Bacteriophages as a Sustainable Food Safety Approach for Vegetable Production in Controlled Environment Agriculture Systems

by

Vania Paula Mickos

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 09, 2023

Keywords: *Salmonella enterica*, Bacteriophage, Aquaponic, Hydroponic, Vegetables, Food Safety, Water Quality, Biological Control

Approved by

Camila Rodrigues, Chair, Assistant Professor, Horticulture
Daniel Wells, Associate Professor, Horticulture
Mark Liles, Professor, Biological Sciences
Stuart B. Price, Associate Professor, Pathobiology

Abstract

Vegetables have frequently been contaminated with *Salmonella enterica, E. coli* O157:H7, and *Listeria monocytogenes*. Food safety concerns have boosted interest in controlled environmental agriculture (CEA) systems, especially as hydroponics and aquaponics have grown in popularity in recent years. Thus, new approaches to control foodborne pathogens are necessary since microorganisms more often become multidrug resistant. Biological control is an example of an alternative to control and prevent microbialcontamination in foods. An example of biological control that can be employed against foodborne pathogens is the bacteriophage, which is the most abundant virus on the earth. Phage cocktails might be a suitable target for specific pathogens in aquaponic, hydroponic, and vertical farming systems to reduce crop contamination and prevent foodborne outbreaks. However, at this point, no research has been conducted to investigate the applicability of phage as a biocontrol for foodborne pathogens in recirculating systems to produce vegetables under hydroponic and aquaponic systems. Thus, this project will investigate the efficiency of a phage cocktail to control *Salmonella enterica* and its applicability in a small-scale setting.

Keywords: *Salmonella enterica*, bacteriophage, aquaponic, hydroponic, vegetables, food safety, water quality.

Acknowledgments

I am profoundly thankful to God for His constant guidance and for bestowing upon me the calm and resilience needed to navigate the challenges of setting up this research. His presence has been a constant source of solace and inspiration.

My deepest gratitude to my mom and dad, whose unwavering love, support, and encouragement have been my constant source of strength throughout this journey. Your belief in me has been the driving force behind my achievements.

I extend my heartfelt appreciation to my mentors and professors at PUCPR in Brazil. I am truly fortunate to have had the privilege of learning from you and still get your guidance.

A sincere thank you goes out to my advisor and committee members for their time, expertise, and valuable input throughout this process.

I would like to extend a special note of gratitude to Steven, whose mentorship has been instrumental in broadening my knowledge and refining my skills. Your dedication to my growth has left an indelible mark on my academic journey.

To Jessica, Fabricio, Zoila, and Sthefani - you have been my extended family far from home. Your unwavering support, friendship, and understanding during the challenging moments of this journey have been my pillar of strength. I am profoundly grateful for your presence in my life.

To Beatriz, Claudismar, Teca and Jackson - your steadfast companionship and support, year after year, have meant the world to me. Your friendship has added depth and warmth to my life, and I am thankful for the enduring bond we share (Love you).

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List of Abbreviation

ANOVA	Analysis of variance
ART	Aligned Rank Transform
ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CEA	Controlled environment agriculture
CFU	Colonies-forming units
DNA	Deoxyribonucleic acid
DWC	Deep-water culture
EOP	Efficiency of plating
FDA	Food and Drug Administration
FSMA	Food Safety Modernization Act
GAPs	Good Agricultural Practice
GM	Genomic Mean
LBM	Luria-Bertani Miller
LED	Light-emitting diode
lmer	Linear mixed models
MBGB	Media-based growing beds
MOI	Multiplicity of Infection
MWQP	Microbial Water Quality
NFT	Nutrient Film Technology
NTS	Nontyphoid and Typhoid *Salmonella*
PBS	Phosphate-buffered saline
PFU	Plaque-forming units

PRRS Pattern – recognition receptors

PSR Produce Safety Rule

RAS Recirculating aquaculture system

RCF Relative centrifugal force

REML Restricted Maximum Likelihood

RPM Revolutions per minute

RT Room temperature

SPI *Salmonella* Pathogenicity Island

STV Statistical Threshold value

TLR9 Toll – like receptor

TSI Triple Sugar Iron

UA Urban Agriculture

UV Ultraviolet

XLD Xylose Lysine Decarboxylase

WHO World Health Organization

1. Introduction

Global food production has increased as a result of the "Green Revolution," with the introduction of novel crop and livestock breeds, artificial fertilizers, synthetic pesticides, and mechanization (Pretty, 2018). Conventional agriculture has led to significant negative environmental impacts caused by the intensification of agriculture practices (Poore & Nemecek, 2018). Healthy eating habits have also contributed to increased fruit and vegetable consumption, requiring advanced agricultural techniques necessary to provide a food supply over time (Wu et al., 2019).

As an alternative, controlled environment agriculture (CEA) employs soilless technology to produce plant crops and/or aquatic animals in an enclosed facility (P. Chen et al., 2020). In hydroponic systems, plants are grown in nutrient-rich water under controlled environmental conditions, providing sustainable climate control to supply fresh vegetable demands throughout the year (Lennard and Ward, 2019). Aquaponics systems uses hydroponic techniques and fish effluent from an aquaculture nutrient solution for plant grown (Greenfeld et al., 2021). In 2018, 82 total farms in the United States were reported to be using aquaponic systems. The global aquaponics market was worth USD 872.7 million in 2021 and is predicted to reach USD 1807.29 million by 2028, accounting for nearly 13% of the market increase (Zion Market Research, 2022).

Over the years, vegetables have often been affected by bacterial contamination, resulting in several outbreaks associated with *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* (López-Gálvez et al., 2021). Concerns about food safety have increased interest in CEA systems, especially as hydroponics and aquaponics have grown in popularity in the United States in recent years. CEA systems were thought to be the solution to foodborne outbreaks linked to fresh produce. However, in 2021, the U.S. Food and Drug

Administration (FDA) reported the first foodborne outbreak caused by *Salmonella* Typhimurium in lettuce grown under a hydroponic controlled environment system (FDA, 2021). This incident has raised concerns about the safety of CEA systems and the search for preventive measures. More recently, new alternatives to control bacterial contamination, including biological resources, have become a sustainable alternative and a valuable tool to use in vegetable production.

Bacteriophage, also known as phage, is an example of biological control that can be used against foodborne pathogens, including *S. enterica*, *E. coli*, or *L. monocytogenes* contamination (Au et al., 2021). The term "bacteriophage" refers to a class of viruses that infect and replicate inside bacteria. Phages are a kind of biological system found almost everywhere in the environment (Broncano-Lavado et al., 2021). Previous studies have demonstrated that phage can reduce *E. coli* levels by 1 log or 90% on lettuce and cantaloupe surfaces after being sprayed with a phage cocktail for two days (Mogren et al., 2018). Accordingly, at least for *E. coli*, *S. enterica*, and *L. monocytogenes*, the use of phages may be beneficial as one of the potential steps to ensure food safety. For phages to be effective, the metabolic activity of the bacteria is critical. Thus, optimal growth conditions need to be explored for bacteriophages to be used in the environment (Mogren et al., 2018). However, little information is available regarding the applicability of phage against foodborne pathogens in agricultural systems as a risk mitigation strategy for CEA systems.

Chapter I

2.0 Literature Review

2.2 Vegetable Production

In 2050, the global population is projected to reach 9.8 billion, and food consumption is estimated to rise by 70% to 100% over present food production, including fruits and vegetables (FAO, 2021). Fruits and vegetables are an essential part of the human diet, such as a natural source of vitamins and minerals. As a result, the consumption of fruits and vegetables has increased over the years as well as the demand in food production. With the increased consumption of fruits and vegetables, ensuring the safety of these products has also been a public health concern, as foodborne illnesses associated with fresh produce have had a substantial impact on the food sector in recent years. Foodborne outbreaks endanger not just public health but also the economy sector (Macieira et al., 2021).

It is estimated that the produce industry has suffered a loss of $600 million due to the two biggest vegetable outbreaks, one in 2006 with an outbreak of *E. coli* contamination in spinach, and another in 2008 with a *Salmonella* contamination in tomatoes (Hussain &Dawson, 2013). The economy in the United States related to fresh fruit and vegetable exports increased by just 4%, going from $7 billion in 2020 to $7.3 billion in 2021. The output of fruit, tree nuts, vegetables, and legumes is projected to have a small rise over the decade, going from 181 million tons in the year 2020 to 187 million tons in the year 2031 (Karst, 2022). The fruit and vegetable production totaled 74,276 farms in 2017 (USDA NASS,2017). The farm production contributed to the output of the United States' gross domesticproduct (GDP) of $134.7 billion or around 0.6 percent (USDA, 2021).

The United States imports a significant number of fresh vegetables from Mexico and Central America. Alabama ranks in the middle of the 50 states in terms of vegetable production,

trailing only adjacent Georgia, which is among the top five. As a result, Alabama has both the potential and the demand to elevate the production of fruits and vegetables over the next few years (Spenser, 2018).

Conventional soil-grown agriculture uses a large growing area and natural resources to supply the large demand for food that exists across the globe (König et al., 2018). However, the use of conventional farming methods has a negative impact on the local ecosystem and land recovery. In addition, certain plant species are unable to thrive in certain geographical areas and seasons, particularly because of changes in the global climate. In addition, it is not possible to cultivate food in many of the world's largest and most inaccessible regions, such as those with low-quality soil or extreme climatic conditions. In this approach, CEA emerges as an alternative that can fulfill the rising demand for food and solve the challenges posed by climate change.

Alternative food production methods are required to meet global demand, reduce environmental issues, and provide fresh produce anywhere, year-round, andwith greater control over diseases and other factors (Jena et al., 2018). Alternatively, aquaponics, hydroponics, and vertical farms are CEA systems that have the potential to grow large quantities of food in a smaller area (Gruda et al., 2019). The advantages of monitoring and controlling the macroenvironment and microenvironment, which in turn enables optimal plant performance (Gruda et al., 2019). The extension of the production period, the induction of early maturity, and the attainment of higher yields of higher quality (Gruda et al., 2019).

2.3 Controlled Environmental Agriculture

More recently, CEA systems have gained popularity because of their effective resource management and food production. Various commercial crops, including leafy greens, tomatoes, cucumbers, peppers, and strawberries, are successfully being produced under CEA systems (Sharma et al., 2018).

The environmental impacts are reduced in water-growing systems (aquaponics, hydroponics, and vertical farms) when compared with conventional soil-based farming, and these systems are considered a more sustainable model for plant cultivation (Lennard and Ward, 2019). It is suggested that CEA systems might be able to support future food demand in amore sustainable manner. CEA systems are an alternative to the growing environment for a variety of plants, which implies that vegetables are generally cultivated inside, i.e., "indoor agriculture" (Gómez et al., 2019).

Indoor growing involves the production of agricultural commodities in a greenhouse or enclosed buildings with a single layer of vegetables exposed to direct sunlight or in numerous vertically stacked racks under artificial light andautomated fertilizer delivery systems (e.g., vertical farming). CEA systems often use a broad range of agricultural production methods, including hydroponics, aeroponics, and aquaponics (Cowan et al., 2022).

2.4 Hydroponic System

The hydroponic system is a technique of growing plants in water through plant roots by immersion in a nutrient solution without soil. The basic hydroponic system contains nutrients delivered via an automated water circulation system (Swain et al., 2021).Vegetables, including tomatoes and leafy greens, are the predominant commodities grown under hydroponic methods using floating rafts and nutrient film techniques (NFT).

Solution culture and media culture are two approaches that are utilized in hydroponic systems. Only the nutrient solution is used in the culture solution, which is also known as solution drip irrigation. There is no solid media used in this method, and the roots are suspended in a nutrient solution as the plant grows. (Kürklü et al., 2018). Media culture or solid medium uses materials such as vermiculite, polystyrene packing peanuts, expanded clay, perlite, coir, and pumice in the plant roots. The sub-irrigation and top irrigation are the primary medium culture types. The term deep-water culture (DWC) systems are included in the category of

media culture systems, and the roots of plants are immersed in a nutrient- and oxygen-rich aqueous solution. (Magwaza et al., 2020). The medium-based system often used in commercial operations uses the NFT system in whicha particular amount of substrate provides root stability and substrate for microorganism adsorption and water-nutritional cycle, which are among the several types of floating raft systems (Bulla, 2022).

The primary benefit of hydroponics systems compared to conventional farming (soil-based culture) is that the plants are not dependent on the soil type or quality of the cultivated area (Gardens, 2021). Compared to the conventional technique of growing plants in soil, hydroponic techniques are regarded as an effective use of resources. Instead, the water is retrieved, sorted, replenished, and recycled, resulting in 70 to 90% water savings compared to conventional production (Sharma et al., 2018).

The expected demand for vegetable greenhouse production is anticipated to increase by 6.50% per year until 2025. (Guo et al., 2019). In 2022, there were 2,360 hydroponic farms in the United States. This represents a growth of 0.9% from the previousyear. Still, hydroponics accounts for a small fraction of the country's $5.2 billion fruit and vegetable industry (IBISWorld, 2021).

2.5 Aquaponic System

Urban areas are expected to have the highest rise in the global population. Aquaponics may be a solution to the issues of food security and infrastructure, which willbecome major concerns in future years. Even in the present day, major cities around the globe confront the difficulty of having a food supply infrastructure (e.g., "food deserts").

Aquaponics used in commercial urban agriculture or community gardening might help decrease food desert areas (Rizal et al., 2018). It may also be utilized for urban rooftop food production, utilizing intense aquaculture in basements and organic hydroponics in rooftop

greenhouses. Rooftop aquaponics may help urban households without land and, is advantageous in overpopulated metropolitan regions where water and green space are scarce. Also, the recirculating aquaculture system (RAS) used in aquaponics reduces environmental impact, including waste handling (Buscaroli et al., 2021). Although pesticides are limited for pest and disease management in plants because of the health of fish due to toxicity concerns, the adoption of biological control practices can be a safe alternative to chemical pesticides in aquaponic systems.

Aquaponics uses fish effluent from an aquaculture component as the main nutritional supplement for the cultivation of plants in a hydroponic system (Greenfeld et al., 2021). The idea of aquaponics originated in the 1970s and 1980s, referred to as "mixedfish and vegetable production in greenhouses" or "combined production of fish and plantsin recirculating water" (Baganz et al., 2022). The essential premise of aquaponics is that fish are cultivated in tanks where plants are grown in a nutrient-rich water circulation system. Plants utilizes nutrients from the water, which is then recirculated back to the fishtanks in a (DWC or NFT in a recirculated system. Sometimes, the water can be discardedafter the plants using a media-based growing beds (MBGB) (Quagrainie et al., 2017).

Aquaponics promotes sustainable agriculture by utilizing water efficiently (95– 99%) and less synthetic fertilizer (Goddek et al., 2015). The system also has an effective management of water usage (Cifuentes-Torres et al., 2021a). Aquaponics has the potentialto help economic growth, improve food supply, and minimize the global impact convention agriculture (Li et al., 2018). Aquaponics have grown in the past few years in the United States totalizing 82 aquaponics farms in 2018, with 25 states having at least onecommercial aquaponics farm (Perdue & Hamer, 2018). Most aquaponic farms are in Florida (13.41 %), Wisconsin (10.97 %), New York (7.32%), and Hawaii (7.32%). Alabama currently has one commercial aquaponic farm combines aquaculture with hydroponic growing methods to maximize resource

efficiency (Little, 2020).

The primary components of a basic aquaponic system include a fish culture tank, biofilter, hydroponics tank, and a sump tank “depositing” (i.e., total feed tank moisture with nutrient replenishment) (Mohapatra et al., 2020). Regularly, the solids can be removed by removing silt from the sedimentation tank or biofilters. The biofilter is a section in an aquaponic system in which most of the beneficial microorganisms, includingnitrifying bacteria, can grow and make nitrogen available to plants (Bracino et al., 2020). The biofilters can be shaped into a variety of forms that can give additional contact surfacearea for the growth of nitrifying bacteria. One might anticipate a shorter maturation periodfor the biofilter when using an inoculum that is already mature. However, the rapid expansion of new biofilm will be restricted due to the intensive competition of resources. (Bracino et al., 2020).

Aquaponic systems are classified into coupled and decoupled systems depending on whether the water is recirculated or not. A fish tank connected to the plant component system may have water recirculated into the aquaponic system in a closed loop, making acoupled system. Decoupled systems demand more water than coupled systems but allow for greater water quality management, leading to higher vegetable yields (Mohapatra et al.,2020). The design of an aquaponic system requires previous knowledge of the size of the facilities and the goal that they will be designed to achieve, each of which gives a referencefor classifying into the three most common techniques of aquaponics farming: media-based growing bed (MBGB), deep water culture (DWC), and nutrient film technique (NFT) (Hao et al., 2020).

The MBGB uses inert solids such as cocopeat, perlite to grow plants, providing root stability and, natural biofiltration. MBGB is less expensive and uses less space but isdifficult to clean and maintain when used in small designs. The DWC system refers to a float raft culture in which the roots of plants are totally submerged in water. The advantageof DWC is that it is easier to clean but it requires more biofilters, aeration devices, and water volume. The

NFT system is the most common used on commercial operations for large scale production in aquaponic and hydroponic systems, where the roots have limitedwater and nutrient contact with low initial cost. NFT offers a greater water usage efficiencybut a lower yield than conventional farming methods to boost plant output, maintain waterquality, and promote fish development (Bracino et al., 2020).

Fish species commonly used in aquaponic systems include tilapia (*Oreochromis niloticus*), catfish (*Clarias gariepinus*), carp (*Cyprinus carpio*), trout (*Oncorhynchusmykiss*), and pacu (*Piaractus mesopotamicus*) (Love et al., 2015). In that order of frequency of usage in commercial systems, tilapia is the most often grown species due to its great tolerance to suspended particles, levels of nitrite above 44.67 mg/L1, and low oxygen concentrations (Cifuentes-Torres et al., 2021). High nitrogen (N) concentrations are available from fish tanks to plant nutrition and shorter growth periods. Relatively low fertilizer requirements promote green vegetables as the most often grown crops in thesystem (Cifuentes-Torres et al., 2021). Commonly used vegetables in aquaponics include tomatoes, leafy greens, herbs, peppers, and cucumbers (Love et al., 2015).

In the United States, hydroponic is more predominant than aquaponic production. Despite the large interest in the production system, large-scale commercial aquaponic operations are difficult to maintain. Aquaponics has grown in popularity in recent years,as well as the number of operations in the United States. However, most of these operationsare small-scale farms (Quagrainie et al., 2017). To consider aquaponics as a sustainable method of food production, a grower needs to consider the "triple-bottom-line", which means environmental, economic, and social implications (Li et al., 2018). Prior to investingin large-scale systems, operators must consider all these factors in addition to accessibility and cost of inputs (i.e., facility maintenance and fish nutrition), cost and reliability of powersupply, and access to an industry willing to pay premium prices for locally grown and pesticide-free vegetables (Li et

al., 2018).

2.6 Vertical Farm

In the twenty-first century, vertical farming grew from single bio-skyscrapers to massive vertical food production buildings for commercial purposes, such as greenhousesto produce hydroponics and container farms with artificial light to grow plants. These systems are supported by cutting-edge technologies that enable rapid growth and plannedproduction in which renewable energy sources supply heat and lighting (Zareba et al., 2021). The vertical farming business in the United States accounted for over 2,000 operations in 2019. Small indoor vertical farms are more prevalent than large-scaleoperations. Vertical farming's market worth was predicted to be $226 million in 2018. Its anticipated value in 2026 is more than six times that amount, or roughly $1.4 billion (Piechowiak, 2019).

Vertical farming is essentially an indoor farm built on a multi-story high-rise manufacturing concept. Typical features include the use of reclaimed water supplementedby rainfall, automated air-temperature, and humidity control, solar panel lighting and heating, and adjustable 24-hour LED lighting. Throughout the growing season, the LED equipment may be set to generate a specific spectrum of light that is best for photosynthesisin various crops, without the need for natural light (Benke & Tomkins, 2017).

Utilizing customized growth media allows for the protection of land and water resources and the achievement of sustainable outputs. In the current scenario, soilless agriculture could be initiated and considered as an alternative method for cultivating healthy food plants, crops, or vegetables (Salim Mir et al., 2022). Air conditioner systemsmaintain a continuous flow of air that can be supplemented with carbon dioxide to promote plant development and growth. Both ambient and nutrient solution temperatures can be kept at specific levels to maximize plant growth rate. Any nutrients and water that the rootsdo not receive can be recycled rather than lost to the system (Benke & Tomkins, 2017).\

Vertical farm systems are an example of proactive thinking that tries to assure the long-term viability of cities by tackling the issue of food security while minimizing exposure to environmental conditions. The cost of food is surging due to rising in oil prices, water constraints, and the depletion of other agricultural resources, which show a viable option for food production in vertical farms (Piechowiak, 2019).

