23 logs less and biofilm-forming *E. coli* was 0,27 logs less in the phage cocktail therapy. In the 5th group, total and biofilm-forming *E. coli* counts were 1 log and 0,70 logs less in phage cocktail therapy. In the 6th group, 0,20

logs less and 0,63 logs less for total and biofilm-forming *E. coli* counts, respectively, in phage cocktail therapy. The total *E. coli* results were visualized in Fig. 4.15 and the biofilm-forming *E. coli* results in Fig. 4.16.

Bacteriophages are effective and have the ability to kill specific target bacteria, however, the feature of this limiting host range limits the bacteriophage application in theory. On the other hand, this setback can be overcome with phage cocktail preparation (Vikram et al., 2020). Results of this study show when phage cocktail therapy and single phage therapy against *E. coli* O104:H4 with the same MOI, phage cocktail therapy is more effective in lowering both total and biofilm-forming *E. coli* counts.

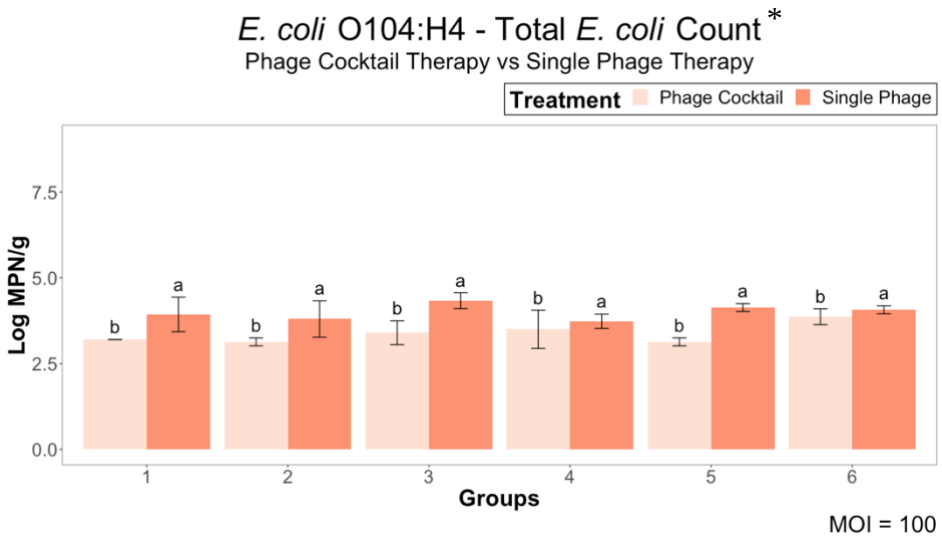


Figure 4.15. Comparison of Total *E. coli* Count on Phage Treated Cresses of Single Phage Therapy or Phage Cocktail Therapy against *E. coli* O104:H4 with MOI = 100

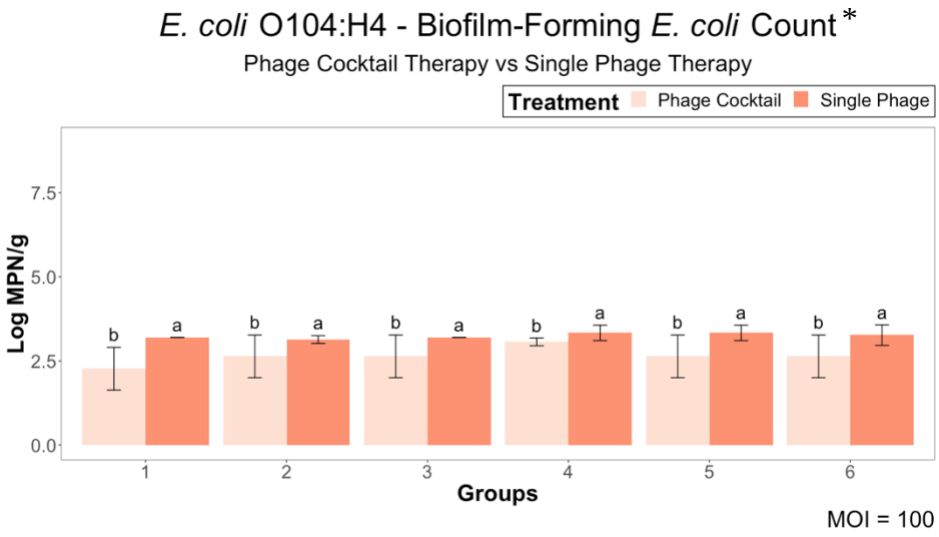


Figure 4.16. Comparison of Biofilm-Forming *E. coli* Count on Phage Treated Cresses of Single Phage Therapy or Phage Cocktail Therapy against *E. coli* O104:H4 with MOI = 100

* Results were shown as a mean of 3 replicates ± standard deviation. Values with different letters represent the statistical difference between therapy and control groups. $P < 0.05$ considered as statistically different.

When all the results for 3 different phage therapy against *E. coli* O104:H4 were considered, a phage cocktail therapy with MOI = 100 was chosen for other phage therapy studies. The reason is that although there were more log reductions in *E. coli* count with a single phage therapy with MOI = -1 (10^8 CFU/mL initial bacterial load), the end *E. coli* O104:H4 count was higher than both single and cocktail phage therapies with MOI = 100 (10^5 CFU/mL initial bacterial load). Also, 10^5 CFU/mL as a bacterial load is closer to the natural bacterial load than 10^8 CFU/mL. Moreover, phage cocktail therapy with the same initial bacterial load, 10^5 CFU/mL, was more effective in reducing *E. coli* O104:H4 counts.

4.4 Phage Therapy Against *E. coli* O157:H7

Application of phage therapy against *E. coli* O157:H7 was done with a cocktail phage solution. From the host range results, 3 different phages were chosen for the

phage cocktail which were METU ID codes MET P1 – 303, MET P1 – 311, and MET P1 – 322, and its titer was adjusted to 10^7 PFU/mL. The initial bacterial load was 10^5 CFU/mL which makes MOI = 100. All 6 therapy groups for each trial were irrigated based on Table 3.6. Each group was planted as 3 replicates and results for *E. coli* counts were calculated as the average of the replicates for each group. On the 30th day, cresses were harvested to collect samples for total and biofilm-forming *E. coli* counts. Results were compared with their control groups. Furthermore, ANOVA and Tukey's test were done to results in order to specify the difference between control and therapy groups.

