ABSTRACT

Title of Dissertation: SURVIVAL AND PERSISTENCE OF LISTERIA AND ESCHERICHIA COLI AND CHANGES IN PHYSICOCHEMICAL PARAMETERS IN AQUAPONICS SYSTEMS DURING LETTUCE PRODUCTION

Ghadah Alhammad, Doctor of Philosophy, 2023

Dissertation directed by: Professor David K. Y. Le
Department of Nutrition and Food Science

Aquaponic (AP) food production systems integrate aquaculture and hydroponics in soilless controlled environments while conserving space and natural resources (soil, water, air). Increasing global demand for high-quality quality nutritious, safe food continues to increase exponentially. Information specific to AP is needed to validate operational and handling practices for AP food safety.

The **first study** is to assess the persistence of *E. coli* TVS 354 in bench-scale aquaponic systems (180 days after primary plant harvest). **Methods:** follow-up on the previous study and evaluate growth and persistence of *E. coli* TVS 354 at 180 days after primary plant harvest. Samples were collected from 10 independent aquaponic systems and bacterial growth was evaluated including aerobic plate count (APC), coliform bacteria (CB), heterotrophic plate count (HPC), and cultural enrichment for *E. coli* TVS 354 levels. **Results:** *E. coli* TVS 354 was not detected in samples from hydrotons (25 g), filters, fish feces, core and roots. The aerobic bacteria count of fish and plant tank samples ranged between 2.5 to 3 log CFU/mL for all treatments, while aerobic bacteria count on the core of the lettuce plant was significantly lower, ranging from 1.44 to 2.08 log CFU/mL.

The **second study** is to evaluate the survival of *Listeria innocua* 2066 in effluent from fish tanks and plant tanks of aquaponic research units. **Methods:** four different treatments: fish tank water, fish tank water-replenished, plant tank water, and plant tank water-replenished. Each bottle was inoculated with *Listeria innocua* 2066, and bacterial growth and water chemistry conditions were evaluated over 7 days to identify physiochemical parameters associated with *Listeria innocua* 2066 survival and growth. **Results:** In 24 hours, a significant reduction ($p<0.05$) in *Listeria inccoua* 2066 populations was observed for all treatments with a total reduction 3.9-3.7 log CFU/mL. *Listeria innocua* 2066 counts were lowest on day 7 for treatment groups, ranging from 1.1 log CFU/mL to 1.0 log CFU/mL. Treatments that were replenished daily from the aquaponic systems had higher counts of *Listeria innocua* 2066 compared to treatments that were non-replenished. The lowest APC on day 0, immediately after inoculation, ranged from 4.16 log CFU/mL to 4.32 log CFU/mL. A significant increase ($P<0.05$) in APC count was observed for all treatments in 24 hours while there is no significant difference in APC values between the treatments ($p=0.35$).

The final and **third study** is to evaluate survival of inoculated *Listeria innocua* 2066 and nonpathogenic *Escherichia coli* and changes in AP physicochemical parameters. **Methods:** A four-week bench-scale AP experiment (n=12) with four goldfish (*Carassius auratus*)/aerated 37L tank, a 3-step biofilter, and four lettuce plants (*Lactuca sativa var. Truchas)*/37L hydroponic tank was conducted. Treatments were high and low-dose (6-log and 2-log CFU/mL, respectively) of *Listeria innocua* 2066-^{Er} or *E. coli*-^{Rifr} TVS 354, and uninoculated controls. *Listeria innocua*-2066-^{Er} and *E. coli*-^{Rifr} populations, mesophilic counts (APC), and physicochemical parameters (pH, temperature, dissolved oxygen, turbidity, ammonia, nitrite, and nitrate) were analyzed in plant tank water, and biofilters until plant harvest. *Listeria innocua* 2066-^{Er} and *E.coli*-^{Rifr}

enumeration and APC (Petrifilm®) from lettuce shoots, roots and rockwool were determined at harvest. **Results:** *Listeria innocua* 2066 and *E. coli* TVS 354 populations declined significantly within 24 hours post-inoculation and were undetectable at day 14 and day 12 respectively ($P < 0.05$). This decline was observed for both high and low-dose treatments. *Listeria innocua* 2066 *and E. coli* TVS 354 were detected in biofilters until week 4. At harvest, *Listeria innocua* 2066 and *E. coli* TVS 354 were recovered from lettuce roots, and rockwool, but not from plant leaves, and 100mL plant tank water grab samples. Lower leaves preharvest had a significantly higher APC (5.1- 6.4 log CFU/mL) relative to harvested lettuce upper leaves (2.8 - 4.2 log CFU/mL). Plant tank water pH had a significant effect ($P<0.05$) on *Listeria innocua* 2066 *and E. coli* TVS 354 survival. **Significance:** These results provide insights into the survival dynamics and sites of *E. coli* TVS 354 and *Listeria innocua* 2066 in AP and associated physicochemical conditions. These findings contribute to our understanding of potential food safety risks and associated risk factors such as inputs, physicochemical factors, and other environmental conditions in aquaponics systems.

SURVIVAL AND PERSISTENCE OF LISTERIA AND ESCHERICHIA COLI AND CHANGES IN PHYSICOCHEMICAL PARAMETERS IN AQUAPONICS SYSTEMS DURING LETTUCE PRODUCTION

by

Ghadah Abdulrahman Alhammad

Dissertation submitted to the Faculty of the Graduate School of the
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Advisory committee:
Professor Jiuzhou Song, Chair
Professor David K. Y. Lei
Professor Abani K. Pradhan
Professor Pat Millner
Professor Jose-Luis Izursa
Professor Seong-Ho Lee
Professor Shaik Rahaman

Dedication

'To my father, who passed away at the beginning of my Ph.D. journey and who always called me a doctor when I was a child and dreamed of seeing me as a doctor. Your words and advice are always with me. Without you, I would have never achieved this.'

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Introduction

The 21st century shows most people enjoy fast and easy access to food. This can be attributed to the new and varying methods of food production, including the aquaponics system that combines aquaculture and hydroponic systems. Aquaponics was introduced to address food demands of the rising number of global urban populations with limited space for traditional agriculture (Bradley, 2014). Aquaponics is one of the agricultural methods of food production that is becoming well-known today as it is quite sustainable and efficient. While producing enough quantity of food is important to feed the growing global population, food safety is equally crucial (Bradley, 2014).

Given the worldwide population is drastically increasing every year, safe food production plays a vital role in sustaining the world's inhabitants. Therefore, it is crucial to ensure food safety. However, there is no standard guideline specifically for aquaponic systems to ensure food safety. According to the Food and Drug Administration's 2005 Food Code, foodborne infections result in around 76 million ailments, 5,000 deaths and 325,000 cases of hospitalization in the United States alone, annually (Castro et al., 2009). In addition, the yearly expenditure related to these diseases is in the middle of $10 billion to $83 billion in regard to decreased efficiency, physical pain and medical fees (Castro et al., 2009).

It is imperative to guarantee the safety of food, starting from farm to plate. All food producers and sellers have a moral and legal responsibility to make sure that food is not contaminated with bacteria and toxic chemicals that may harm consumers (Castro et al., 2009). Despite the advancement of technology in producing food, it is still important to ensure that food delivered or sold to buyers is safe for consumption. Thus, food producers including aquaponics growers should

become more mindful and responsive to the factors that may pose risks to their food production system and should uphold safe and quality agricultural methods.

Cases of food contamination are increasing and should be given more attention as it may carry dangers to the health of the population, this research will discuss the growth, transfer, and persistence of non-pathogenic surrogate strains of typically enteric bacteria in replicated, bench-scale, aquaponic systems dosage trials.

Hypotheses

1. *Listeria innocua* 2066 and *E. coli* TVS 354 survival and persistence in a bench-scale aquaponics system are associated with the initial bacterial inoculate (e.g., high-dose inoculate will persist for longer than low-dose inoculate).
2. Plant tank water physiochemical parameters (i.e., pH, temperature, dissolved oxygen, and total ammonia nitrogen, nitrites & nitrates) play a key role in bacterial growth and persistence in aquaponic systems and are thus key parameters to consider for aquaponics system food safety management.

Problem Definition

Leafy vegetables are often eaten uncooked, making bacterial pathogen contamination of leafy vegetables a considerable food safety issue. Studies have reported pathogen contamination of lettuce, basil, and other vegetables grown in controlled agriculture environments, including Aquaponics (AP) and Hydroponic (HP) systems (Wang et al., 2020). In 2021, two soilless leafy greens (LGs) producers issued product recalls for potential contamination with *Salmonella* and

Listeria monocytogenes raising concerns about the safety of soilless operations and practices, which are rapidly expanding in the USA and globally (FDA, 2021). These observations suggest a pressing need for data on potential food safety risks and risk factors to inform the development of pre-harvest and post-harvest risk management strategies. The proposed project would generate data on growth and persistence of inoculated pathogen surrogates in AP systems and interactions with background microflora and physicochemical factors. The results of this study will provide useful data for producers, regulators, and researchers.

Objectives

The objectives of this dissertation are to:

1. Evaluate survival and persistence of *E. coli* TVS 354 and *Listeria innocua* 2066 in bench-scale aquaponics (AP) systems.
2. Examine bacterial localization in the systems (plant tank, filter sponge, roots, leaves and rockwool).
3. Assess changes in water physicochemical parameters (temperature, pH, dissolved oxygen, total ammonia nitrogen, nitrites, nitrates) over time in bench-scale AP systems.

Chapter 1: Literature Review

One of the most significant accomplishments of humankind was the development of agriculture to sustain the growing global population in producing nutritious food. Originating over 12,000 years ago, agriculture revolutionized the way that people lived and allowed humans to transition from a hunter-gatherer lifestyle to a sedentary one. Because of agriculture, civilizations were able to grow, and the human population continues to grow, resulting in healthy society (Kennett & Winterhalder, 2006). Throughout the years, there have been countless developments in farming and agriculture that have allowed humans to improve the way they grow and raise their food. Aquaponics is a recently coined term describing an agricultural system with ancient roots. The system integrates fish farming and vegetable production into a closed loop system that allows for an efficient use of resources. Modern aquaponics has been praised for its sustainable nature, but the system of agriculture is not without issue.

The purpose of this study is to shine a light on the microbial aspects of food safety in aquaponic systems.

1.1. History and Importance of Aquaponics

Modern aquaponics emerged in the 1970s, but the earliest examples of aquaponics can be traced back to the Aztec and Mayan civilizations in about 1,000 A.D. These societies grew plants on rafts and created a system of agricultural islands where nutrient-rich waste (also known as liquid effluent) from aquatic life was dredged from the canals to the plants on the islands above. The liquid effluent from the canals fertilized the crops, allowing them to grow. Aquaponics systems also existed in the Far East, where rice paddy fields were integrated with systems to

raise different species of fish. Ancient systems in China were even found to incorporate ducks into this system to create a symbiotic relationship between multiple species of plants and animals (Bradley, 2014).

Aquaponics saw a resurgence in the 1970s with farmers who were attempting to reduce their dependence on water, land, and other resources. Fish were traditionally farmed in large ponds or in ocean pens made of netting, but these methods are often costly and are not particularly efficient. Recirculating Aquaculture Systems (RAS) were later introduced that allowed fish to be kept in denser populations than in traditional fish farms. Currently, the three main species reared by RAS in North America are salmon, rainbow trout and channel catfish (Chalmers, 2004). RAS works by filtering water from fish farms so that the water can be reused in the tank. Filtration involves solids removal, ammonia removal, carbon dioxide removal and oxygenation. These systems yield more fish than traditional systems, but fish are more susceptible to disease and infection because they are kept so densely. To counteract this, fish raised through RAS are often given large doses of antibiotics to keep them healthy, which have lasting impacts on the environment (Bradley, 2014). Even after the development of RAS, farmers still needed to find a way to efficiently raise healthy fish using as few resources as possible.

One of the first systems bred from this desire to create an efficient fish farm was developed by Dr. Mark McMurty at North Carolina State University. In the mid-1980s, Dr. McMurty and team created a closed loop aquaponics system where liquid effluent from fish tanks was used to irrigate cucumber and tomato plants in sand beds that acted to filter the water. Liquid effluent is derived from fish manure and decomposing organic matter and is used to provide essential nutrients for plant growth. After the effluent was filtered through the plants, the water was recalculated back to the fish tanks (Rakocy, 2012). The aquaponics system is similar

to many other RAS systems, but rather than just focusing on fish farming it focuses on plant farming as well. Soon after this system was perfected, it was put into place in commercial settings such as the Bioshelters in Amherst, MA. At this facility, herbs and vegetables are grown in gravel beds that are irrigated by Tilapia tanks. This system has been frequently duplicated and is now an essential component to the future of food security (Rakocy, 2012).

As the human population grows, food production becomes even more important. Globally there are over 870 million people that are currently undernourished, which is about one eighth of the population (Tscharntke, et. al., 2012). The demand for food is expected to increase by 14% every decade, which is particularly detrimental in developing countries where food scarcity is already a serious issue (Tscharntke, et. al., 2012). Aquaponics is a system that allows people to produce multiple different types of food while using less space and fewer resources than many other systems. In the near future, aquaponics or similar systems will be essential in order to keep up with the high demand of food. These systems will also be essential in limiting the negative impact that commercial farming has on the environment. Of the 140 million metric tons of fish and shellfish consumed yearly, about 20 million are produced through some form of aquaculture. The other 120 million metric tons of shellfish and fish are harvested from naturally occurring populations, but because these populations are rapidly being depleted fish farming is becoming more and more essential (Chalmers, 2004). As natural fisheries dwindle and collapse, aquaculture and particularly aquaponics becomes more important. Across the globe, aquaculture is expected to increase to produce over 55 million metric tons of fish and shellfish by 2025 (Chalmers, 2004). The increases of these systems have a great deal of benefits, but they also face a number of challenges.

1.2. Microbial Contamination in Aquaponic Systems

Despite the potential for success with aquaponic systems, there are still a number of issues that must be addressed. There are economic issues at play that determine whether or not the system will be cost effective. These issues relate to socio-ecological challenges such as energy consumption, water use, and overfishing that determine whether the system can be effective and functional in certain regions. Finally, there are technical challenges such as pH stabilization, nutrient balance, phosphorus management, or pest and disease management that must be handled in order to ensure that the system is physically functional (Goddek, et. al., 2015). Of all the issues surrounding aquaponics, the largest and most persistent issue is the overall safety of the food product. The purpose of implementing aquaponic systems is to farm food that will ultimately be consumed by people and animals. For this system to be practical in the real world, it must consistently yield food that is safe to eat. Because of the nature of this closed loop system, however, there are multiple aspects that affect the overall safety of the raw vegetable and fish products. There are chemical aspects such as pesticide, herbicide and antibiotic use that must be factored in to ensure that the system can yield enough healthy products. There are also physical aspects that must be dealt with such as contamination from foreign objects, metal of bone fragments or large pieces of soil and rock. Finally, there are relevant biological aspects of food safety, which will be the main focus of this paper. The biological aspects of food safety in aquaponics are essential to understand in order to have a fully functioning agricultural system that produces safe and healthy food products.

There are a number of different types of biological contamination that are relevant in aquaponic systems. Of all the potential contaminants, the main contaminants of concern are *E. coli*, *Listeria*, *Aeromonas*, and *Staphylococcus*. These bacteria are all relevant concerns in many

forms of agriculture and are particularly dangerous for humans. If food is contaminated with any one of these bacteria, it could cause serious and potentially life-threatening issues for the consumers. Because these bacteria all have the potential to be deadly, it is essential to fully understand their threat to aquaponic systems to determine whether or not the systems can be a reliable method for large-scale food production in the future.

1.2.1 *Escherichia coli.*

E. coli are bacteria that are found in the lower intestines of most mammals and birds. They are a large group of gram-negative, anaerobic, rod-shaped bacteria and many serotypes of the bacteria are harmless to humans. There are currently 150 to 200 serotypes of the bacteria, separated by somatic, capsular, fimbrial, and flagellar antigens (Totsika et al., 2012). Even though most types of *E. coli* are harmless to humans, types exist that are pathogenic to their hosts. When these pathotypes are ingested, it can result in serious illness in humans and can lead to death. Of the many types of *E. coli,* six of them are diarrheagenic *E. coli* and are commonly associated with illness in humans. These pathotypes are Shinga toxin-producing *E. coli* (the most common of the pathotypes), enterotoxogenic *E. coli*, enteropathogenic *E. coli,* enteroaggregative *E. coli,* enteroinvasive *E. coli,* and diffusely adherent *E. coli*.

Pathogenic *E. coli* has a specific ecology that makes it difficult to control. The bacteria are constantly being shed by most mammals and birds through their waste. The waste products are then mixed with soil into fertilizer, which is used to treat crops. *E. coli* can be swept from land to ground and surface water, and it can live without a host for over ten weeks (Totsika et al., 2012). Humans can ingest *E. coli* from the water they drink, from food treated with animal waste or through the ingestion of contaminated meat.

Because of the potential for food poisoning, the presence of *E. coli* is often the cause for food recalls. Because *E. coli* is found in the lower intestines of warm-blooded animals and fish are cold-blooded, aquaponic systems are less likely to be contaminated with *E. coli* than other, soil-based agricultural systems (Fox et al., 2013). Although this has been the consensus thus far, the large-scale commercialization of aquaponics is relatively new and there is limited research on food safety. Aquaponics is a water-based system, and the main way that bacteria are carried through the system is through the water. Because of this, it is important to understand how easily bacteria like *E. coli* can be transmitted through the water, and how much of the bacteria in the water is a sign of danger. *E. coli* bacteria are often used as "indicator microbes "by government agencies and industries to assess water safety in agricultural systems. Agencies look at the levels of generic *E. coli* in the water and use those levels to predict how much pathogenic *E. coli* might be in the water. There are currently no specific standards for how much *E. coli* is allowed to be in irrigation water for it to be considered safe. The Food Safety Modernization Act produces a safety rule that specifies microbiological water quality criteria for irrigation water, which is based on the presence of generic E. coli, which can indicate fecal contamination. The criteria are based on two values, the geometric mean (GM) and the statistical threshold (STV). The GM value of the water samples should be 126 or less CFU of generic E. coli per 100 mL of water and the STV of samples should be 410 CFU or less in 100 mL of water (Rock, 2019). These standards have no basis in food safety, and furthermore, recent research shows that measuring generic *E. coli* is a poor indicator for foodborne illness. Because there are no meaningful tests to determine how much *E. coli* in the water is dangerous, aquaponic systems have an undetermined level of risk for contamination by *E. coli.*

1.2.2. *Listeria* monocytogenes.