2.7 Food Safety

Unsafe foods represent a risk and have an economic impact globally, including contamination by pathogenic microorganisms or chemicals. Technologies, regulations, and risk mitigation strategies for improving food safety are essential in public health (Funget al., 2018). Food contamination can occur via infectious organisms in food that release toxins into the digestive system or itself can penetrate and cause illness. Bacteria, viruses,parasites, protozoa, and fungi are the agents behind foodborne illnesses causing intoxications, infections, and other health issues (Gallo et al., 2020). Bacteria such as *Escherichia coli, Salmonella enterica, Campylobacter* spp., *Vibrio cholera*, and *Listeria monocytogenes* are responsible for most of the foodborne illnesses in the United States. Foodborne illness can occur from consuming under cooked meat, poultry, eggs, and poorly pasteurized milk. Fresh fruits and vegetables can also be sources of contamination becausethey are commonly consumed raw. Poor water quality can contribute to fresh produce contamination. Symptoms of foodborne illnesses include fever, headache, nausea, vomiting, abdominal pain, and diarrhea. In some cases, health complications can occur in sick individuals leading to hospitalizations and even death (World Health Organization, 2020).

According to the World Health Organization (WHO), microorganisms and chemicals are responsible for nearly 200 diseases, leading to 600 million people illnesses and 420,000 deaths per year. Children under 5 years represent 125,000 of these cases (World Health Organization, 2020). In the United States, the Center for Diseases Control and Prevention

(CDC) estimates 48 million cases every year of foodborne illness in which 9,4 million is caused by pathogenic microorganisms. Viruses represent high cases of disease transmission through food and water. Hospitalization is usually related to bacterialorganisms, making food safety a public health concern (Fung et al., 2018).

Food can become contaminated at any point of the food production chain from farm to table. Mandatory regulations have been established to ensure that foods are safely produced and handled and are free from contamination, reducing the likelihood of illnesses. Most foodborne diseases are associated with foods from questionable safety practices, including production processes with poor individual hygiene, insufficient food sanitation, and inadequate storage temperature (Nguyena, 2021). Fruits and vegetables account for nearly half of foodborne outbreaks in the United States, mostly due to the lackof a “kill step” to eradicate microbial hazards (Mohammad et al., 2019). These outbreaks are bound to have an impact on the economy as well as the productivity and health of the people. Foodborne disease in the United States has been linked to outbreaks of *S. enterica, Escherichia coli* O157:H7, and *Listeria monocytogenes* contamination of fruits and vegetables since 1998 (Gálvez et al., 2021). As a result of fresh produce outbreaks linked to tomatoes, spinach, and lettuce, producers are taking preventive steps to ensure that theirfresh products are safe to consume. Initiatives of Good Agricultural Practice (GAPs) have been formed in response to the outbreaks, and the FDA has implemented measures to identify possible sources of contamination and recommend preventative actions as part of contamination reduction (Bennett et al., 2018).

In 2011, the FDA established the Food Safety Modernization Act (FSMA) to regulate foods and beverages of human and animal consumption. In 2016, as part of the FSMA, the FDA published the Produce Safety Rule (PSR) with standards for growing, harvesting, packing, and holding produce for human consumption (Food and Drug Administration, 2021). The regulation specifies best practices for preventing hazardous disease contamination of

"covered produce," which is defined as produce consumed uncooked. Growers that comply with the regulation must achieve specified criteria for theuse of organic soil additives of animal sources, personnel hygienic procedures, microbial water quality, cleaning and sanitizing of equipment, and monitoring wild and domesticatedanimals' activities in the farm environment (Misra & Gibson, 2021).

The PSR excludes small farm operations, local farms, or fresh markets. The regulation does not apply to farmers whose average yearly produce sales over the preceding three years were less than $25,000, which includes most hydroponic and aquaponic farm operations in the Southeast. Thus, it's vital to address food safety concerns for these farmers, especially due to their unique setting systems and limited available resources for CEA operations (Fusco et al., 2022).

2.8 Food Safety in CEA Systems

Fruits and vegetables cultivated in CEA are often seen to be safer to eat than field-grown produce since it is not exposed to the soil or any wild animals. Water, substrates, and human interaction are just a few of the potential entry points for human pathogens into CEA systems (Gómez et al., 2019). Since CEA often utilizes recirculating nutrient solutions, it is potential for human pathogens to be introduced into the solutions (i.e., agricultural water or nutrients) to quickly spread throughout the production system. Studiesconducted in hydroponic systems demonstrate that some microbes, such as *Escherichia coli* O157:H7, *E. coli* non-O157 STEC, and *S. enterica*, can live and reproduce even after being introduced into the system for a short period of time (48 hours) (Gómez et al., 2019).In 2021, *S. enterica* Liverpool was identified in a lettuce farm's indoor pond. No one became sick from this strain. The epidemic strain of *S. enterica* Typhimurium was also detected in a farm pond as well as a genomic match to the strain that. associated to the consumption of pre-packaged leafy greens grown by Bright Farms and sold by thecompany at its Rochelle, Illinois plant. (CDC, 2021).

Produce contamination related to food safety may occur during production, harvest, handling, wholesalers storing, transportation, sale, and cooking. Physical factors (e.g., substrate, water, air, post-harvest apparatus), wildlife, or operators are also sources of contamination (Riggio et al., 2019). Food safety and animal health are important concernswhen it comes to obtaining public preference for an aquaponic system. One of the most common concerns raised by food safety experts over aquaponics is the risk of foodborne contamination when using fish effluent as plant fertilizer (Joyce et al., 2019). The FDA isresponsible for enforcing the provisions of the FSMA PSR, which regulates the water quality used by aquaponic farmers in the United States (Fogarty, 2021).

Water is an important part of all production systems, and many *E. coli* O157:H7, *S. enterica* outbreaks are linked to contaminated water. Also, water sources can be put intothree groups: low-, medium-, and high-risk. Even though cities and towns test their waterfor *E. coli* on a regular basis, municipal water sources are still thought to be low risk. Ground water is relatively safe compared to open water sources, but it requires a proper constructed and maintained system to avoid infiltration and water contamination. Water sources like ponds and streams are high-risk because these sources are open to the environment and can easily become contaminated (Gómez et al., 2019). Thus, water testing is essential to providing information on the microbial quality of the water used to grow produce. Through food safety evaluations and advancements in quality control programs, urban agriculture (UA) such as vertical farms, aquaponics, or hydroponics facilities that use hazardous inputs must constantly evaluate the potential of bacteria contaminations on their harvest and choose water sources that do not pose high risks to produce safety. Water and nutrient solution quality parameters should be regularly evaluated (e.g., implementing sensors for water quality parameters) (Buscaroli et al., 2021).

2.9 Microbial Water Quality in Recirculating Systems

Water is the major contributor to microbial contamination in fruits and vegetables

(Lynch et al., 2009). Water contamination from various sources includes feces from animals, birds, and poor employee's hygiene (Goddard and Fatma, 2018). Water that is used in aquaponic and hydroponic systems can pose a risk to produce safety. Currently, there is no specific regulation that specifically applies to these operations in terms of waterquality. The PSR regulates agricultural water in the subpart E for all covered produce farms, regardless of the farming method (Sallenave, 2016). In aquaponic operations, water has a crucial role including different factors able to affect the system such as water sourceswhich can impact microbial quality and chemical composition. For example, municipal water contains chlorine and chloramines that must be removed before use. Regardless of the water source, growers must check the quality of the water, including nutrient analysis and microbial to verify if it can support fish and plants growth and produce safe food (Sallenave, 2016).

The Subpart E of the PSR has changed over time, where the initial approach of the regulation required produce growers to evaluate generic *E. coli* levels in water used for fresh produce activities; if such water is meant for or expected to meet a product that is subject to regulatory oversight. Initially, growers were required to develop a microbial water quality profile (MWQP) with generic *E. coli* levels of or below 126 CFU in 100 mL of water for the geometric mean (GM), and a statistical threshold value (STV) of 410 or fewer CFU of generic *E. coli* in 100 mL of water (FDA, 2017). However, in December 2021, the FDA has proposed changes in the new agricultural water requirements where microbial tests becomes optional, but it is highly recommended as part of a new water assessment (FDA, 2022).

To overcome the obstacles of water contamination issues, growers are currently adopting water treatment to their water sources, including the use of chemical sanitizers, and physical devices like membranes and UV-light systems (Riggio et al., 2019). However,these approaches cannot be used in aquaponic systems because it can affect the biofilter efficacy. Common pathogenic foodborne bacteria such as *E. coli, S. enterica* , and *Campylobacter*

appear to be increased by organic matter and chemicals like phosphate, while physical interference such as solar radiation appears to reduce some bacteria such as*Vibrio, Shigella flexneri, E. coli*, as well as *S. enterica* Typhimurium (Rodrigueset al., 2019). Additional factors that impact the microbial quality of water include physicaland chemical factors, such as water temperature that affects the fish health, plant development, and biofilter efficacy (Sallenave, 2016).

2.10Salmonella

Salmonella is a gram-negative, rod-shaped bacterium that belongs to the Enterobacteriaceae family (Schneider et al., 2018). *S. enterica* is one of the specie responsible for 1.2 million illnesses and 450 deaths per year in the United States (Sodagariet al., 2020). These bacteria are aerobic and facultatively anaerobic, oxidase negative for the most part, and negative to urease which means they survive and are resistant in different conditions of temperature, air availability, and antibiotics. There are over 2,500 different serotypes of *S. enterica*, of which 2,000 may cause infection in humans and are classified as pathogenic (Sodagari et al., 2020). *S. enterica* infections may be divided into two main categories: nontyphoidal and typhoid *S. enterica*, which include gastroenteritis-causing bacteria like *S. enterica* Enteritidis and *S. enterica* Typhimurium (Johnson et al.,2018).

S. enterica has been a public health concern since it is linked to human illnesses. Gastroenteritis, fever, and bacteremia are among the most common symptoms caused by *S. enterica* infections, as well as moderate watery diarrhea (Kadhi, 2020). *S. enterica* virulence factors are encoded in specific loci known as *Salmonella* Pathogenicity Island. At various stages of infection, virulence factors are implanted, and coding is conducted, transporting proteins from the afflicted cells to the cytoplasm of the host cells. For bacteria to survive within the body, they must exchange proteins (Askoura et al., 2021). Liao et al. (2019) found that the pathogenicity of *S. enterica* is affected by its genome, which comprises characteristics that

come from evolution and genetic virulence.

Prevention of *S. enterica* contamination includes cooking food at high temperatures; pasteurization; storing food at the appropriate temperatures; and hygiene when processing food (Schneider et al., 2018). Antimicrobial treatments are adopted to manage illnesses caused by *S. enterica* infections. Even if some drugs, such as cotrimoxazole, chloramphenicol, and ampicillin, are no longer the primary substances in the treatment due to the resistance presented by *S. enterica*, drugs such as cephalosporins and fluoroquinolones have become effective in this case. However, due to an increase in antibiotic use for treatment and in the food industry, the population of bacteria has emergedand disseminated with resistance to the controls currently adopted in the past few years asa result of multidrug resistance (Breurec, 2019). Multidrug resistance represents 8% of *S. enterica* species that are resistant to three or more types of antimicrobials or are not affected by antibiotic treatments (McMillan et al., 2019).

2.11 Fresh Produce Outbreaks Caused by S. enterica in the United States

Foodborne diseases are commonly associated with fresh produce, and several countries have reported outbreaks caused by *S. enterica*. *S. enterica* Newport comes in third place after *S. enterica* Enteritidis and *S. enterica* Typhimurium, among the main *S. enterica* serovars linked to foodborne disease in the United States (Gurtler et al., 2018).

Foodborne outbreaks related to *S. enterica* account for nearly 1.2 million illnessesand 540 deaths annually in the United States (Gurtler et al., 2018). *S. enterica* contamination is commonly associated with chicken and eggs, but fresh produce is one ofthe major sources of Salmonellosis affecting Americans. The consumption of fresh tomatoes has been previously linked to several foodborne outbreaks related to different *S. enterica* serovars (Gurtler et al., 2018). In 1990, a multistate outbreak linked tocontaminated tomatoes resulted in 176 illnesses caused by *Salmonella* Javiana affecting four Midwest States in the United States (CDC, 2007). Three years later, the same four states were implicated in a subsequent outbreak causing 100

Salmonellosis cases linked to tomatoes (CDC, 2005). In 1999, the rare *S. enterica* Baildon was linked to a tomato outbreak in California, affecting 86 individuals (Gurtler et al., 2018). Packinghouseoperations were responsible for two tomato outbreaks between 2004 and 2007 from *S.enterica* contamination in the United States (Boltena et al., 2018). Another incident was linked to *S. enterica* Braenderup contamination of tomatoes in 2005 in eightstates (West Virginia, Pennsylvania, Michigan, Massachusetts, Ohio, Kentucky, Illinois, and Indiana). In 2008, *S. enterica* Saintpaul related to tomatoes was responsible for over 1,000 illnesses and 286 hospitalizations in the United States (CDC, 2008)

Another crop that has been previously linked to *S. enterica* contamination was cucumber. In 2015, a routine test was positive for *S. enterica* Newport in cucumbers (Dyda et al., 2020). Contamination of *S. enterica* Saintpaul was identified in 2013 in the United States from Mexican cucumbers, and the outbreak resulted in 17 hospitalizations out of 84cases (CDC, 2013). In 2014, *S. enterica* Newport was linked to contaminated cucumbers leading to 275 individual cases, 48 hospitalizations, and one death in 29 states (CDC, 2015). Between 2015 and 2016, multistate Salmonellosis outbreaks were reported to havecaused 907 people to be contaminated, 204 hospitalizations, and the deaths of 6 individualsfrom eating contaminated cucumbers (CDC, 2016). Still, in 2016, an outbreak in the UnitedStates sickened 14 people and led to two hospitalizations from cucumbers contaminated with *S. enterica* Oslo in 8 states (CDC, 2016a).

Sprouts have also been documented as the source of *S. enterica* outbreaks in the United States. In 2009, a *S. enterica* Newport outbreak led to 235 reported cases (CDC, 2009). In 2010, sprouts contaminated with *S. enterica* caused 44 illnesses (CDC, 2010). Between 2010 and 2011, sprouts have been linked to 140 illnesses caused by *S. enterica* (CDC, 2011). In 2016, two multistate outbreaks linked to alfalfa sprouts contaminated with different *S. enterica* serovars occurred in the United States, leading to 62 cases and 15 hospitalizations in total

(CDC, 2016b, 2016c). In 2018, *S. enterica* Montevideo sickened 10 individuals that consumed raw sprouts from three states (CDC, 2018).

Although foodborne outbreaks linked to leafy greens are normally caused by *E. coli* infections in the United States, *Salmonella* has been implicated in some casesincluding the first outbreak documented in CEA. Lettuce outbreaks of *Salmonella spp*. contamination was linked in 2009, 2013, and 2015 with 162, 15, and 42 cases of illness, respectively, in the U.S. (Krishnasamy et al., 2020). Romaine lettuce was reported as the source of *S. enterica* in 2017, causing 151 illnesses and 31 hospitalizations in 36 states inthe United States (CDC, 2017). Foodborne infection linked with *S. enterica* in leafy greens occurred in a controlled growing system in 2021, as the first case related to the indoor hydroponic system in the United States, which resulted in 31 illnesses and 4 hospitalizations (FDA, 2022).

Fresh onions have never been reported in previous foodborne outbreaks until September 2021, when onions imported from Mexico contaminated with *S. enterica* Oranienburg caused 1040 illnesses and 260 hospitalizations in the United States (FDA, 2022).

2.12 Sources and Colonization of Salmonella in Plants

Fresh produce can be contaminated at any point in the food supply chain, however,most contamination occurs in the field and during harvesting post-harvesting practices. Inthe field, produce can become contaminated via water, soil, animals, and humans. Irrigation water has been one of the major sources of produce contamination (Liu et al., 2018). During harvesting and post-harvesting practices, water, and contaminated food- contact surfaces pose the greatest threat to produce safety. Workers can spread microorganisms during produce handling, such as during harvest and postharvest operations, which has the potential to contaminate fresh produce with poor hand hygiene (Koukkidis and Freestone, 2018). In bagged salads, the moisture in the bag can promote *S. enterica* growth over 3 logs if exposed to temperatures higher than 5º C (Koukkidis and Freestone, 2018).

S. enterica is an opportunist bacterium with high adaptability to colonizing several plants in a variety of settings (Ku et al., 2019). *S. enterica* produces enzymes responsible for breaking down plant tissues in its periplasm. Meanwhile, pectin, polygalacturonate, and weakened stomata are required for plant penetration. *S. enterica* may colonize hydathodes and roots as well (Ehuwa et al., 2021). Leafy greens are primarily composed of water and carbohydrates. After harvesting, cell degradation can occur quickly, and microbial colonization by spoilage might increase the *S. enterica* population (Koukkidis and Freestone, 2018). Previous studies have demonstrated that *S. enterica* Typhimurium can survive in lettuce for 63 dayswhile in carrots, radishes, and parsley bacteria can survive up to 260 days (Kljujeva et al.,2017). *S. enterica* may infiltrate tomato fruits through infiltration, leading to *S. enterica* internalization to the fruit (Kljujeva et al., 2018).

S. enterica can grow in extreme environmental conditions, including low temperature, low pH, and have the ability to adapt to diverse hosts via lipopolysaccharides (LPS) and nucleotides, such as amino acid biosynthesis, being considered a persistent pathogen in foods (Liu et al., 2018). *S. enterica* can exhibit possible survival for a periodof days in different surfaces, such as on tomato tissues for 18 days and the exponential growth decreases after 7 days (Dyda et al., 2020).

2.13 Reduction of Vegetable Contamination by Salmonella

The key to reducing *S. enterica* contamination is the prevention of infections transmitted through food. To prevent contamination in fruits and vegetables proper storageat adequate temperature is required, also adding a sanitizing process to post-harvest washing helps to minimize microbial hazards (Koukkidis and Freestone, 2018). One of thevarious methods that may be used to reduce pathogenic load and prevent cross- contamination is chorine. Following the guidelines for tomato wash with 25 ppm of free chorine, *S. enterica* might be effectively controlled; however, cross-contamination may be avoided by using 100 ppm of free

chorine (Bolten et al., 2019).

Other sanitizing methods include high-pressure processing, UV radiation, and temperature treatments. Sanitizing methods can also be used to treat agricultural water forcrop production. Fruits and vegetables in general can be effectively washed with chorine and other antimicrobial chemicals when the appropriate concentration is used in a post- harvest process. More recently, biological control is another method being used to reduce or control *S. enterica* contamination such as the use of *Lactobacillus plantarum* and phage (Arena et al., 2016).

2.14 Biological Control of Salmonella

Biological control, commonly known as biocontrol, is the employment of biological agents (including fungi, bacteria, viruses, or insects) to manage pathogenic organisms for a wide range of human-beneficial purposes (Stenberg et al., 2021). Biological control also includes the interactions among microorganisms that cause growthand nutrient competition. Biological control has the potential to manage the mechanism of *S. enterica* contamination which is an advantage when it comes to providing safe vegetable production without the use of chemicals (Stenberg et al., 2021).

Several studies have reported the use of antagonist drugs to control harmful organisms in humans, which have the potential to be used to minimize foodborne infections. One example of this is the use of *Pseudomonas* strain to reduce the populationof *S. enterica* on alfalfa seeds by 1-2 logs while they are sprouting (Liao, 2008). For controlling *S. enterica*, effective control was achieved when lactic acid and bacteriocin derived from *Lactobacillus* spp. were used in meat products (Liu et al., 2021). In addition, *L. lactis* was found to be a colonizer and persistent reducer of *S. enterica* growthinside the rinds of melons without causing any phytotoxic effects. *L. lactis* is frequently used in the preparation of food, which results in its secure usage as a biocontrol agent (Mcgarvey et al., 2019).

Phages are used as a biocontrol against *S. enterica* Enteritidis. In carrot salads, experiments had shown those phages as a reducer considering the high contamination related to this vegetable (Kumar et al., 2020). On fresh cucumbers, a commercial bacteriophage cocktail outperforms chlorine treatment for *S. enterica* Newport population reduction (Zhang et al., 2019). In this case, phages were more efficient at 2 and 10°C than at 25°. In another study, the effectiveness of phage therapy was lost after 72 hours of immersion treatment on lettuce and sprouts with the phage cocktail "SalmoFresh TM" against *S. enterica* population and decreased by 1 log CFU/g *S. enterica* growth (Zhang et al., 2019).

2.15 Bacteriophage Overview

Bacteriophage is the most abundant virus on earth that colonizes bacteria and can be used to reduce the risk of human infections (Gurtler et al., 2018). The antimicrobial application of phage therapy offers the benefits of self-replication and non-toxicityin the environmental system. The virus has shown effectiveness against multidrug-resistant bacteria and biofilms, targeting pathogeneses-requiring host receptors to multiply strains utilized in food prevention (Sieiro et al., 2020a).