When control and therapy groups were compared, total *E. coli* was reduced by 1,60 logs with 3414,28 MPN/g, and biofilm-forming *E. coli* was reduced by 1,77 logs with 354,81 MPN/g in the 1st group. In the 2nd group, there were 1,93 logs of reduction in total *E. coli* with 1584,89 MPN/g and 2 logs of reduction in biofilm-forming *E. coli* 184,79 MPN/g. In the 3rd group, the total *E. coli* 1,4667 logs were reduced by 4641,94 MPN/g, and biofilm-forming *E. coli* 1,67 logs were reduced by 398,10 MPN/g. Total *E. coli* was reduced by 2,60 logs with 2928,86 MPN/g and biofilm-forming *E. coli* was reduced by 3,53 logs with 158,48 MPN/g in the 4th group. In the 5th group, there was a 2,33 log reduction in total *E. coli* with 5411,27 MPN/g and 2,97 log biofilm-forming *E. coli* 584,38 MPN/g. In the 6th group, total and biofilm-forming *E. coli* counts were reduced by 1,73 logs with 4641,94 MPN/g and 2,27 logs with 215,42 MPN/g, respectively. The results were shown in Table 4.5. The total *E. coli* results were visualized in Fig. 4.17 and the biofilm-forming *E. coli* results in Fig. 4.18.

According to the Turkish Food Codex, *E. coli* O157:H7 has a zero-tolerance policy in washed, sliced, and packaged, separate, or mixed raw vegetables (*Regulation on Turkish Food Codex Microbiological*, 2011). Also, in Commission Regulation (EC) No 2073/2005, *E. coli* O157:H7 bacterial load limitation in sprouts is zero (*Commission Regulation (EC) No 2073/2005 on Microbiological Criteria for Foodstuffs*, 2020). On the other hand, in this study, the elimination of *E. coli* O157:H7 to zero was not observed in any groups. The lowest total bacterial load was

log3,20 MPN/g which is 1584,90 MPN/g and the lowest biofilm-forming *E. coli* count was log2,20 MPN/g which is 158,48 MPN/g. In order to eliminate all *E. coli* O157:H7 load for the zero-tolerance policy, additional substances could be used to enhance the efficiency of bacteriophages which was mentioned in Part 4.2.3.

The SEM images for control groups are in Fig. 4.19 and for therapy groups are in Fig. 4.20.

Table 4.5 Total and Biofilm-Forming *E. coli* Counts of Phage Cocktail Therapy Against *E. coli* O157:H7

	Total E. coli Count				*Biofilm-Forming E. coli Count*			
	Contaminated Cresses		*Phage Treated Cresses*		*Contaminated Cresses*		*Phage Treated Cresses*	
Groups	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*
1st group	5,13	135925,20	3,53	3414,28	4,27	18479,91	2,55	354,81
2nd group	5,13	135925,20	3,20	1584,89	4,27	18479,91	2,27	184,79
3rd group	5,13	135925,20	3,67	4641,94	4,27	18479,91	2,60	398,10
4th group	6,07	1166003,89	3,47	2928,86	5,73	541127,99	2,20	158,48
5th group	6,07	1166003,89	3,73	5411,27	5,73	541127,99	2,77	584,38
6th group	5,40	251188,64	3,67	4641,94	4,60	39810,71	2,33	215,42

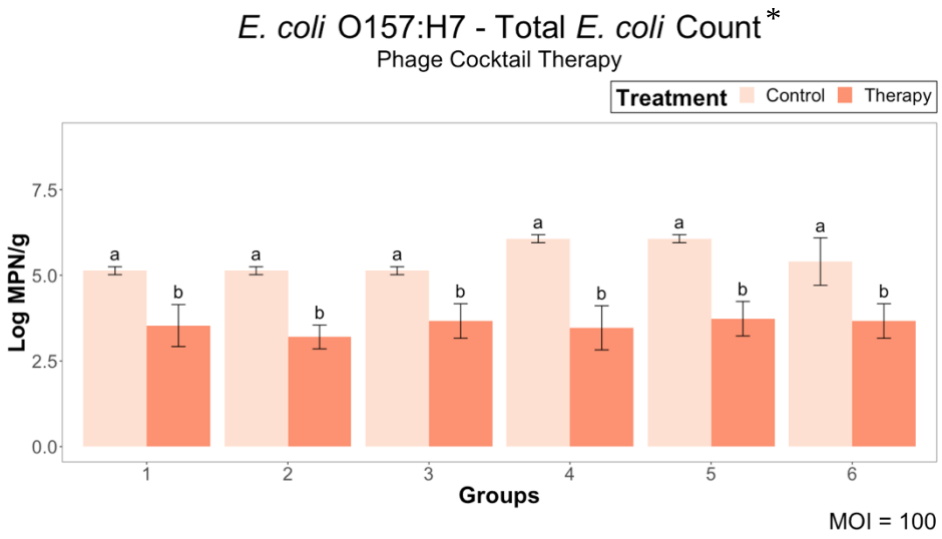


Figure 4.17. Total *E. coli* Count of Contaminated (Control) and Phage Treated (Therapy) Cresses of Phage Cocktail Therapy against *E. coli* O157:H7

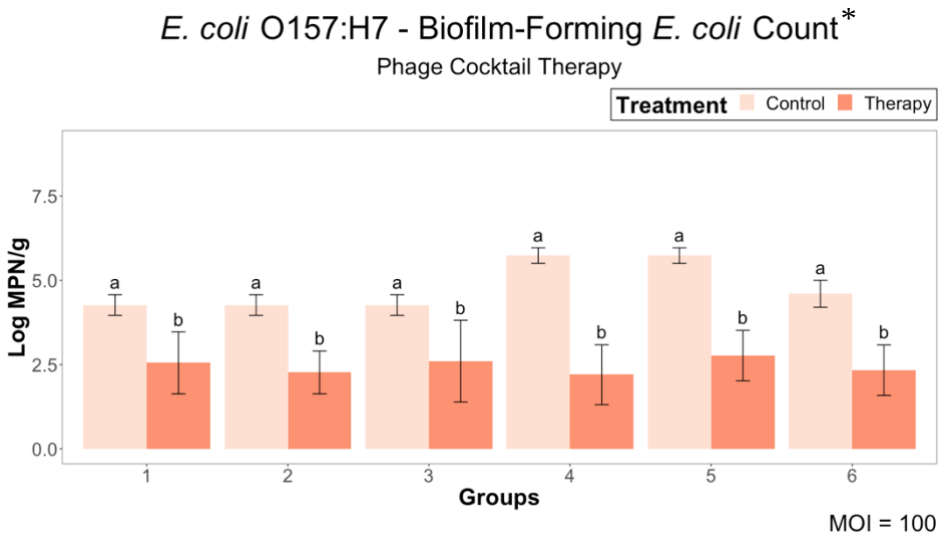


Figure 4.18. Biofilm-Forming *E. coli* Count of Contaminated (Control) and Phage Treated (Therapy) Cresses of Phage Cocktail Therapy against *E. coli* O157:H7

* Results were shown as a mean of 3 replicates ± standard deviation. Values with different letters represent the statistical difference between therapy and control groups. $P < 0.05$ considered as statistically different.