Listeria is a genus of gram-positive, rod-shaped, anaerobic bacteria. Currently, there are fifteen different species of *Listeria* that are known to science. Of the different species, one of the most concerns in terms of foodborne illness is *Listeria monocytogenes*. This species of bacteria has the potential to cause Listeriosis in humans. Listeriosis primarily affects the elderly, newborns and infants, pregnant women, and adults that suffer from a weakened immune system, but healthy adults can contract the infection as well. If Listeriosis is contracted, there is a case-fatality rate of about twenty percent. This makes the infection the third-leading cause of death among foodborne bacterial pathogens (Chalmers, 2004). It is possible to treat Listeriosis with antibiotics, but even with rapid treatment, the infection can still result in death. Because of the serious nature of *Listeria*, the outbreaks of this infection are carefully monitored. In the United States when two or more individuals contract this infection, it is considered an outbreak by the Center for Disease Control.

Listeria monocytogenes has a unique biology that causes it to be particularly dangerous. The bacteria are found in soil and in water and can be carried by animals that do not present any symptoms of infection. It has been found in products such as unpasteurized dairy and meat and presents the largest threat in foods that are uncooked. When present, *Listeria* is often found living on the surface of vegetables. Specifically, the bacterium resides in biofilm, which is a living coating on a plant's roots and leaves that contains harmful and beneficial microbes. Vegetables like leafy greens are the most susceptible to bacteria like *Listeria* because they have large surface areas with many folds. In a 1998 study, *Listeria* was cultivated from hybrid striped bass that was raised in three different freshwater systems in Maryland (Chalmers, 2004). This proves that it is possible for *Listeria* to be present in farmed fish. *Listeria* has been found in a number of fishery products such as raw and processed fish that have been collected from retail establishments and

processing facilities. Another review, however, found no presence of the species in freshly harvested fish from wild or aquacultural sources (Dillon and Patel, 1992). It can be concluded that it is possible for processing to be the cause for contamination rather than the aquaponics farming process itself. In order to prove an association between processing and *Listeria* and to prove a lack of association between *Listeria* and aquaponics farming, further research must be done.

One of the most important aspects of the bacterium to understand is the fact that it is capable of surviving without the presence of oxygen. It is also able to grow in temperatures as low as 0 degrees Celsius, which is unusual for most common bacteria. This means that even if a product is refrigerated, the bacteria could still be living on the product. If the bacterium is present in a facility, it is particularly difficult to manage and remove (Chalmers, 2004). Some scientists believe that because of its qualities, *Listeria* is the greatest microbial threat to aquaponics food safety (Pattillo, 2015). Still, there is a lack of research to confirm how much of a threat the bacteria are to hydroponic systems.

1.2.3. *Aeromonas*

Aeromonas is a genus of bacteria that has been separated into fourteen different species. Of the different species, almost all of them have been associated with human diseases. It is a gram-negative anaerobic, rod-shaped bacterium that is structurally very similar to *E. coli, Salmonella*, and other Enterobacteria. Of the pathogenic strains of *Aeromonas*, the most common are *A. hydrophila, A. caviae, and A. veronii.* Of the strains, *A. hydrophila* is the one that is most commonly associated with fish. These bacteria are present in most bodies of fresh and brackish water. Diseases associated with the bacteria include gastroenteritis for those who have consumed the bacteria through contaminated water or food, and wound infections, which, in their most severe form, can present as necrotizing fasciitis. Gastroenteritis from *Aeromonas* is self-limiting,

and with supportive care people who are infected with the bacteria typically recover. The people who are most susceptible to *Aeromonas* infection are the young, the elderly, and the immunocompromised (Janda and Abott, 2010).

One of the greatest threats to aquaponics that *Aeromonas* presents is the fact that it is associated with both fish and with water. Like *Listeria, Aeromonas* can be found in the biofilm of plants, particularly leafy greens. Since it is present in most bodies of fresh and brackish water, it is also associated with fish. *A. hydrophila* is common on the mucosal surfaces and organs of many healthy fish, but some strains of Aeromonas have been found to be pathogenic to fish. There have been reported cases of disease in eels and with outbreaks of furunculosis in salmonids (Chalmers, 2004). The dose of *Aeromonas* must be relatively high to be infectious in humans, however, which makes it less dangerous than many other bacteria (Pattillo, n.d.).

1.2.4. *Staphylococcus*

Staphylococcus is a genus of bacteria that includes at least forty different species. They are gram-positive bacteria that derive their name from the Greek word for grape, because they are round and group in clusters. Most *Staphylococcus* bacteria are harmless, and the bacteria have been found to be present in up to twenty-five percent of people (Foster, 1996). These bacteria are found worldwide and are often a component of the microbial flora of soil. *Staphylococcus aureus* is the most commonly observed strain and the one that is most likely to cause infection in humans. When ingested, the bacteria are able to make several different toxins in the body that can all be responsible for gastrointestinal illness.

Staphylococcus is a bacterium that is a larger concern in the processing stage than it is in the farming stage. It is most often transferred to food by workers who directly come into contact

with food without washing their hands. Once on the food product, the bacteria can grow and produce toxins. The toxins are destroyed with high heat, so foods that are consumed raw are the ones that are most dangerous to consume. There are currently no studies that have produced statistics about the presence of *Staphylococcus* in aquaponic systems (LeChevallier and Seidler, 1980).

Methicillin-resistant *Staphylococcus aureus*, also known as MRSA, has also been found in bodies of water, and even in swimming pools, which are treated to kill bacteria (Tolba, et. al., 2008). In Brazil, a study found 175 samples of fish fillets, shrimp, and crabmeat that were infected with *Staphylococcus,* but it was undetermined at what stage the food was infected with the bacteria (Ayulo, et. al., 1994). It is extremely important for researchers to explore the presence of *Staphylococcus* in aquaponic systems to better understand the safety of the systems.

1.3. Hazard Analysis and Critical Control Points

Microbial contaminants are an extremely serious concern when dealing with aquaponic systems. Because of the threats associated with these contaminants, it is essential that there are food safety and quality plans in place to ensure that food produced in aquaponic systems is safe to consume. In the United States, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) was created with help from the U.S. Department of Agriculture (USDA), the Department of Health and Human Services, the National Marine Fisheries Service, the Department of Defense, industry, employees and academia to achieve this goal. The NACMCF adopted the Hazard Analysis and Critical Control Points system as an "effective and rational means of assuring food safety from harvest to consumption" ("HACCP Principles & Application Guidelines," 1997). HACCP is used in basic agriculture, food processing, catering, and in other

similar situations involving food to eliminate or significantly reduce hazards, to prevent or minimize microbial growth and toxin production and to control contamination ("HACCP Principles & Application Guidelines," 1997).

The HACCP system was first developed in the 1960s by Pillsbury, a popular food company that was working on producing safe foods for the NASA space exploration program. The program was used publicly for the first time in 1971 and by 1973 it was being used for low-acid canned foods. Twelve years later, the National Academy of Science recommended that the entire food industry should be using HACCP, and so should food sanitary agencies. Later the United Nations and the *Codex Alimentarius* Commission decided to use the HACCP as the recommended International Code of Practice for the General Principles of Food Hygiene ("HACCP Principles & Application Guidelines," 1997). This decision shows how important HACCP procedures are for food safety.

Industry members cannot simply implement HACCP in their workplace; there are a number of prerequisites that must be met. The workplace must follow and meet Good Manufacturing Practices and Sanitation Standard Operating Procedures. Management must take preliminary steps to control the conditions in the workplace such as identifying their products, sanitizing equipment, conducting preventative maintenance and training employees ("HACCP Principles & Application Guidelines," 1997). After these preliminary requirements and conditions are met, HACCP principles may be adopted in a workplace.

HACCP consists of seven major principles. The first principle pertains to hazard analysis (HA). Management must conduct a hazard analysis of their facilities and identify the preventative measures that should be used to avoid the contamination of food. In order to

conduct this analysis, first a multidisciplinary team must be assembled with members who have insight into how a food production or food handling operation is run. This team must describe the product, including its composition, packaging, storage and delivery methods, and then construct a flow diagram that includes all of the steps in the operation of how that product is handled. After a diagram has been created and the process is understood, the team must then identify any and all potential hazards associated with each step of the operation. Hazards can be biological, chemical or physical, and once these hazards are identified, preventative measures to minimize these hazards must be considered.

The second principle involved in HACCP is to identify the critical control points (CCP) in the process. A critical control point is described as, "a step at which control can be applied and where it is essential to prevent or eliminate a food safety hazard or to reduce it to an acceptable level" ("HACCP Principles & Application Guidelines," 1997). When considering microbial contaminants, times when raw material is introduced into the operation are typically the most significant critical control points. Often, the final product can avoid microbial contamination with the addition of preservatives, adjustment in pH levels, or thermal processing such as refrigeration or freezing. The CCP that are involved in an operation are specific to the product and to the process involved. Factors such as changes in product formulation, changes in equipment or operational flow, or even the size and shape of packaging affect the critical control points and must be addressed in order to successfully use HACCP.

Once critical control points have been fully established, the next principle in HACCP is to establish critical limits for each critical control point. A critical limit is defined as, "a criterion which should be met for each measure associated with a CCP, to ensure safety" ("HACCP Principles & Application Guidelines," 1997). These limits are control points that are set so that

organizations understand how much contamination is acceptable before it is considered dangerous. In a food production or processing operation, it is virtually impossible to contain one hundred percent of contaminants. Because of that fact, industry members must determine scientifically based factors that, when followed, produce a safe product. Critical limits can be based on temperature, time, moisture level, viscosity, or a number of other factors. An example of a critical limit is meat, which must be cooked to a certain temperature in order to be considered safe. Industries put these limits on meat because it has been determined that cooking to a certain temperature kills bacteria that could potentially contaminate the meat.

The next principle involves establishing monitoring systems for each CCP so that management can regularly assess whether a CCP is under control. Organizations must determine what needs to be monitored, how it should be monitored, how often it should be monitored and how frequently it should be monitored. All these components must be explicitly expressed as part of a fully functioning HACCP. Next, organizations must establish corrective actions that should be taken if the monitoring systems indicate that critical limits are not met. An important part of HACCP is to establish critical limits that, when followed, eliminate or reduce food safety hazards. If these critical limits are not met by a food production or processing facility, corrective actions must take place so that the facility does meet these critical limits. Individuals evaluating must determine whether a product is a public health risk based on expert evaluation and chemical and microbiological analysis. If the product is considered a risk, it must be reworked or reprocessed, and if this is impossible the product must be destroyed ("HACCP Principles & Application Guidelines," 1997).

Principle six of HACCP instructs users to establish procedures to verify that HACCP is working correctly. Verification is defined as the "application of methods, procedures, tests and

other types of evaluation, in addition to monitoring, to determine compliance with the HACCP plan" ("HACCP Principles & Application Guidelines," 1997). There are verifications for the critical control points such as the calibration of monitoring equipment and targeted sampling and testing, and there are verifications for the HACCP plan as a whole, such as audits and microbiological analysis of the final product. Both levels of verification are equally important in order to yield a safe final product. Verification must be performed by the HACCP team or individuals that are qualified by training or experience. This step exists to ensure that the rest of the HACCP principles have been successfully carried out and to ensure that food is produced and processed in a safe way.

The final principle of HACCP is to establish an effective record keeping procedure that documents the HACCP system as a whole. HACCP is an extremely important and detailed system that requires a number of intricate steps in order to be successful. In order to ensure that this system can work successfully over a long period of time, the system must be thoroughly documented. Records must include the product identification, materials and equipment used, critical criteria and limits, corrective actions and instances when they were taken, information about the operator involved in the system, and any other relevant data to food production, processing, or HACCP in general.

1.3.1. HACCP in Aquaponic Systems

HACCP procedures are an essential part of the food production and processing industries but in most situations, the procedures are not applied to farming. For much of the time that HACCP was being used, it was under the general assumption that farming was too messy of a

process and that it would be impossible to control all of the factors necessary to make HACCP effective (Ryan, 2012). Aquaponics provides the new and unique opportunity to apply HACCP to the farming process, which could potentially lead to food products becoming safer and more regulated than ever before. Because there are hundreds of different set-ups for aquaponic systems, each systems management must establish their own HACCP that is specific to their system. There is, however, a general outline of necessary components for a HACCP that can be used for aquaponic systems.

Before a HACCP plan can be put in place, a flow diagram must be established that provides a simple outline of the steps involved in the food handling process. In the situation of aquaponics farming, one flow diagram must be developed for food safety, and another must be created for food quality. Worker controls, facility controls, and management controls must all be considered for these diagrams. Each of the potential food quality process concerns must be thoroughly explored in order for the HACCP team to develop an effective HACCP plan. Fish, water, fish food, the medium used as a biofilter, and the plants grown in the grow-beds are all examples of inputs that are necessary to study and regulate in an aquaponics system. Each input comes with its own set of controls that are necessary in order to operate at HACCP standards (Ryan, 2012).

1.3.2. Controls in Aquaponic Systems

Of the many factors that require control in an aquaponics system, one of the most significant and well-studied is water. Virtually every type of farming system uses water at some point, but the way that water is used in aquaponic systems is unlike many others. This is because

in an aquaponics system, water starts in a pond and then is moved through a filter and into grow-beds that contain vegetable plants. This means that unlike many other systems, the water being used is re-circulating and has been recycled (Tyson & Simonne, 2014). Because of the nature of the system, it is recommended that well water or municipal water sources be used. This is so that the water put into the system is as pure as possible. In developing countries, experts recommend using rainwater to reduce the cost of the system, but the use of rainwater can cause higher levels of contamination because it is not regulated the way that other sources of water are (Nichols & Savidov, 2011).

Another area where aquaponic systems must be controlled is in the biofiltration system. In order for water to be re-circulated through an aquaponics system, it must go through the process of biofiltration. Biofiltration involves the disciplines of mechanical engineering, microbial ecology, and aquaculture husbandry to filter water from the fishponds so that it can safely be used to nurture plants. Nitrifying bacteria are used in biofilters to consume nitrogen as part of the filtering process. The biofilter must be made from safe and non-toxic materials, and the frequency that the filter is cleaned must be strictly controlled to maintain the health of the biofilm of nitrifying bacteria. It is very important to strictly control the support media for the biofilter because if it is not cleaned enough the filter will not be effective, but if it is cleaned too much it could also become ineffective (Horowitz & Horowitz, 2000). This support media can easily harbor pathogens if it is not properly maintained, but the use of HACCP is an effective way to ensure that the system remains healthy. When nitrogen filters are used effectively, it has been proven that the nitrogen concentrations in vegetables grown in aquaponic farms are the same as the concentrations in vegetables grown in hydroponic farms (Pantanella, et. al., 2010).

Using HACCP, the filtration process can be consistently regulated, suggesting that aquaponics is a valid production system for vegetables, despite the use of recycled water.

1.3.3. UV Sterilization

UV sterilization is an important process in order to maintain the health of an aquaponics system. It can be used in conjunction with HACCP procedures to ensure that the system suffers as little contamination as possible. The system itself is relatively simple, but it yields very positive results. UV sterilization consists of one or multiple lamps that radiate Ultraviolet (UV) beams. UV is a type of electromagnetic radiation that has a wavelength between 10 and 380 nm. UV radiation has enough energy that it is able to cause many chemical reactions to occur. The energy that comes from UV radiation alters or disrupts the DNA or RNA of many single-celled organisms such as algae, bacteria or protozoa. The radiation effectively kills many different types of bacteria, allowing the aquaponics system to be much more sterile. UV sterilization has a significant benefit because it does not leave behind any potentially harmful residuals that other systems may leave behind (Bell, 2015), There are many ways in which one can sterilize an aquaponics system, but other methods, such as chemical sterilization, may leave behind residue that would negatively affect the quality of the system and the quality of the foods produced in the system. Since UV sterilization works through radiation, there is no sort of potentially harmful residue left behind.

It is important to understand that although UV sterilization is the most effective and safe way to sterilize an aquaponics system, it cannot completely sterilize the system. Because of the nature of bacteria, the nature of the system, and the presence of living organisms, there is no method that can completely sterilize the system and still leave the system functional. The best way to maintain a healthy aquaponics system is to use UV sterilization and to create and follow

HACCP procedures. By analyzing hazards and developing critical control points and by incorporating sterilization into the system, aquaponic systems have a large opportunity for commercial success in the future.

1.3.4. HACCP Plan for UMD Aquaponic system

In order to have a safe and successful aquaponic system at UMD, a HACCP plan must be implemented so that people who work with the system can understand how to properly manage the system. Having an established HACCP plan will help to prevent contamination and increase the overall safety of the system. Because there are limited HACCP plans for aquaponic systems established and because most aquaponic systems are slightly different, it is most beneficial to create a plan unique to the UMD aquaponic systems.

When establishing a HACCP plan, the first principle that must be addressed is conducting a hazard analysis. One must look at the system as a whole and identify areas where significant hazards occur. Hazards can be physical, chemical, biological, or any other property that may make the food produced in the system unsafe for human consumption. In the UMD Aquaponic systems, there are multiple areas that could be considered hazardous.