Bacteriophages have started to be applied in industrial applications for the management ofplant disease, animal health, food conservation or safety, and ecosystem biocontrol (Pathak-Vaidya et al., 2021). The European Union is considering phages as food additives.Each bacterial cell is expected to have several phages able to control the bacterial population. The mechanisms of action to control *S. enterica* consist of the phage promotingmultiplication and disintegration by rupturing the cell barrier or membrane within bacteria. The virulence of phages can be responsible for controlling the bacteria without replication.Bacteriophages can be used in both the processes of pre-harvest and postharvest and has been shown to reduce 90% of *S. enterica* in the lytic cycle life of bacteriophages in lettuce and cantaloupe (Mogren et al., 2018).

The life cycle of bacteriophages is a crucial characteristic to determine the efficiency

of bacterial control (Tikunova et al., 2022). The equilibrium of bacterial proliferation in the environment is maintained through the phage lytic life cycle (López-Cuevas et al., 2021). The difference (**Figure 2.14.1**) in their behavior in host cells is based on their ability to lyse the bacterial cell among lytic and lysogenic cycles (Tikunovaet al., 2022). During the lytic cycle, bacteriophages infect a bacterial cell and use its biosynthetic and genomic machinery for progeny production and lysis of the bacteria. In the lysogenic cycle, the viral genome fuses with the bacterial host, leaving behind multiple signatures such as virulence and antibiotic resistance genes (López-Cuevas et al., 2021). Enzymes called lysins are created by bacteriophages that can infect the bacterial cell wall. However, bacterial metabolism determines whether the virus can reproduce. After phages identify a host and consume its supplies, they kill the bacteria by releasing their progeny (Ramos-Vivas et al.,2021). The scientific community has recognized 22 official families of bacteriophages based on their morphological and genetic characteristics (López-Cuevas et al., 2021).

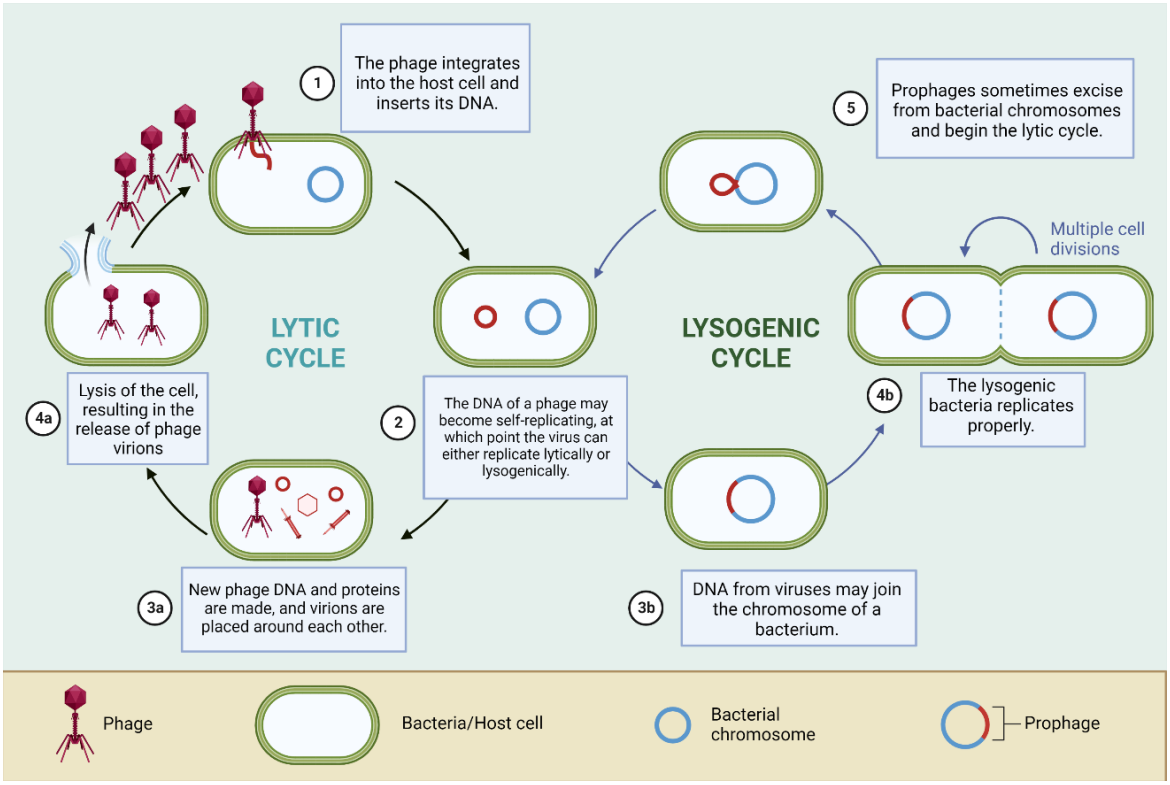

Figure 2.14. 1 Lytic and lysogenic bacteriophages cycles. Adapted from "Lytic and Lysogenic Cycle" by BioRender.com (2022). Created with BioRender.com

Strains that are host-specific are categorized as monophagous or poli phages and are able to recognize one or more receptors with pattern-recognition receptors (PRRs), which are responsible for detecting molecular patterns associated with pathogens, may be stimulated when bacteriophages are present in phagosomes or endosomes (Bodner et al., 2021). Toll-like receptor 9 (TLR9) is a DNA detector that particularly looks for unmethylated 5'—C—phosphate— G—3 (CpG) dinucleotides inside endosomes (Bodner et al., 2021). The life cycle of phages is a crucial characteristic to determine the efficiency of bacterial control. The difference intheir behavior in host cells is based on their ability to lyse the bacterial host between lyticand lysogenic cycles (Tikunova et al., 2022).

Due to the limited lytic spectrum and host range of many bacteriophages, a single bacteriophage cannot be used to control a single bacterial cell. Bacterial strains may gain resistance to phages due to alterations in their bacteriophage receptor (s) or other mechanisms (Tikunova et al., 2022). Different bacteriophage strains may be needed to treat the same bacterial disease. Reduction of the treatmenteffects (including during a treatment) results in bacteriophage cocktail applicability (by discoveryor adaptation/engineering). Several phage combinations are tested to handle resistant variations of bacteria as a strategy over the cocktails, providing safety testing and regulatory approval (Nikolich & Filippov, 2020). Approaches are used for developing phages treatment in pre-defined phage cocktails with consistent formulations. Phage cocktail banks and individual phages allow phages can handle bacterial resistance (Nikolich & Filippov, 2020). Phage cocktails are more effective at targeting a broad range of bacteria and conditions than individual phage isolates. They may also improve the treatment of different bacterial strains or species (Tikunova et al., 2022).

Bacteriophages isolated from the environment, also known as wild phages, are commonly used in recent research studies because using well-established procedures, bacteriophages may be target bacterial hosts with relative ease and low cost. Commercial

bacteriophages are used as preventive bacterial contamination. Commercial bacteriophage therapy does not include any chemicals or preservatives either. Instead, they are mostly water-based solutions with purified bacteriophages and low salt levels (Moye et al., 2018). The bioactivity of lytic bacteriophages is an attractive method for improving the safety of foods since bacteriophages are found in abundancein the environment and are non-toxic. Organizations and industries are immersed in bacteriophage biocontrol development as a reflection of popularity and control approval against bacteria (Moye et al., 2018).

Bacteriophage instability in the environment is a result of external influences such as temperature, dehydration, and UV light. Bacteriophage sensitivity is one of the primary obstacles to applying agricultural controls. The profitable applicability of bacteriophages on a broadscale in the natural environment is one approach to a biological solution to bacterial diseases, but there is a need for further studies (Sieiro et al., 2020). Some bacteriophages are sensitive to multiplying in the environment, even though they have a lytic circle of life. *Invitro,* research has been widely used to ensure their efficiency and validate the specificity of each bacteriophage (Tikunova et al., 2022). The advantages and disadvantages of bacteriophage therapy is related to validation in methods on a large scale, whether by injection, as a food additive,or externally by immersion, and must be evaluated as in industry trials showing that *S. enterica* after 24 hours was unable to achieve exponential growth, as a bacteriophage control result (Hagens et al., 2019). Guidelines for the use and development of bacteriophage therapy in a few years against bacterial diseases are the goals of current research (Sieiro et al., 2020).

Several studies have explored the use of bacteriophage to control different strains of *S. enterica*. The efficacy of phage therapy is variable between the strains, as log reduction can be low in a specific strain and high in another, as they correlate with the host plant as well (Seo et al., 2018). A previous study demonstrated that bacteriophage therapy against *S. enterica* Enteritidis in carrots had a reduction of 1 log CFU/cm^2 after 4 hours of incubation (Kumar et

al., 2020). On fresh cucumbers, a commercial bacteriophage cocktail outperforms chlorine treatment in terms of *S. enterica* Newport reducing up to 5 log CFU/mL (Zhang et al., 2019).

Bacteriophage cocktails are currently being tested to overcome the obstacles of microbial resistance to a single bacteriophage and to amplify the control of foodborne illness using the potential strains of the target species (Wong et al., 2020). Thus, more studies are necessary to evaluate the use of bacteriophages as a biocontrol method to prevent *S. enterica* contamination in ŒAproduction and provide safe products for a growingpopulation.

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Chapter II

3.0 Research Justification

This research aims to establish a sustainable food safety strategy to control microbial contamination in vegetables grown under CEA systems. Compared to conventional farming systems, growers do not have a sustainable and effective method for controlling foodborne pathogens in CEA systems other than chemical sanitizers. Currently, chemical sanitizers such as chlorine and peroxyacetic acid are utilized to prevent microbial contamination of irrigation water in the field (Mendoza et al., 2022). However, the use of chemical sanitizers in aquaponics might have an adverse effect on the beneficial bacteria necessary for plant growth and development. Additionally, local production and pesticide-free products have an important role in consumers' choices. Thus, sustainable alternatives to chemical sanitizers are required for CEA systems in order to reduce the use of chemical pesticides, maintain the microbiological balance in the ecosystem, and reduce food safety risks.

It has been proposed that CEA systems, including hydroponics, aquaponics, and vertical farming, can be built to maximize productivity per unit space (Gruda et al., 2019). Despite their modest size, indoor farms can produce year-round fresh produce on a limited plot with low environmental impact. Additionally, indoor gardening is on the rise in the southeast as a result of consumers' demand for locally grown fresh vegetables. Some vegetables, such as leafy greens, are difficult to cultivate outdoors due to hot and humid summers; hence, hydroponics and aquaponics have emerged as viable alternatives.

Changes in agricultural methods, such as indoor cultivation, require the adoption of new management techniques in order to keep plants free of diseases and pests, provide nutritious and safe food, and protect environmental health. The interaction between water

and bacteria is of public concern and increases the risk of disease-causing contamination, as bacteria's ability to adapt to water serves as a static reservoir for foodborne pathogens (Ferelli & Micallef, 2019). Foodborne infections, including pathogenic *E. coli* and *S. enterica*, have been detected in aquaponic and hydroponic water systems, demonstrating that these farming methods are not immune to microbial contamination (Sawyer, 2021).

Globally, foodborne diseases are the leading cause of morbidity and mortality. Each year, nearly half (46%) of foodborne illnesses in the United States are caused by the contamination of fresh produce (Fung et al., 2018). Due to prior foodborne outbreaks that resulted in tens of thousands of illnesses, leafy greens were the principal fresh produce commodity of concern for contamination.

Food safety concerns in vegetable agriculture have reached CEA operations. In 2021, lettuce cultivated hydroponically indoors was the source of 33 *S. enterica* Typhimurium-related infections and 4 hospitalizations (FDA, 2022). Water is a major source of produce contamination, particularly in CEA systems; however, chemical water treatments are currently available for field crops and CEA except for aquaponics. The use of chemical sanitizers in aquaponic systems is minimal due to the integration system with fishes, requiring other measures to manage human infections such as *E. coli* and *S. enterica*.

Bacteriophage is a pervasive virus that can infect, replicate, and restrict the growth of bacteria. Bacteriophage habitat is where bacterial organisms live, including water sources (Mogren et al., 2018). As a biocontrol agent, the usage of bacteriophage in the food sector has significantly increased during the past few years. However, the application of bacteriophages to control foodborne pathogens in CEA systems has not yet been investigated. bacteriophages can be utilized in both preharvest and postharvest procedures to suppress foodborne pathogens without affecting the native microbiota of

the system. Consequently, the creation of bacteriophagecocktails has the potential to target specific pathogenic microorganisms in aquaponic, hydroponic, and vertical farming systems and prevent crop contamination and future foodborne outbreaks.

This approach will provide a valuable and sustainable option for growers to prevent foodborne illnesses linked to fresh vegetables. Bacteriophage therapy has not shown any negativeeffects on the environment or the health of plants, animals, or humans. In the southern United States, CEA is significantly increasing. Also, this practice will raise the alternatives with sustainable agents, contributing to reducing the use of chemical pesticides whileimplementing effective food safety strategies.

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Chapter III

4.0 Objectives

The main objective of this research is to investigate the use of a bacteriophage cocktail as a biocontrol for *Salmonella enterica* in recirculating systems of vegetable production. Thespecific objectives of this project are:

Objective 1 – Evaluate the effectiveness of bacteriophages against *S. enterica serovars* under different conditions.

Objective 2 – Evaluate the bacteriophage cocktail efficiency against *S. enterica* in a recirculating water system for reduce or eliminate bacterial population.

Chapter IV

5.0 Evaluation of Lytic Bacteriophages to Control *Salmonella enterica* under Different Concentrations and Temperature Conditions

5.1 Abstract

There is a need to identify novel ways to control disease due to *Salmonella enterica*. This study isolated, characterized, and evaluated three virulent bacteriophages (S7, S10, and S13) as biocontrol agents against a range of *Salmonella enterica* serovars, with a focus on *S. enterica* serotypes Braenderup, Enteritidis, Kentucky, Newport, and Typhimurium. These bacteriophages were identified as Myoviridae members of the Caudovirales order. Moreover, the effects of temperature, multiplicity of infection (MOI), *S. enterica* serovars, and bacteriophage interactions on bacterial growth inhibition were also determined. These phages effectively suppressed *S. enterica* growth, with significant effects observed due to temperature and MOI ($p < 0.05$). The growth inhibition was higher at 30 °C compared to inhibition observed at 25 °C or at 20 °C. The largest inhibitory effect was observed at MOI 0.1 Phage: Bacteria at all temperatures. Bacteriophages S7 and S13 showed stronger lytic activity than S10 ($p < 0.05$). *S. enterica* serotype Newport had the lowest growth among *S. enterica* serovars ($p < 0.05$) in the presence of phage treatment. A phage cocktail comprising these phages managed bacterial co-infections and reduced bacterial resistance. The highest reductions of 73.3 % for *S.* Braenderup, 48.0% for *S. enterica* serotype Enteritidis, 62.1% for *S. enterica* serotype Kentucky, 62.3 for *S. enterica* serotype Newport and 67.5 % for *S. enterica* serotype Typhimurium, occurred at MOI 1 at 30 °C. Among these serovars, *S. enterica* serotype Typhimurium exhibited the lowest growth rate ($p < 0.05$). These results highlight the potential of these bacteriophages as biocontrol agents against diverse serotypes of *S. enterica*, enabling phage-based food safety and public health

solutions. Bacteriophages have significant promise, but the study also highlights the need to address concerns including continuous phage exposure and environmental changes that may impair phage-based biocontrol efficacy.

Keywords: Bacteriophage, foodborne pathogens, *Salmonella enterica*, lytic.

5.2 Introduction

Salmonella enterica is a leading cause of foodborne illnesses, posing a significant threat to public health (CDC, 2023a). In the United States, *S. enterica* is estimated to cause 1.35 million infections annually, resulting in 26,500 hospitalizations and 420 deaths (CDC, 2023b). Food contamination can occur at any stage of production, processing, and distribution that can lead to foodborne outbreaks (CDC, 2022). While *S. enterica* outbreaks are commonly associated with animal-derived products such as meat, poultry, and eggs, it is well documented that fresh vegetables can also contribute to foodborne outbreaks (Sadekuzzaman et al., 2018).

Chemical sanitizers such as chlorine and peroxyacetic acid are commonly used in the produce industry to control *S. enterica*. However, there is a concern about the potential negative effects of these chemicals on human health and development of bacterial resistance (Giacometti et al., 2021). Additionally, the use of antibiotics in food production is discouraged due to their non-specific antimicrobial activity and potential long-term environmental stability (Chattopadhyay, 2014). As an alternative to traditional antimicrobial agents, bacteriophages, also known as phages, have emerged as promising biocontrol agents for controlling foodborne pathogens, including *Salmonella* spp., *Escherichia coli,* and *Campylobacter* spp. in food products (Golkar et al., 2014).

Bacteriophages are viruses that exhibit a selective ability to infect and kill bacterial hosts, preserving the quality of the final product and protecting the environment (Goodridge & Bisha, 2011; Summers, 2012; Zaczek et al., 2015). Phage are specific in their action, targeting only their host bacteria without harming humans, animals, or plants (Golkar et al., 2014; Principi et al., 2019). Moreover, bacteriophages have a self-replicating ability and can rapidly adapt to overcome bacterial resistance, especially when

used as a cocktail with multiple phage strains (Abedon et al., 2021). These characteristics make phages an attractive candidate for controlling foodborne pathogens, including *S. enterica*. Studies have shown that phage interventions can significantly reduce *S. enterica* contamination in various foods, including fresh produce (Guenther et al., 2012; Kocharunchitt et al., 2009) and meat products (Aguilera et al., 2022; Yeh et al., 2017).

The lytic activity of bacteriophage against *S. enterica* strains provides valuable insights to advance the development of bacteriophage-based control methods. To maximize the effectiveness of bacteriophages against multiple *S. enterica* serovars, it is important to have a thorough understanding of their optimal growth conditions. Studies have shown that different phages exhibit specific temperature and multiplicity of infection (MOI) requirements for optimal lytic activity against *S. enterica* serovars (Huang et al., 2018; Nale et al., 2021a; Robeson et al., 2014). Some bacteriophages exhibit higher activity levels at lower temperatures, while others demonstrate increased activity at higher temperatures (Huang et al., 2014;Abhisingha et al., 2020). Generally, a higher bacteriophage MOI leads to faster and more efficient bacterial lysis. However, a limited quantity of phage might be effective for achieving successful outcomes in bacteriophage therapy (Yin et al., 2018). The use of a bacteriophage cocktail is an alternative approach to prevent the emergence of bacteriophage-resistant bacteria when used as a biocontrol method.

Therefore, this study aimed to isolate and identify three *S. enterica*-specific bacteriophages from environmental sources. The single bacteriophages and a cocktail of bacteriophages were evaluated *in vitro* at different temperatures (20, 25, and 30 °C) and MOIs (0.001, 0.01, 0.1, 1, and 10) to determine the lytic activity against five *S. enterica* serovars Braenderup, Enteritidis, Kentucky, Newport, and Typhimurium. The lytic activity was assessed at 30-minute intervals over a total period of 6 h.

5.3 Material and methods

5.3.1 Bacterial Strains and Culture Conditions

The following *S. enterica* serovars were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA): *S. enterica* serovar Braenderup ATCC BAA-664, *S. enterica* serovar Enteritidis ATCC 13076, *S. enterica* serovar Kentucky ATCC 9263, *S. enterica* serovar Newport ATCC 6962, and *S. enterica* serovar Typhimurium ATCC 14028. The freeze-dried bacteria were resuspended in Nutrient Broth (BD Difco™ Nutrient Broth, Becton Dickinson and Company, Sparks, MD, USA) and incubated at 37 °C for 24 h. Overnight cultures (500 µL) were added to 500 µL of sterile glycerol:dH_2O (1:1 v/v; EMD Chemicals, Darmstadt, Germany) in 2 mL screw-cap tubes, mixed, and stored at -80 °C.

S. enterica serovars were gradually adapted to 50 ppm nalidixic acid (Sigma-Aldrich, Saint Louis, MO, USA) and 50 ppm rifampicin (Sigma-Aldrich, Saint Louis, MO, USA) according to the method described by Cimowsky et al. (2022). The adaptation process was conducted in triple sugar iron agar (TSI; BD Difco, Sparks, MD, USA), with the antibiotic concentrations increased by 10 ppm at each transfer and incubation at 37 °C for 24 h. The adaptation continued until the cultures were able to grow in TSI containing 50 ppm nalidixic acid and 50 ppm rifampicin.