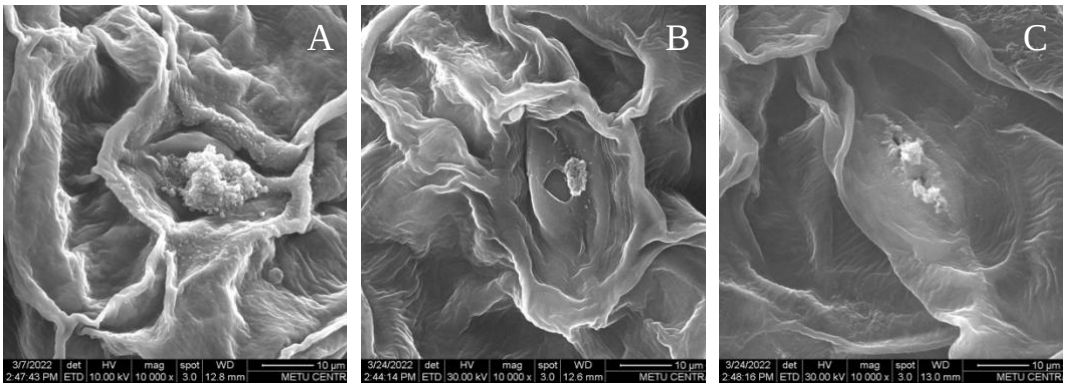
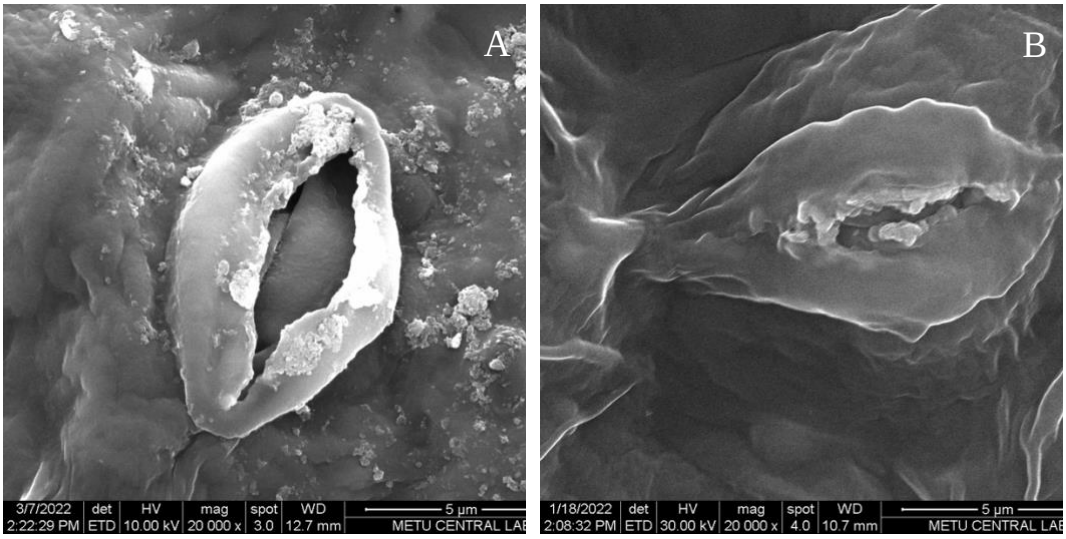


Figure 4.19. SEM images of control groups of phage cocktail treated groups against *E. coli* O157:H7. Bacterial growth and biofilm in and above the stoma and on the surface of the leaf in the 1st group (A), bacterial growth and biofilm above the stoma in 2nd group (B), and bacterial biofilm in the stoma and bacterial growth on the surface of the leaf in the 3rd group (C).



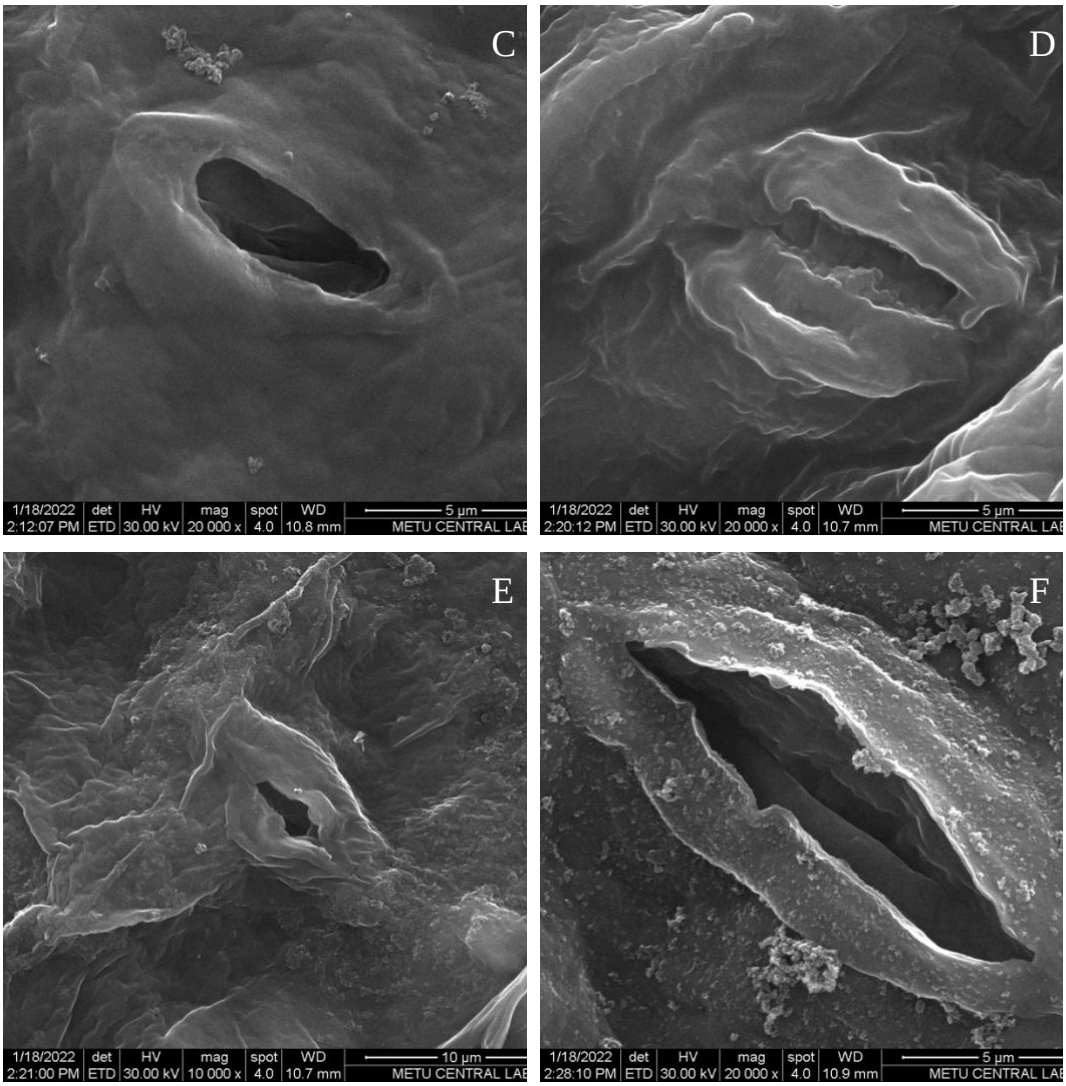


Figure 4.20. SEM images of phage cocktail treated groups agaisnt *E. coli* O157:H7. Bacterial colonies on the stoma's guard cells and the surface of the leaf in the 1st group (A), bacterial growth in the stoma in the 2nd group (B), bacterial colonies on the surface of the leaf in the 3rd group (C), bacterial growth in the stoma in the 4th group (D), bacterial biofilm on the surface of the leaf in 5th group (E), and bacterial colonies above and around the stoma in the 6th group (F).