Of the potential hazards in the UMD aquaponic systems, one of the most significant and most obvious sources for potential contamination is the water. Water is the one component that flows through the entire system. It is necessary to support the lives of the fish, and the lives of the plants. The same water for the fish is used for the plants, so if the water is contaminated it has the potential of polluting the entire system. There are many aspects of water that must be considered in order to develop a safe system. The first point to consider is the source of the water. In an aquaponics system, there are many potential sources for water. Some systems use

rainwater. Although rainwater is often the most contaminated source of water, it is typically the most sustainable source as well. Filtered water can be fed through the system, and so can city water. All these sources have their own strengths and weaknesses, and it is important to use a water source that is practical and safe. Water levels are also another important aspect to consider because too much water is wasteful, but too little water can increase the chance of contamination, especially in the fish tanks.

Fish are another potential hazard in aquaponic systems. Depending on where the fish came from, they could be harboring dangerous bacteria or parasites that could harm the fish and potentially harm the consumers. The source of the fish must be considered thoroughly tested in order to make sure that the fish are healthy before entering the system. At the UMD aquaponics farm, there was an incident where goldfish were introduced into the fish tank. This may have seemed like a harmless prank at the time. But the introduction of goldfish that were not screened for contamination is a very large problem. It is possible that these goldfish were carrying contaminants that will ultimately affect the food quality from the farm in a negative way, and it is difficult for researchers to know for sure that they were the cause of contamination in the tanks.

Relating to fish, the fish food introduced in the system is also a potential hazard. Depending on the source of the food, it could be contaminated with different bacteria or undesirable materials. If the food is kept for too long, it is possible that it could expire, and the food could become contaminated over time. If, for example, mold grows on the food and that food is fed to the fish, it would contaminate the system and possibly bring negative consequences to every part of the system.

The seeds used to grow vegetables and herbs are yet another hazard. Seeds could come into the system contaminated, and they could also contaminate the system if they are stored or handled improperly. A process must be developed where seeds are routinely sterilized before they are introduced into the environment in order to assure that they will grow into food that is safe for human consumption.

With all the potential sources of contamination, preventative measures must be incorporated into the system that will increase the safety of the food. UV sterilization is an important way to kill many types of bacterial contamination that can pollute a system. By making UV sterilization a standard part of an aquaponics system, even if bacterial contamination occurs, there is a very good chance that it will be eliminated before the food leaves the system and goes to the consumers.

Similar to UV sterilization, the use of biofilters is an important way to reduce contamination in an aquaponics system. A biofilter is a medium that facilitates the conversion of nitrites to nitrates. The nitrogen cycle is an important part of the aquaponics system, but because of the density of the fish population it is possible for the cycle to move too slowly and for too many nitrates to pollute the system. Having a proper functioning biofilter is extremely helpful in an aquaponics system in order to maintain the health of the plants and animals in the system.

Sanitization needs to be a fundamental component of an aquaponics system in order to develop a successful HACCP plan and in order to have a healthy system. Equipment such as hoses, scissors, tanks, tank filters, must be regularly sanitized in order to reduce the spread of contamination. Coupled with sanitization, the facility should be maintained to ensure that no pests such as mice, frogs, roaches, etc. could enter the system. Management of the system should

have a regular and structured plan in place for sanitization and for pest control, and employees must be trained in how to properly conduct these procedures and how to properly interact with the system in general. They must be trained to wear gloves, to practice good hygiene, and to follow proper planting and feeding procedures. The better trained the employees in a system are, the less likely problems will occur in the system.

1.3.5. Regulation

1.3.5.1. The FDA Food Safety Modernization Act (FSMA) Stander

The FDA Food Safety Modernization Act (FSMA) provides science-based minimum standards for the safe growth, harvest, packaging, and handling of fruits and vegetables for human consumption (US FDA, 2022). The final rule embraces a broad approach to water quality, with some changes, compared to the supplemental rule (US FDA, 2022). The final rule includes two sets of criteria for microbial water quality, which are focused on the presence of generic *E. coli* that indicates the incidence of fecal contamination. Firstly, the rule says that generic *E. coli* should not be detectable in certain uses of agricultural water because, if present, it can have potentially hazardous microbes which would contaminate produce through direct or indirect interaction. Examples include water used for drinking or washing hands during harvest, and water used to directly contact produce (US FDA, 2022). Secondly, the rule sets the numerical criteria for agricultural water that is relevant to growing produce (other than sprouts) (US FDA, 2022). The criteria use two values, the geometric mean (GM) and the statistical threshold (STV): "The GM of samples is 126 colony-forming units (CFU) or less of generic *E. coli* per 100 mL of water and the STV of samples is 410 CFU or less of generic *E. coli* in 100 mL of water" (US

FDA, 2022). In this study, the geometric mean (GM) which is 126 CFU or less generic *E. coli* per 100 mL of water was applied.

1.3.5.2. U.S. Environmental Protection Agency (EPA) Stander

Presently, government and industry agencies approximate food safety risk factors in agricultural system irrigation waters through the measurement of waterborne indicator microbes, including generic (commensal) *E. coli*, which are not automatically pathogenic to humans. When national or state irrigation water-quality standards are not present, water-quality standards for agriculture will follow those set by the U.S. Environmental Protection Agency (EPA) for recreational uses, which covers bodies of water that humans use (Fox et al., 2012). These standards refer to the following: "1) geometric mean of <126 CFU/100 mL, or 2) <235 CFU/100 mL-for any single water sample (Fox et al., 2012).

Listeria is a genus of bacteria, but only one of them, *Listeria monocytogenes*, is of concern for food safety. The USDA threshold for *L. monocytogenes* is zero tolerance (Chen et al., 2003). The presence or absence of *Listeria spp*. in a sample, as it was measured for this study, indicates that there is a suitable environment and exposure risk for *L. monocytogenes.*

1.4. Water physicochemical parameters

Aquaponics relies on a thorough knowledge of water chemistry to ensure that fish, plants, and microorganisms all thrive in the same environment (Hager et al., 2021). For aquaponics, temperature, pH, dissolved oxygen, and total ammonia nitrogen are the five most critical water chemistry factors. It is essential for all living organisms to have high quantities of oxygen. The dissolved oxygen (DO) in water is used to measure the amount of oxygen in a given volume of water. Aquaponic systems need the addition of oxygen because of their high concentration of

nutrients. Diffusers in the surrounding water or movement at the water's surface may both introduce oxygen into the system. Stocking densities, plant types and numbers, organic solids, and biological oxygen demand all influence how much-dissolved oxygen is required. The link between DO and temperature is significant. Because dissolved oxygen is more easily dissolved in cold water than in warm water, cold water may hold more oxygen than warm water (Hager et al., 2021).

Organism	Temperature (°C)	pH	Ammonia (mg/L)	Nitrite (mg/L)	Nitrate (mg/L)	DO (mg/L)
Warm water fish	22 – 32	6 – 8.5	< 3	< 1	< 400	4 – 6
Cold water fish	10 – 18	6 – 8.5	< 1	< 0.1	< 400	6 – 8
Plants	16 – 30	5.5 – 7.5	< 30	< 1	< 250	> 3
Bacteria	14 – 34	6 – 8.5	< 3	< 1	-	4 – 8
Compromise for Aquaponics	18 – 30	6 – 7	< 1	< 1	< 150	5 – 8

*Reproduced and adapted from FAO small-scale aquaponic food production (Somerville *et al.* 2014).

Table 1. 1 Recommended water quality parameters for aquaponics.

Aquaponics places more emphasis on water temperature than on ambient temperature. The level of dissolved oxygen and the quantity of hazardous ammonia (un-ionized) in the water are only two examples of water chemistry variables that are influenced by temperature. As well as fish and plants, it has a direct effect on their well-being and survival. Poikilothermia, or cold-bloodedness, characterizes fish. As a result, the temperature of their bodies is influenced by the water they are swimming in. Fish will cease feeding under severe temperatures, becoming weak and vulnerable to sickness. Temperature increases in plants have been shown to impair the absorption of important plant nutrients such as calcium, drive early blooming in cool-weather crops, as well as increase the risk of plant root infections like *Pythium spp*. becoming established. Because of this, it's crucial to keep the temperature consistent throughout the day. Passive or solar

heating in greenhouses, covering or concealing water surfaces, insulation of fish tanks and plant beds, and other similar techniques are all often used by greenhouse farmers (Hager et al., 2021). To save money on heating and cooling, farmers in temperate regions might rotate their agricultural planting and fishing operations on a seasonal basis. A solution's acidity or basicity may be measured by its pH. A solution's acidity level is dictated by the number of free hydrogen ions (H+) in the solution. Acids have a low pH because of the chemical reactions they undergo. The pH scale ranges from 1 to 14, with 7 indicating a neutral pH. An acidic solution has a pH of less than 7 while a basic solution has a pH of more than 7. A lot of practitioners struggle with pH because of its logarithmic scale. Different species of animals and microorganisms have different pH tolerances. Even if they can put up with parameters that are outside of their ideal range, poor environments may have a significant impact on their ability to d survive. The pH is influenced by multiple factors present in the system. The production of H+ and CO by nitrification as well as fish stocking density is associated with decreasing in the water. It's essential that the pH be raised to a level where it's good for culture. Monitoring and documenting pH levels is an essential management activity for aquaponics producers. Due to nitrification, aquaponics farmers don't have to worry about lowering their pH levels. In certain cases, producers may have to add hard water or compounds to their water supply to raise the alkalinity, which in turn stabilizes or raises the pH. Even after cycling, if the pH of the system remains high, the first action to do is to ensure that particles aren't building up in the system. Nitrate is re-converted back into ammonia when anaerobic circumstances arise, via the process known as denitrification. During this process, alkalinity is liberated, allowing the pH to be stabilized. Nitrogen is introduced to the aquaponics system through the fish meal, which is a crude protein source. Ammonia or urea are ejected via the gills, while 70% is digested and eliminated as solid waste (Timmons and Ebeling, 2013).

Ammonia (NH_3) is hazardous to fish) and ionized ammonia (NH_4^+), which is not toxic to fish, makes up most of the total ammonia nitrogen (TAN) in the tank water. Depending on the pH and temperature, one form is more prevalent than the other. Toxic ammonia concentrations rise with increasing pH and temperature (Hager et al., 2021).

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Chapter 2: Experimental Design

The experiment consists of 12 independent recirculating aquaponic systems (RAS) (Figures 2.1 and 2.2). Two sets of six tanks were placed on two 1.8 m shelves. Each system was composed of a 38-liter fish tank, a 3-tier biofiltration system that contains sponge and bio balls to promote colonization and nitrification by indigenous bacteria, a 15-liter, deep-water, plant grow bed, and one LED grow light. Each fish tank contains four goldfish, one 144 GPH water pump, and one air pump. Each deep-water plant grows in bed contains a floating raft with four lettuce plants. The entire shelf was covered in mylar film to provide a higher level of light reflection in the system.

Fish were raised in a tank and dechlorinated water is pumped to the biofiltration system that discharges water to the plant tanks. The naturally occurring bacteria colonizing the biofilter and/or plants' grow bed convert total ammonia nitrogen (TAN) and nitrite to nitrate (fertilizer). Plants absorb nutrient-rich, oxygenated water and use nitrate as their essential nitrogen source. Filtered water (clean) is recirculated to the fish tank. Micronutrients in the water are also derived from fish waste and any residual decomposed fish feed.

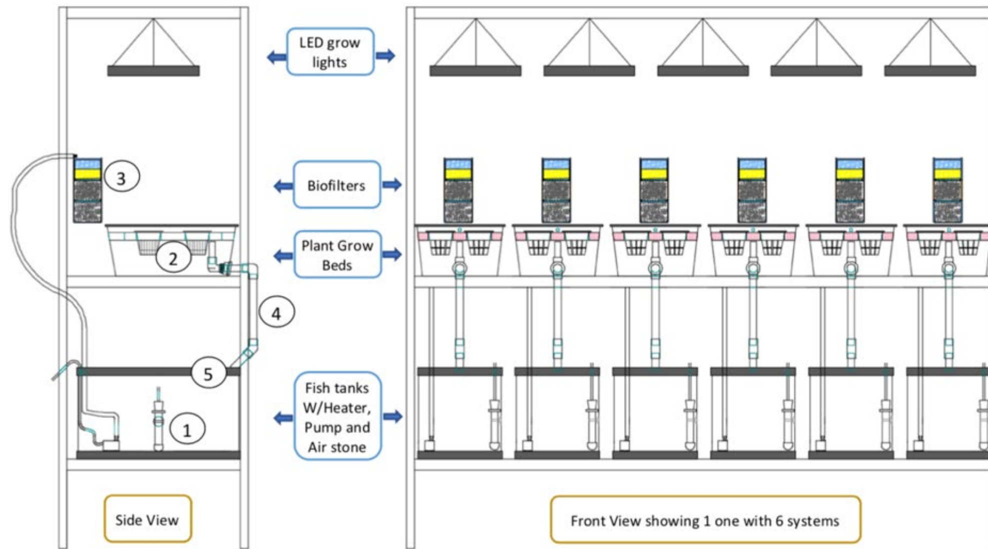

Figure 2. 1. Aquaponics System Details: 1. Fish Tank; 2. Plant Grow Bed; 3. Water Pump; 4. Bell Siphon; 5. Water Discharge

Figure 2. 2. One of the two rack systems used in the experiment.

2.1. Experiment Hazard Management

Bacterial strains used in this experiment are classified as BSL-1. The *Listeria innocua* 2066 strain used in these studies is genetically modified to express green fluorescent protein and resistant to erythromycin to facilitate detection on agar media. No fish were used for human consumption. Personal protective equipment (PPE) was used by lab personnel while conducting the experiments, including face masks, gloves, lab coats, and hand washing pre- and post-experimental procedures. All measures were taken to prevent direct contact with the aquaponic system setup. All sampled water and plants, spent media, pipettes and disposable labware used in the experiment were autoclaved to prevent environmental contamination. Eating and drinking is prohibited in the lab. All PPE and consumables were disposed of in autoclave bins, later attended for appropriate autoclaving and disposal in accordance with university protocols.

2.2. Lettuce and Goldfish

Romaine lettuce (*Lactuca sativa* var. Truchas) was chosen because it has been shown to grow well in aquaponic systems and is a crop that is in universal demand. Goldfish are known for their robustness and resilience to a variety of environmental conditions and are not prone to cannibalism or disease when obtained from high-quality stock and housed in non-crowded settings.

2.3. Fish feeding

Fish were bought at 1.5-2-month-old and were approximately 5 cm long and 1g in weight. Fish of a similar age and size were selected to reduce variability. Fish were fed once a day using

commercial TetraFin Goldfish Flakes[1], a food diet containing 44% crude protein, at a nominal rate of 5% of body weight per day (bw/d) for the first two weeks, followed by 2.5% bw/d for the remaining 6 weeks. Fish were weighed and sized weekly, and measurements were used to calculate appropriate food amount for that week.

2.4. Seeds germination and Transplantation

Seeds were watered and germinated in rockwool two weeks before being transferred into a Styrofoam raft or net pots filled with expanded clay (hydroton). Seeds, rockwool, and expanded clay were disinfected prior to the start of the experiment by rinsing with sterile distilled water and heating at 55ºC overnight to eliminate any preexisting *E. coli* or *Listeria*. To germinate the lettuce seeds, two seeds were placed in the center of a rockwool square and then placed into a germination chamber. Fifty rockwool were germinated with 100 seeds for this experiment, which only needs 48 total seedlings. This includes an excess to compensate for any failed seeds. The chamber includes ~1cm of water that will be absorbed into the rockwool. The wool is left in the chamber for two weeks and lightly misted daily with water. After two weeks, the smallest seedling is carefully removed from the wool, leaving only the stronger one. To transplant each of four rockwool cubes, a seedling is placed into a Styrofoam raft in which a one-inch diameter hole has been cut to hold one rockwool cube. This raft is placed on top of the water in the plant tank compartment of the system, which constitutes the deep-water hydroponic compartment of the total system.

[1] http://www.tetra-fish.com/products/nutrition/goldfish-flakes.aspx

2.5. Bacterial inoculum preparation

2.5.1. Escherichia coli TVS 355

A cocktail of three non-pathogenic, generic, *E. coli* strains with rifampicin resistance were used for inoculation. Bacterial strains were stored at USDA-ARS-BARC, Beltsville, MD in the Pat Millner Lab (Bldg. 001) at -80 °C freezer until used for this study. Antibiotic resistance was used to distinguish the *E. coli* strains from all other strains that may be present in the purchased fish. These include *E. coli* TVS 353 (isolated from surface irrigation water), TVS 354 (isolated from romaine lettuce), and TVS 355 (isolated from sandy-loam soil near the central coast of California (Tomás-Callejas et al., 2011). All strains were provided by Dr. Trevor Suslow from the University of California (Davis, CA) and were used in several other studies, including greenhouse, growth chamber, and field plot studies. Briefly, a pure colony of each strain was obtained by streaking the bacterial archive onto tryptic soy agar supplemented with 80ppm rifampin and incubating at 37 °C for 24 hours. The colony was transferred into 10 mL of tryptic soy broth (Difco) supplemented with 80ppm rifampin (TSB-R) and incubated at 37 °C for 24 hours. Three individual inoculums were mixed to make the cocktail inoculum.

2.5.2. *Listeria innocua* 2066

Listeria innocua (6a) modified from NCTC 11288 (Microbiologics, MN, USA) were used in this study. The stock culture was stored at -80 °C in Tryptic Soy Broth (TSB) with 15% glycerol. The working culture was prepared by streaking onto Tryptic Soy Agar (TSA) and incubating at 37 °C for 24 hours. A colony was transferred into 10 mL of tryptic soy broth (Difco) and incubated at 37 °C for 24 hours. For each experiment, 10mL of each strain with a final inoculum of 8-9 log CFU/mL was prepared. This culture contains a Green Fluorescent Protein (GFP) marker that produces fluorescence of the culture visible under UV light (475 nm-495 nm).