5.3.2 Bacteriophage Propagation

Three bacteriophages (S7, S10, and S13; family in the Caudovirales order, Myoviridae) from Auburn University College of Veterinary Medicine (AUCVM) Diagnostic Bacteriology and Mycology Laboratory's collection of *S. enterica* isolates of clinical veterinary samples. Each phage was isolated and characterized by the Auburn University College of Veterinary Medicine. Bacteriophages were individually amplified

as described by Chen et al. (2018). The phage host propagating strain used in this study was *S.* Enteritidis ATCC 13076. Briefly, a 10 µL loopful of *S.* Enteritidis was added to 1.5 mL of Luria-Bertani Miller broth (LBM; Becton, Dickinson and Company Sparks, MD, USA) with a pH of 7.0. The culture was incubated for 24 h at 37 °C in a shaker (C24 Incubator Shaker, Edison, NJ, USA) at 150 rpm. Then, 125 µL of the overnight culture was transferred to 12.5 mL LBM broth (pH 7.0) supplemented with 150 µL of phage stock. The mixture was shaken for approximately 3 h at 37 °C until it reached an optical density measured at a wavelength of 595 nm (OD_{595}) of 1.0 (equivalent to approximately 3.0 x 10^8 colony-forming units per mL; CFU/ mL). The resulting culture was transferred to a sterile 50 mL centrifuge tube and mixed with 50 µL of chloroform (Sigma-Aldrich, Saint Louis, MO, USA). The amplified phages were then centrifuged at 10,000 x *g* for 20 min and filtered through a 0.20 µm membrane (Acrodisc® Syringe Filters, St Columb Major, Conmwall, UK) to remove bacterial lysates or debris.

5.3.3 Plaque Assay

The bacteriophage titers were determined using the double agar overlay plaque technique, following the method described by Jagannathan et al. (2020) and Panec & Sue Katz (2016). In brief, the amplified phages were serially diluted (1:10) with SM buffer at pH 7.5 (Thermo Fisher Scientific, Waltham, MA, USA), which consisted of 50 mM Tris-HCl, 8 mM magnesium sulfate, 100 mM sodium chloride, and 0.01% gelatin. Then, 10 µL of each diluted bacteriophage was added to 200 µL of *S. enterica* Braenderup, *S. enterica* Enteritidis, *S. enterica* Kentucky, *S. enterica* Newport, or *S. enterica* Typhimurium cells, each with an OD_{595} of 0.5 using a McFarland standard (Thermo Fisher Scientific, Waltham, MA, USA). This step was performed in triplicate.

The bacteriophage-bacteria mixtures were incubated in a water bath at 56 °C for 20 min, followed by mixing with 3 mL of soft agar (LBM plus 0.7% Bacto™ agar; BD

Difco, Sparks, MD, USA). The resulting mixture was then poured over pre-warmed LBM agar plates and incubated at 37 °C for 24 h. After incubation, the plaques formed by the bacteriophage were enumerated to determine the plaque-forming units per milliliter (PFU/mL). Individual bacteriophages with titers greater than 10^9 PFU/mL were selected for use in all subsequent experiments (Jagannathan et al., 2020).

The efficiency of plating (EOP) was assessed to determine the relative efficiency or infectivity of the bacteriophages compared to the host bacteriophage, following the methodology described by Hosny et al. (2022). This involved dividing the average bacteriophage titer by the average titer of the host phage average. Based on the obtained EOP, the phages were classified into different categories: high (EOP ≥ 0.5), medium (0.5 < EOP ≥ 0.1), low (0.1 < EOP > 0.001), and inefficient (EOP ≤ 0.001).

5.3.4 In vitro Lytic Activity of Bacteriophage

Growth curves were generated for three bacteriophages (S7, S10, and S13) as individual treatments and as a cocktail consisting of equal proportions of each bacteriophage (1:1:1). The experiments were conducted at different temperature conditions (20, 25, and 30°C) and using various MOIs (0.001, 0.01, 0.1, 1, and 10), against five *S. enterica* serovars at an initial concentration of 10^8 CFU/mL (OD_{595} = 1.0). Bacterial growth was assessed by measuring the absorbance at OD_{595} at 30-minute intervals over a total period of 6 h, as well as at 24 h and 48 h (**Figure 5.3.4.1**).

Figure 5.4.3 1 Schematic illustration of in vitro experimental design of the lytic activity of phages S7, S10, S13, and cocktail against *S. enterica* Braenderup, *S. enterica* Enteritidis, *S. enterica* Kentucky, *S. enterica* Newport, and *S. enterica* Typhimurium at different MOIs (0.001, 0.01, 0.1, 1, and 10) and temperatures (20, 25, and 30 °C). The initial concentration of the bacterial strains was 10^8 CFU/mL.

5.3.5 In vitro Lytic Activity of Isolated Bacteriophages

Individual cultures of *S. enterica* serovars were grown and incubated at 37 °C with shaking at 150 rpm until they reached an OD_{595} of 1.0, which typically required approximately 3 h. At time 0 h, 100 µL of individually diluted phages at different MOIs (0.001, 0.01, 0.1, 1, and 10) were added to 100 µL of the bacterial culture with an OD_{595} of 1.0 in a 96-well plate with flat bottom (VWR, Radnor, PA, USA). The mixtures were then incubated at temperatures of 20, 25, and 30 °C, with shaking at 150 rpm. The OD_{595} was measured at 30-minute intervals over a total period of 6 h, as well as at 24 h and 48 h, using a 96-well iMark™ Microplate Reader (BIO-RAD, Hercules, CA, USA). The obtained OD_{595} values were plotted against time to visualize the antimicrobial activity of

isolated phages against each *S. enterica* serovar. To establish the baseline for bacterial growth, positive control was set up by inoculating 100 µL of sterile LBM broth with 100 µL of each bacterial culture. Each experiment was conducted in triplicate.

5.3.6 In vitro Lytic Activity of Bacteriophage Cocktail

A bacteriophage cocktail was prepared by combining three phages (S7, S10, and S13) in equal concentrations. At time 0 h, 100 µL of the phage cocktail at different MOIs (0.001, 0.01, 0.1, 1, and 10) were added to 100 µL of each of the five *S. enterica* serovars with an initial OD_{595} of 1.0 in a 96-well plate with a flat bottom. The mixtures were then incubated at temperatures of 20, 25, and 30 °C, with shaking at 150 rpm. The OD_{595} was measured at 30-minute intervals over a period of 6 h, as well as at 24 h and 48 h, using a 96-well iMark^{TM} Microplate Reader. The obtained OD_{595} values were plotted against time to visualize the antimicrobial activity of the bacteriophage cocktail against each *S. enterica* serovar. The results of the OD_{595} obtained from the positive control of the *in vitro* lytic activity of isolated ohages experiment were utilized to establish the baseline for bacterial growth for each *S. enterica* serovar.

5.3.7 Statistical Analysis

The effect of *S. enterica* serovars, phages, MOIs, temperature, and their interactions on bacterial growth was evaluated through statistical analysis using R Statistical Software (version 4.2.2). The normality and homoscedasticity of the data were assessed using Shapiro-Wilk's and Levene's tests, respectively. Non-parametric factorial data analysis was performed using the Aligned Rank Transform (ART) (Wobbrock et al., 2011) with the ARTool package. To evaluate differences in pairwise combinations of factor levels and interactions, the Aligned Rank Transform Contrasts (ART-C) were conducted using the "art.con" function (Elkin et al., 2021). A $p < 0.05$ was considered statistically significant.

5.4 Results

5.4.1 Enumeration of Bacteriophages and Efficiency of Plating

The average phage titers for each bacteriophage (S7, S10, and S13) hosted by *S. enterica* Enteritidis amplification performed the highest target against *S. enterica* Braenderup, *S. enterica* Enteritidis, *S. enterica* Kentucky, *S. enterica* Newport, or *S. enterica* Typhimurium ranged from 1.19 x 10^{10} to 9.86 x 10^{10} PFU/mL (**Table 5.4.1.1**). The presence of small clear plaques indicated the ability of these bacteriophages to effectively lyse the target bacterial strains (**Figure 5.4.1.1**). Furthermore, the EOP values for phages S7, S10, and S13 were classified as high (≥ 0.5) for each *S. enterica* serovar.

Table 5.4.1. 1 Bacteriophage infectivity, average titers, and efficiency of plating against different *S. enterica* serovars.

		Bacteriophages					
		S7		S10		S13	
Salmonella serovars	Lysis[a]	Titer (x 10^{10} PFU/mL)[b]	EOP[c]	Titer (x 10^{10} PFU/mL)	EOP	Titer (x 10^{10} PFU/mL)	EOP
Braenderup	+	8.56 ± 0.57	1.09	6.66 ± 0.57	0.77	9.16 ± 1.15	0.94
Enteritidis	+	7.86 ± 0.57	1.00	8.66 ± 0.57	1.00	9.76 ± 0.57	1.00
Kentucky	+	6.96 ± 0.57	0.89	9.03 ± 0.57	1.04	7.56 ± 0.57	0.77
Newport	+	9.86 ± 0.57	1.09	5.53 ± 0.57	0.64	1.19 ± 0.57	1.23
Typhimurium	+	9.03 ± 0.57	1.15	6.06 ± 0.57	0.70	9.76 ± 0.57	1.00

[a] Plaque formation.

[b] Bacteriophage titer values are presented as mean ± standard deviation. PFU/mL: Plaque forming unit per milliliter.

[c] Efficiency of plating.

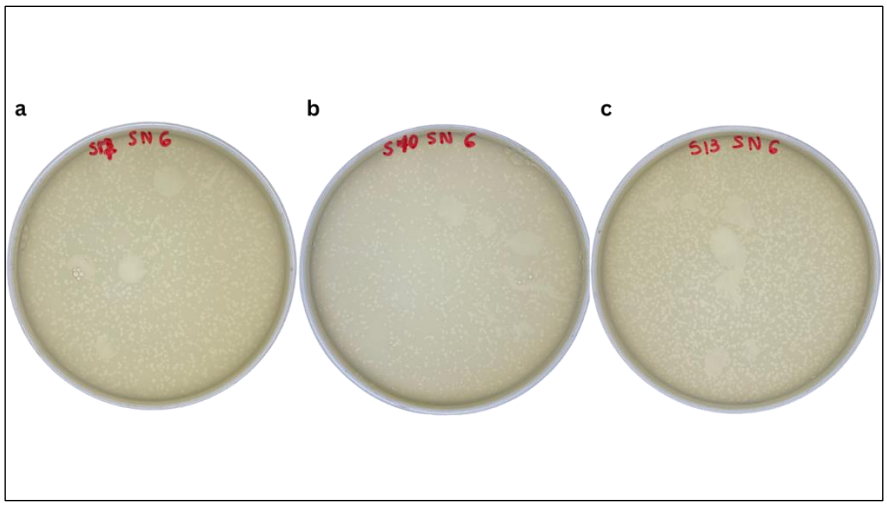

Figure 5.4.1. 1 Double agar overlay plaque assay results for bacteriophages (A) S7, (B) S10, and (C) S13 against *S.enterica* Newport.

5.4.2 In vitro Lytic Activity of Individual Bacteriophages

The lytic activity of individual bacteriophages was evaluated *in vitro*, revealing significant interactions between phages, temperature, MOI, and *S. enterica* serovars ($p < 0.05$). As illustrated in **Figure 5.4.1. 2**, the positive controls of the five *S. enterica* serovars exhibited typical growth patterns, indicating normal growth behavior. Phages S7, S10, and S13 consistently inhibited the growth of all *S. enterica* serovars within a 6-h time frame across all temperatures (20, 25, and 30 °C) and MOIs (0.001, 0.01, 0.1, 1, and 10). The inhibitory effect was evident from the lower absorbance readings at OD_{595} in the presence of the bacteriophages compared to the positive control groups. During the 6-h period, phages S7 and S13 exhibited higher lytic activity than S10 ($p < 0.05$), but no significant difference was observed between S7 and S13 ($p > 0.05$). A temperature of 30 °C had a stronger inhibitory effect on bacterial growth compared to 25 °C and 20 °C ($p < 0.05$), and a MOI of 0.1 demonstrated the highest inhibitory effect compared to other MOIs ($p < 0.05$). Among the bacterial strains, *S. enterica* Newport exhibited the lowest growth compared to other *S. enterica* serovars ($p < 0.05$). In general, all *S. enterica* serovars experienced re-growth after 6 h to 48 h of incubation.

Evident differences in lysis curves were observed for bacteriophages S7, S10, and

S13 in relation to different *S. enterica* serovars during the 6-h incubation period. Overall, *S. enterica* Braenderup (**Figure 5.4.2.1**) at 25 °C with MOI 0.1, phages S13 and S7 achieved a reduction in OD_{595} below the starting concentration at 5 h and between 1.5 to 5.5 h of incubation, respectively. Particularly, at 30 °C, significant differences ($p < 0.05$) in absorbance results were observed for all MOIs of S7 and S10 compared to the control. Bacteriophage S7 displayed the highest lytic activity at 30 °C, specifically at MOI 10, resulting in a 34.0% reduction in absorbance compared to the control. At 25 °C, S7 at MOIs 0.1 and 1; and S13 at MOIs 0.01, 0.1, 1, and 10 showed statistically significant differences from the control ($p < 0.05$). The highest inhibitory effect was observed at MOI 0.1 for phage S7, resulting in a 65.9% reduction in absorbance compared to the control. Finally, at 20 °C, only S10 with MOI 0.1 exhibited a statistically significant difference from the control ($p < 0.05$), resulting in a 30.6% decrease in absorbance compared to the control. Furthermore, between 6 h and 48 h, only phages S10 at MOI 0.001 and S13 at MOI 0.01 at a temperature of 25 °C exhibited inhibitory effects on the growth of *S. enterica* Braenderup.

For *S. enterica* Enteritidis (**Figure 5.4.2.2**), during the 6-h incubation at 20 °C, bacteriophages S7 at MOIs 0.01 and 0.1; S10 at MOIs 0.001, 0.01, 0.1, and 10; and S13 at MOI 0.01 showed statistically significant differences from the control ($p < 0.05$). The highest lytic activity at 20 °C was observed for S10 at MOI 0.1, resulting in a 41.0% reduction in absorbance compared to the control. At 25 °C, only bacteriophage S13 at MOIs 0.01, 0.1, and 10 demonstrated statistically significant differences ($p < 0.05$) compared to the control, with the highest lytic activity observed at MOI 0.1, resulting in a 40.3% reduction in absorbance compared to the control. Moreover, at 30 °C, statistically significant differences ($p < 0.05$) in absorbance results were observed for all MOIs of phages S7 and S10 compared to the control. The highest lytic activity at 30 °C was

observed at MOI 1 for S7, resulting in a 34.4% decrease in absorbance compared to the control. No inhibitory growth was observed by any of the bacteriophages between 6 h and 48 h of incubation.

For *S. enterica* Kentucky (**Figure 5.4.2.3**), the following bacteriophages achieved a reduction in OD_{595} below the starting point at 25 °C: S10 (MOI 0.001) at 2h, S13 (MOI 0.001) at 1 h, and S13 (MOI 1) at 2.5 h. Particularly, at 20 °C, statistically significant differences ($p < 0.05$) in absorbance results compared to the control were observed for S7 at MOIs 0.01 and 0.1; S10 at MOIs 0.01, 0.1, 1, and 10; and S13 at MOI 0.01. The highest lytic activity at 20 °C was observed for S10 at MOI 0.1, resulting in a 38.9% reduction in absorbance compared to the control. At 25 °C, phages S7 at MOIs 0.001 and 0.1; S10 at MOI 0.001; and S13 at MOIs 0.01, 0.1, and 1, showed statistically significant differences from the control ($p < 0.05$), with the highest lytic activity observed for S10 at MOI 0.001, resulting in a 42.6% reduction in absorbance compared to the control. Moreover, at 30 °C, statistically significant differences ($p < 0.05$) in absorbance results were observed for all MOIs of bacteriophages S7, S10, and S13 compared to the control. The highest lytic activity at 30 °C was observed for S13 at MOI 10, resulting in a 31.9% reduction in absorbance compared to the control. Additionally, between 6 h and 48 h, only bacteriophages S10 at MOI 0.1, at a temperature of 25 °C, exhibited inhibitory effects on the growth of *S. enterica* Kentucky.

Regarding *S. enterica* Newport (**Figure 5.4.2.4**), bacteriophage S13 achieved a reduction in OD_{595} below the initial value after 5 h at 25 °C for MOI 0.1. Similarly, for MOI 10 at 30 °C, S13 achieved a reduction in OD_{595} below the initial value from 1 to 1.5 h, 2.5 to 3.5 h, 5 h, and 6 h. Furthermore, during the 6-h incubation period, statistically significant differences ($p < 0.05$) in absorbance results compared to the control were observed at 20 °C for S7 at MOI 0.01, and for S10 at MOIs 0.01 and 0.1. The highest

lytic activity at 20 °C was observed for S10 at MOI 0.1, resulting in a 32.2% reduction in absorbance compared to the control. At 25 °C, phage S13 at MOIs 0.01, 0.1, and 1 exhibited statistically significant differences ($p < 0.05$) compared to the control, with MOI 1 showing the highest lytic activity, resulting in a 37.4% reduction compared to the control. Lastly, at 30 °C, statistically significant differences ($p < 0.05$) in absorbances were observed for all MOIs of S7, S10, and S13 compared to the control. The highest lytic activity resulting in a 59.8% reduction in absorbance compared to the control, was observed for bacteriophage S13 at MOI 10. Only phage S10 at 30 °C with MOI 0.01 exhibited inhibitory effects on the growth of *S. enterica* Newport from 6 h to 48 h.

Finally, for *S. enterica* Typhimurium bacteriophage S13 at MOI 0.1 and 25 °C reduced the OD_{595} below the initial point from 5 to 6 h (**Figure 5.4.2.5**). Similarly, phage S7 at MOI 1 had a reduction in the absorbance results below the starting point between 2 and 4.5 h of incubation at 30 °C. Moreover, during the 6-h incubation period, statistically significant differences ($p < 0.05$) in absorbance results compared to the control were observed at 20 °C for S10 at MOIs 0.001, 0.01, and 0.1, with MOI 0.1 presenting the lowest absorbance, resulting in a 31.1% decrease compared to the control. At 25 °C, phage S7 at MOI 10 and S13 at MOIs 0.01, 0.1, and 10 exhibited statistically significant differences ($p < 0.05$) compared to the control. At this temperature, the lowest absorbance was observed for S13 at MOI 0.1, resulting in a 50.3% decrease compared to the control. At 30°C, statistically significant differences ($p < 0.05$) in the absorbance results were observed for all MOIs of S7, S10, and S13 compared to the control. The highest lytic activity at 30 °C was observed for S7 at MOI 1, resulting in a 50.9% decrease in the absorbance compared to the control. Between 6 h and 48 h, only bacteriophages S7 at MOI 10 and S10 at MOIs 0.01 and 10, at a temperature of 25 °C, exhibited inhibitory effects on the growth of *S. enterica* Typhimurium

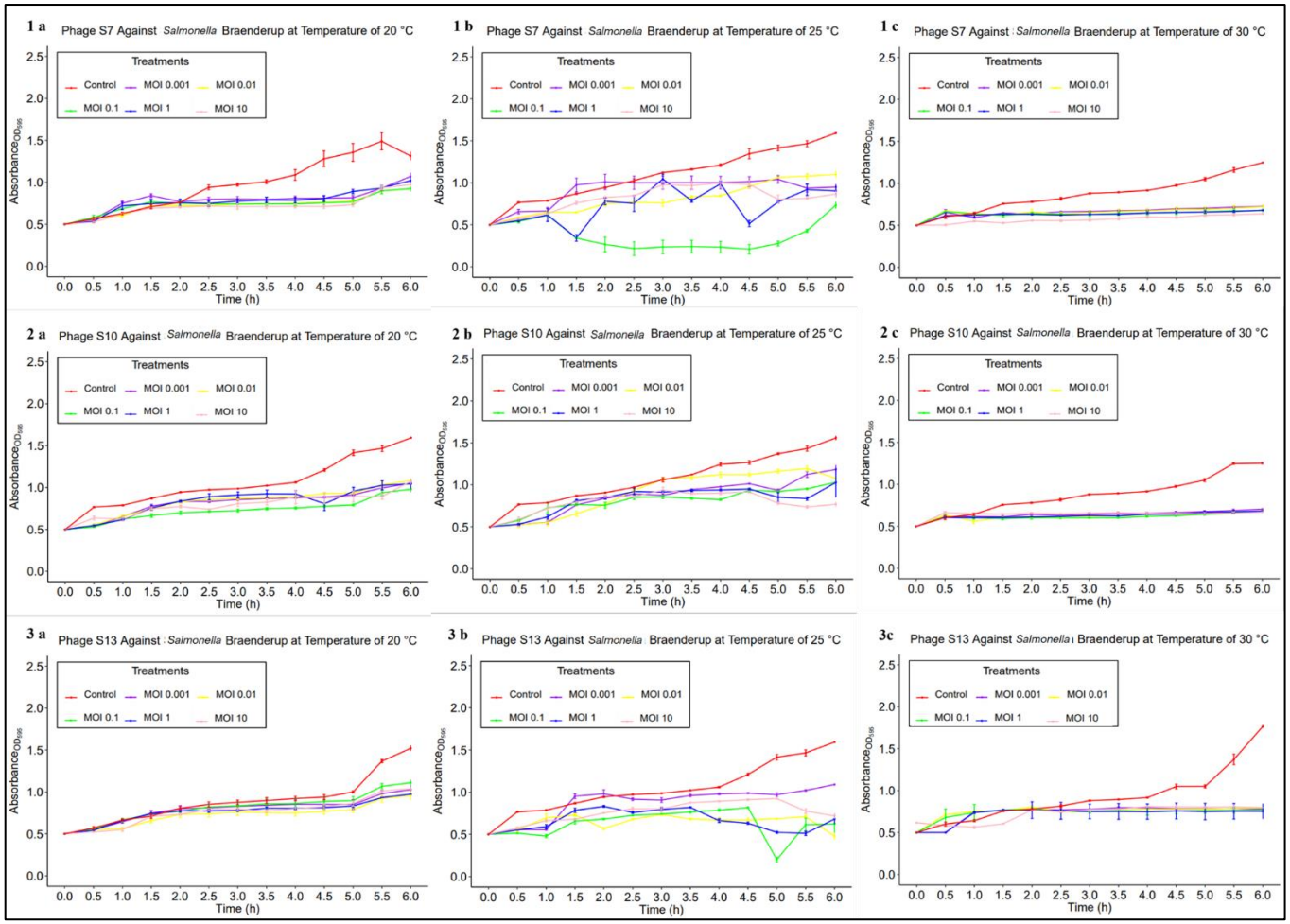

Figure 5.4.3.1 Growth inhibition of *S. enterica* Braenderup by bacteriophages S7 at 20 °C (1a), 25 °C (1b), and 30 °C (1c); S10 at 20 °C (2a), 25 °C (2b), and 30 °C (2c); and S13 at 20 °C (3a), 25 °C (3b), and 30 °C (3c). The data points represent the mean values obtained from triplicate replications, and the errors bars represent the standard deviations of three independent experiments for each phage, using different multiplicities of infection (MOIs) of 0.001, 0.01, 0.1, 1, and 10, in comparison to the control group. The growth curve exhibits data collected at 30-minute intervals over a period of 6 hours.