4.5 Phage Therapy Against *E. coli* O26

Application of phage therapy against *E. coli* O26 was done with a single phage solution. The solution was made with METU ID code MET P1 – 346, and its titer was adjusted to 10^7 PFU/mL. The initial bacterial load was 10^5 CFU/mL which makes MOI = 100 All 6 therapy groups for each trial were irrigated based on Table 3.3. Each group was planted as 3 replicates and results for *E. coli* counts were calculated as the average of the replicates for each group. On the 30th day, cresses were harvested to collect samples for total and biofilm-forming *E. coli* counts. Results were compared with their control groups. Moreover, ANOVA and Tukey's test were done to results in order to specify the difference between control and therapy groups.

When the control and therapy groups' results were compared, there were 1,53 log reductions in total *E. coli* with 11660,03 MPN/g and 1,27 log reductions in biofilm-forming *E. coli* with 1711,19 MPN/g in the 1st group. In the 2nd group, total *E. coli* was reduced by 1,20 logs with 25118,86 MPN/g, and biofilm-forming *E. coli* was reduced by 0,37 logs with 13592,52 MPN/g. In the 3rd group, total and biofilm-forming *E. coli* counts were reduced by 3,33 logs with 184,79 MPN/g and 2,87 logs with 42,98 MPN/g, respectively. In the 4th group, total *E. coli* was decreased by 2,57 logs with 794,32 MPN/g, and biofilm-forming *E. coli* was decreased by 3,30 logs with 58,43 MPN/g. There were 0,60 log reductions in total *E. coli* with 73569,87 MPN/g and 1,87 log reductions in biofilm-forming *E. coli* with 1584,89 MPN/g in the 5th group. In the 6th group, total *E. coli* was reduced by 1,80 logs with 7356,98 MPN/g, and biofilm-forming *E. coli* was reduced by 1,73 logs with 2154,26 MPN/g. The results were shown in Table 4.6. The total *E. coli* results were visualized in Fig. 4.21 and the biofilm-forming *E. coli* results in Fig. 4.22.

Based on the Turkish Food Codex, *E. coli* satisfactory level is <10 CFU/g and the maximum level is 10 CFU/g in all RTE salads, and *E. coli* satisfactory level is 100 CFU/g and the maximum level is 1000 CFU/g in pre-cut and RTE fruits and vegetables (*Regulation on Turkish Food Codex Microbiological,* 2011). Moreover,

in Commission Regulation (EC) No 2073/2005 *E. coli* satisfactory level is ≤ 100 CFU/g and the maximum level is 1000 CFU/g in pre-cut and RTE fruit and vegetables which is the same as the Turkish Food Codex (*Commission Regulation (EC) No 2073/2005 on Microbiological Criteria for Foodstuffs*, 2020). Even though there are no specific regulations for EPEC bacterial load, total *E. coli* O26 counts were reduced below the *E. coli* limitations in the two groups. Total *E. coli* O26 count was log2,27 MPN/g which is 184,79 MPN/g in the 3rd group and log2,90 MPN/g which is 794,32 MPN/g in the 4th group. Furthermore, the biofilm-forming *E. coli* O26 counts were within the satisfactory level in two groups and the best result was log1,63 MPN/g which is 42,98 MPN/g.

As mentioned in Part 4.2.5, the preparation of a phage cocktail has advantages. Nonetheless, the phage cocktail solution against *E. coli* O26 which was prepared for this study did not affect as much as a single phage solution. The reason for this could be because of the phage-phage antagonist relationships as interactions of phage-phage are as important as interactions of phage-host a phage cocktail. An example of the most well-recognized antagonistic relationship is the temperate phages that mediate super-infection immunity. In addition, islands of pathogenicity or phage satellites can limit their helper phages' spread. If bacteriophages have the same receptor sites or abortive infection mechanisms, antagonistic relationships can happen (Molina et al., 2022).

The SEM images for control groups are in Fig. 4.23 and for therapy groups are in Fig. 4.24.

Table 4.6 Total and Biofilm-Forming *E. coli* Counts of Single Phage Therapy Against *E. coli* O26

	Total E. coli Count				*Biofilm-Forming E. coli Count*			
	Contaminated Cresses		*Phage Treated Cresses*		*Contaminated Cresses*		*Phage Treated Cresses*	
Groups	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*	*LogMPN/g*	*MPN/g*
1st group	5,60	398107,17	4,07	11660,03	4,50	31622,77	3,23	1711,19
2nd group	5,60	398107,17	4,40	25118,86	4,50	31622,77	4,13	13592,52
3rd group	5,60	398107,17	2,27	184,79	4,50	31622,77	1,63	42,98
4th group	5,47	292886,93	2,90	794,32	5,07	116600,38	1,77	58,43
5th group	5,47	292886,93	4,87	73569,87	5,07	116600,38	3,20	1584,89
6th group	5,67	464194,51	3,87	7356,98	5,07	116600,38	3,33	2154,26

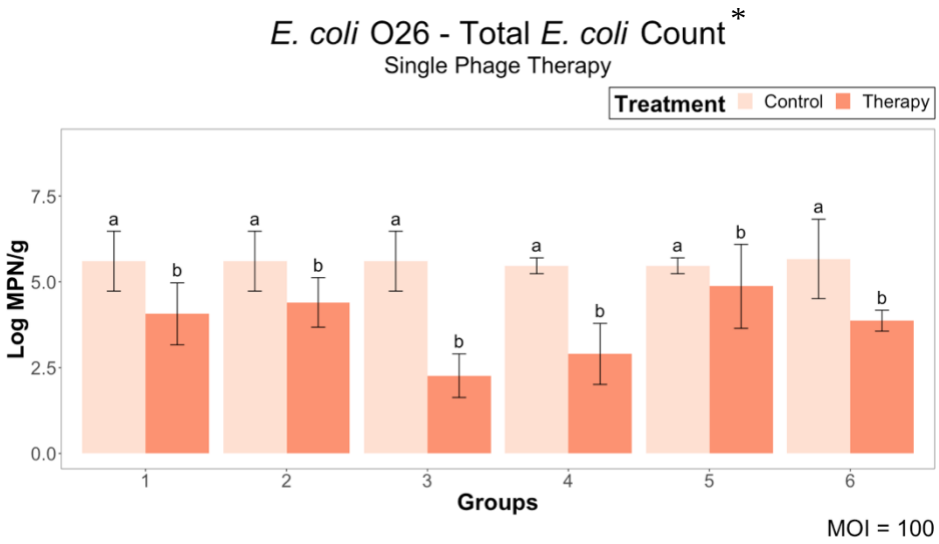


Figure 4.21. Total *E. coli* Count of Contaminated (Control) and Phage Treated (Therapy) Cresses of Single Phage Therapy against *E. coli* O26