2.6. Bacterial inoculation in the systems

At the start of the experiment, the 38-liter tanks were inoculated with *E. coli* TVS 354 or *Listeria innocua* 2066 at a dose of 2 or 3 log CFU/mL (low-dose) or 4 or 6 log CFU/mL (high-dose), respectively. Four liters of dechlorinated water were added to each fish tank each week to maintain water levels at 1 inch below the top edge for the course of the experiment. One uninoculated control group and two treatment groups (low-dose and high-dose), with randomized allocation of each tank, were included in the experiment, and summarized below:

- **Control group (UN)**: Uninoculated
- **Treatment one (Low-Dose; LD):** 2 or 3 log CFU/mL inoculation of *E. coli* or *Listeria.*
- **Treatment two (High-Dose; HD):** 4 or 6 log CFU/mL inoculation of *E. coli* or *Listeria.*

2.7. Analytical methods

2.7.1. Most Probable Number (MPN) Determinations

Most probable number (MPN) estimations were conducted in 48-well polypropylene deep-well blocks (5-mL capacity wells) or 96-well polypropylene deep-well blocks (2 mL capacity wells). All blocks were autoclaved for 15min at 121°C and 15 min dry cycle, prior to use. Serial dilutions were conducted, beginning at 1:10, until the appropriate endpoint concentration was reached for *Listeria, E. coli*, and other microbial groups. For the 1:10 dilution, 1.0 mL of the primary sample was aseptically transferred into 1.0 mL of 2x TSB containing 160 g/mL erythromycin. Serial dilutions were performed by transferring 0.2 mL of the prior dilution to 1.8 mL of 1xTSB containing 80 g/mL erythromycin. The blocks were covered with sterile foil and incubated overnight at 37°C, without shaking. After incubation, all MPN wells were mixed by pipetting up and down 10 times and then drop-spotted on Tryptic Soy Agar-Erythromycin (TSE^{ER})

plates (3 μL from each well). Prior to drop-spotting, the plates were marked with equidistant lines using a visible template to create 6 rows with 8 spots each. Plates were incubated at 37 °C overnight and then analyzed for bacterial growth and typical morphological characteristics. Fluorescent colonies when viewed with UV light indicated the presence of *Listeria*. Results were recorded and the MPN calculations were computed using MPNcalc v1.2.0 (galaxytrakr.org) based on the number of wells positive for *Listeria innocua* 2066 growth. MacConkey Agar-Rifampicin plates (MAC ^{Rifr}) were used for *E.coli*-^{Rifr} enumeration.

2.7.2. APC Petrifilm

3M Petrifilm Aerobic Count Plates were used for the enumeration of aerobic bacteria. Briefly, 3M Petrifilm Aerobic Count Plates were placed on a level surface and the top film was lifted. One mL of diluted sample was placed onto the center of the bottom of the film with a 3M™ Electronic Pipettor or equivalent held perpendicular to the plate. The top film was then dropped down onto the sample. Pressure was gently applied on the top film with a 3M™ Petrifilm™ Spreader to distribute the inoculum over a circular area before the gel is formed. Then, the plates were incubated at 37ºC for 24 hours with the clear side up in stacks of up to 20 plates. Bacterial colonies were counted following this 24-hour incubation (*3M™ Petrifilm™* 2021*)*

2.7.3. IDEXX Quanti-Tray®/2000

The Colilert IDEXX test was used to analyze water samples for *E. coli*. The Quanti-Tray 2000 system is designed to quantify bacterial counts in 100 mL of the sample using IDEXX reagents and was used when quantifying coliforms, *E. coli*, Enterococci, Pseudomonas, and *Aeruginosa*. The manufacturer's recommendations were followed for processing water samples. Briefly, 100 mL of the sample was allocated into a sterile container and the Colilert 18 media

was dispensed directly into the container. The lid was secured onto the container and the sample was shaken vigorously until all the media was dissolved. The sample mixture was poured directly into the Quanti-Tray. The sample-filled Quanti-Tray was then placed onto the rubber insert Quanti-Tray Sealer. The Quanti-Tray was then incubated at 37 °C for eighteen hours. After incubation, large and small positive wells were counted in the sample and compared with the Quanti-Tray 2000 MPN counts to determine the Most Probable Number (MPN) of total coliform and *E. coli* per 100 mL sample (Weller et al., 2020)

2.8. Preparing enrichment media

2.8.1. MacConkey agar- Rifampicin (MAC Rifr). Protocol

Fifty grams of MacConkey Agar were mixed with 1L of autoclaved distilled water in a sterile container with a sterile magnet. The bottle was placed onto a stir/hot plate and heated with frequent agitation and boiling for approximately 1 minute to completely dissolve the powder. The solution was then autoclaved at 121ºC for 15 mins and cooled to 45-50ºC. Rifampicin was then added to the solution, followed by mixing for 30 seconds on the stir/hot plate (without heat). The solution was then poured into 20-25 mL sterile Petri plates and allowed to solidify.

2.8.2. Brilliance *Listeria* Agar - Erythromycin Protocol

The amount of 33.6 grams of Brilliance Listeria Agar Base (CM1080) were added to 480 milliliters of distilled water. A magnetic stirrer was used to mix the solution until completely dissolved. The solution was autoclaved at 121ºC for 15 mins and then cooled to approximately 45-50 ºC. Erythromycin was added to the solution, mixed, and then the solution was poured into sterile Petri dishes and allowed to be set overnight.

2.8.3. Tryptic Soy Broth

Nineteen grams of Tryptic Soy Broth were added to 600 mL of distilled water in a sterile bottle and mixed via a magnetic stirrer. The bottle was autoclaved at 121ºC for 15 minutes. Rifampicin was added after the bottle cooled to 45-50ºC. The stock was left in the cold room/refrigerator until use.

2.8.4. Listeria Enrichment Broth (LEB^{ER})

A solution of LEB^{ER} broth was created by mixing 36.1 grams in 1L of distilled water, dissolving on a magnetic stir plate with low heat, and autoclaving for 15 minutes at 121ºC. Erythromycin was added after allowing the solution to cool to 45-55º. The stock was left in the cold room/refrigerator until use.

2.9. Change in physicochemical parameters and Water Quality Metrics

The following parameters were measured to assess water quality in the aquaponic systems: temperature, pH, dissolved oxygen, total ammonia nitrogen, nitrites, and nitrates. Calcium, Phosphorus, Potassium, Magnesium, Boron, and Iron levels were examined in case fish or plants showing signs of deficiency. The equipment used to measure the indicators and the testing frequency for each physiochemical parameter are provided in **Table 2.1.** and their corresponding optimal ranges in **Table 2.2**.

Parameter	Equipment	Frequency*
Temperature	YSI Pro 20	Daily
pH	Accumet AP110	Daily
Dissolved Oxygen	YSI Pro 20	Daily
Total Ammonia	YSI D3900	Weekly
Nitrites	YSI D3900	Weekly

Nitrates	YSI D3900	Weekly
Calcium, Phosphorus, Potassium, Magnesium, Boron, Iron	Send to a specialized laboratory	As needed

Table 2. 1 Water quality parameters to be measured and frequency.

When starting up the systems, pH, ammonia, nitrites, and nitrates should be tested daily so that adjustments can be made quickly when needed. Once nutrient cycles are balanced, weekly testing will be performed. In case ammonia concentration reaches a toxic level (> 0.5 mg/L), water will be replaced, using fresh, dechlorinated water, and a new food dose will be administered.

Parameter	Expected Range	If out of range: Contact Lab Manager and PI to preform actions below
Temperature	17.8 – 23.3ºC	Increase ambient room temperature
pH	6.8 – 7.0	Increase the rate of buffer transfer
Dissolved Oxygen	6-8 mg/L	Ensure proper air flow through air stones. Ensure that bumps and bell siphon are working properly.
Total Ammonia	0 ppm	Clean & drain dirty tanks. Ensure system has enough plants
Nitrites	0 ppm	Clean & drain dirty tanks. Ensure system has enough plants
Nitrates	<20 ppm	Clean & drain dirty tanks. Ensure system has enough plants

Table 2. 2 Expected ranges for water parameters.

2.10. Statistical analysis

The bacterial populations were converted to log CFU/mL or CFU/g. Reductions or changes in bacterial populations at different time intervals were computed as the difference between the count observed at time 0 (*N0*, log CFU/mL or CFU/g) and the count observed at a sampling point (*N*, log CFU/mL or CFU/g). ANOVA (proc mixed feature) was used to evaluate statistical

significance. Normality and homogeneity of variance were tested before running ANOVA. Pearson correlation and Fisher z-transformation were used to determine the correlation between *Listeria innocua* 2066*, E. coli* TVS 354 or APC count and physicochemical parameters. In all cases, $p<0.05$ was considered statistically significant. All analyses were run on SAS 9.4 (SAS Institute, Cary, NC, USA.).

Chapter 3: Persistence of inoculated *E. coli TVS 354* in bench-scale aquaponic systems (180 days after primary plant harvest (Study 1)

Abstract

Aquaponics is a food production system that combines aquaculture and hydroponics. Although aquaponics farming is gaining popularity around the world, few research studies have identified the sources of microbial contamination and associated food safety risk factors. Studies on the survival and persistence of human pathogens in aquaponics systems are limited. This project seeks to address this knowledge gap by investigating the growth, transmission, and persistence of human pathogen surrogates in bench-scale aquaponics systems. The objectives of this project are: (**1**) assess the persistence of *E. coli* TVS 354 in bench-scale aquaponic systems (180 days after primary plant harvest), and (**2**) examine differences in bacterial survival within discrete aquaponic system compartments (*i.e.*, fish feces, plant tank water, filters, leaves, roots & hydroton). A bench-scale aquaponics was previously established and inoculated with *E. coli* TVS 354 at two doses (low-dose: 3 log CFU/mL and high-dose: 4 log CFU/mL). In this study, we follow-up on the previous study and evaluate growth and persistence of *E. coli* TVS 354 at 180 days after primary plant harvest. Samples were collected from 10 independent aquaponic system and bacterial growth was evaluated including aerobic plate count (APC), coliform bacteria (CB), heterotrophic plate count (HPC), and cultural enrichment for *E. coli* TVS 354 levels. *E. coli* TVS 354 was not detected in samples from hydrotons (25 g), filters, fish feces, core and roots. The aerobic bacteria count of fish and plant tank samples ranged between 2.5 to 3 log CFU/mL for all treatments, while aerobic bacteria count on the core of the lettuce plant was significantly lower, ranging from 1.44 to 2.08 log CFU/mL. These findings contribute to our understanding of

potential food safety risks and associated risk factors such as inputs, physicochemical factors, and other environmental conditions in aquaponics systems. Results from this project may be useful while developing pre-harvest and post-harvest produce safety risk management strategies.

3.1. Introduction

Consumption of leafy green vegetables contaminated with *E. coli O157:H7* poses an important risk for foodborne disease in humans. Infection with *E. coli* O157:H7 can cause various forms illness, from self-limiting diarrhea to live-threatening hymolytic uremic syndrome, particularly in infants and elderly people (Delaquis et al., 2007). Contamination of edible tissues in leafy green vegetables is often due to the transfer of pathogens from soil or water (Delaquis et al, 2007). Several factors impact *E. coli* survival on plants, including nutrient availability, competition with indigenous microflora, UV radiation, and relative humidity (Brandl, 2006). Islam et al. (2004) reported survival of *E. coli* on lettuce and parsley for up to 77 and 177 days, respectively, in an experiment that exposed lettuce and parsley plots to contaminated compost or water (Islam et al. 2004). In greenhouse experiments, *E. coli* was detected on lettuce plants 28-35 days post high-dose (10^4 CFU/mL) spray-inoculation (Salomon et al., 2003; Moyne et al., 2011). While the persistence and fate of *E. coli* and other bacteria have been evaluated in various growing conditions (Islam et al., 2004; Moyne et al., 2011; Tomás-Callejas et al., 2011), less is known about survival and transmission of pathogenic bacteria in plants grown and harvested from aquaponics systems.

The purpose of this study was to evaluate the survival of *E. coli* TVS 354, a generic, non-pathogenic, *E. coli* TVS 354 strains, on lettuce plants grown and harvested from bench-scale aquaponic (AP) systems. This study further sought to evaluate AP system compartments that are enriched for bacterial growth to identify critical nodes for bacterial transfer within the AP

system. The results of this study will inform policies and management practices related to preventing pathogenic bacterial contamination and overall AP system food safety.

3.2. Objectives

1. Evaluate persistence of *E. coli* TVS 354 in bench-scale aquaponics (AP) systems, up to 180 days after primary plant harvest.
2. Examine bacterial growth localization in the AP systems (*i.e.*, filter, grow bed, fish tank, and plant roots).

3.3. Hypothesis

E. coli TVS 354 will survive on aquaponic systems for up to >180 days.

3.4. Methods

3.4.1. Systems Setup

This study was conducted using 10 individual recirculating research aquaponic systems placed on two metal racks, each rack containing six systems spaced ~2cm apart. Each system was composed of a 38 L fish tank and a 15 L deep-water grow bed (**Figure 3.1**). Each system contained two lettuce, 2 basil plants, and four goldfish (*Carassius auratus*). The lettuce and basil seeds were germinated in rockwool for 14 days in a growth chamber kept damp at ~24°C, then transferred into net pots filled with expanded hydroton clay pebbles and growth media.

Figure 3. 1 setup of recirculating research aquaponics systems and discharge tube connecting grow bed to fish tank. Mature lettuce and basil are shown in the raft.

3.4.2. Bacterial inoculum preparation

A cocktail of three non-pathogenic, generic, *E. coli* TVS 354 strains with rifampicin resistance were used for inoculation. Bacterial strains were stored at USDA-ARS-BARC, Beltsville, MD in the Pat Millner Lab (Bldg. 001) at -80 °C freezer until used for this study. Antibiotic resistance was used to distinguish the *E. coli* strains from all other strains that may be present in the purchased fish. These include *E. coli* TVS 353 (isolated from surface irrigation water), TVS 354 (isolated from romaine lettuce), and TVS 355 (isolated from sandy-loam soil near the central coast of California (Tomás-Callejas et al., 2011). All strains were provided by Dr. Trevor Suslow from the University of California (Davis, CA) and were used in several other studies, including greenhouse, growth chamber, and field plot studies. Briefly, a pure colony of each strain was obtained by streaking the bacterial archive onto tryptic soy agar supplemented

with 80ppm rifampin and incubating at 37 °C for 24 hours. The colony was transferred into 10 mL of tryptic soy broth (Difco) supplemented with 80ppm rifampin (TSB-R) and incubated at 37 °C for 24 hours. Three individual inoculums were mixed to make the cocktail inoculum.

3.4.3. Sample Preparation and procedures

E. coli TVS 354 was inoculated into the aquaponics systems to a final level of 3 log (low-dose) or 4 log CFU/mL (high-dose) in September 2021. This study was conducted in March 2022 to follow up on the persistence of *E. coli* TVS 354 on lettuce plants (basil plants were excluded from analysis because most of the basil plants died). Approximately 130 samples were collected from ten systems, including two control and eight inoculated systems. Appropriate dilutions were made for bacterial isolation and enumeration using Petrifilm (Aerobic Bacteria Count, APC), and IDEXX (coliform and *E. coli*, HPC). Cultural enrichment of MPN block was used to increase the chance of detecting *E. coli.* McConkey Agar with rifampicin was used for the presumptive detection of *E. coli.* Different procedures were used to evaluate bacterial load in different compartments of the aquaponics systems **(Figure 3.2)**. The procedure for sampling and evaluating each compartment is described below:

a) ***Roots procedure.*** Two lettuce plants from each system were extracted from their net pots. The roots were cut off from the plant and placed in a sterile Whirl-Pak bag, weighed, and then filled with 30 mL phosphate-buffered saline (PBS). The bags were placed in a stomacher for 1 min at 260 rpm. The samples were then analyzed for APC and *E. coli* TVS 354 using Petrifilm and MPN block methods, respectively (Patel et al., 2010).
b) ***Hydroton procedure.*** After the plants were removed from the systems, the roots and hydroton were separated. A total of 12-13 hydroton balls of various sizes (~25g) were taken from each lettuce plant from the system and placed into a Whirl-Pak bag. PBS (25 mL)

was added to each bag and hand-rolled for 2 mins. The samples were then analyzed for APC and *E. coli* TVS 354 using Petrifilm and MPN block methods.

c) ***Core procedure.*** Two lettuce plants from each system were removed from the beds and carefully extracted from their net pots. Any excess leaves from the bottom inch of the stem were removed. The internal portion of the bottom inch of the stem was cut out using a sterile scalpel, making sure to remove all excess outer tissue from the core. After weighing the core tissue, the core was then placed into a sterile Whirl-Pak bag with 30 mL of PBS and then stomached for one minute at 260 rpm. APC and *E. coli* TVS 354 in the samples were analyzed by the methods previously described.

d) ***Tank water (fish and plant tank).*** Water from both the deep-water plant tank and the primary fish tank was collected. The samples were analyzed for APC and *E. coli* TVS 354 using Petrifilm and MPN block methods, respectively.

e) ***Filter procedure.*** The upper filter layer (a 3x3 blue and white sponge) was removed from the main filter system and put in a sterile Whirl-Pak bag with 50 mL of PBS and stomached for 1 min at 260 rpm. A total of 250 µL of each solution from the Whirl-Pak bag was spread-plated on MacConkey Agar-Rifampicin plates and incubated for 24 hours at 37 °C before counting colonies.

f) ***Fish feces procedure.*** Fish feces were collected for microbial analysis to estimate the internal bacterial population of the fish. All the fish from one tank system were put into a separate tank with fresh dechlorinated water. The fish were then fed 0.4g of fish feed (TetraFin Goldfish Flakes) and after 30 mins the excreted fecal matter (that sunk to the tank floor) was removed via pipetting. The water and feces from the pipette were poured on a piece of filter paper laid on top of a beaker, allowing the feces and residual fish feed

to be strained from the water. *E. coli* TVS 354 and APC analyses were conducted as previously described.