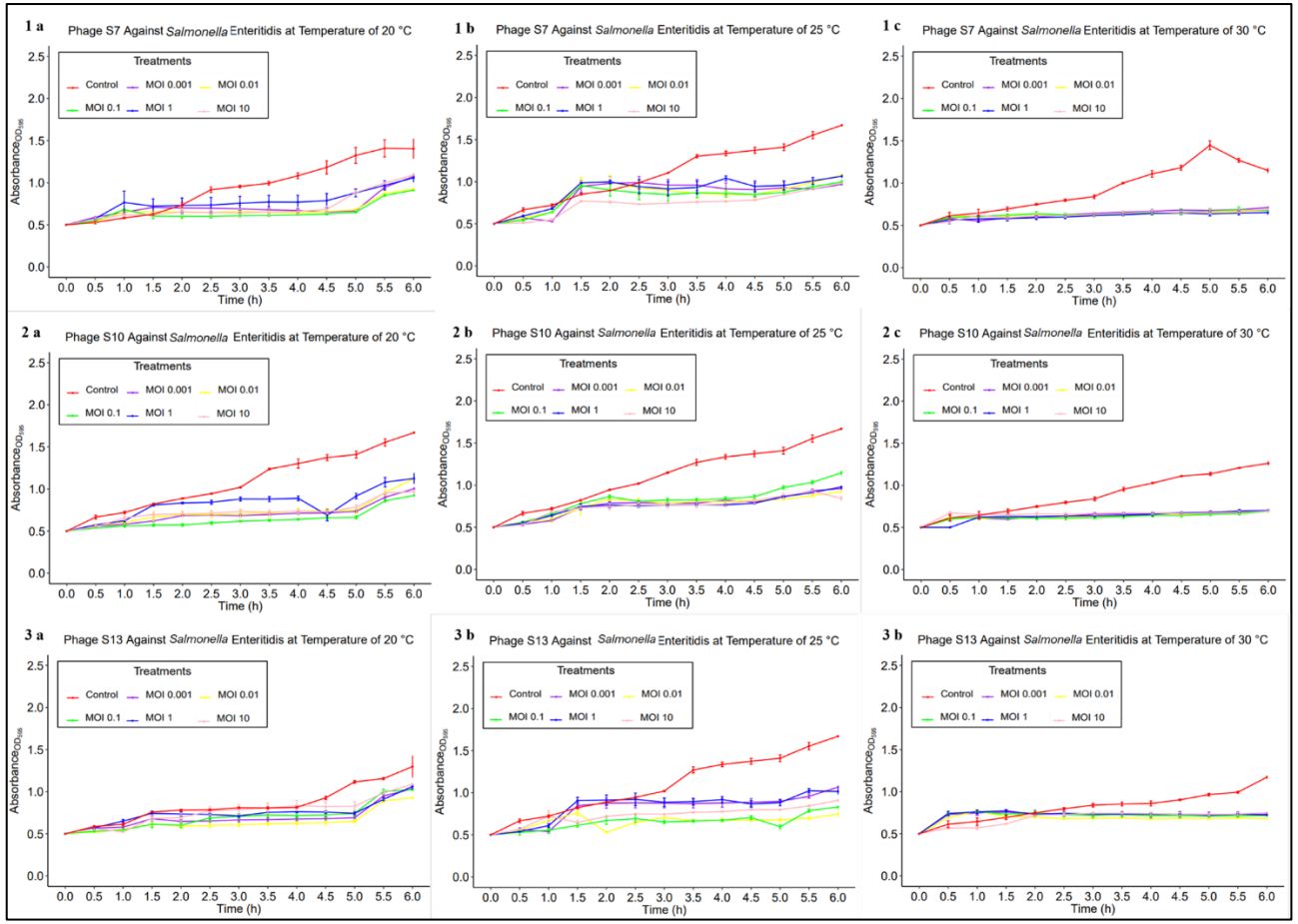

Figure 5.4.2.2 Growth inhibition of *S. enterica* Enteritidis by bacteriophages S7 at 20°C (1 a), 25°C (1 b), and 30°C (1 c); S10 at 20 °C (2 a), 25°C (2 b), and 30°C (2 c); and S13 at 20°C (3 a), 25°C (3 b), and 30°C (3 c). The data points represent the mean values obtained triplicate replications, and the errors bars represent the standard deviations of three independent experiments for each phage, using different multiplicities of infection (MOIs) of 0.001, 0.01, 0.1, 1, and 10, in comparison to the control group. The growth curve exhibits data collected at 30-minute intervals over a period of 6 hours.

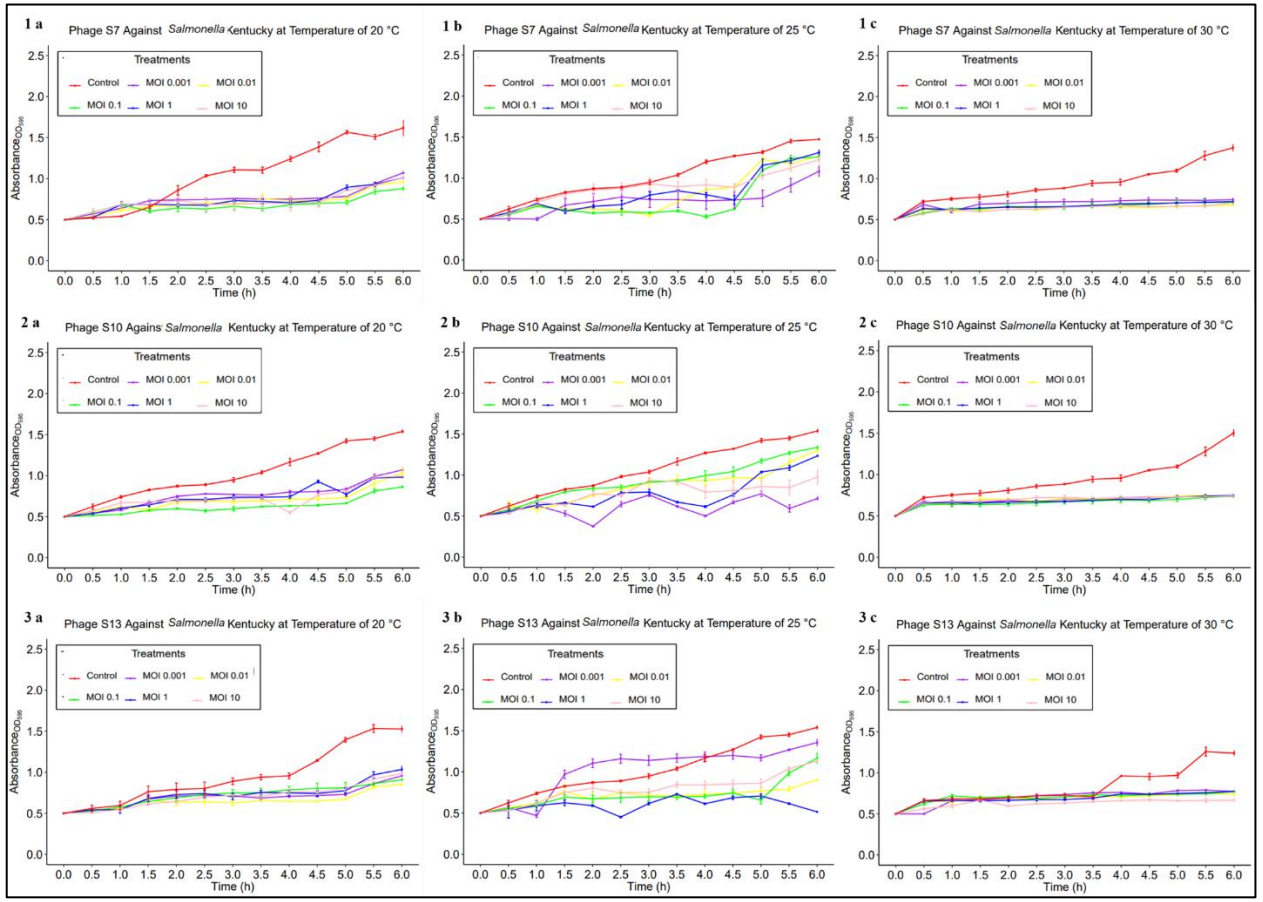

Figure 5.4.2.3 Growth inhibition of *S. enterica* Kentucky by bacteriophages S7 at 20°C (1 a), 25°C (1 b), and 30°C (1 c); S10 at 20 °C (2 a), 25°C (2 b), and 30°C (2 c); and S13 at 20°C (3 a), 25°C (3 b), and 30°C (3 c). The data points represent the mean values obtained from triplicate replications, and the errors bars represent the standard deviations of three independent experiments for each phage, using different multiplicities of infection (MOIs) of 0.001, 0.01, 0.1, 1, and 10, in comparison to the control group. The growth curve exhibits data collected at 30-minute intervals over a period of 6 hours.

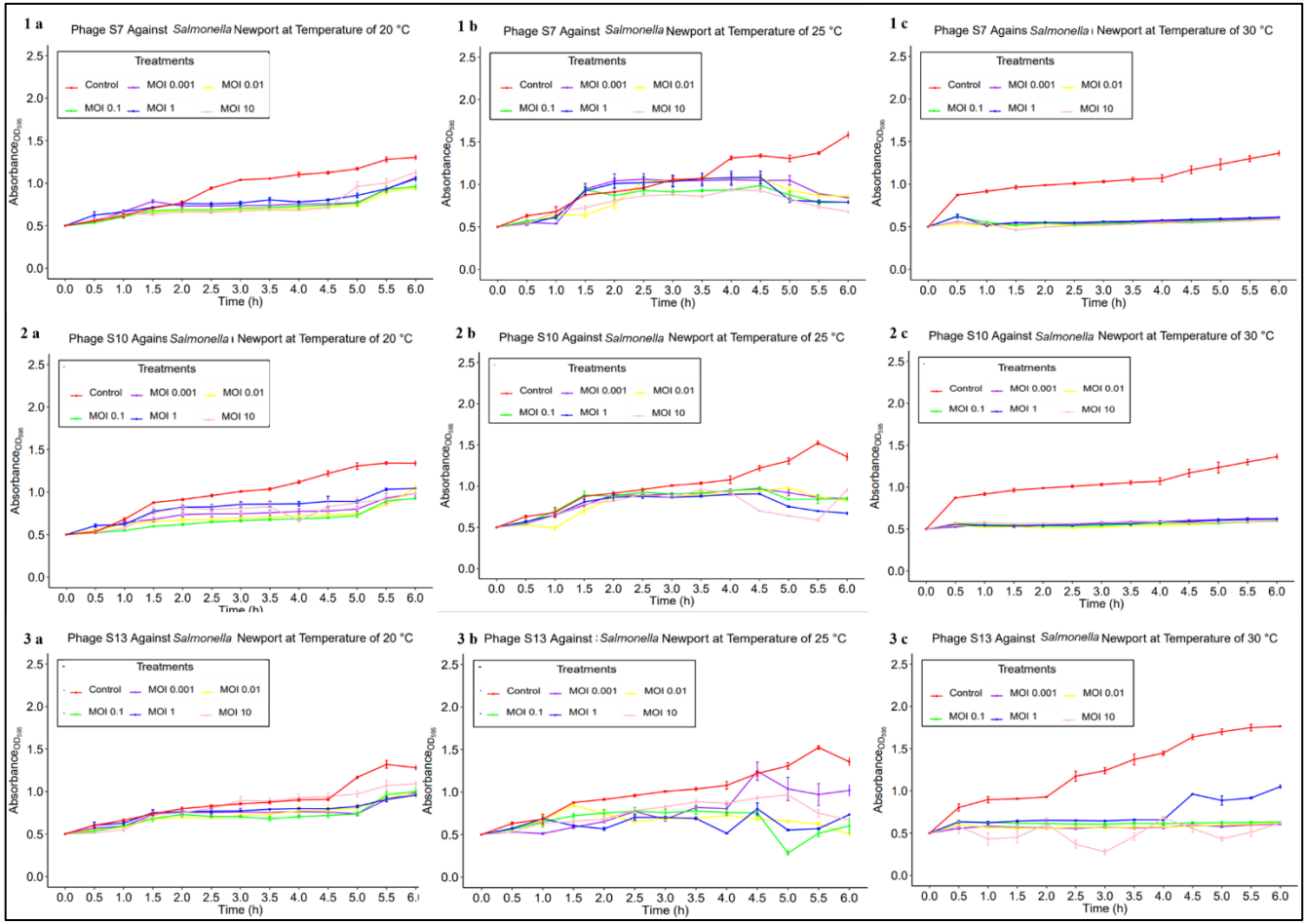

Figure 5.4.2.4 Growth inhibition of *S. enterica* Newport by bacteriophages S7 at 20°C (1 a), 25°C (1 b), and 30°C (1 c); S10 at 20 °C (2 a), 25°C (2 b), and 30°C (2 c); and S13 at 20°C (3 a), 25°C (3 b), and 30°C (3 c). The data points represent the mean values obtained from triplicate replications, and the errors bars represent the standard deviations of three independent experiments for each phage, using different multiplicities of infection (MOIs) of 0.001, 0.01, 0.1, 1, and 10, in comparison to the control group. The growth curve exhibits data collected at 30-minute intervals over a period of 6 hours.

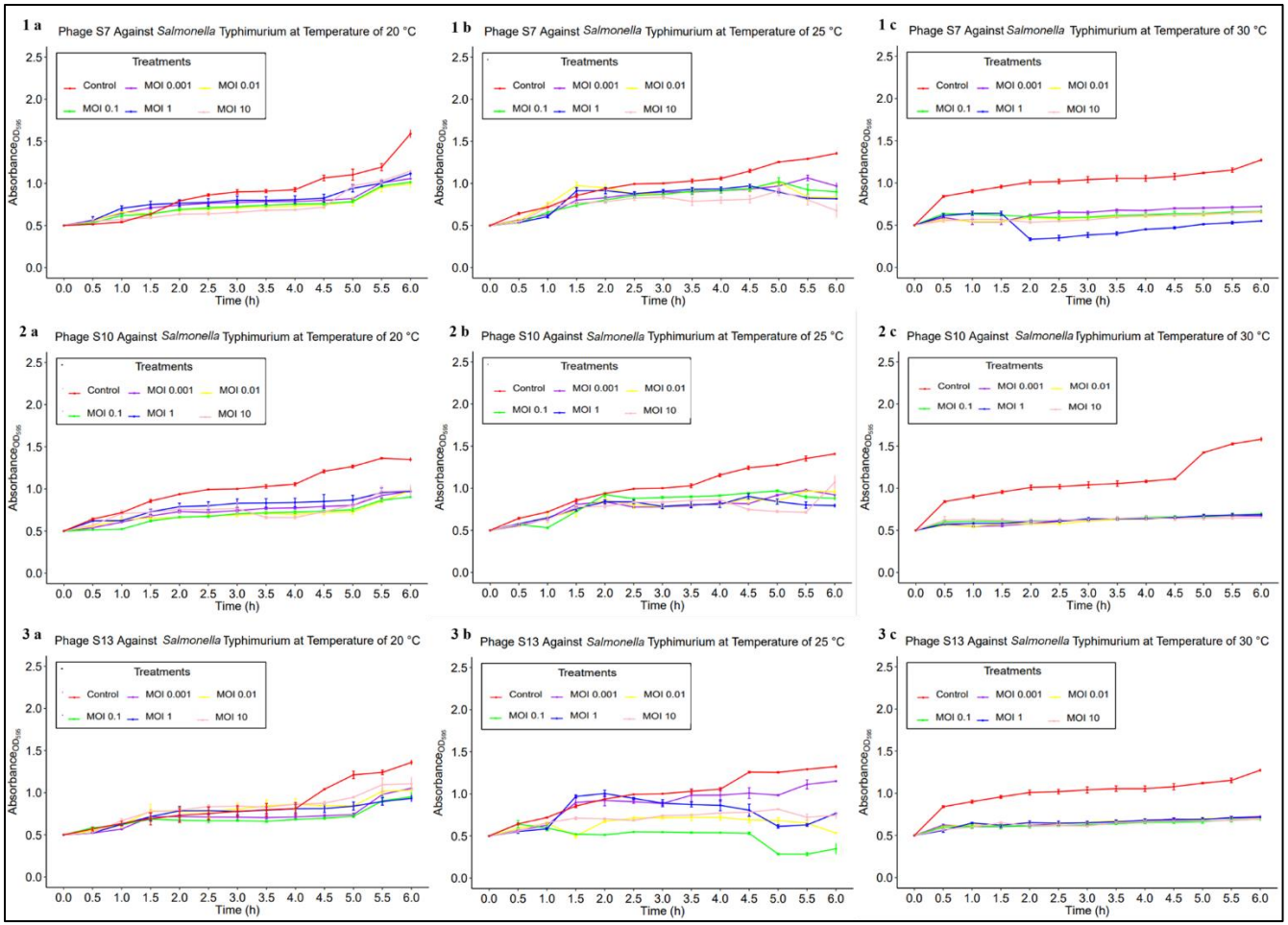

Figure 5.4.2.5 Growth inhibition of *S. enterica* Typhimurium by bacteriophages S7 at 20°C (1 a), 25°C (1 b), and 30°C (1 c); S10 at 20 °C (2 a), 25°C (2 b), and 30°C (2 c); and S13 at 20°C (3 a), 25°C (3 b), and 30°C (3 c). The data points represent the mean values obtained from triplicate replications, and the errors bars represent the standard deviations of three independent experiments for each phage, using different multiplicities of infection (MOIs) of 0.001, 0.01, 0.1, 1, and 10, in comparison to the control group. The growth curve exhibits data collected at 30-minute intervals over a period of 6 hours.

1.1.1. In vitro Lytic Activity of Bacteriophage Cocktail

Significant three-way and two-way interactions were observed among temperature, MOI, and *S. enterica* serovars. The positive control group showed a typical growth pattern curve (**Figure 5.4.3.1**), while the presence of the phage cocktail led to lower absorbance readings at OD_{595} compared to the positive control groups. Temperatures of 25°C and 30 °C exhibited higher inhibitory effects on bacterial growth compared to 20 °C ($p < 0.05$), with no significant difference between the results obtained at 25 °C and 30 °C ($p > 0.05$). Among the *S. enterica* serovars, *S. enterica* Typhimurium displayed the lowest growth ($p < 0.05$), and MOI 1 showed the highest inhibitory effects compared to the other MOIs ($p < 0.05$). Overall, all *S. enterica* serovars experienced re-growth after the past 6 h to 48 h.