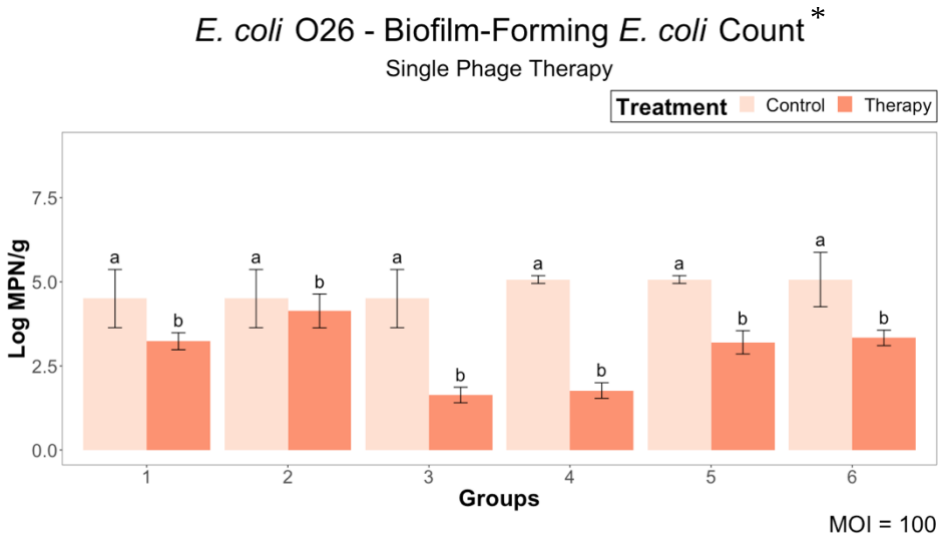


Figure 4.22. Biofilm- Forming *E. coli* Count of Contaminated (Control) and Phage Treated (Therapy) Cresses of Single Phage Therapy against *E. coli* O26

* Results were shown as a mean of 3 replicates ± standard deviation. Values with different letters represent the statistical difference between therapy and control groups. $P < 0.05$ considered as statistically different.

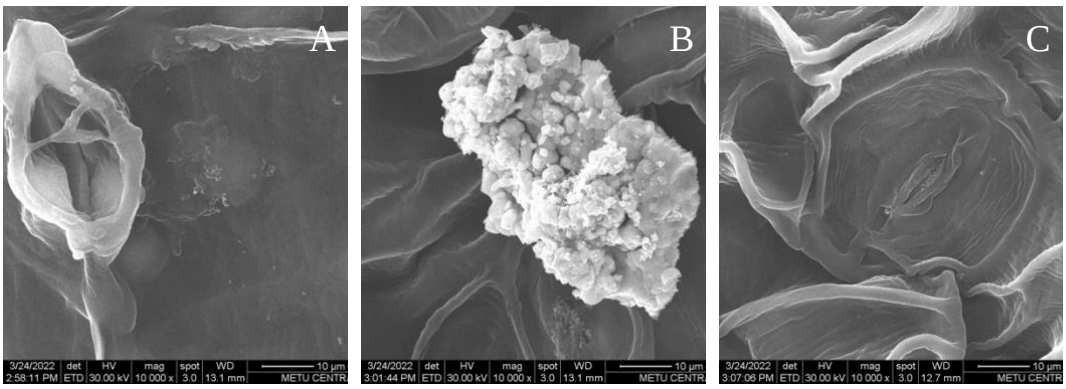


Figure 4.23. SEM images of control groups of single phage treated groups against *E. coli* O26. Bacterial growth on the guard cells of stoma and the surface of the leaf in the 1st group (A), bacterial biofilm in and above the stoma and on the surface of the leaf in the 2nd group (B), and bacterial growth above the stoma in the 3rd group (C).