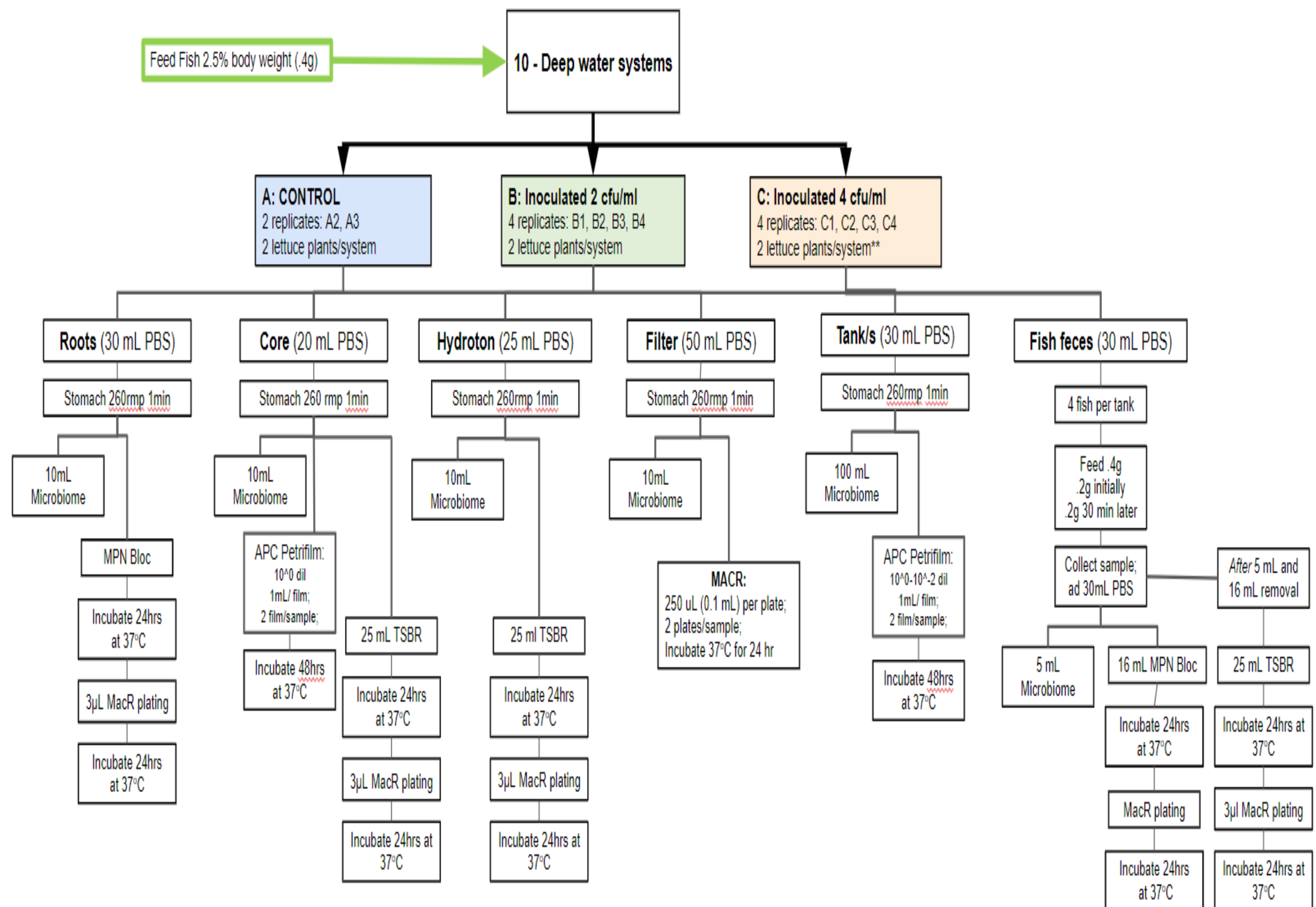

Figure 3. 2 Process Flow diagram for the bench-scale study on *E. coli* TVS 354 survival in aquaponics systems.

3.5. Results

We obtained baseline results on the persistence of inoculated *E. coli* TVS 354 in fish feces, tank water, biofilter, and lettuce plant (roots and core) compartments of aquaponic research system units at 180 days post-harvest of the primary study. The *E. coli* TVS 354 inoculation level in the primary study was 3 log CFU/mL (low-dose) or 4 log CFU/mL (high-dose). pH (7.8-8) and dissolved oxygen (6.5-8 mg/L) were monitored during the experiment, and both were within the optimal range.

3.5.1. Total Aerobic Plate count (APC), heterotrophic count, coliforms, and *E. coli* TVS 354 in fish tank water, plant tank water, and core lettuce at 180 days post-harvest

We evaluated the levels of aerobic, heterotrophic, coliform and *E. coli* TVS 354 bacteria in ten aquaponics systems at 210 days post-inoculation (or 180 days post-harvest). The weights of root, core, and hydroton samples that were analyzed are provided in **Table 3.1.** Treatments A, B, and C refer to control samples, low dose *E. coli* TVS 354 inoculation (3 log CFU/mL), and high dose *E. coli* TVS 354 inoculation (4 log CFU/mL), respectively. We did not detect *E. coli* TVS 354 in fish tank water, plant tank water, or core lettuce. Coliforms and heterotrophic bacteria were detected in all the samples from all treatment groups (**Table 3.2**), and aerobic mesophilic counts were comparable between treatment groups. The aerobic bacteria count of fish and plant tank samples ranged between 2.5 to 3 log CFU/mL for all treatments, while aerobic bacteria count on the core of the lettuce plant was significantly lower, ranging from 1.44 to 2.08 log CFU/mL.

Treatments	Roots		Core		Hydroton
	Lettuce 1	Lettuce 2	Lettuce 1	Lettuce 2	Lettuce 1 + 2
1-C4	3.35	3	1.13	1.15	25.98
4-B1	6.32	7.24	1.35	1.7	26.79
5-C2	5.7	2.21	2.91	1.11	27.21
6-B2	5.36	12.6	2.48	2.4	33.9
7-B4	5.29	5.8	0.78	1.6	32
8-C3	5.03	4.7	2.66	1	33.6
9-B3	5.2	5.4	2.9	0.95	39
10-A3	5.18	4.3	3.66	1.4	37.5
11-A3	5.15	5.1	3.7	1	28.6
12-C1	5.8	5.3	3.16	0.92	26.3

Table 3. 1 Weight of roots, core and hydroton in different treatment conditions. *Note.* A: control; B= 3 log CFU/mL; C= 4 log CFU/mL.

Treatment	Fish tank water				Plant tank water				Core lettuce
	APC (log CFU/mL	MPN-coliform	*E. coli*	MPN-HPC	APC (log CFU/mL	MPN-coliform	*E. coli*	MPN-HPC	APC log CFU/g
1-C4	2.7	>2419.6	ND	>2419.6	2.8	>2419.6	ND	>2419.6	1.91
4-B1	2.7	>2419.6	ND	>2419.6	2.6	>2419.6	ND	>2419.6	2.08
5-C2	3.0	>2419.6	ND	N/A	2.8	>2419.6	ND	>2419.6	1.77
6-B2	2.7	>2419.6	ND	>2419.6	2.7	>2419.6	ND	>2419.6	1.64
7-B4	2.7	>2419.6	ND	N/A	2.5	>2419.6	ND	>2419.6	1.44
8-C3	2.5	>2419.6	ND	N/A	2.7	>2419.6	ND	>2419.6	1.85
9-B3	2.9	>2419.6	ND	N/A	2.6	>2419.6	ND	N/A	N/A
10-A3	2.7	>2419.6	ND	>2419.6	2.4	>2419.6	ND	N/A	2.07
11-A3	2.7	2419.6	ND	>2419.6	2.7	42.8	ND	N/A	1.68
12-C1	2.8	>2419.6	ND	>2419.6	3.0	>2419.6	ND	>2419.6	1.87

Table 3. 2 Aerobic plate count (APC), coliforms (MPN), heterotrophic bacteria (HPC), and *E. coli* TVS 354 at 180 days post-harvest in different treatments. *Note.* A: control; B= 3 log CFU/mL; C= 4 log CFU/mL

3.5.2. *coli* in hydroton, filter, fish Feces and roots at 180 days post-harvest.

E. coli TVS 354 was not detected in hydrotons (25g), filters, fish feces, or roots in all aquaponic systems (n=10) **(Table 3.3).**

System code	Hydroton	Filter	Fish Feces	Roots
1-C4	Neg.	Neg.	Neg.	Neg.
4-B1	Neg.	Neg.	Neg.	Neg.
5-C2	Neg.	Neg.	Neg.	Neg.
6-B2	Neg.	Neg.	Neg.	Neg.
7-B4	Neg.	Neg.	Neg.	Neg.
8-C3	Neg.	Neg.	Neg.	Neg.
9-B3	Neg.	Neg.	Neg.	Neg.
10-A3	Neg.	Neg.	Neg.	Neg.
11-A3	Neg.	Neg.	Neg.	Neg.
12-C1	Neg.	Neg.	Neg.	Neg.

Table 3. 3 *E. coli* TVS 354 in hydroton, filter, fish feces, and root samples from different treatment conditions. *Note.* A: control; B= 3 log CFU/mL; C= 4 log CFU/mL

3.6. Discussion

In this study, we determined that *E. coli* TVS 354 can survive on the roots of lettuce, grown and harvested from bench-scale aquaponic systems, for up to 32 days post-harvest but not 180 days post-harvest. We did not detect *E. coli* TVS 354 *in* fish (feces) or in the plant (crown of the lettuce). These results suggest that *E. coli* TVS 354 does not survive for long periods in a bench-scale AP system. We observed significantly less aerobic bacteria growth on the core of the lettuce plant compared to the fish and plant tank water samples, suggesting that bacteria are less likely to transmit and replicate in the lettuce plant core compared to other compartments in the system and that the plant tank water acts as a carrier for bacterial transmission.

A previous study in a similar system showed the presence of *E. coli* TVS 354 for up to 32 days post-inoculation. In this study, the *E. coli* TVS 354 strains used did not survive this long in

the simulated aquaponics research systems. Contrary to this finding, *E. coli* TVS 354 has also been reported to persist for up to 77 and 177 days in lettuce and parsley, respectively (Islam et al., 2004). In our study, we detected algae and parasites within the AP systems via microscope (data not shown), which may be competing *E. coli* TVS 354 and other bacteria for the limited nutrients available within the aquaponics systems, potentially contributing to the observed decline in bacterial populations over the course of the experiment. Further research on the effects of algae and parasites on the survival of *E. coli* is needed.

3.7.Conclusion

In this study, the survival of *E. coli* TVS 354 at 180 post-harvest (210 post-inoculation) was assessed. *E. coli* TVS 354 was detected on lettuce and basil roots at 28 and 32 days, but not at 180 days post-harvest. We did not detect *E. coli* in fish (feces) or in the plant (crown of the lettuce). These results suggest that *E. coli* TVS 354 can't survive for long periods in a bench-scale AP system. Algae and parasites were also detected via microscope and may be competing with *E. coli* and other bacteria for the limited nutrients available within the aquaponics systems, potentially contributing to the observed decline in bacterial populations over the course of the experiment. Further research on the effects of algae and parasites on the survival of *E. coli* TVS 354 is needed. Results from this project can directly inform pre-harvest and post-harvest produce safety risk management strategies and will be useful to the USDA-Agricultural Marketing Service GAPS certification program for policy making.

Chapter 4: Evaluate the Survival of *Listeria innocua* 2066 in Aquaponic effluent (Study 2)

Preliminary Bottle Study

Abstract

The survival and transfer of foodborne pathogens, specifically *Listeria*, is a concern for aquaponics systems. Few research studies have aimed to evaluate aquaponics and food safety. The purpose of this study is to evaluate the survival of *Listeria innocua* 2066 in effluent from fish tanks and plant tanks of aquaponic research units. In this microcosm study, we evaluated the impact of four treatment conditions on *Listeria innocua* 2066 growth: fish tank water, fish tank water with daily nutrient replenishment, plant tank water, and plant tank water with daily nutrient replenishment. Each bottle was inoculated with *Listeria innocua* (6a) modified from *NCTC 11288* with a final inoculum size of 8-9 log CFU/mL. Water samples were examined on days 0, 1, 2, 3, and 7 for microbial and physiochemical parameters, including water and air temperatures, pH, dissolved oxygen, total ammonia nitrogen, nitrites, nitrates, and lighting. Appropriate dilutions were made for bacterial isolation and enumeration using aerobic plate count (APC), and cultured enrichment for the level of *Listeria innocua* 2066. The water temperature throughout the entire experiment was monitored and ranged between 22.3 °C and 24.43 °C. *Listeria innocua* 2066 counts were the highest on day 0, immediately after inoculation, ranging from 5.57 log CFU/mL to 6.5 log CFU/mL. In 24 hours, a significant reduction ($p<0.05$) in *Listeria innocua* 2066 populations was observed for all treatments with total reduction 3.9-3.7 log CFU/mL. *Listeria innocua* 2066 counts were lowest on day 7 for treatment groups, ranging from 1.1 log CFU/mL to 1.0 log CFU/mL. *Listeria innocua* 2066 populations decreased as holding time increased ($r=-0.68$). Treatments that were replenished daily from the aquaponic systems had higher counts of *Listeria innocua* 2066

compared to treatments that were non-replenished. The lowest APC on day 0, immediately after inoculation, ranging from 4.16 log CFU/mL to 4.32 log CFU/mL was observed. A significant increase ($P<0.05$) in APC count was observed for all treatments in 24 hours while there is no significant difference in APC values between the treatments ($p=0.35$). In conclusion, we have developed a simulated aquaponic system and identified key microbial and physiochemical parameters that impact *Listeria innocua* 2066 growth in aquaponic systems.

4.1. Introduction

Listeriosis is a serious infection commonly caused by eating food contaminated with *Listeria monocytogenes*. According to the Center for Disease Control and Prevention (CDC, 2023), *Listeria monocytogenes* is responsible for 1,600 infections and about 260 deaths each year. Pregnant women, newborns, adults ≥ 65 years old, and immunocompromised individuals are especially at risk. Vegetables like leafy greens are a common environment for bacteria like *Listeria* to thrive because they have large surface areas with many folds.

In a 1998 study, *Listeria* was cultivated from hybrid striped bass that was raised in three different freshwater systems in Maryland (Chalmers, 2004). The results of this study showed that *Listeria* can grow on farmed fish. *Listeria* has been found in several fishery products including raw and processed fish that have been collected from retail establishments and processing facilities. In contrast to these findings, another review reports no presence of the bacteria in freshly harvested fish from wild or aquacultural sources (Dillon and Patel, 1992). Taken together, it can be surmised that processing is the cause for contamination rather than the aquaculture farming process itself. Further research must be conducted to determine the association between *Listeria,* processing, and aquaponics farming.

Listeria is capable of surviving without the presence of oxygen and grows in temperatures as low as 0°C, which is unusual for most common bacteria. If a product is refrigerated, for example, the bacteria could still be living on the product. Furthermore, Listeria contamination in facilities is especially difficult to manage and remove (Chalmers, 2004). Some scientists believe that *Listeria* is the greatest microbial threat to aquaponics food safety (Pattillo, 2015). This study seeks to evaluate *Listeria* development and survival in effluent from aquaponic systems to estimate baseline survival, replication, and population dynamics.

Monitoring chemistry in aquaponics is critical to provide optimal growth conditions for fish, plants, and bacteria. Key aquaponic chemistry parameters include water pH, temperature, and dissolved oxygen. Fish, plants, and bacteria require high levels of oxygen in water and dissolved oxygen (DO) is measured to quantify oxygen content (expressed as mg/L). Dissolved oxygen is recommended to be maintained between 5-8mg/L (Hager et al., 2021). The pH is a measure of the acidity or basicity of a solution**.** The optimal pH level for aquaponics water and bacteria ranges from 6-8.5. Water temperature impacts the health and survival of both fish and plants. In aquaponics, water temperature is more important than air temperature, and it needs to be monitored daily. Total ammonia (ammonia, nitrite, and nitrate) tests are used to monitor the function of the nitrifying bacteria and the performance of the biofilter. Ammonia and nitrite should be close to 0 mg/L while nitrate should be <150 mg/L (Somerville et al., 2014).

4.2. Objective

1. Determine the survival of *Listeria* in aquaponic effluent (research units).
2. Evaluate the association between physicochemical water quality parameters.

4.3.Mehods

4.3.1. *Listeria innocua* 2066 inoculum preparation

Listeria innocua (6a) modified from *NCTC 11288* (Microbiologics, MN, USA) were used in this study. The stock culture was stored at -80 °C in Tryptic Soy Broth (TSB) with 15% glycerol. The working culture was prepared by streaking onto Tryptic Soy Agar (TSA) and incubating at 37 °C for 24 hours. A colony was transferred into 10 mL of tryptic soy broth (Difco) and incubated at 37 °C for 24 hours. For each experiment, 10 mL of each strain with a final inoculum of 8-9 log CFU/mL was prepared. This culture contains a Green Fluorescent Protein (GFP) marker that produces fluorescence of the culture visible under UV light (475 nm-495 nm).

4.3.2. System Setup

12,500 mL glass bottles were arranged and fixed onto a shaker (VWR Scientific). We evaluated four different treatment conditions with three repeats each. Treatment A bottles contained 350 mL of fish tank water inoculated with *Listeria innocua* 2066. Treatment B bottles contained 350 mL of fish tank water inoculated with *Listeria innocua* 2066 and was replenished with new fish tank water at the end of every day. Treatments C and D were the same as A and B but contained plant tank water rather than fish tank water. All bottles were shaken at 150rpm for 7 days, with a grow light fixed above the bottles set on a 12-hour timer (**Figure 4.1**).