Evident differences in lysis curves were observed for the bacteriophage cocktail in relation to different *S. enterica* serovars during the 6-h incubation period. In Figure 5.4.7-1a, -1b, and -1c, for *S.* Braenderup, the phage cocktail at MOI 1 resulted in a reduction in OD_{595} below the starting point at 0.5 h at a temperature of 20 °C and at 5 and 6 h at a temperature of 30 °C. Statistically significant differences ($p < 0.05$) in absorbance results were observed for all MOIs of the bacteriophage cocktail compared to the control during the 6-h incubation period at temperatures of 20, 25, and 30 °C. The highest lytic activity observed in each temperature at 6 h was 50.5 % at 20°C, 57.9% at 25°C and 73.3% at 30 °C of reduction in absorbance compared to the control, was observed at MOI 1.

In Figure 5.4.7-2a, -2b, and -2c, for *S. enterica* Enteritidis, the bacteriophage cocktail resulted in a reduction of OD_{595} below the initial value at a temperature of 20 °C and MOI 1. During the 6-h incubation period, statistically significant differences ($p < 0.05$) in absorbance results compared to the control were observed at temperatures of 20, 25, and 30 °C. Specifically, at 20 °C, the bacteriophage cocktail at MOIs 0.001, 0.1, and 1 exhibited significant differences from the control ($p < 0.05$). At 25 °C, significant differences ($p < 0.05$)

were observed for the phage cocktail at MOIs 0.001, 0.1, 1, and 10 compared to the control. Similarly, at 30 °C, significant differences ($p < 0.05$) were observed for the bacteriophage cocktail at MOIs 0.001, 0.01, 0.1, and 1 compared to the control. The highest lytic activity, resulting in a 48.0% decrease in absorbance, was observed at MOI 1 and a temperature of 30 °C. Additionally, at 6 h, the bacteriophage cocktail at MOI 1, at a temperature of 20 and 30 °C, exhibited inhibitory effects on the growth of *S. enterica* Enteritidis of 45.5% and 68%, respectively. The highest inhibition at 25°C occurred at MOI 1, resulting in a 56.9% of reduction.

Similarly, for *S. enterica* Kentucky (Figure 5.4.7-3a, -3b, and -3c), the bacteriophage cocktail exhibited a decrease in OD_{595} below the starting point at a temperature of 20 °C and MOI 0.001 at 0.5 h. Additionally, at 20 °C, statistically significant differences ($p < 0.05$) in absorbance results were observed compared to the control at MOIs 0.001, 0.01, 0.1, and 1. At 25 °C, statistically significant differences ($p < 0.05$) in absorbance results compared to the control were observed for MOI 0.001, 0.1, 1, and 10. Moreover, at 30 °C, statistically significant differences ($p < 0.05$) in absorbance results were observed for MOIs 0.001, 0.1, and 1 compared to the control. The highest lytic activity, resulting in a 43.5% decrease in absorbance, was observed at 30 °C and MOI 1. In addition, at 6 h, the bacteriophage cocktail at MOI 1, at a temperature of 20, 25, and 30 °C, exhibited inhibitory effects on the growth of *S. enterica* Kentucky, resulting in 50.4 %, 58.0, %, and 62.1 % reduction, respectively.

Regarding *S. enterica* Newport (Figure 5.4.7-4a, -4b, and -4c), the bacteriophage cocktail displayed the ability to reduce OD_{595} below the initial value after 0.5 h, at MOI 1 and temperatures of 20 and 25 °C. Furthermore, during the 6-h incubation period, statistically significant differences ($p < 0.05$) in absorbance results were observed at 20 °C for MOIs 0.001, 0.01, 0.1, and 1. At 25 °C, significant differences ($p < 0.05$) were observed for MOIs 0.001, 0.1, 1, and 10 compared to the control. Similarly, at 30 °C, significant differences ($p < 0.05$) in

absorbance results were observed for MOIs 0.001, 0.01, 0.1, and 1 compared to the control. The highest lytic activity, resulting in a 49.7% decrease in absorbance, was observed at a temperature of 25 °C and MOI 1. Moreover, at 6 h the bacteriophage cocktail at MOI 1, at a temperature of 20, 25 and 30 °C exhibited a reduction of 51.3%, 62.1%, and 62.3% and MOI 10, at 25 °C exhibited inhibitory effects on the growth of *S. enterica* Newport of 62.2 % of reduction.

Finally, for *S. enterica* Typhimurium (Figure 5.4.7-5a, -5b, and -5c), the bacteriophage cocktail showed reductions in OD_{595} below the initial value after 0.5 h, at MOIs 0.001 and 1, at temperatures of 25 and 20 °C, respectively. Moreover, during the 6-h incubation period, statistically significant differences ($p < 0.05$) in absorbance results were observed at 20°C for MOIs 0.001, 0.01, 0.1, and 1. At 25 °C, the bacteriophage cocktail at MOIs 0.001, 0.1, 1, and 10 exhibited significant differences ($p < 0.05$) compared to the control. Lastly, at 30 °C, statistically significant differences ($p < 0.05$) in absorbance results were observed for MOIs 0.001, 0.01, 0.1, and 1 compared to the control. The highest lytic activity, resulting in a 49.4% decrease in absorbance, was observed at a temperature of 30 °C and MOI 1. Additionally, at 6 h the bacteriophage cocktail at MOI 1, at a temperature of 20, 25, and 30 °C, exhibited inhibitory effects reducing 43.3 %, 61.8 %, and 67.5 %, respectively on the growth of *S. enterica* Typhimurium.

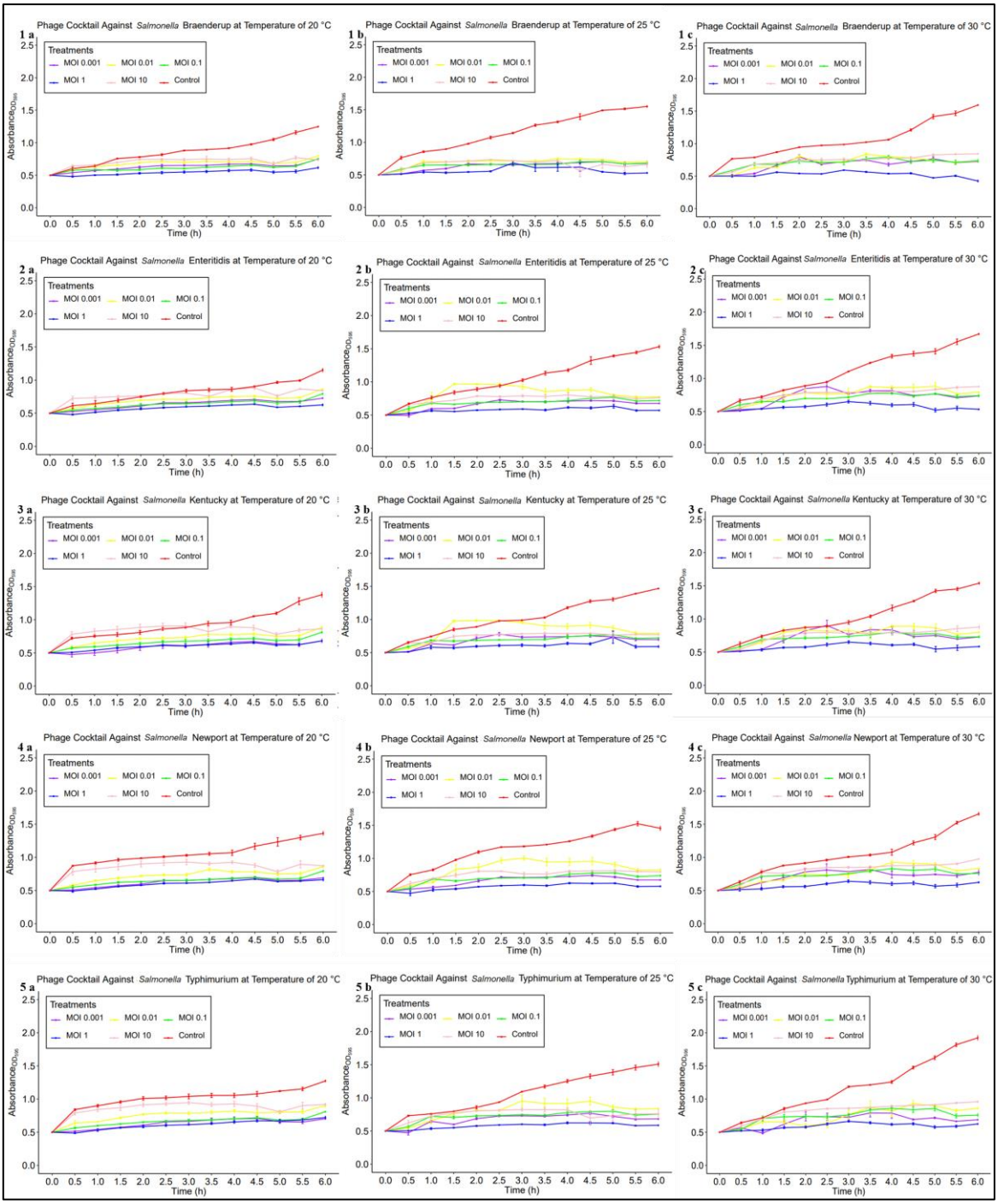

Figure 5.4.3.1 Growth inhibition of *S. enterica* Braenderup by bacteriophage cocktail at 20°C (1 a), 25°C (1 b), and 30°C (1 c); *S. enterica* Enteritidis by bacteriophage cocktail at 20 °C (2 a), 25°C (2 b), and 30°C (2 c); *S. enterica* Kentucky by bacteriophage cocktail at 20°C (3 a), 25°C (3 b), and 30°C (3 c); *S. enterica* Newport by bacteriophages cocktail at 20 °C (4 a), 25°C (4 b), and 30°C (4 c); *S. enterica* Typhimurim by bacteriophages cocktail at 20°C (5 a), 25°C (5 b), and 30°C (5 c). The data points represent the mean values obtained from triplicate replications, and the error bars represent the standard deviations of three independent experiments for each bacteriophage cocktail, using different multiplicities of infection (MOIs) of 0.001, 0.01, 0.1, 1, and 10, in comparison to the control group. The growth curve exhibits data collected at 30-minute intervals over a period of 6 hours.

5.5 Discussion

In recent years, bacteriophage therapy has been recognized as a promising alternative to chemical sanitizers and antibiotics for controlling foodborne pathogens, especially in food products (Aguilera et al., 2022; Guenther et al., 2012; Kocharunchitt et al., 2009; Yeh et al., 2017). Previous studies have focused on isolating bacteriophage from the environment and evaluating their activity as either bacteriophage cocktails or single preparations to reduce bacterial populations *in vitro* and *in vivo* (Esmael et al., 2021a; Pelyuntha et al., 2021; Shang et al., 2021; Yeh et al., 2017). This study specifically demonstrated the effectiveness of environmental bacteriophages (S7, S10, and S13) as an alternative approach to control the growth of *S. enterica*. These single bacteriophages showed lytic activity against five *S. enterica* serovars, including *S. enterica* Enteritidis, *S. enterica* Typhimurium, and *S. enterica* Newport, which are commonly associated with foodborne illnesses in the United States (CDC, 2023b)

In this study, all three phages effectively lysed *S. enterica* Braenderup, *S. enterica* Enteritidis, *S. enterica* Kentucky, *S. enterica* Newport, and *S. enterica* Typhimurium, as evidenced by small clear plaque formation on double agar plates (**Figure 5.4.1.1**) and high EOP (**Table 5.4.1.1**), indicating these bacteriophages exhibited a high level of efficacy in terms of its ability to infect and lyse the bacterial from a variety of serogroups. The EOP has significant importance in comprehending the efficacy of bacteriophage S7, S10, and S13 in selectively targeting the *S. enterica*. Other studies have also reported the ability of certain bacteriophages to infect multiple *S. enterica* serovars, further highlighting their potential in controlling this pathogen. In similar studies, Hosny, Shalaby, Nasef, et al. (2022) found that bacteriophage WP109 and 110 exhibited high efficiency in infecting multiple *S. enterica* serovars, including *S.* Gallinarum, *S.* Cape, *S.* Enteritidis, *S.* Montevideo, *S.* Uno, *S.* Oritamerin, *S.* Belgdam, *S.* Agona, *S.* Daula, and *S.* Aba. Gomez-Garcia et al. (2021) also demonstrated that bacteriophage S1 showed high EOP on *S.* Pullorum, *S. enterica* Gallinarum, and *S. enterica*

Enteritidis. Additionally, Lu et al. (2022) reported that phage ΦEnt was capable of lysing nine different *S. enterica* serovars (*S. enterica* Braenderup, *S. enterica* Belem, *S. enterica* Cerro, *S. enterica* Typhimurium, *S. enterica* Enteritidis, *S. enterica* Kentucky, *S. enterica* Hadar, *S. enterica* Thompson, and *S. enterica* Infantis) and three *Shigella* strains of two other species (*S. dysenteriae* and *S. sonnei*).

In the *in vitro* analysis, both single phages and the phage cocktail effectively inhibited the growth of tested *S. enterica* serovars. However, the lytic activity of single bacteriophages varied against the *S. enterica* serovars, deviating from the typical bacteriophage behavior where higher MOIs usually result in greater lytic activity (Lu et al., 2022). For *S. enterica* Kentucky, phages S7, S10, and S13 showed high inhibitory effects at MOI 0.1, 0.001, and 1, respectively, but not at MOI 10. Similar findings have been reported in other studies (Abdelsattar et al., 2023; Kim et al., 2021; Lee et al., 2020; Wang et al., 2022). Kim et al. (2021). In general, it was observed that bacteriophages have the capacity to regulate bacterial populations. In addition, it was found by Kim et al. (2021), that phages pSp-J and pSp-S had a more significant antimicrobial effect at lower MOIs compared to high MOI groups. This particular case was attributed to pseudo lysogeny induced by high MOIs, where virulent bacteriophages exhibit characteristics similar to lysogenic bacteriophages, enabling their genome to survive in unfavorable propagation conditions and the isolation and characterization of novel bacteriophages that can mitigate antibiotic-resistant strains and disrupt biofilms present a promising opportunity for the development of effective treatments against bacteria. In another study, Abdelsattar et al. (2023) the implementation of a time-killing curve experiment, which yielded findings indicating that the phage ZCSE9 exhibited significant efficacy in inhibiting bacterial proliferation in the planktonic condition. The authors also demonstrated that phage ZCSE9 had lower activity at MOI 0.01 compared to MOI 0.001, 0.1, 1, and 10 against *S. enterica* Typhimurium. Similarly, Wang et al. (2022) found that the optimal MOI for phage

SP76 against *S. enterica* Enteritidis SA215 was 0.0001, while the worst was 1. In the bacterial challenge assay conducted by Lee et al. (2020), phage KFS-EC inhibited the growth of *E. coli* O157:H7 for 8 h across different MOIs (0.01, 0.1, 1.0, 10, and 100) without significant differences. Shang et al. (2021) observed a significant inhibitory effect of bacteriophage vB_SalP_TR2 against *S.* Albany at MOI 0.01 and 0.001, while the inhibitory effect was not prominent at MOI 0.1. According to these authors, this discrepancy could potentially be attributed to the use of a small measurement volume (200 µL) and the adoption of a static culture condition during the experiments. It is important to note that all these studies evaluated the lytic activity of phages at a single temperature and with specific bacteria, without considering the effect of different levels for each factor and their interactions. In contrast, in the present study, factors such as temperature, MOI, *S. enterica* serovar, and bacteriophage significantly influenced bacterial growth inhibition ($p < 0.05$). In general, lower MOI was found to lead to higher lytic activity compared to higher MOI, depending on temperature, *S. enterica* serovar, and bacteriophage type.

For the phage cocktail, increasing the MOI from 0.001 to 1 significantly enhanced its lytic activity during the 6 h-incubation period. However, at MOI 10, the lytic activity of the cocktail decreased significantly compared to MOI 1 ($p < 0.05$). This dose-dependent response has also been observed in other studies. Lu et al. (2022) demonstrated a time-dose-dependent reduction in *S. enterica* Typhimurium, with phage ST-3 effectively inhibiting bacterial growth at MOI 10 within 6 h and suggesting that phage ST-3 might prove beneficial in reducing *S. enterica* Typhimurium infections. ST-3's ability to suppress bacterial growth, eliminate biofilms, and synergize with antibiotics provides novel ways to treat drug-resistant bacteria. This research sheds light on phage ST-3's biological characteristics and predicts its potential role in bacteriophage-antibiotic infection control. Abdelsattar et al., (2023) also reported a dose-dependent reduction in *S. enterica* Typhimurium following a 5-h exposure to

bacteriophage ZCSE9 treatment. Additionally, Ni et al. (2020) found that bacteriophages PN05 and PN09 completely inhibited the growth of *Pseudomonas syringae* pv. *actinidiae* (Psa) strain (SCJY02-1) for 12 h at different MOIs (0.1, 1, 10, and 100) using single bacteriophage suspensions of bacteriophages PN05 and PN09, as well as a cocktail of both bacteriophages. The single bacteriophages effectively inhibited Psa growth for 24 hours.

The lytic activity against *S. enterica* in both single bacteriophage treatment and the bacteriophage cocktail in different concentrations resulted in the inhibition of bacterial growth compared to each control. The use of a phage cocktail is preferred in managing bacterial co-infections, as it can potentially enhance the overall lytic activity and reduce the chances of bacterial resistance development (Almutairi et al., 2022; Lin et al., 2017; Nale et al., 2021b; Thanki et al., 2022; Wei et al., 2019; Wong & Wang, 2022a).

Moreover, bacterial regrowth was observed as the treatment time increased, which could be due to the emergence of bacteriophage-resistant bacteria. Continuous exposure to bacteriophages can select resistant strains, posing challenges to the long-term effectiveness of phage therapy (Ni et al., 2020). Environmental variables such as temperature, pH, and nutrient availability can significantly impact bacteriophage efficiency and stability (Silva et al., 2014). Changes in environmental conditions can lead to decreased stability and effectiveness of bacteriophages (Fister et al., 2016; Jończyk-Matysiak et al., 2019; Li et al., 2022). Therefore, it is essential to consider these factors when utilizing bacteriophage cocktails as antimicrobial agents.

5.6 Conclusion

This study highlights the potential of bacteriophage as a promising alternative to chemical sanitizers for controlling foodborne pathogens, particularly *S. enterica*. The single bacteriophages S7, S10, and S13 exhibited high lytic activity against multiple *S. enterica* serovars, including those commonly associated with foodborne illnesses. The findings also

emphasize the importance of considering factors such as temperature, MOI, *S. enterica* serovar, and phage interactions in assessing bacterial growth inhibition. Furthermore, phage cocktails have shown comparable efficacy to single bacteriophage treatments, suggesting their efficacy in managing bacterial co-infections and reducing the development of bacterial resistance. However, continuous exposure to phages and variations in environmental conditions can pose challenges to long-term effectiveness. Therefore, further research and careful consideration of these factors are essential for effectively utilizing bacteriophage cocktails as antimicrobial agents. Overall, this study provides valuable insights into the potential application of phages for controlling foodborne pathogens and underscores the need for comprehensive investigations in this field. Further research is warranted to explore the application of bacteriophages *in vivo* and their potential for mitigating foodborne illnesses.

5.7 References

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Chapter V

6 Controlling *Salmonella enterica* in Water Systems for Lettuce Production using a Bacteriophage Cocktail

6.1 Abstract

Controlled environment agriculture has gained popularity in recent years, especially for hydroponics, vertical farms, and aquaponics, in which plants develop without soil using nutrient solutions and substrates. However, concerns have emerged regarding food safety, requiring new strategies to mitigate risks of produce contamination. This study investigates the efficacy of a bacteriophage cocktail as a biocontrol agent against *S. enterica* contamination in lettuce-growing recirculating systems. *S. enterica* Newport and *S. enterica* Typhimurium were inoculated into nutrient solutions of aquaponic and hydroponic systems to simulate contamination following a phage cocktail treatment (S7, S10, and S13) at different multiplicity of infection (MOI 0.01 and MOI 1). Results showed a significant reduction in *S. enterica* Newport and *S. enterica* Typhimurium populations in both MOI 0.01 and MOI 1 ($p < 0.05$) in aquaponic and hydroponic nutrient solutions, reaching values below the limit of detection (LOD) after 3 to 4 days post-bacteriophage cocktail inoculation. Also, there were significant reductions in *S. enterica* Newport and *S. enterica* Typhimurium levels in plant roots from the aquaponic nutrient solution treatment (p-values <0.05) but not a very consistent reduction in plugs. Contrarily, the microbial population of *Salmonella* serovars in plugs and roots from the hydroponic nutrient solution had a significant reduction reaching levels below the LOD in both phage cocktail treatments after 2-day inoculation. These findings highlight the potential of using bacteriophage as a tool to

improve food safety in indoor-grown lettuce by controlling *S. enterica* and the need for future research to understand the microbial interactions within each type of system.