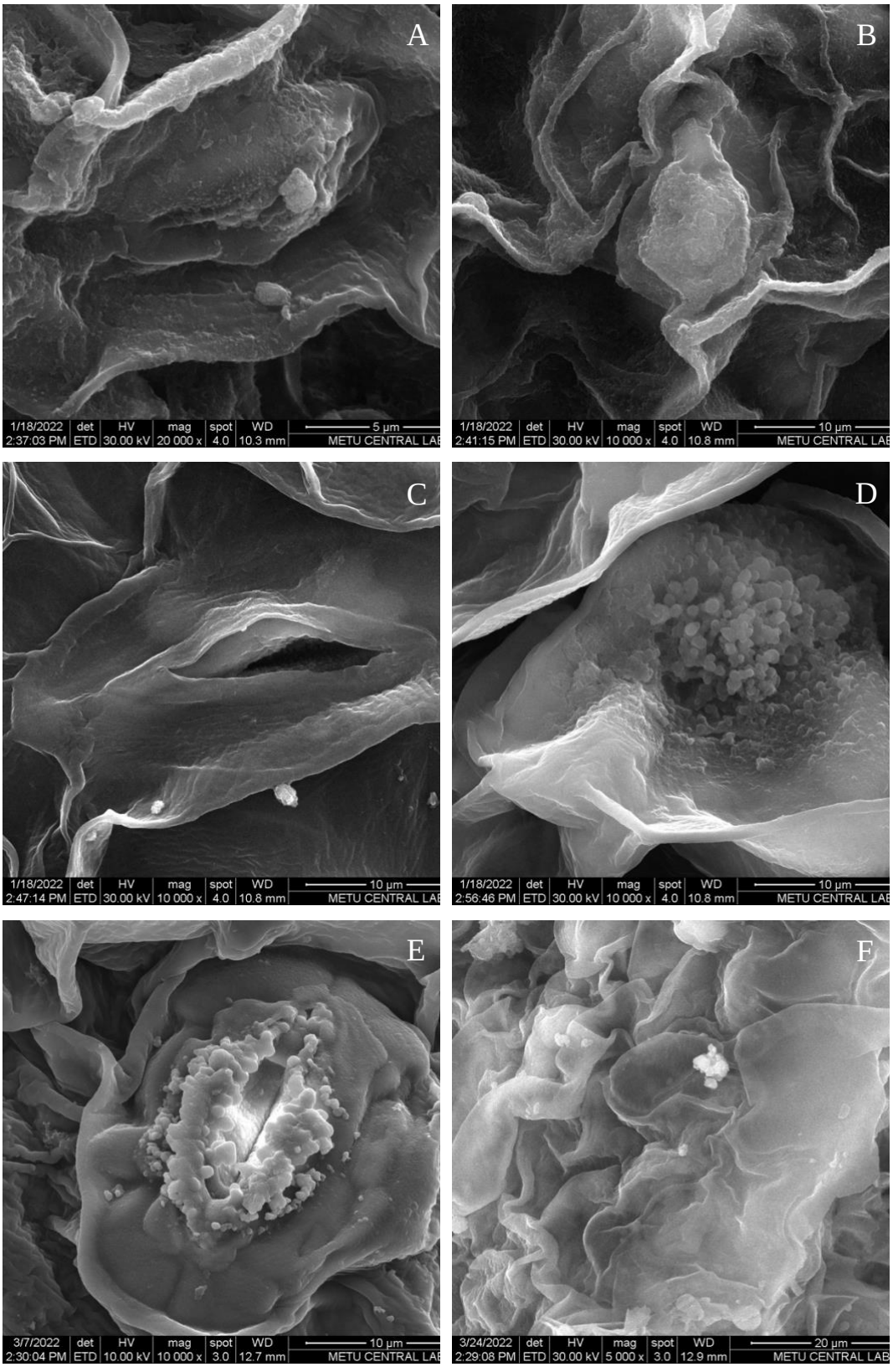

A
1/18/2022 | det | HV | mag | spot | WD | 5 µm
2:37:03 PM | ETD | 30.00 kV | 20 000 x | 4.0 | 10.3 mm | METU CENTRAL LAB
B
1/18/2022 | det | HV | mag | spot | WD | 10 µm
2:41:15 PM | ETD | 30.00 kV | 10 000 x | 4.0 | 10.8 mm | METU CENTRAL LAB
C
1/18/2022 | det | HV | mag | spot | WD | 10 µm
2:47:14 PM | ETD | 30.00 kV | 10 000 x | 4.0 | 10.8 mm | METU CENTRAL LAB
D
1/18/2022 | det | HV | mag | spot | WD | 10 µm
2:56:46 PM | ETD | 30.00 kV | 10 000 x | 4.0 | 10.8 mm | METU CENTRAL LAB
E
3/7/2022 | det | HV | mag | spot | WD | 10 µm
2:30:04 PM | ETD | 10.00 kV | 10 000 x | 3.0 | 12.7 mm | METU CENTRAL LAB
F
3/24/2022 | det | HV | mag | spot | WD | 20 µm
2:29:08 PM | ETD | 30.00 kV | 5 000 x | 3.0 | 12.9 mm | METU CENTRAL LAB

Figure 4.24. SEM images of single phage treated groups against *E. coli* O26. Bacterial biofilm in and above the stoma in the 1st group (A), bacterial biofilm on the surface of the leaf in 2nd group (B), bacterial colonies on the surface of the leaf, and bacterial growth in the stoma in the 3rd group (C), bacterial colonies on the surface of the leaf in the 4th group (D), bacterial growth in and above the stoma in the 5th group (E), and bacterial growth on the surface of the leaf in the 6th group (F).

CHAPTER 5

CONCLUSION

Foodborne outbreaks associated with the consumption of fresh produce reveal the need for more effective choices for eliminating foodborne pathogens from raw foods. Commercial chemical disinfectant usage has demonstrated that they have not been significantly effective in reducing foodborne pathogens which are attached to fresh produce's surface (López-Cuevas et al., 2021). Also, using disinfectants is one of the main stressors that bacteria could encounter. Bacterial tolerance increase could enhance the adaptive resistance to antibiotics and bacterial virulence which causes the bacterial disinfectant tolerance to increase that becomes an important point for food safety (Sun et al., 2019). Moreover, antibiotic resistance gradually increases due to the overuse and misuse of antibiotics in humans, animals, and agriculture. Because of this, once treatable bacteria become untreatable or need the last line of antibiotics (Paitan, 2018). Herewith, a more effective disinfectant agent is needed. As an alternative biocontrol agent, bacteriophages can be used. There are studies on bacteriophages' usage against bacterial diseases not only for human or veterinary but also for agricultural purposes since their discovery (López-Cuevas et al., 2021). To reduce illnesses due to foodborne pathogens, we should eliminate pathogens at the field level since pathogens can be colonized in the stomata in the plant tissue. Therefore, they cannot be eliminated.

To our knowledge, there is no study on the application of phage therapy against foodborne pathogens on fresh produce during the vegetation state of leafy greens with irrigation water. In this study, it was tried to determine if there is a difference in when contamination of cresses occurred and when and how many times cresses received phage therapy in six different irrigation schedules with three *E. coli* strains during 30 days trials.

This study demonstrates the potential of applications of phage therapy against foodborne pathogens during leafy greens' vegetation state with irrigation water. Both total and biofilm-forming *E. coli* O157:H7, *E. coli* O104:H4, and *E. coli* O26 counts have reduced significantly. The enumeration results were also confirmed with SEM images in which regular, rod-shaped cells have not been observed. On the other hand, confocal microscopy could be used to get clearer images of pathogens on the leafy greens' surface. Enumeration of *E. coli* O157:H7 and *E. coli* O104:H4 have been reduced significantly, however, counts were higher than bacterial load limitations on fresh produce. To increase the reduction, additional substances such as essential oils could be used to enhance the killing efficiency of bacteriophages. Additionally, the natural pathogen loads in leafy greens are not as high as the pathogens' loads in the experimental setups. Thereof, phage therapy against the natural load of pathogens might get better reductions.

Phage therapy against foodborne pathogens is more advantageous than disinfectants that are used today. Although there are studies on phage therapy against foodborne pathogens on leafy greens, there are no studies on phage therapy against foodborne pathogens during vegetation of leafy greens. Consequently, further investigations are required to optimize phage therapy against foodborne pathogens with natural loads during the vegetation stage of leafy greens with irrigation water. Also, the effect of the natural environment and weather conditions on the phage therapy of leafy greens should be investigated and optimized in order to be applied in the field. Lastly, phage therapy with additional substances such as essential oil against foodborne pathogens during leafy green vegetation via irrigation water should be investigated.