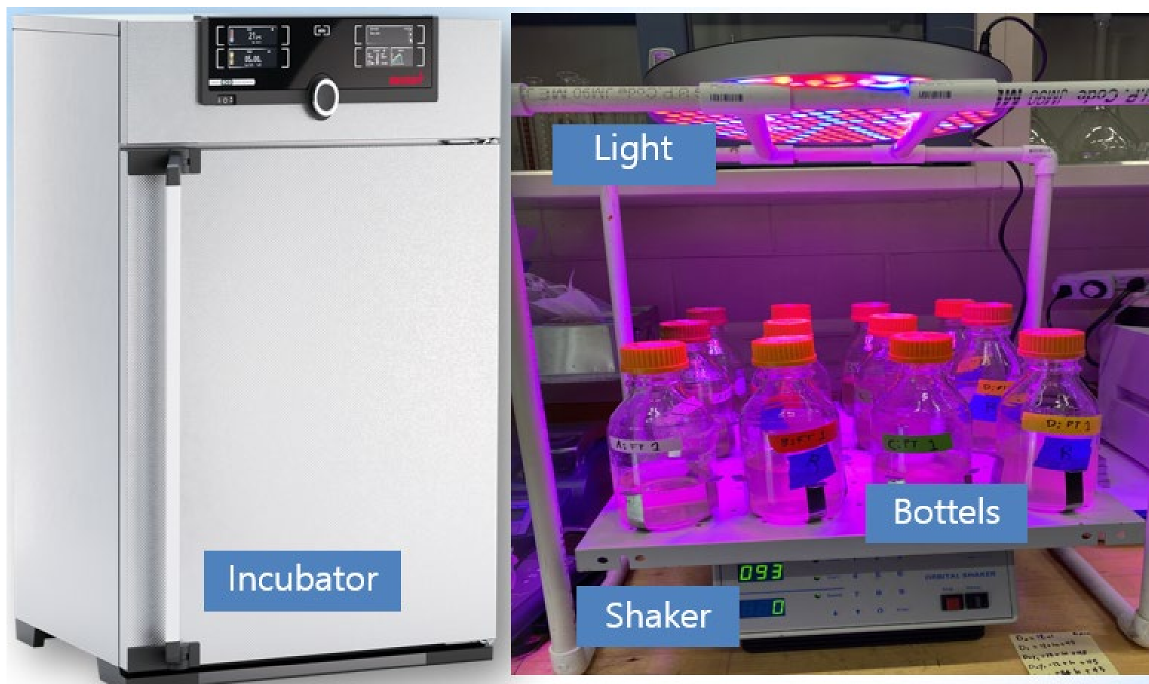

Figure 4.1. The set-up for the experiment contains an incubator for microbial enumeration analysis and a shaker to incubate samples with the inoculated effluent contained in glass bottles.

4.3.3. Survival of *Listeria innocua* 2066:

A preliminary experiment was conducted in self-contained bottles inoculated with *Listeria innocua* 2066 to track the persistence of *Listeria innocua* 2066 survival in an aquaponics environment with and without nutritional supplements. First, 25 mL from each of the 12 systems' fish tanks were transferred to one large beaker to create a sample of fish tank aquaponics parent water (FAPw) source. The same was done for the 12 systems' plant tanks to create the plant tank aquaponics parent water (PAPw) source. This water was used to run four different treatments conditions: A) 350 mL of FAPw; B) 350 mL of FAPw, with daily replacement of 100 mL with fresh FAPw; C) a bottle with 350 mL of PAPw, and D) 350 mL of PAPw, with daily replacement of 100 mL with fresh PAPw. For treatments B and D, 100 mL were removed and replaced with

100 mL from the respective APw daily to evaluate the association between nutrients and growth of inoculated *Listeria innocua* 2066. Each treatment had three replicates. Each of the twelve bottles, comprising four treatment conditions and three replicates, was inoculated with *Listeria innocua* 2066 and placed on a shaker set to 150 rpms and 20 °C for seven days, with a light overhead. Temperature, dissolved oxygen and pH were measured daily. Water samples were collected daily and analyzed for aerobic plate count (APC) and *Listeria innocua* 2066 using Petrifilm and MPN block methods, respectively **(Figure. 4.2)**.

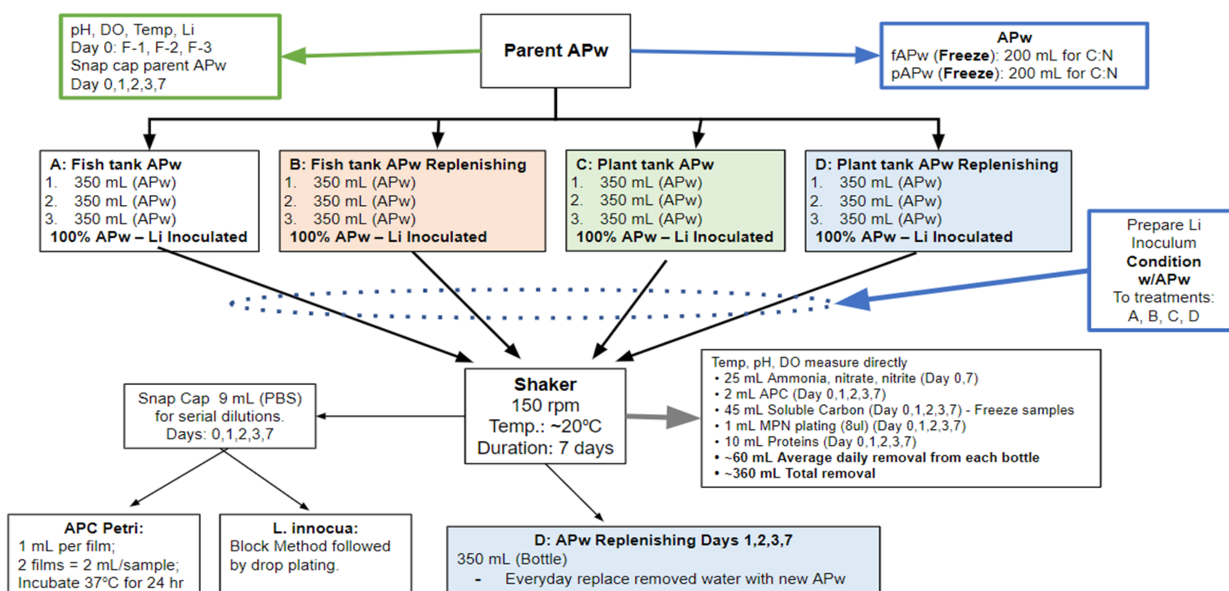

Figure 4.2. Process flow diagram describing the methodology for *Listeria inccoua* 2066 Preliminary experiment.

4.3.4. Physicochemical testing

Water pH, temperature and dissolved oxygen were measured on days 0, 1, 2, 3, and 7 and recorded by inserting probes directly into the. Chemical testing was conducted on days 0 and 7

and involved removing water from the bottles and testing the ammonia, nitrite, and nitrate levels using a Hach DR 3900 laboratory spectrophotometer (Hach Company, Loveland, CO).

4.3.5. Statistical analysis

The bacterial populations were converted to log CFU/mL or CFU/g. Reductions or changes in bacterial populations at different time intervals were computed as the difference between the count observed at time 0 (*N0*, log CFU/mL or CFU/g) and the count observed at a sampling point (*N*, log CFU/mL or CFU/g). ANOVA (proc mixed feature) was used to evaluate statistical significance. Normality and homogeneity of variance were tested before running ANOVA. Pearson correlation and Fisher z-transformation was used to determine the correlation between *Listeria innocua 2066* or *E. coli* TVS 354 with APC count and physicochemical parameters. $P < 0.05$ was considered significant in all cases. All analyses were run on SAS 9.4 (SAS Institute, Cary, NC, USA.).

4.4. Results

4.4.1. Survival of *Listeria innocua* 2066

We observed a rapid decline in *Listeria inoccua* populations from day 1 to 7 for all treatment conditions (**Figure 4.3. and Appendix C1**). The reduction in *Listeria innocua* 2066 was statistically significant at 24 hours ($p<0.05$) for all treatment groups but only significant for the replenished fish tank water condition at 48 hours ($p<0.05$).

Listeria innocua 2066 counts were the highest on day 0, immediately after inoculation, ranging from 5.57 log CFU/mL to 6.5 log CFU/mL. *Listeria innocua* 2066 counts were generally greater in the replenished treatment groups compared to the non-replenished groups. The

replenished fish tank and plant tanks yielded 6.5 log CFU/mL and 6.1 log CFU/mL respectively, compared to 5.57 log CFU/mL from non-replenished fish tanks and 5.93 log CFU/mL from non-replenished plant tanks.

On day 1, the populations of *Listeria innocua* 2066 in fish tanks and plant tanks that were replenished decreased by 3.07 log CFU/mL and 2.87 log CFU/mL, respectively, while the non-replenished fish and plant tanks decreased by 3.77 log CFU/mL and 3.6 log CFU/mL, respectively. Treatments that were replenished daily from the aquaponic systems had higher counts of *Listeria innocua* 2066 compared to treatments that were non-replenished, likely due to the nutrients introduced daily from the fish food and fish waste in the systems. Although the population of *Listeria innocua* 2066 on all treatments was slightly decreased on day 2, there was a consistent spike on day 3 for samples from fish tanks, fish tanks replenished, and plant tanks albeit not reaching statistical significance. All treatment conditions displayed the lowest *Listeria innocua* 2066 counts on Day 7, ranging from 1.0 log CFU/mL and 1.1 log CFU/mL. We observed a strong negative correlation between *Listeria innocua* 2066 population and holding time ($r=-0.68$, $p<.0001$).

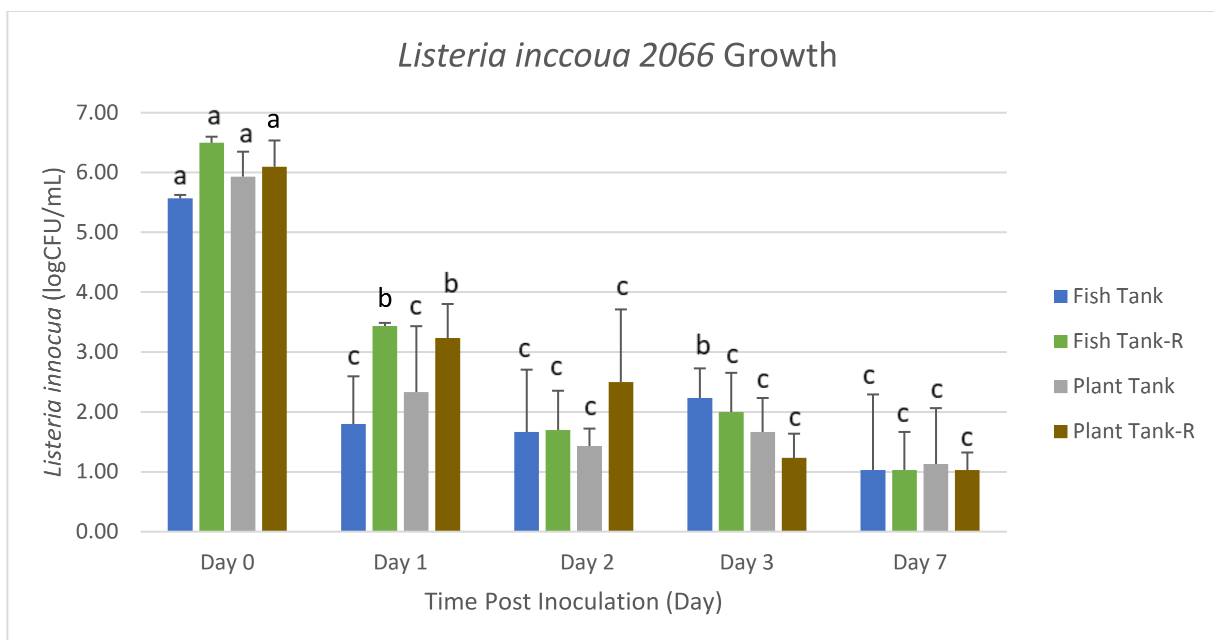

Figure 4. 3. Persistence of *Listeria inccoua* 2066 inoculated in four different treatments from aquaponic systems effluent. The data are presented as average counts ± standard deviation (n=3). Different lowercase letter on the top of the bar diagrams indicates significant differences (P<0.05) in between the means for each treatment among the post inoculation times (ranked by LSD post-hoc test).

4.4.2. Enumeration of Aerobic Plate Count (APC)

The four treatments were tested for general coliform counts. A significant increase in APC count was observed for all treatments at 24 hours (p<0.05) (**Figure 4.4. and Appendix C2**). We did not observe any significant differences between treatment groups.

We observed the lowest APC on day 0, immediately after inoculation, ranging from 4.16 log CFU/mL to 4.32 log CFU/mL. This was followed by a steady increase in APC on days 1, 2 and 3 (4.90 log CFU/mL to 5.17 log CFU/mL) followed by a slight decline on day 7 (**Figure 4.4**). We observed a modest negative correlation between the APC and *Listeria inccoua* 2066 populations (r= -0.59, p<.0001) (**Figure 4.5 and 4.6**). As the population of *Listeria inccoua* 2066 decreases the aerobic bacteria count increases.

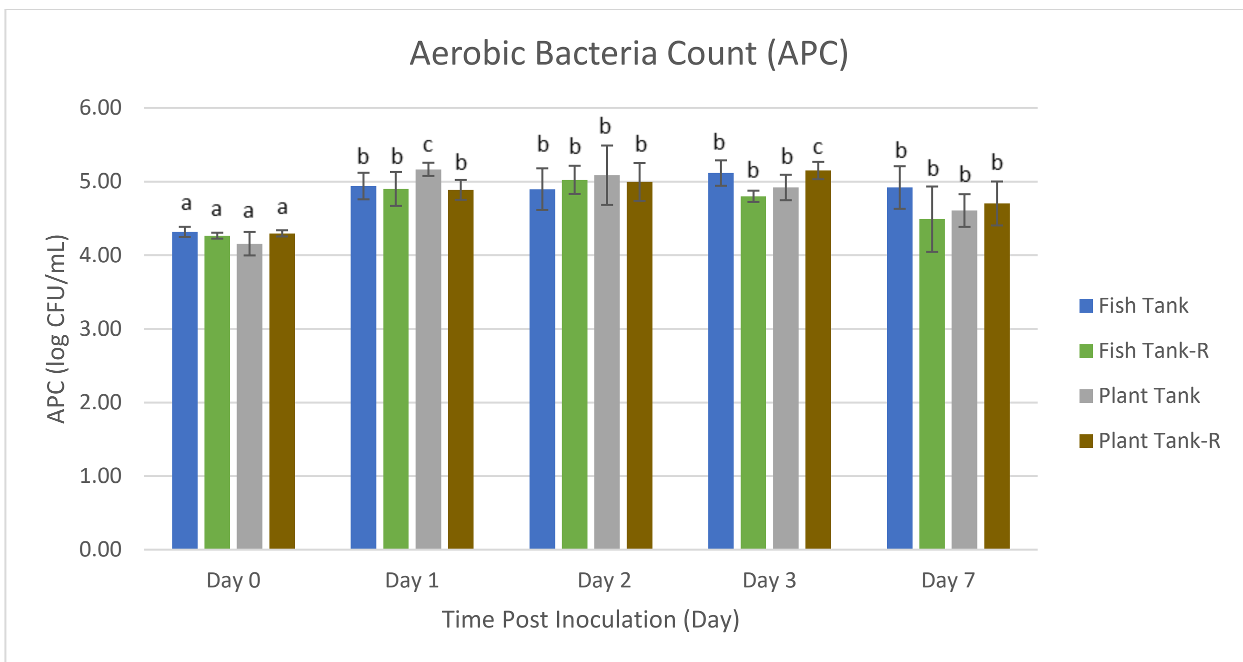

Figure 4.4. Aerobic plate count (APC) in four different treatments from aquaponic systems effluent. The data are presented as average counts ± standard deviation (n=3). Different lowercase letters on the top of the bar diagrams indicate significant differences (P<0.05) between the means for each treatment among the post inoculation times (ranked by LSD post-hoc test).

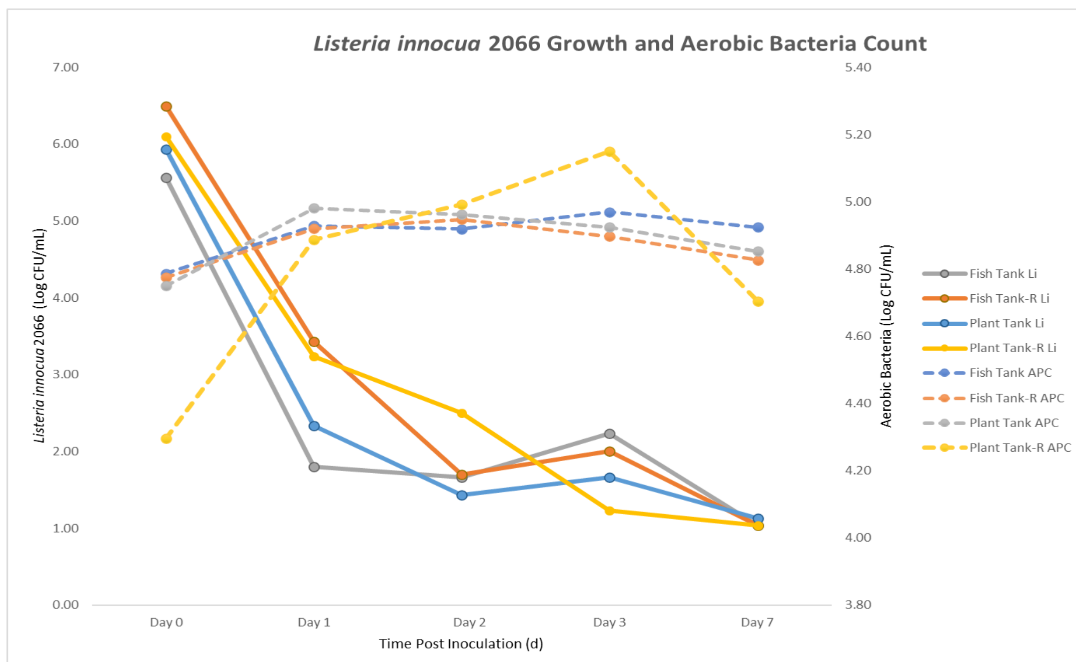

Figure 4.5. Correlation between *Listeria innocua* 2066 and aerobic bacteria count.