Keywords: food safety, hydroponic, aquaponic, *Salmonella*, bacteriophage, water quality, leafy greens.

6.2 Introduction

Controlled environment agriculture (CEA) is a modern farming approach that precisely manages environmental factors as temperature, light, and nutrients, with hydroponics being a key method where plants grow without soil, using water and substrates (Srivani et al., 2019, Ahamed et al., 2023). This technique is also used in aquaponics and vertical farming operations (Kloas et al., 2015, Sharma et al., 2023). CEA operations primarily use recirculating water systems, which can potentially spread foodborne pathogens that may occur within the system. The sources of water contamination can include feces from animals and birds, as well as poor employee hygiene. Recent concerns about food safety highlight the potential risk of contamination in CEA operations of harmful microorganisms (Dong & Feng, 2022), especially as hydroponics and aquaponics have grown in popularity in the United States in recent years (Broad et al., 2022).

Several foodborne outbreaks have been linked to contaminated water, which is a major concern for the production of fresh produce (Liu et al., 2018;Broad et al., 2022). In 2021, the U.S. Food and Drug Administration (FDA) reported the first foodborne outbreak caused by *S. enterica* Typhimurium in lettuce grown under a hydroponic indoor system (FDA, 2022a). In 2022, another hydroponic operation voluntarily recalled 633 cases of crunch, butter, and romaine whole-head lettuce due to potential *S. enterica* contamination (FDA, 2022b). In the following year, several hydroponically grown lettuces were recalled over concerns of a potential *Listeria monocytogenes*

contamination (FDA, 2023a). These events have raised questions about the safety of indoor agriculture systems and the search for preventive measures. Although current food safety standards emphasize prevention, specific treatments utilizing multidisciplinary approaches are needed to solve fresh produce safety issues (Raffo & Paoletti, 2022; Vågsholm et al., 2020). More recently, alternatives to control bacterial contamination, including biological resources, have become a sustainable option and a valuable tool for vegetable production (Sellitto et al., 2021).

Bacteriophages, also known as phages, are viruses that infect bacteria and can perform as antimicrobial agents against multiple types of pathogens (Abedon et al., 2021). Using bacteriophage as a biocontrol agent in the food sector has significantly increased during the past few years (Endersen & Coffey, 2020; Hudson et al., 2005). Bacteriophage cocktails have been explored as a potential postharvest strategy to mitigate food safety concerns for lettuce operations (Wong et al., 2019). Bacteriophages have also the potential to be utilized in both preharvest and postharvest practices to control foodborne pathogens without affecting the native microbiota of the system (Endersen & Coffey, 2020). Bacteriophages have been suggested as potential indicators and alternative treatments to adequately manage infections, biofilms, and foaming inside wastewater treatment facilities; while protecting beneficial bacteria within an ecosystem (Jassim et al., 2016). However, the application of bacteriophages to control foodborne pathogens in CEA, specifically in recirculating systems for vegetable production, has not yet been explored.

There is a lack of data necessary to define effective good agricultural practices and recommendations for using bacteriophage for CEA businesses. The objective of this study was to evaluate the efficiency of a phage cocktail against *S. enterica* serovars in recirculating systems for indoor lettuce production.

6.3 Materials and Methods

6.3.1 Salmonella Preparation

S. enterica Newport (ATCC 6962) and *S. enterica* Typhimurium (ATCC 14028) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and used for inoculation into the recirculating water systems to simulate contamination. Serovars were isolated from frozen stock cultures and incubated with Triple Sugar Iron (TSI; BD Difco™, Sparks, Maryland, United States) in tubes containing 50 ppm nalidixic acid and 50 ppm rifampicin for 24 h at 37 °C. A a 10 µL loopful of bacterial cells was transferred to a 12.5 ml tube containing Luria-Bertani Miller (LBM; BD Difco™, Becton, Dickinson and Company Sparks, MD, USA) broth and overnight shaken at 150 RPM at 37 °C. The liquid culture was then poured into a sterile glass tube and adjusted to a McFarland scale of 0.5 using approximately 300 mL of phosphate-buffered solution (PBS) before inoculating the system.

6.3.2 Bacteriophage Cocktail Preparation

The previously identified bacteriophages used as a cocktail in this study (S7, S10, and S13) were isolated from environmental samples and obtained from the laboratory of Dr. Stuart Price at the Auburn University Veterinary School (Chapter IV). For the amplification phase, *S. enterica* Enteritidis (ATCC 13076) was used as a host for all three bacteriophages, in accordance with Chen et al., 2018. Briefly, *S. enterica* Enteritidis was inoculated into 1.5 mL of LBM broth (Difco™, Becton, Dickinson and Company Sparks, MD, USA) at pH 7.0 and incubated overnight at 37 °C at 150 RPM. A 125 mL of the overnight culture was then transferred into 12.5 mL of LBM broth (BD Difco™, Becton, Dickinson and Company Sparks, MD, USA) at pH 7.0, containing 150 mL of each phage stock. The mixture was incubated at 37 °C and shaken at 150 RPM for approximately 3 hours.

The resulting culture was transferred to a sterile 50 mL centrifuge tube, mixed with 100 µL of chloroform (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged for 20 minutes at 10,000 x g (RCF) at room temperature. The supernatant was subsequently filtered using a 2 µm membrane filter (Acrodisc® Syringe Filters,St Columb Major, Conmwall, UK) and stored at 4 °C. The titer of the suspension was measured using the double-layer agar technique previously described by Jagannathan et al. (2020) and Panec & Sue Katz (2016). A 200 µL aliquot of *S. enterica* Newport and *S. enterica* Typhimurium were each mixed into sterile tubes with 100 µL of each bacteriophage supernatant. The tubes were incubated in a water bath at 56 °C for 20 minutes. Subsequently, the mixtures were combined with 3 mL of soft agar LBM supplemented with 0.7% Bacto™ Agar (BD Difco™, Becton, Dickinson and Company Sparks, MD, USA). The resulting mixture was carefully poured onto pre-warmed LBM agar (BD Difco™, Becton, Dickinson and Company Sparks, MD, USA) plates and subjected to further incubation at 37 °C for 24 hours. Following the incubation period, the plaques formed by bacteriophages were counted and enumerated to determine the plaque-forming units per milliliter (PFU/mL) and kept under incubation until further use.

6.3.3 Nutrient Film Technique System

Six small-scale nutrient film technique (NFT) systems were built based on a typical commercial-scale system. Each system consisted of two 1 m PVC channels with removable covers, each having five plant spaces, accommodating a total of 10 plants per system (**Figure 6.3.3.1**). Treatments were added to a 5-gallon bucket of nutrient solution (e.g., aquaponic and hydroponic) equipped with a 155-GPH submersible aquarium pump (Fountain Pump, Thompson Way, Santa Maria, CA, USA) for effective recirculation of the water and an aquarium heater 150 W, 9.5” range from 20 to 35.5 °C (Orlushy, Submersible Aquarium Heater, Guangdong, CN) to maintain the nutrient solutions at a fixed temperature of 25 ± 1 °C.

Figure 6.3.3.2 Small-scale nutrient film technique (NFT) systems with 2 channels containing five plant spaces fitting 10 plants per system in a 5-gallon bucket were equipped with a pump for water recirculation efficiency and a water heater to maintain the nutrient solution at a fixed temperature of 25 °C. The systems were set in a Biosafety Level-2 (BSL-2) laboratory at Auburn University (Auburn, AL, USA).

6.3.4 Lettuce Seedling and Production

Lettuce seeds (*Lactuca sativa* 'Salanova Red Oakleaf'; Johnny's Selected Seeds, Winslow, ME 04901, USA) were seeded into 200 Grodan Rockwool Cubes (Grodan, P.O, NL) and grown in the Vertical Farm at Auburn University (Auburn, AL, USA) for approximately three weeks before being transplanted into the NFT systems (**Figure 6.3.4.1**). A fertilizer solution containing 8.2 g of Hort America's Hydroponic Fertilizer (9-37-7; Hort Americas LLC, Bedford, Tx, USA), 4 g of Epsom Salt (EpsoTop Magnesium Sulfate K+S, Kassel, DE) and 3.5 g of calcium nitrate (Yara International Drammensveien, Oslo, NO; pH range: 6.8 to 7.0) was used until plants reached 3 weeks.

Figure 6.3.4.1 Salanova Lettuce Red Oakleaf seedling onto 200 Grodan Rockwool Cubes at the Vertical Farm at Auburn University (Auburn, AL, USA).

6.3.5 Experimental Design

The experiment was conducted in a Biosafety Level-2 (BSL-2) laboratory at Auburn University (Auburn, AL, USA). *S. enterica* Newport and *S.enterica* Typhimurium were used to assess contamination dynamics in hydroponic and aquaponic systems and evaluate the efficacy of a bacteriophage cocktail in mitigating *S. enterica* proliferation. The hydroponic nutrient solution, formulated according to established guidelines of 8.2 g of Hort America's Hydroponic Fertilizer (9-37-7) (Hort Americas LLC, Bedford, Tx, USA), 4 g of Epsom Salt (EpsoTop Magnuesium Sulfate K+S, Kassel, DE) and 3.5 g of calcium nitrate (Yara International Drammensveien, Oslo, NO) was prepared in the laboratory on the same day the experiment was set. Simultaneously, the aquaponic solution, representing an existing aquaponic ecosystem, was obtained from the Aquaponic Research Center at Auburn University on the same day the experiment was set. A ProDSS Multiparameter Digital Water Quality Meter (YSI, Brannum Lane Yellow Springs, Ohio, USA) was used to measure pH, electrical conductivity (EC), turbidity, dissolved oxygen (DO),

and temperature during the experiment period. A 50 mL volume of each nutrient solution was sent to the Soil, Forage, & Water Testing Laboratory at Auburn University for nutrient analysis (**Table 6.3.5.1**).

Prior to introducing each nutrient solution to the system, the aquaponic and hydroponic solutions were tested for endemic antibiotic-tolerant *S. enterica* using the methods outlined by the Food and Drug Administration (2023).

Aquaponic and hydropic nutrient solutions were added to each experimental design unit in triplicate. The 3-week lettuce plants were immediately transplanted into each system under sterile conditions and left for 24 hours for plant acclimation to the systems. The system was constantly kept at a fixed temperature of 25 °C ± 1°C and pH ~7. After the 24-hour period, *S. enterica* strains were inoculated into each system individually at an initial concentration of 10^3 CFU/mL to mimic sporadic contamination. Subsequently, the bacteriophage cocktail composed of equal proportions of bacteriophages S7, S10, and S13 (1:1:1) at two multiplicities of infection (MOI) of 1 and 0.01 was introduced into the systems 30 minutes after *S.enterica* inoculation. The phage cocktail treatments were determined by phage dilution from the initial phage stock to achieve each MOI dose. Over a 6-day period, the *S. enterica* population was monitored by collecting samples regularly from water, plant tissues (roots and leaves), and the growing medium (plugs).

It is important to note that immediately after each treatment was complete, a 10% bleach solution was flushed through each system and left for 24 hours, followed by a triple wash with sterile water to ensure proper cleaning and sanitizing of each experimental unit. Water samples were collected and tested for *S. enterica* to ensure no bacteria remained in each system. Also, *S. enterica* strains were used in the same systems throughout the whole experiment to minimize cross-contamination.

Table 6.3.5.1 Physicochemical parameters and nutrient profile of nutrient solutions.

Parameters	Aquaponic	Hydroponic
pH	7.0	7.0
Conductivity[1]	1235 µS/cm	1496 µS/cm
Turbidity [2]	21.52 mg/L	1.99 mg/L
DO [2]	8054.45 mg/L	3.82 mg/L
Nitrate-N*	100,2	131.6
Sulfate-S*	-	51.5
Phosphorus- P_2O_5*	16.0	25.7
Potassium-K_2O*	149	255.9
Magnesium-Mg*	57.5	39.1
Calcium-Ca*	37	69.4
Boron -B*	<0.1	0.8
Copper -Cu*	<0.1	0.3
Iron-Fe*	<0.1	2.6
Manganese-Mn*	<0.1	1
Molybdenum-Mo*	-	0.05
Zinc -Zn*	<0.1	0.8
Soluble Salts*	680	1.28

*ppm; [1]µS/cm; [2]mg/L; - no reference values.

6.3.6 Evaluation of Salmonella Growth and Bacteriophage Cocktail Behavior

Nutrient solution samples were collected after 12 hours of the initial inoculation of bacteriophage and at every 24 hours for six days. The water sample was collected from the NFT channels through a side opening in the system. An aliquot of 100 µL was spread plated in plaques containing Xylose Lysine Decarboxylase agar (XLD, Oxoid Ltd, Lasingstoke, Hants, UK). To increase the limit of detection (LOD), an additional aliquot of 100 mL was collected from each reservoir and filtered using membrane filters 0.45 µm (Whatman, Amersham Place, Little Chalfont, Bucknghamshire, UK) and placed in XLD agar (Oxoid Ltd, Basingstoke, Hants, UK). The samples from the spread plate and membrane filter were incubated for 24 hours at 37 °C, and

the colonies were counted by hand to determine the bacterial population levels. The bacterial population levels were expressed as log CFU/mL. Each experiment was performed in triplicate.

The leaf tissue, roots, and plugs were collected from one aleatory plant per NFT system at days 2, 4, and 6 and placed in sterile Whirl-Pak® bags. Samples were diluted in a ratio of 1:5 (weight/volume) with PBS solution containing 0.2% Tween 80 and 0.1% sodium thiosulfate (Fisher Scientific, Fair Lawn, NJ, USA) and placed in a stomacher (Stomacher® 400CIRCULATOR, Seward Inc, Bohemia, NY) at 300 RPM for 30 seconds. Subsequently, 100 µL of each sample was spread-plated onto plates using a sterile L-spreader. The plates were then incubated at 37 °C for 24 hours, after which the colonies were counted by hand, and the results were expressed as log CFU/mL.

6.3.7 Evaluation of Final Bacteriophage Cocktail Population

The final bacteriophage population was determined after the 6-day incubation period. An aliquot of 12 mL from each reservoir was placed into a sterile 50 mL centrifuge tube, mixed with 80 µL of chloroform, and centrifuged for 20 minutes at 10,000 x g (RCF) at room temperature. The samples were filtered using a 2 µm membrane and stored at 4 °C. The double-layer agar technique previously described by Jagannathan et al., (2020) and Panec & Sue Katz, (2016) was used to measure the final bacteriophage population in the samples, and results were expressed as plaque forming unit (PFU) per mL.

6.3.8 Data analysis

A generalized linear model (GLM) was performed using R Statistical Software (version 4.2.2). In the GLM, the *S. enterica* population values (Log CFU/mL) were analyzed using phage cocktail treatments, nutrient solution, *S. enterica* serovar, and time as fixed effects. Separate analyses were conducted in leaf tissue, roots, and plugs. A multifactorial analysis of variance

(ANOVA) with paired means analysis and significance letters were conducted to examine differences among treatments and days, where $p < 0.05$ was considered statistically significant.

6.4 Results

The three-way interaction among treatment, nutrient solution, and days was shown to be statistically significant ($p < 0.001$). This observation implies that the *S. enterica* population is significantly influenced by the combined factors. Furthermore, we noted substantial two-way interactions. The study found a significant interaction between treatment and water ($p < 0.001$), there was a significant correlation between treatment and days ($p < 0.001$), and the interaction between water and days ($p < 0.05$).

The research identified significant three-way interactions among treatment, days, and nutrient solutions. In the aquaponic nutrient solution, *S. enterica* Newport and *S. enterica* Typhimurium in the control started at mean concentrations of 2.10 and 2.46 log CFU/mL, respectively, after 12 hours of inoculation into the system; while the initial mean concentrations of *S.* Newport and *S.* Typhimurium after 12 hours inoculation in the hydroponic nutrient solution were 1.42 and 1.17 log CFU/mL, respectively (**Figure 6.4.1** and **Table 6.4 1**). Overall, in the results of *S. enterica* control, there was no significant difference in the mean log CFU/mL of *S. enterica* Newport and *S.enterica* Typhimurium over time for both aquaponic and hydroponic nutrient solutions ($p > 0.05$). In general, phage treatments (MOI 0.01 and MOI 1) demonstrated a statistically significant reduction of *S. enterica* Typhimurium and *S. enterica* Newport populations over time when compared to the control treatment ($p < 0.001$) in both aquaponic and hydroponic nutrient solutions.

For a MOI of 0.01, significant reductions in *S. enterica* Newport populations were observed in both aquaponic and hydroponic nutrient solutions. In the aquaponic solution, a 1.34 log CFU/mL reduction compared to the control occurred after 2 days, with levels reaching below the LOD after 3 days. In the hydroponic solution, a significant difference was noted after just 1 day, with a reduction of 1.17 log CFU/mL compared to the control. Levels also reached below the LOD by day 3 of inoculation. For *S. enterica* Typhimurium, a significant 1.3 log CFU/mL reduction compared to the control was observed in the aquaponic nutrient solution after 2 days, reaching levels below the LOD after 3 days. In the hydroponic nutrient solution, a significant difference was only observed after 3 days, with a reduction of 1.51 log CFU/mL compared to the control, with levels reaching below the LOD after 4 days.

For MOI 1, in the aquaponic nutrient solution, there was a significant reduction of 1.55 log CFU/mL in *S. enterica* Newport populations after 3 days when compared to the control, with levels reaching below the LOD after 4 days. In the hydroponic nutrient solution, a significant reduction below the LOD was observed for *S. enterica* Newport populations after 3 days. For *S. enterica* Typhimurium in the aquaponic nutrient solution, there was a significant reduction of 1.26 CFU/mL after 3 days, with bacterial levels reaching below the LOD after 6 days. While in the hydroponic nutrient solution, a significant reduction of 1.61 log CFU/mL was observed after 3 days, with bacterial levels reaching below the LOD after 4 days.

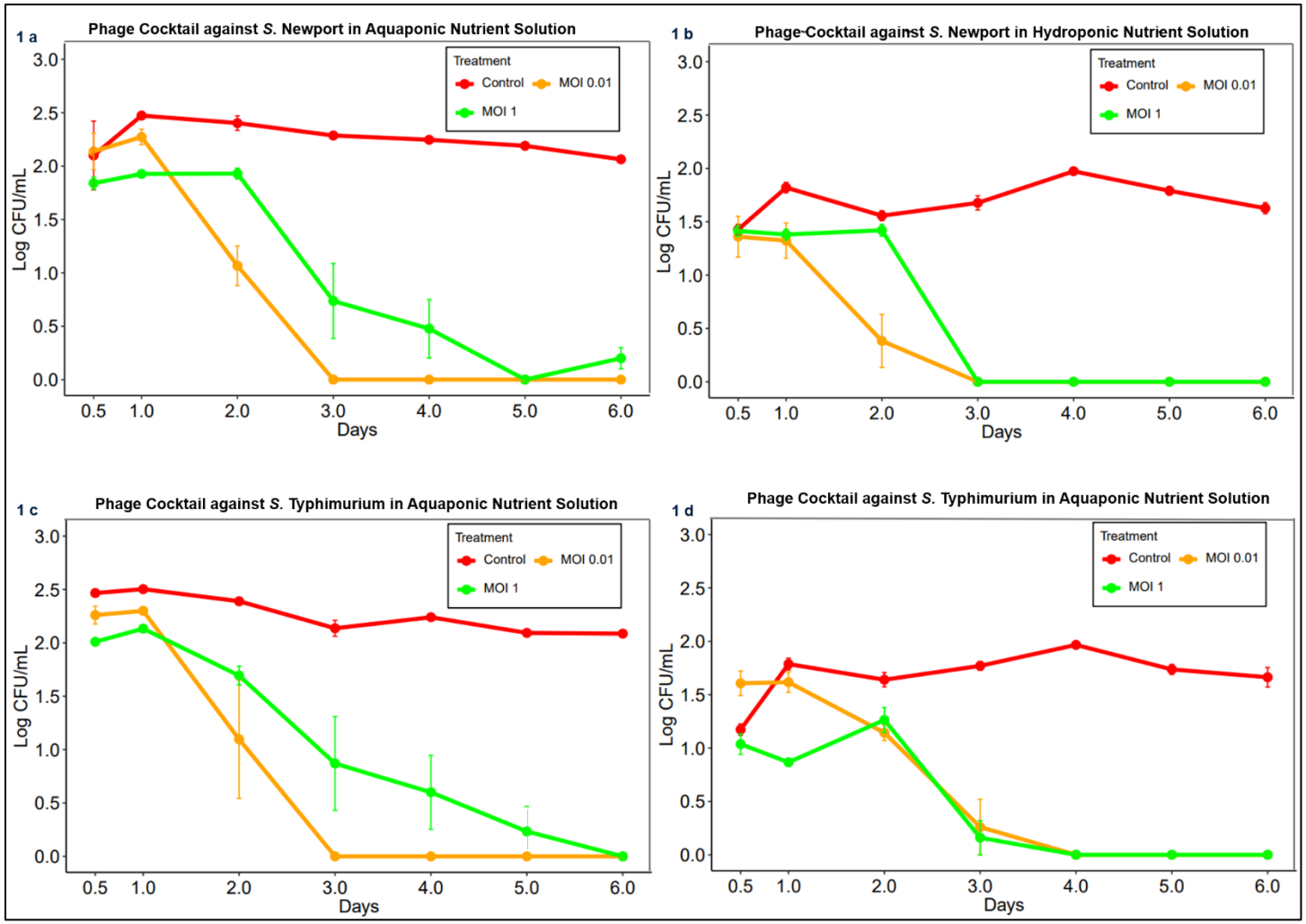

Figure 6.4. 1 The graphs represent the interactions among *S. enterica* Newport under phage treatments (MOI 0.01 and MOI 1) in aquaponic nutrient solution (1 a) and hydroponic nutrient solution (1 b), and *S. enterica* Typhimurium under phage treatments (MOI 0.01 and MOI 1) in aquaponic nutrient solution (1 c) and hydroponic nutrient solution (1 d) over a 6-day incubation period.