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APPENDICES

A. ANOVA and Tukey's Test Results

Table A.1 ANOVA Results of Single Phage Therapy against *E. coli* O104:H4 with MOI = 100

	Total E. coli Count		*Biofilm-Forming E. coli Count*	
	ind	*Residuals*	*ind*	*Residuals*
Df	1	10	1	10
Sum Sq	0.6847	0.6704	0.3333	0.4030
Mean Sq	0.6847	0.0670	0.3333	0.0403
F-value	10.21		8.271	
Pr(>F)	0.00956**		0.0165*	

*Significant codes: 0 (***), 0.001 (**), 0.01 (*), 0.05 (.), 0.1 ( ), 1

Table A.2 Tukey's Test Results of Single Phage Therapy against *E. coli* O104:H4 with MOI = 100

	Total E. coli Count	*Biofilm-Forming E. coli Count*
Diff	-0.477775	-0.3333333
Lwr	-0.8108275	-0.5915915
Upr	-0.1446725	-0.07507514
P adj	0.0095574	0.0165023

*Diff: mean the difference between groups, Lwr: lower end point of the interval, Upr: upper endpoint, P adj: p-value after adjustment

Table A.3 ANOVA Results of Single Phage Therapy against *E. coli* O104:H4 with MOI = -1

	Total E. coli Count		*Biofilm-Forming E. coli Count*	
	ind	*Residuals*	*ind*	*Residuals*
Df	1	10	1	10
Sum Sq	20.365	3.103	18.09	2.03
Mean Sq	20.365	0.31	18.09	0.203
F-value	65.62		89.09	
Pr(>F)	1.06e-05***		2.69e-06***	

*Significant codes: 0 (***), 0.001 (**), 0.01 (*), 0.05 (.), 0.1 ( ), 1

Table A.4 Tukey's Test Results of Single Phage Therapy against *E. coli* O104:H4 with MOI = -1

	Total E. coli Count	*Biofilm-Forming E. coli Count*
Diff	-2.605467	-2.455583
Lwr	-3.322105	-3.035254
Upr	-1.888829	-1.875913
P adj	1.05e-05	2.7e-06

*Diff: mean the difference between groups, Lwr: lower end point of the interval, Upr: upper endpoint, P adj: p-value after adjustment

Table A.5 ANOVA Results of Phage Cocktail Therapy against *E. coli* O104:H4 with MOI = 100

	Total E. coli Count		*Biofilm-Forming E. coli Count*	
	ind	*Residuals*	*ind*	*Residuals*
Df	1	10	1	10
Sum Sq	3.378	0.945	26.133	0.6919
Mean Sq	3.378	0.095	26.133	0.0692

Table A.5 (continued)

F-value	35.75	37.77
Pr(>F)	0.000136***	0.000109***

*Significant codes: 0 (***), 0.001 (**), 0.01 (*), 0.05 (.), 0.1 ( ), 1

Table A.6 Tukey's Test Results of Phage Cocktail Therapy against *E. coli* O104:H4 with MOI = 100

	Total E. coli Count	*Biofilm-Forming E. coli Count*
Diff	-1.061083	-0.9333333
Lwr	-1.45663	-1.271721
Upr	-0.6655369	-0.5949459
P adj	0.0001362	0.0001089

*Diff: mean the difference between groups, Lwr: lower end point of the interval, Upr: upper endpoint, P adj: p-value after adjustment

Table A.7 ANOVA Results of Phage Treated Cresses of Single Phage Therapy against *E. Coli* O104:H4 with MOI = 100 or MOI = -1

	Total E. coli Count		*Biofilm-Forming E. coli Count*	
	ind	*Residuals*	*ind*	*Residuals*
Df	1	10	1	10
Sum Sq	3.929	1.933	2.551	1.870
Mean Sq	3.929	0.193	2.551	0.187
F-value	20.33		13.64	
Pr(>F)	0.00113**		0.00415**	

*Significant codes: 0 (***), 0.001 (**), 0.01 (*), 0.05 (.), 0.1 ( ), 1

Table A.8 Tukey's Test Results of Phage Treated Cresses of Single Phage Therapy against *E. Coli* O104:H4 with MOI = 100 or MOI = -1

	Total E. coli Count	*Biofilm-Forming E. coli Count*
Diff	-1.14445	-0.9222333
Lwr	-1.709978	-1.478579
Upr	-0.5789222	-0.3658872
P adj	0.0011272	0.0041528

*Diff: mean the difference between groups, Lwr: lower end point of the interval, Upr: upper endpoint, P adj: p-value after adjustment

Table A.9 ANOVA Results of Phage Treated Cresses of Single Phage Therapy or Phage Cocktail Therapy against *E. Coli* O104:H4 with MOI = 100

	Total E. coli Count		*Biofilm-Forming E. coli Count*	
	ind	*Residuals*	*ind*	*Residuals*
Df	1	10	1	10
Sum Sq	10.208	0.5358	1.0800	0.3541
Mean Sq	10.208	0.0536	1.0800	0.0354
F-value	19.05		30.5	
Pr(>F)	0.00141**		0.000254***	

*Significant codes: 0 (***), 0.001 (**), 0.01 (*), 0.05 (.), 0.1 ( ), 1

Table A.10 Tukey's Test Results of Phage Treated Cresses of Single Phage Therapy or Phage Cocktail Therapy against *E. Coli* O104:H4 with MOI = 100

	Total E. coli Count	*Biofilm-Forming E. coli Count*
Diff	-0.5833333	-0.6
Lwr	-0.8811116	-0.8420642
Upr	-0.285555	-0.3579358
P adj	0.0014105	0.0002535

*Diff: mean the difference between groups, Lwr: lower end point of the interval, Upr: upper endpoint, P adj: p-value after adjustment

Table A.11 ANOVA Results of Phage Cocktail Therapy against *E. coli* O157:H7

	Total E. coli Count		*Biofilm-Forming E. coli Count*	
	ind	*Residuals*	*ind*	*Residuals*
Df	1	10	1	10
Sum Sq	11.342	1.245	16.685	2.877
Mean Sq	11.342	0.125	16.685	0.288
F-value	91.08		57.99	
Pr(>F)	2.44e-06***		1.81e-05***	

*Significant codes: 0 (***), 0.001 (**), 0.01 (*), 0.05 (·), 0.1 ( ), 1

Table A.12 Tukey's Test Results of Phage Cocktail Therapy against *E. coli* O157:H7

	Total E. coli Count	*Biofilm-Forming E. coli Count*
Diff	-1.944433	-2.358333
Lwr	-2.3984	-3.048344
Upr	-1.490466	-1.668323
P adj	2.4e-06	1.81e-05

*Diff: mean the difference between groups, Lwr: the lower end point of the interval, Upr: upper endpoint, P adj: p-value after adjustment

Table A.13 ANOVA Results of Single Phage Therapy against *E. coli* O26

	Total E. coli Count		*Biofilm-Forming E. coli Count*	
	ind	*Residuals*	*ind*	*Residuals*
Df	1	10	1	10
Sum Sq	10.144	4.736	10.830	5.279
Mean Sq	10.144	0.474	10.830	0.528
F-value	21.42		20.52	
Pr(>F)	0.000939***		0.00109**	

*Significant codes: 0 (***), 0.001 (**), 0.01 (*), 0.05 (.), 0.1 ( ), 1

Table A.14 Tukey's Test Results of Single Phage Therapy against *E. coli* O26

	Total E. coli Count	*Biofilm-Forming E. coli Count*
Diff	-1.838883	-1.900033
Lwr	-2.724223	-2.834685
Upr	-0.9535441	-0.9653813
P adj	0.0009391	0.0010922