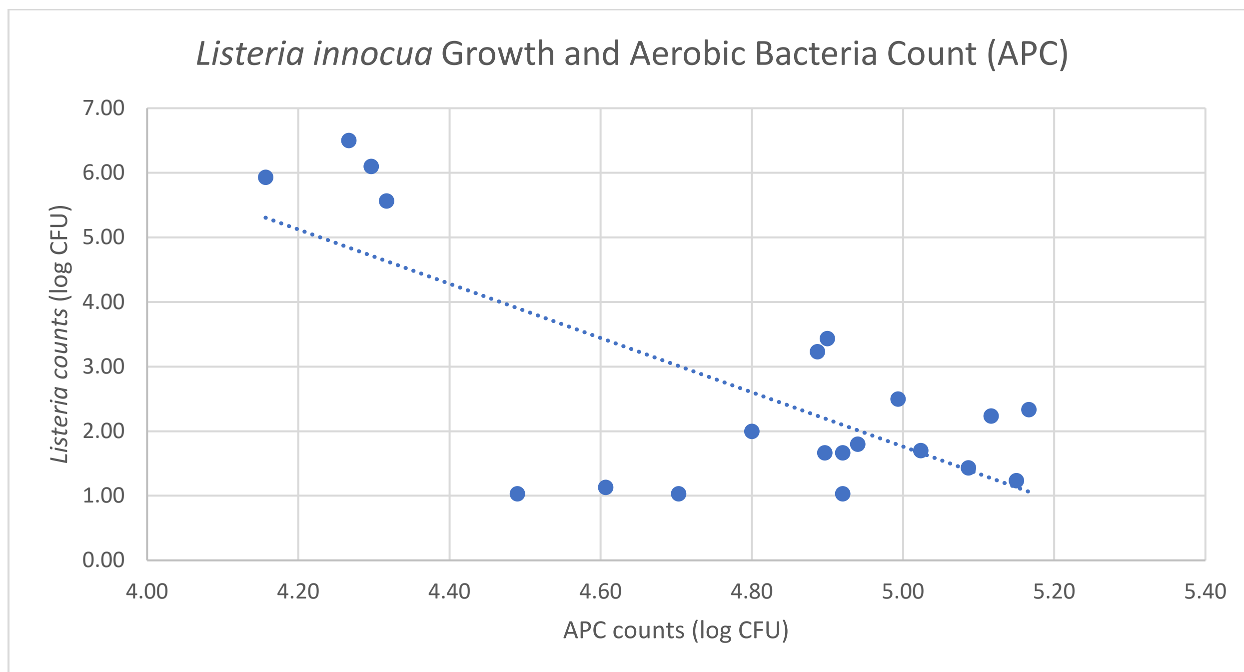

Figure 4. 6 Correlation between *Listeria innocua* 2066 and aerobic bacteria count.

4.4.3. Physiochemical Parameters

4.4.3.1. Dissolved oxygen

Dissolved oxygen is recommended to be maintained between 5-8 mg/L (Hager et al., 2021). The average dissolved oxygen for all treatments ranged from 5.21 to 8.13 mg/L (**Table 4.1**). All samples were within the recommended levels except two samples on day 2 from the replenished fish and plant tank treatment conditions (8.13 mg/L and 8.01 mg/L).

	Dissolve oxygen, Mean (mg/L)			
Time	Fish tank	Fish tank R	Plant tank	Plant tank R
Day 0	5.29 ± 0.11	5.21 ± 0.60	5.21 ± 0.10	5.3 ± 0.253
Day 1	7.59 ± 0.46	7.76 ± 0.14	7.65 ± 0.32	7.5 ± 0.11
Day 2	8.13 ± 0.06	7.7 ± 0.33	7.81 ± 0.20	8.01 ± 0.09
Day 3	7.56 ± 0.43	7.54 ± 0.73	7.68 ± 0.19	7.46 ± 0.71
Day 7	6.53 ± 0.58	6.40 ± 0.33	6.68 ± 0.46	6.8 ± 0.10

Table 4.1. Average ± standard deviation of dissolved oxygen for each treatment group and time point.

We observed a fairly strong negative correlation between *Listeria innocua* 2066 count and dissolved oxygen (r=-0.65, p<.0001) (**Figure 4.7**). *Listeria* is *an* anaerobic bacteria that can survive without oxygen (Chalmers, 2004). As expected, we also observed a fairly strong positive correlation between APC counts and dissolved oxygen (r=0.74, p<.0001) (**Figure 4.8**).

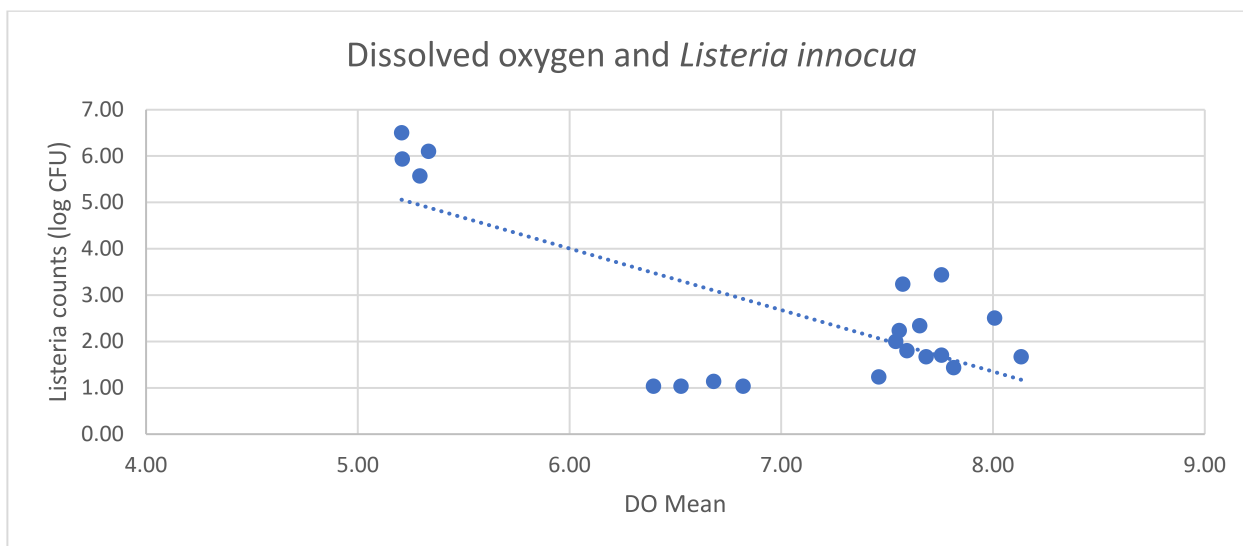

Figure 4.7. Correlation between dissolved oxygen and *Listeria innocua* 2066

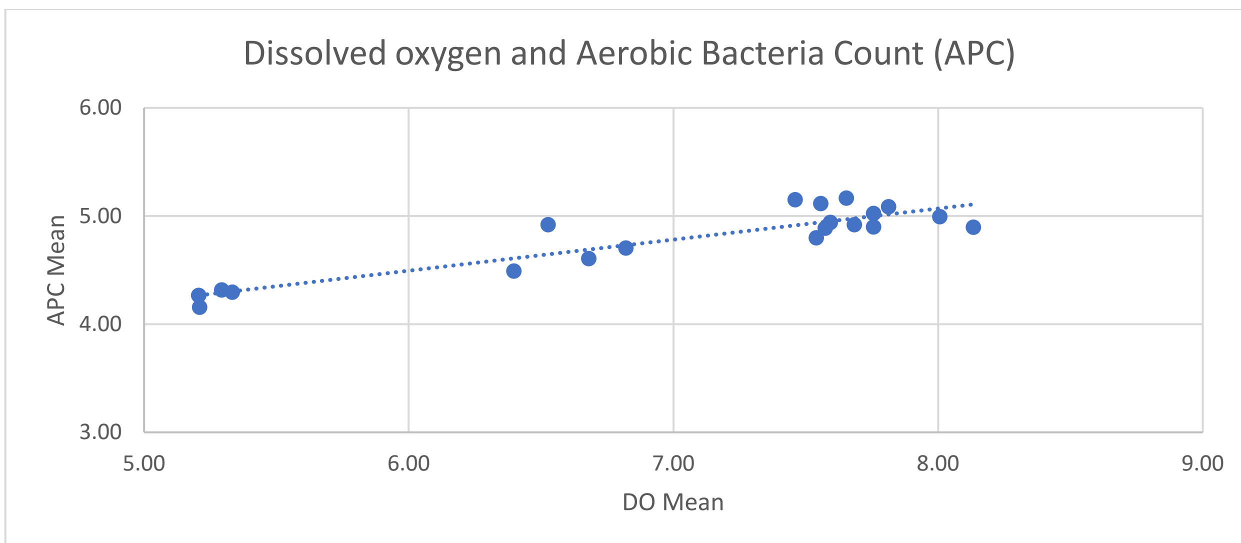

Figure 4. 8. Correlation between dissolved oxygen and aerobic bacteria count

4.4.3.2. pH

The optimal pH level for aquaponics water and bacteria ranges between 6-8.5 mg/L. The average pH for all treatments ranged from 7.06 to 10.71 mg/L (**Table 4.2**). All samples of the four treatments were within the recommended level except those taken on day 7, which displayed pH values from 10.15 to 10.71 mg/L, which might be the reason for the decline of the *Listeria innocua* 2066 populations since bacteria function adequately through a pH range of 6–8.5 which is a compromise between all the organisms within this ecosystem (Somerville et al., 2014). We observed a weak negative correlation between *Listeria innocua* 2066 count and pH (r=-0.36, p=0.0036) (**Figure 7**).

	pH, Mean (mg/L)			
Time	Fish tank	Fish tank **R**	Plant tank	Plant tank **R**
Day 0	7.06 ± 0.72	7.89 ± 0.00	7.66 ± 0.40	7.5 ± 0.52
Day 1	7.61 ± 0.17	7.37 ± 0.23	7.27 ± 0.06	7.13 ± 0.06
Day 2	7.16 ± 0.13	7.22 ± 0.01	7.32 ± 0.07	7.35 ± 0.05
Day 3	8.45 ± 0.14	7.52 ± 0.78	7.52 ± 0.56	7.66 ± 0.68
Day 7	10.15 ± 0.67	10.43 ± 0.35	10.71 ± 0.60	10.17 ± 0.55

Table 4.2. Average pH ± standard deviation for all treatment conditions and time points.

We observed a weak negative correlation between pH and aerobic bacteria counts with (r =- 0.18, p=0.169) (**Figure 4.9**). pH increased as aerobic bacteria decreased, potentially due to the bacteria using the nutrients in the water. pH had a similar effect on *Listeria innocua* 2066 count, where we observed a weak negative correlation between *Listeria innocua* 2066 count and pH (r=-0.36, p=0.0036) (**Figure 4.10**).

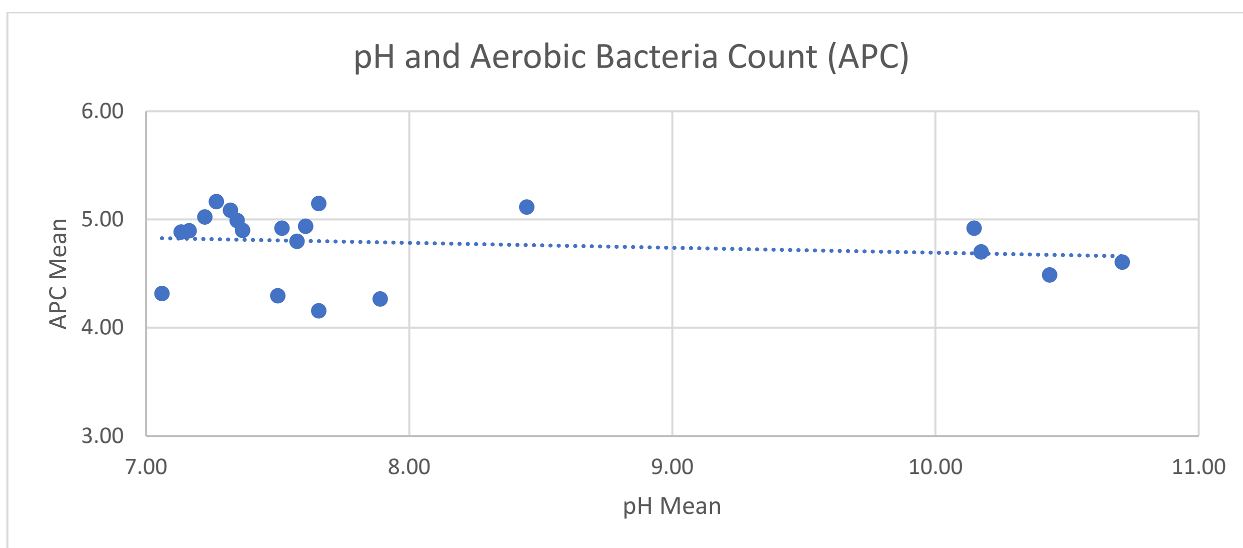

Figure 4.9. Correlation between pH and aerobic bacteria count

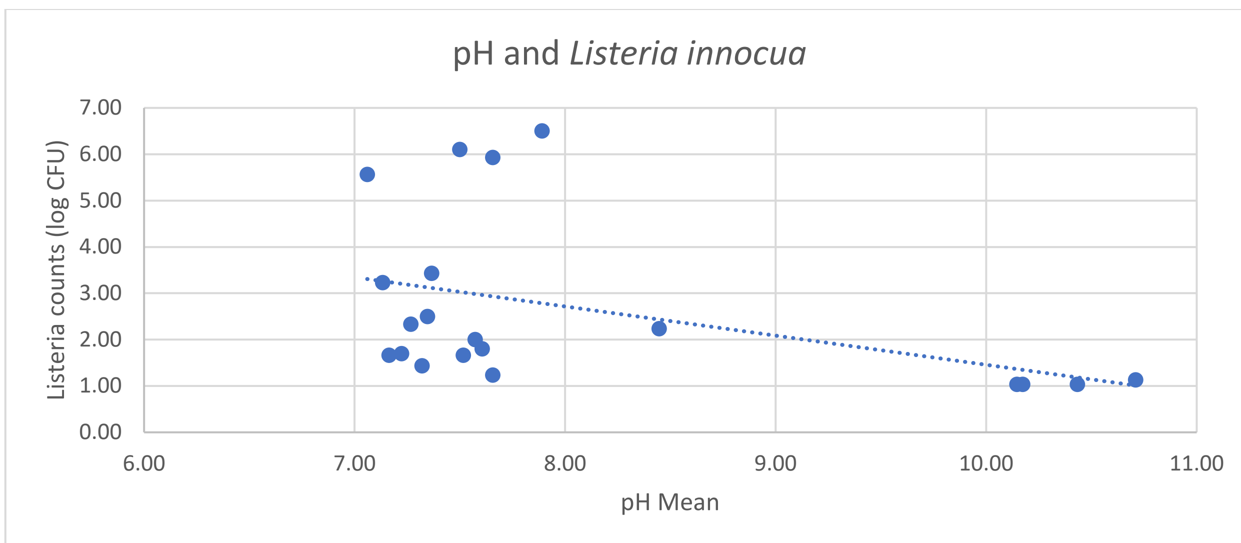

Figure 4.10. Correlation between pH and *Listeria innocua* 2066 population

4.4.3.3. Temperature

The average water temperature throughout the entire experiment ranged from 22.3 °C to 24.43 °C (**Table 4.3**). We observed a modest negative correlation between *Listeria innocua* 2066 count and temperature (r=-0.58).

	Water temperature, Mean (°C)			
Time	Fish tank	Fish tank R	Plant tank	Plant tank R
Day 0	22.3 ± 0.17	22.6 ± 0.50	22.83 ± 0.15	22.4 ± 0.20
Day 1	22.57 ± 0.15	23.00 ± 0.26	22.87 ± 0.25	22.37 ± 0.23
Day 2	22.87 ± 0.12	23.2 ± 0.17	23.47 ± 0.23	23.1 ± 0.20
Day 3	23.67 ± 0.35	23.43 ± 0.45	23.40 ± 0.35	23.53 ± 0.01
Day 7	23.40 ± 0.53	24.27 ± 0.59	23.83 ± 0.70	24.43 ± 0.46

Table 4.3. Average water temperature ± standard deviation for all treatment conditions and time points.

4.4.3.4. Total Ammonia-Nitrogen

Total Ammonia was analyzed on days 0 (pre-inoculation) and 7 (post-inoculation) of the experiment. The average ammonia and nitrite and nitrate for all treatments are shown in table 4. All samples from four treatments on day 0 were within the recommended level of Total Ammonia. Ammonia yielded 0.44 and .04 mg/L, Nitrite yielded .004 and .005 mg/L. Nitrate yielded 137.6 and 139 mg/L. On day seven, for Ammonia, all samples were found within the recommended level except three samples from Plant tanks non-replenished were found at 1.1, 2.4, and 3.0 mg/L. A nitrite test was not conducted while nitrate ranged between 70.93 and 83.5 mg/L on day 7. (**Table 4.4**).

Treatment	**Day 0**			**Day 7**		
	Ammonia	**Nitrite**	**nitrate**	**Ammonia**	**Nitrite**	**nitrate**
Fish tank	0.44	.005	137.6	0.67 ± 0.21	NA	71.3 ± 28.8
Fish tank R	0.44	.005	137.6	0.5 ± 0.26	NA	83.5 ± 23.69
Plant tank	0.4	.004	139	2.17 ± 0.97	NA	70.93 ± 18.15
Plant tank R	0.4	.004	139	0.47 ± 0.06	NA	84.10 ± 6.18

Table 4.4. Average Total Ammonia ± standard deviation for all treatment conditions and time points.