Table 6.4.1 Log population of *S. enterica* Newport and *S. enterica* Typhimurium recovered from nutrient solutions over a 6-day incubation period of bacteriophage cocktail inoculation in aquaponic and hydroponic nutrient solutions.

		Aquaponic			Hydroponic		
		Treatments[1]					
Salmonella enterica	Time (days)[2]	Control	MOI 0.01	MOI 1	Control	MOI 0.01	MOI 1
S. Newport	0.5	2.10± 0.55A[3]a[4]	2.13 ± 0.29Aa	1.84 ± 0.09Aa	1.42 ± 0.08Ac	1.36 ± 0.33Aa	1.41 ± 0.08Aa
	1	2.47 ± 0.01Aa	2.27 ± 0.12ABa	1.92 ± 0.05Ba	1.82 ± 0.08Abc	1.32 ± 0.28Ba	1.38 ± 0.08Ba
	2	2.40 ± 0.16Aa	1.06 ± 0.32Cb	1.93 ± 0.08Ba	1.55 ± 0.08Acb	0.38 ± 0.43Bb	1.42 ± 0.09Aa
	3	2.28 ± 0.05Aa	<LOD B[5]c	0.73 ± 0.60Bb	1.67 ± 0.11Aabc	<LOD Bc	<LOD Bb
	4	2.24 ± 0.01Aa	<LOD Cb	0.47 ± 0.01Bbc	1.97 ± 0.03Aa	<LOD Bc	<LOD Bb
	5	2.19 ± 0.03Aa	<LOD Bc	<LOD Bc	1.79 ± 0.07Aabc	<LOD Bc	<LOD Bb
	6	2.06 ± 0.03Aa	<LOD Bc	<LOD Bc	1.62 ± 0.09Aabc	<LOD Bc	<LOD Bb
S. Typhimurium	0.5	2.46 ± 0.01Aa	2.26 ± 0.14Aa	2.01 ± 0.03Aa	1.17 ± 0.09Bb	1.60 ± 0.19Aa	1.03 ± 0.16Bab
	1	2.50 ± 0.02Aa	2.30± 0.03Aa	2.13 ± 0.05Aa	1.78 ± 0.09Aa	1.61 ± 0.16Aa	0.86 ± 0.02Bb
	2	2.39 ± 0.02Aa	1.09 ± 0.95Ba	1.69 ± 0.15Ba	1.64 ± 0.11Aa	1.14 ± 0.12Bb	1.26 ± 0.20Ba
	3	2.13 ± 0.13Aa	<LOD Cb	0.87 ± 0.76Bb	1.77 ± 0.07Aa	0.26 ± 0.45Bc	0.16 ± 0.27Bc
	4	2.24 ± 0.01Aa	<LOD Bc	0.60 ± 0.60Bbc	1.96 ± 0.01Aa	<LOD Bc	<LOD Bc
	5	2.09 ± 0.47Aa	<LOD Bc	0.23 ± 0.40Bbc	1.73 ± 0.08Aa	<LOD Bc	<LOD Bc
	6	2.08 ± 0.03Aa	<LOD Bc	<LOD Bc	1.66 ± 0.15Aa	<LOD c	<LOD Bc

[1] Each data point represents the combined LS-means (log CFU/mL) of three replicates, ± SD
[2] Time post-inoculation
[3] Values in rows followed by different capitalized letters are significantly different at $p < 0.05$ among treatments.
[4] Values in columns followed by different letters are significantly different at $p < 0.05$ over time for each treatment.
[5] Limit of detection (LOD

The following results of the bacteriophage cocktail against *S. enterica* Newport and *S. enterica* Typhimurium for plant parts evaluation in aquaponic nutrient solution are demonstrated in Table 6.4. 2. Throughout all treatment groups and nutrient solutions, except for the *S.enterica* Newport control in the aquaponic nutrient solution at day 6 post-inoculation, the presence of *S. enterica* Newport and *S. enterica* Typhimurium in the leaves remained below the LOD. In the aquaponic nutrient solution, both *S. enterica* Newport and *S. enterica* Typhimurium were detected in plugs and plant roots two days after inoculation, with population levels of 1.20 and 2.07 CFU/mL in the plugs, and 1.83 and 1.99 CFU/mL in the roots, respectively. In the hydroponic nutrient solution, on day 2, *S. enterica* Newport and *S. enterica* Typhimurium populations.

Overall, there was a significant reduction in the mean log CFU/mL of *S. enterica* Newport and *S. enterica* Typhimurium controls over time for both aquaponic and hydroponic nutrient solutions ($p > 0.05$), except for *S. enterica* Typhimurium recovered from plugs and roots in the hydroponic nutrient solution (**Table 6.4.2**).

In the aquaponic nutrient solution, there were no statistically significant differences in the mean CFU/mL values when compared to the control for both *S. enterica* serovars over time in both bacteriophage cocktail treatments ($p > 0.05$). The only exception was for *S. enterica* Typhimurium recovered from the roots in the MOI 0.01 treatment, where a slight decrease (0.72 log CFU/mL) in bacterial populations occurred after 4 days of inoculation into the system. Contrarily, in the hydroponic solution, both *S. enterica* serovar levels exhibited a significant reduction compared to the control, to the extent that bacterial populations could not be recovered above LOD (**Table 6.4.2**).

Table 6.4. 3 Log population of *S. enterica* Newport and *S. enterica* Typhimurium recovered from leaves, plugs, and roots of lettuce after 2, 4, and 6 days of bacteriophage cocktail inoculation in aquaponic and hydroponic nutrient solutions.

			Treatments and Recovery Times								
			Control [1]			MOI 0.01			MOI 1		
Salmonella enterica	Nutrient solution	Plant Parts	2 days	4 days	6 days	2 days	4 days	6 days	2 days	4 days	6 days
S. Newport	Aquaponic	Leaves	<LOD[2]A[3]a[4]	<LOD Aa	0.10 ± 0.17Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa
		Plugs	1.20 ± 1.04Aa	1.46 ± 0.10Aa	0.56 ± 0.48Aa	1.67 ± 0.52Aa	0.79 ± 0.44Aab	0.54 ± 0.28Ab	1.86 ± 0.14Aa	1.67 ± 0.30Aab	0.74 ± 0.64Ab
		Roots	1.83 ± 0.14Aa	0.63 ± 0.71Ab	0.54 ± 0.47Ab	1.31 ± 0.50Aa	0.36 ± 0.39Ab	0.32 ± 0.27Ab	2.03 ± 0.01Aa	0.77 ± 0.40Ab	0.54 ± 0.47Ab
	Hydroponic	Leaves	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa
		Plugs	1.26 ± 0.27Aa	0.93 ± 0.21Ab	<LOD Bc	<LOD Ba	<LOD Ba	<LOD Aa	<LOD Bb	<LOD Bb	1.46 ± 0.14Aa
		Roots	1.42 ± 0.71Aa	0.16 ± 0.27Ab	<LOD Bb	<LOD Ba	<LOD Aa	<LOD Ba	<LOD Bb	<LOD Ab	1.26 ± 0.38Aa
S. Typhimurium	Aquaponic	Leaves	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa
		Plugs	2.07 ± 0.17Aa	1.11 ± 0.29Ab	0.49 ± 0.50Ab	2.02 ± 0.16Aa	0.16 ± 0.27Bb	0.63 ± 0.54Ab	1.69 ± 0.16Aa	1.33 ± 0.57Aa	0.96 ± 0.33Aa
		Roots	1.99 ± 0.07Aa	0.92 ± 0.45Ab	0.50 ± 0.52 Ab	0.72 ± 0.31Ba	<LOD Ba	<LOD Aa	1.92 ± 0.12Aa	0.55 ± 0.49ABb	0.30 ± 0.51Ab
	Hydroponic	Leaves	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa	<LOD Aa
		Plugs	1.50 ± 0.07Aa	1.19 ± 0.34Ab	1.62 ± 0.04Aa	<LOD Ba	<LOD Ba	<LOD Ba	<LOD Ba	<LOD Ba	<LOD Ba
		Roots	1.13 ± 0.74Aa	0.33 ± 0.35Ab	0.44 ± 0.76Aab	<LOD Ba	<LOD Aa	<LOD Aa	<LOD Ba	<LOD Aa	<LOD Aa

[1] Each data point represents the combined LS-means (log CFU/mL) of three replicates, ± SD.
[2] Limit of detection (LOD).
[3] Values in rows followed by different upper-case letters are significantly different at $p < 0.05$ among treatments.
[4] Values in rows followed by different lower-case letters are significantly different at $p < 0.05$ over time within each treatment.

Lastly, the final population of bacteriophage cocktail titer in aquaponic nutrient solution was presented as a mean ± standard deviation of $3.45 \pm 0.57 \times 10^7$ PFU/mL for MOI 0.01 and $4.12 \pm 1.15 \times 10^8$ PFU/mL for MOI 1. In the hydroponic nutrient solution, the final phage cocktail population was $2.32 \pm 0.57 \times 10^6$ PFU/mL for MOI 0.01 and $2.78 \pm 0.57 \times 10^7$ PFU/mL.

6.5 Discussion

Our study aimed to address the lack of information regarding lettuce contamination in recirculating water systems production. As the agricultural industry undergoes transformation, there is a growing need to embrace innovative strategies in order to ensure food quality and safeguard public health. According to Pal (2015) and Sieiro et al. (2020), bacteriophage therapy presents a viable and environmentally friendly approach to addressing the issue of infectious bacteria in aquaculture, which poses a threat to aquatic organisms. The significance of this antibiotic alternative has grown due to the emergence of antimicrobial-resistant bacteria (Wagh et al., 2023).

This study has offered significant insights into the effects of treatment methods, nutrient solution, and time duration on *S. enterica* growth. The differences in results between aquaponic and hydroponic nutrient solutions show the significant impact of the nutrient solution on the persistence of *S. enterica*. Liu et al. (2018) studied the presence and persistence of *S. enterica* in water, demonstrating that aquatic sources can act as a reservoir for *S. enterica* contamination in produce, thereby facilitating its transmission. Mayton et al., (2019) also explored the adhesion of *S. enterica* Typhimurium to spinach leaf surfaces and interactions among water chemistry and nutrient availability. The findings of the study on spinach leaf surfaces by Mayton et al. (2019) indicate that bacterial adhesion is influenced by growth conditions and solution complexities.

Similarly, the differential behavior of *S. enterica* in aquaponic and hydroponic nutrient solutions implies that the properties of the nutrient solution have a substantial effect on the persistence of *S. enterica.*

The survival of bacteria in the environment is influenced by the availability of nutrients, such as nitrogen, and energy sources (Hoagland et al., 2018). In aquaponic nutrient solutions, the composition includes nutrient-rich liquid effluent derived from fish feces, decomposing organic matter, and fish waste (Fox et al., 2012). Environmental factors, including nutrient availability, pH levels, and water temperature, have been found to influence the population dynamics of *S. enterica* by Liston, 1972. *S. enterica* exhibits optimal growth in soil within a pH range of 6.5 to 7.5 and a temperature range of 20 to 30 °C (Podolak et al., 2010). Despite the favorable conditions for the growth of *S. enterica*, such as optimal pH, temperature, as well as sufficient nutrient levels in both systems, the presence of the bacteria was detected in higher concentrations under an aquaponic nutrient solution. Culot et al., (2019) and Liu et al., (2022) aimed the use of bacteriophages in aquatic farming exhibits potential as a viable approach to mitigate the alteration of the natural microbiota in aquatic environments, due to phage-specificity. Regardless of limited ongoing interactions with environmental phage treatment, existing literature suggests that there is a lack of evidence associated with phage treatments and microbial communities' interactions.

The observed differences between the control group and the treatment groups (MOI 0.01 and MOI 1) indicate the potential effectiveness of bacteriophage-based treatments in reducing *S. enterica* contamination in aquaponic and hydroponic nutrient solutions. This suggests that such treatments could be a promising strategy for addressing bacteria contamination in leaf greens produced under nutrient solution. The study's findings are consistent with previous research conducted by Kocharunchitt et al. (2009);

Żbikowska et al. (2020); Bao et al. (2015); Capparelli et al. (2010) on the effectiveness of bacteriophages in controlling bacterial populations, specifically in the context of treatment using phages.

The study by Au et al. (2021) presents a thorough analysis of data from the past decade, offering valuable insights into the effectiveness of phage therapy in addressing common foodborne pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., and *Campylobacter jejuni*. The findings presented by Au et al. (2021) highlight both the benefits and difficulties associated with phage therapy. Furthermore, Kuek et al. (2022) explored bacteriophages and found them to have significant potential in reducing bacterial populations, with applications in food processing and livestock management. Our study reveals a significant decrease in *S. enterica* Newport and *S. enterica* Typhimurium in aquaponic and hydroponic nutrient solutions. This reduction is particularly evident when the MOI is 0.01. In the aquaponic system, a significant reduction of 1.34 log CFU/mL in bacterial populations was observed within a short duration of 2 days post-inoculation when compared to the control group. Remarkably, the population of bacteria in the aquaponic nutrient solution decreased below the LOD within just 3 days post-inoculation. This highlights the rapid and effective effect of phage treatment in this particular context as explored by Lee & Harris (2001), who showed that treatment with *Salmonella* Felix 0-1 phage lysate resulted in a significant decrease in the population of *S.* Typhimurium in pigs within 3 hours post-*S. enterica* infection. The study conducted by Gong & Jiang (2017) aimed to simulate the greenhouse conditions in order to investigate the effectiveness of bacteriophage treatment under temperature and relative humidity, and reported *S. enterica* Typhimurium strain 8243 exhibited a decrease over a time of 4 days, followed by an abrupt and significant decrease after 7 days. In the bacteriophage treatment group, there was a consistent decrease in the population of *S.*

enterica attachment and the formation of biofilm throughout the investigation's time period (Gong & Jiang, 2017). In addition, the experimental group showed a substantial decrease in bacterial count compared to the control group. Specifically, a reduction of approximately 3.4 log CFU/cm² was observed on the 7th day. This reduction resulted in a bacterial population below the detection limit of 0.4 log CFU/cm² (Gong & Jiang, 2017).

The findings from our study into the impact of bacteriophage cocktail treatment on the presence of *S. enterica* Newport and *S. enterica* Typhimurium in different plant parts (leaves, plugs, and roots) within aquaponic and hydroponic nutrient solutions reveal several significant trends and effects. The plugs and roots had a significantly higher bacterial population compared to the leaves due to direct contact with the inoculated nutrient solution. Barak & Schroeder (2011) specifically highlighted the risk of human pathogens spreading from contaminated nutrient solutions to the edible parts of plants. It is highlighted by Dankwa et al. (2020) that the substrates could be a major part of a potential source of contamination in hydroponic systems, consequently promoting the transmission of microorganisms to the harvested crops.

Our research findings are consistent with observed patterns in bacteriophage survival and effectiveness in aquatic ecosystems, highlighting the potential of bacteriophage treatments to reduce bacterial contamination. Abedon (2015) and Batinovic et al. (2019) have conducted studies that consistently show the efficacy of bacteriophages in controlling bacterial proliferation in complex ecosystems. This suggests that bacteriophages have the ability to effectively control bacterial growth under different conditions. Pereira et al. (2011) and Duarte et al. (2018) corroborate these findings, demonstrating consistency in the survival and efficacy of bacteriophages within aquatic ecosystems. Al-Ishaq et al. (2020) highlight the importance of conducting additional research to identify the most suitable bacteriophages and thoroughly assess

their growth characteristics and propensity for resistance development. In summary, our research aligns with prior studies (López-Gálvez et al., 2021; Possas & Pérez-Rodríguez, 2023;Dankwa et al., 2020; Moye et al., 2018) and highlights the potential of bacteriophage treatments to minimize bacterial contamination, specifically in water-based settings.

Our research suggests that bacteriophages have great potential to be explored to mitigate foodborne pathogens in the agriculture industry focused on hydroponic and aquaponic systems. Furthermore, additional researchers are required to achieve the goals of enhancing the process of bacteriophage selection while developing an in-depth knowledge of their growth dynamics and mechanisms of resistance.

6.6 Conclusion

In conclusion, this research offers significant contributions to our understanding of the intricate dynamics among *S. enterica* serovars, bacteriophage cocktails, and various nutrient solutions. The results emphasize the potential of using bacteriophage treatment as a strategy for reducing bacterial contamination in aquatic settings, while variability in effectiveness among various bacteriophage strains and doses is apparent. This study effectively demonstrates the efficacy of bacteriophage treatment in reducing bacterial populations across different plant parts. This implies that bacteriophage therapy can potentially be a valuable strategy for improving food safety within agricultural systems. Additional research is required to optimize bacteriophage cocktails for improved effectiveness against *S. enterica* and bacteriophage-resistant mutants. Furthermore, exploratory studies are essential before bacteriophage treatment can be considered a highly effective method for controlling *S. enterica* under a recirculating water system.

Future investigation is required to aim at the microbial interaction in different nutrient solutions.

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Chapter VI

7.0 Future Considerations

The use of phages in the food production has the potential to effectively mitigate *S. enterica* infection within the food industry. Nevertheless, it is important to consider the pragmatic elements associated with implementing this microorganism, including its cost-effectiveness, simplicity of use, and adherence to regulatory requirements**.**

Customized phage cocktails have an important level of potential for effectively eliminating certain *S. enterica* strains. The primary difficulty is in the identification of the most efficacious combinations and comprehending their interactions with both one another and the specific bacteria they are intended to target. The investigation of various sources for *Salmonella*-specific phages in the context of environmental bacteriophage sourcing may contribute to the expansion of available resources for the management of *S. enterica*. This strategy needs to consider the ethical and ecological ramifications to ensure the responsible obtaining of phages.

Safety and Regulatory Approval: Although phage treatment approaches are usually considered safe, it is important to undergo thorough safety studies and get regulatory clearances to establish confidence among industry stakeholders and consumers. Additional investigation is required to ascertain the most efficient phages for the purpose of managing certain strains of *S. enterica* in CEA systems. This entails evaluating phage characteristics that are associated with treatment efficacy *in vivo*.

Assessment of the environmental impact aims to evaluate the environmental consequences associated with using phage treatment within CEA systems, specifically focusing on the possible impacts on beneficial bacteria and ecological equilibrium. In addition, it examines the potential scalability of bacteriophage treatment in the context of commercial controlled environment agriculture (CEA) operations, with the objective of

addressing the requirements of large-scale food production.

The establishment of global cooperation in phage research enables the consolidation of resources, knowledge, and data, hence expediting advancements in the realm of food production that prioritize safety and sustainability on a global scale.

7.1 Conclusion

Overall, the findings highlight the potential effectiveness of bacteriophages as biocontrol agents for mitigating *S. enterica* contamination in food products. The behaviors of these organisms are highly targeted, posing no harm to individuals of the human, animal, or plant species. Moreover, their adaptability enables them to effectively counteract bacterial resistance, particularly when used in combination. It is critical to understand the optimum development conditions for bacteriophages and *Salmonella* serotypes, since this knowledge serves as crucial for their effective use in the control of contaminated food.

The outcomes presented in the study provide significant insights into the use of bacteriophage treatment in the context of food safety, emphasizing the need to take into consideration factors such as temperature and MOI when implementing bacteriophage-based treatments. In additional, this study provides significant findings about the efficacy of bacteriophage cocktails as a biocontrol mechanism for mitigating *S. enterica* contamination in water recirculating systems, including hydroponic and aquaponics. It is essential to acknowledge the future issues to fully exploit the potential of bacteriophage treatment in augmenting food safety and security within the realm of contemporary agriculture. These endeavors possess the capacity to transform CEA systems, mitigate occurrences of foodborne outbreaks, and provide consumers agricultural goods that are both safer and more sustainable.