*Diff: mean the difference between groups, Lwr: lower end point of the interval, Upr: upper endpoint, P adj: p-value after adjustment

B. PREPARATION OF MEDIA

Table B.1 0.85% NaCl Solution

NaCl	0.425 g
dH_2O	50 mL

Table B.2 Brain Heart Infusion (BHI) Broth

BHI Medium	22.2 g
dH_2O	600 mL

Table B.3 Brain Heart Infusion (BHI) Agar

BHI Medium	14.8 g
Agar Bacteriological	6 g
dH_2O	400 mL

Table B.4 Luria-Bertani (LB) Broth

LB Medium	1.25 g
dH_2O	50 mL

Table B.5 Luria-Bertani (LB) Agar

LB Medium	10 g
Agar Bacteriological	6 g
dH_2O	400 mL

Table B.6 Semi-Solid Luria-Bertani (LB) Agar

LB Medium	6.25 g
Agar Bacteriological	1.5 g
dH_2O	250 mL

Table B.7 Buffered Peptone Water (BPW)

BPW Medium	25.5 g
dH_2O	1 L

Table B.8 Brilliant-Green Phenol-Red Lactose Sucrose (BPLS) Agar

BPLS Medium	51.5 g
dH_2O	1 L

C. CHEMICALS AND MATERIALS

Table C.1 The list of chemicals and materials with their suppliers

Chemicals	*Producers*
American Bacteriological Agar	Condalab (Madrid, Spain)
Luria Bertani (LB) Broth	Condalab (Madrid, Spain)
Buffered Peptone Water	Merck (Darmstadt, Germany)
Brain Heart Infusion Broth	Merck (Darmstadt, Germany)
Sodium chloride	Merck (Darmstadt, Germany)
BPLS Agar Modified	Merck (Darmstadt, Germany)
Pot	San (Istanbul, Turkey)
Cress Seeds	Zenitt Tohumculuk (Balikesir, Turkey)
Acetone	Sigma-Aldrich (Darmstadt, Germany)
Ethanol Absolute	ISOLAB (Eschau, Germany)
Methanol	ISOLAB (Wertheim, Germany)

D. IMAGES OF *E. COLI* BACTERIOPHAGES

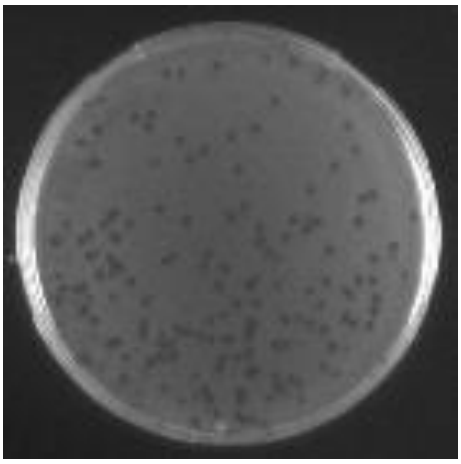

Figure D.1. MET P1 – 303 Double Plaque Assay Result

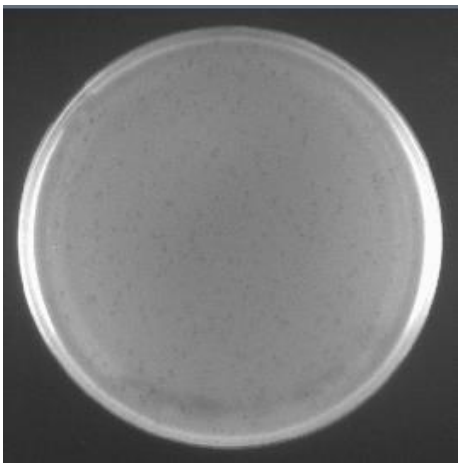

Figure D.2. MET P1 – 311 Double Plaque Assay Result

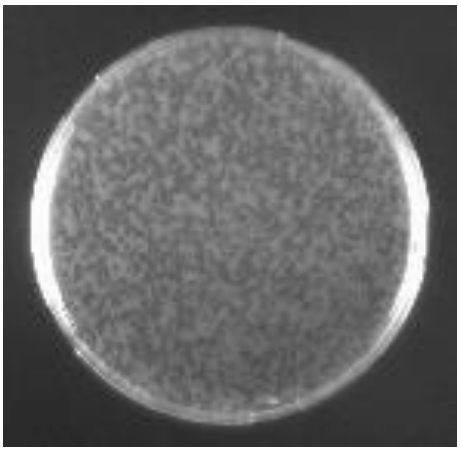

Figure D.3. MET P1 – 316 Double Plaque Assay Result

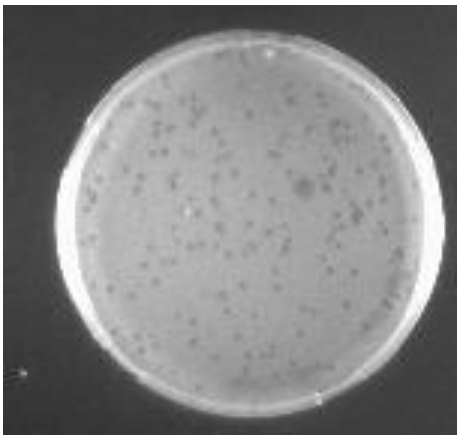

Figure D.4. MET P1 – 322 Double Plaque Assay Result

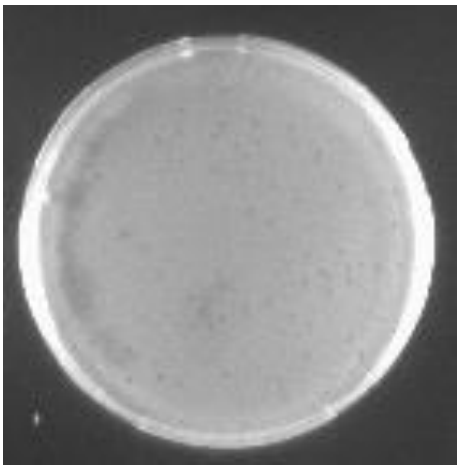

Figure D.5. MET P1 – 346 Double Plaque Assay Result

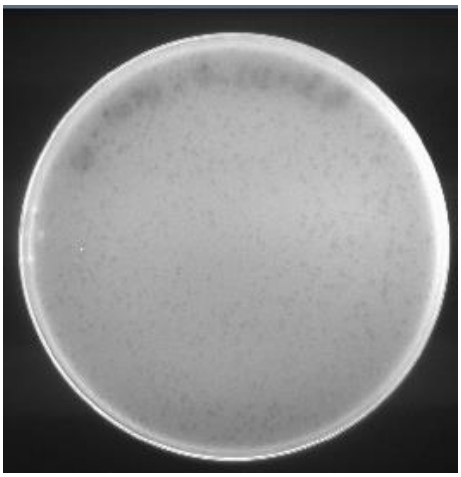

Figure D.6. MET P1 – 349 Double Plaque Assay Result