4.5. Discussion

The objective of this preliminary study was to evaluate the survival and persistence of *Listeria innocua* 2066 in effluent from fish tanks and plant tanks of aquaponic research. We evaluated four different treatment conditions: fish tank water, fish tank water-replenished, plant tank water, and plant tank water-replenished. Each bottle was inoculated with *Listeria innocua*

2066, and bacterial growth and water chemistry conditions were evaluated over 7 days to identify physiochemical parameters associated with *L. innocua* survival and growth.

While *Listeria innocua* 2066 can survive for up to 7 days in a recirculating aquaculture system, the results of this preliminary study show a significant decline in *L. innocua* population over time in both plant and fish tank effluent conditions. This reduction was also apparent in the replenished treatment conditions, although the replenished conditions generally showed greater *Listeria innocua* 2066 populations compared to non-replenished conditions. This is likely due to the daily re-introduction of nutrients in replenished conditions. Samples from day 7 for all treatments displayed pH values from 10.15 to 10.71 mg/L, which could explain the decline of the *Listeria innocua* 2066 population that persists in pH ranges of 6–8.5 (Somerville et al., 2014).

Listeria innocua 2066 is an anaerobic bacterium that can survive without oxygen (Chalmers, 2004). We observed a negative correlation between *Listeria innocua* 2066 and dissolved oxygen (r=-0.65). The validity of the experiment was supported by a strong positive correlation between APC count and dissolved oxygen (r=0.74). Ammonia levels in samples from the plant tank water (non-replenished) were found above-recommended levels at 1.1, 2.4, and 3.0 mg/L on day seven post-inoculation. In conclusion, we have developed a simulated aquaponic system and identified key microbial and physiochemical parameters that impact *Listeria innocua* 2066 growth in aquaponic systems.

Chapter 5: Survival and Persistence of Inoculated *E. coli TVS 354* and *Listeria innocua 2066* in bench-scale Aquaponics Systems (Study 3)

Abstract

E. coli and *Listeria* have been associated with multiple food-borne outbreaks linked to the consumption of fresh green leafy vegetables. The leaves of leafy green vegetables provide a large surface area with many folds, an environment where *E. coli* and *Listeria* can thrive. In this study, we evaluated the survival and transmission dynamics of *E. coli* TVS 354 and *Listeria innocua* 2066 within the lettuce (*Lactuca sativa* var. Truchas) during production and harvest over one aquaponic production cycle. In this study, we seek to: (1) evaluate the survival and persistence of *E. coli* TVS 354 and *Listeria innocua* 2066 in a bench-scale aquaponics system; (2) examine differences in bacterial survival within discrete aquaponic system compartments (*i.e.*, plant tank water, filters, leaves, roots & rockwool), and; (3) determine the relationship between water physicochemical parameters (*i.e.*, water and air temperatures, pH, dissolved oxygen, total ammonia nitrogen, nitrites, nitrates, turbidity) and *E. coli* TVS 354 or *Listeria innocu*a 2066 growth and persistence. In our experiment, *E. coli* TVS 354 and *Listeria innocua* 2066 were inoculated separately in 12 plant tank systems at two different doses (low-dose and high-dose). Uninoculated plant tanks were used as controls. Bacterial counts and physiochemical parameters were measured from various compartments in the aquaponic systems over a 25-day time course. The results showed a rapid decline of *E. coli* TVS 354 *and Listeria innocua* 2066 in plant tank water and filter sponges over the experimental time course. We observed a modest negative correlation between sampling time and *E. coli* TVS 354 ($r = -0.483$, $p<.0001$) and *Listeria innocua*

2066 (r = -0.45, p<0.0002). Targeted bacteria populations decreased as holding time increased. The reduction in *Listeria innocua* 2066 and *E. coli* TVS 354 was statistically significant at 24 and 72 hours (p<0.05) for both LD and HD conditions, displaying a total reduction of 1-4 logs. *E. coli* and *Listeria innocua* 2066 were detected for up to 5 and 7 days in plant tank water, respectively. Our study suggests that *E. coli* and *Listeria* can survive longer on filter (sponge-like matrix) than in plant tank water and can persist on filter for 14 and 19 days, respectively. During harvest day (25 days post-inoculation for *E. coli* TVS 354, 20 days for *Listeria innocua* 2066), no *E. coli* TVS 354 *or Listeria innocua 2066 was* detected on leaves. Both were detected on roots and rockwool, with higher counts on the backs compared to the fronts. The filter sponge displayed the highest APC counts for all treatments, ranging from 5.98 to 8.50 log CFU/mL while the plant tank water displayed the lowest APC for all treatments, ranging from 2.93 to 3.02 log CFU/mL. Lettuce lower leaf samples preharvest had a significantly higher APC (5.1- 6.4 log CFU/mL) relative to harvested lettuce upper leaves (2.8 - 4.2 log CFU/mL). The impact of pH and dissolved oxygen on *E. coli* TVS 354 and *Listeria innocua* 2066 survival was significant (p<.0001). A negative correlation was observed between the survival of *Listeria innocua* 2066 and pH (r = -0.524, p<.0001). A weak positive correlation was observed on DO with E. *coli* TVS 354 *counts* (r = 0.31, p<0.01), and *Listeria innocua* 2066 counts (r =0.28, p<0.05). In this study, the ability of low population of *E. coli* TVS 354 and *Listeria innocua* 2066 to survive during production and postharvest operations in Lettuce of AP has been demonstrated. However, Field-based trials, under real-world conditions, will be needed to corroborate and strengthen these findings. Follow-up experiments/surveys will be necessary to evaluate the public health concern related to these findings. In summary, this research contributes to understanding potential food safety risks and factors associated with bacterial growth and persistence, including physicochemical factors, and other environmental

conditions in aquaponics systems. Results from this project can directly inform pre-harvest and post-harvest produce safety risk management strategies and useful to the USDA-Agricultural Marketing Service GAPS certification program.

5.1. Introduction

Consumption of leafy green vegetables contaminated with *E. coli O157:H7* and *Listeria monocytogenes* poses an important risk for foodborne disease in humans. Infection with *E. coli* O157:H7 can cause various forms of illness, from self-limiting diarrhea to life-threatening hymolytic uremic syndrome, particularly in infants and elderly people (Delaquis et al., 2007). Contamination of edible tissues in leafy green vegetables is often due to the transfer of pathogens from soil or water (Delaquis et al., 2007). Several factors impact *E. coli* survival on plants, including nutrient availability, competition with indigenous microflora, UV radiation, and relative humidity (Brandl, 2006). Islam et al. (2004) reported survival of *E. coli* on lettuce and parsley for up to 77 and 177 days, respectively, in an experiment that exposed lettuce and parsley plots to contaminated compost or water (Islam et al., 2004). In greenhouse experiments, *E. coli* was detected on lettuce plants 28-35 days post high-dose (10^4 CFU/mL) spray-inoculation (Salomon et al., 2003; Moyne et al., 2011). While the persistence and fate of *E. coli* and other bacteria have been evaluated in various growing conditions (Islam et al., 2004; Moyne et al., 2011; Tomás-Callejas et al., 2011), less is known about survival and transmission of pathogenic bacteria in plants grown and harvested from aquaponics systems.

Listeriosis is a serious infection commonly caused by eating food contaminated with *Listeria monocytogenes*. According to the Center for Disease Control and Prevention (CDC, 2023), *Listeria monocytogenes* is responsible for 1,600 infections and about 260 deaths each year. Pregnant women, newborns, adults ≥ 65 years old, and immunocompromised individuals are

especially at risk. Vegetables like leafy greens are a common environment for bacteria like *Listeria* to thrive because they have large surface areas with many folds.

In a 1998 study, *Listeria* was cultivated from hybrid striped bass that was raised in three different freshwater systems in Maryland (Chalmers, 2004). The results of this study showed that *Listeria* can grow on farmed fish. *Listeria* has been found in several fishery products including raw and processed fish that have been collected from retail establishments and processing facilities. In contrast to these findings, another review reports no presence of the bacteria in freshly harvested fish from wild or aquacultural sources (Dillon and Patel, 1992). Taken together, it can be surmised that processing is the cause for contamination rather than the aquaponics farming process itself. Further research must be conducted to determine the association between *Listeria,* processing, and aquaponics farming.

Monitoring chemistry in aquaponics is critical to provide optimal growth conditions for fish, plants, and bacteria. Key aquaponic chemistry parameters include water pH, temperature, and dissolved oxygen. Fish, plants, and bacteria require high levels of oxygen in water and dissolved oxygen (DO) is measured to quantify oxygen content (expressed as mg/L). Dissolved oxygen is recommended to be maintained between 5-8mg/L (Hager et al., 2021). The pH is a measure of the acidity or basicity of a solution**.** The optimal pH level for aquaponics water and bacteria ranges from 6-8.5. Water temperature impacts the health and survival of both fish and plants. In aquaponics, water temperature is more important than air temperature, and it needs to be monitored daily. Total ammonia (ammonia, nitrite, nitrate) tests are used to monitor the function of the nitrifying bacteria and the performance of the biofilter. Ammonia and nitrite should be close to 0 mg/L while nitrate should be <150 mg/L (Somerville et al. 2014).

The purpose of this study was to evaluate the survival of *E. coli* TVS 354 and *Listeria innocua* 2066, non-pathogenic strains, on lettuce plants grown and harvested from bench-scale aquaponic (AP) systems. This study further sought to evaluate AP system compartments that are enriched for bacterial growth to identify critical nodes for bacterial transfer within the AP system. The results of this study will inform policies and management practices related to preventing pathogenic bacterial contamination and overall AP system food safety.

5.2. Research questions

1. How long can surrogate pathogens bacteria survive in aquaponics system?
2. Which compartments of the aquaponic systems are enriched for surrogate pathogen bacteria survival and persistence?
3. Does the filter sponge serve as a matrix for bacterial growth and effectively purify the AP system water of bacteria?
4. Can the plant naturally eliminate the pathogenic bacteria within one cultivation cycle?
5. What is the relationship between AP system physicochemical parameters (*i.e.*, water and air temperature, pH, dissolved oxygen, total ammonia nitrogen, nitrites & nitrates, and turbidity) and *E. coli* TVS 354 and *Listeria innocua* 2066 growth and persistence?
6. Do leaves at lower positions on the plant, where they can come into contact with the plant tank water and rockwool, display greater bacteria growth relative to leaves at higher positions on the plant?
7. Do the roots and rockwool from the back plants, situated in close proximity to the out spout of the filter, display greater bacterial growth and persistence than roots and rockwool from the front plants?

8. Do Good Agricultural Practices (GAPs) and polycarbonate sheets between each AP system prevent cross-contamination?

5.3. Hypothesis

1. *Listeria* and *E. coli* survival and persistence in a bench-scale aquaponics system are associated with the initial bacterial inoculate (e.g., high-dose inoculate will persist for longer than low-dose inoculate).
2. Plant tank water physiochemical parameters (*i.e.*, pH, temperature, dissolved oxygen, and total ammonia nitrogen, nitrites & nitrates) play a key role in bacterial growth and persistence in aquaponic systems and are thus key parameters to consider for aquaponic system food safety management.

5.4. Problem Definition

Leafy vegetables are often eaten uncooked, making bacterial pathogen contamination of leafy vegetables a considerable food safety issue. Studies have reported pathogen contamination of lettuce, basil, and other vegetables grown in controlled agriculture environments, including Aquaponics (AP) and Hydroponic (HP) systems (Wang et al., 2020). In 2021, two soilless leafy greens (LGs) producers issued product recalls for potential contamination with *Salmonella* and *Listeria monocytogenes* raising concerns about the safety of soilless operations and practices, which are rapidly expanding in the USA and globally (FDA, 2021). These observations suggest a pressing need for data on potential food safety risks and risk factors to inform the development of pre-harvest and post-harvest risk management strategies. The proposed project would generate data on growth and persistence of inoculated *E- coli* TVS 354 and *Listeria innocua* 2066 surrogates

in AP systems and interactions with background microflora and physicochemical factors. The results of this study will provide useful data for producers, decision makers, and researchers.

5.5. Objectives

a) Evaluate survival and persistence of *E. coli* TVS 354 and *Listeria innocua* 2066 in bench-scale aquaponics (AP) systems.

b) Examine bacterial localization in the systems (plant tank, filter sponge, roots, leaves and rockwool).

c) Assess changes in water physicochemical parameters (temperature, pH, dissolved oxygen, total ammonia nitrogen, nitrites, nitrates) over time in bench-scale AP systems.

5.6. Statistical analysis

The bacterial populations were converted to log CFU/mL or CFU/g. Reductions or changes in bacterial populations at different time intervals were computed as the difference between the count observed at time 0 (*N0*, log CFU/mL or CFU/g) and the count observed at a sampling point (*N*, log CFU/mL or CFU/g). ANOVA (proc mixed feature) was used to evaluate statistical significance. Normality and homogeneity of variance were tested before running ANOVA. Pearson correlation and Fisher z-transformation were used to determine the correlation between *Listeria innocua* 2066*, E. coli* TVS 354 or APC count and physicochemical parameters. $p<0.05$ was considered statistically significant in all cases. All analyses were run on SAS 9.4 (SAS Institute, Cary, NC, USA.).

5.7. Methods

5.7.1. Systems Setup

The study was conducted using 12 individual recirculating bench-size aquaponic systems placed on two metal racks, each rack containing six systems evenly spaced ~2cm apart. Each system contained four lettuce plants (*Lactuca sativa*, var. Truchas) and four goldfish (Carassius auratus). Each system was composed of a 38 L fish tank and a 15 L deep-water grow bed with 1.25 cm insulation foam boards acting as floating rafts which allowed the lettuce plants to grow above the water while keeping their roots submerged in the water (**Figure 5.1**). Each raft had four 1 inch-diameter holes cut into them. The lettuce seeds were germinated in rockwool for 14 days in a germination chamber kept damp at ~24ºC, then transferred into the holes made in the Styrofoam raft. Polycarbonate sheets were placed between each system, extending from the tops of the filters to the bottom of the plant tanks. These were installed to prevent cross-system contamination of water particles and bacteria.

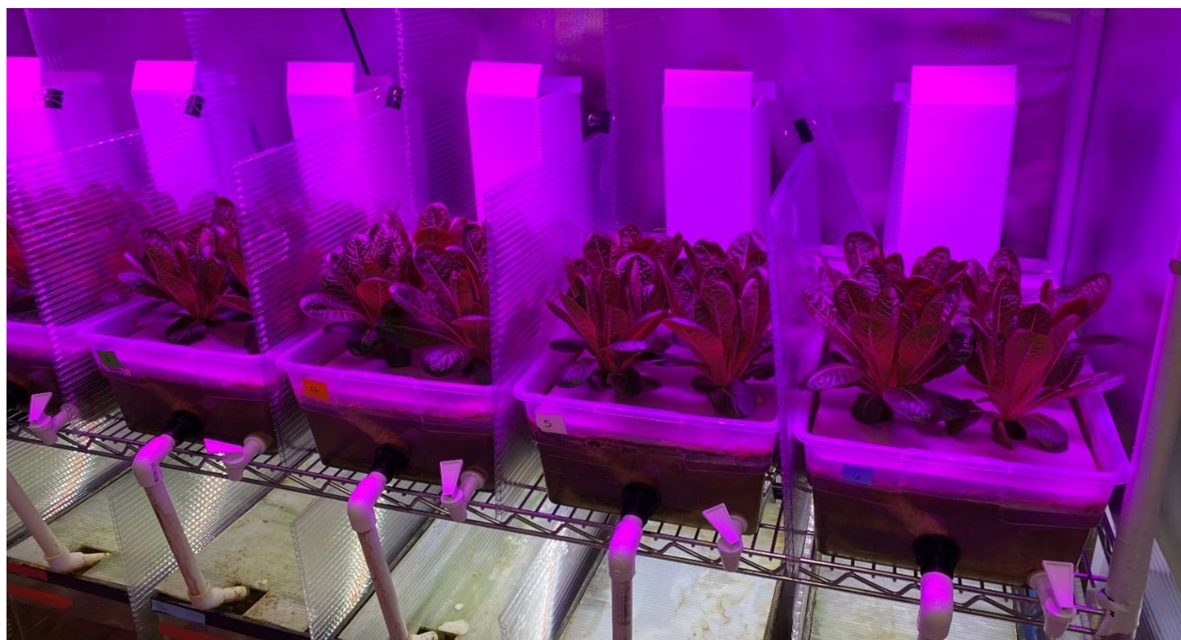

Figure 5.1. Discharge tube connecting grow bed to fish tank and 4 Truchas lettuce in the raft.

5.7.2. Inoculation procedure

Out of the 12 systems, four were inoculated with a high dose of *Listeria innocua* 2066 (6 log CFU/mL) and E. *coli* TVS 354 (4 log CFU/mL). Another 4 were inoculated with a low dose of *Listeria innocua* 2066 (2 Log CFU/mL) and *E. coli* TVS 354 (3 Log CFU/mL). The remaining four AP systems were not inoculated and served as controls.

Listeria innocua 2066, a non-pathogenic strain of *Listeria* with erythromycin resistance, was used for inoculation. *Listeria innocua* 2066 growth dynamics were evaluated in high-dose and low-dose conditions. Each High-dose inoculation was vortexed in two 8 mL tubes, and 2 mL from each tube was distributed into four designated high-dose (HD) systems, the first 1 mL in the plant tank and the second 1 mL in the fish tank. Low-dose inoculate was vortexed in two 6 mL tubes and distributed to four designated low-dose (LD) systems, 1 mL in the plant tank and 1 mL in the fish tank. The water in each tank was stirred with a sterile pipette after being inoculated. *E. coli* TVS 354, a non-pathogenic strain of *E. coli* with rifampin resistance, was also used for inoculation in independent AP systems using the aforementioned method.

Four of the 12 systems were controls, 4 were LD conditions and 4 were HD conditions, each block containing one of each treatment with a masked position (**Figure 5.2)**. The position of each treatment condition was masked to prevent investigator bias.

BLOCK 1			BLOCK 2			BLOCK 3			BLOCK 4		
H	C	L	L	C	C	C	L	H	L	C	H

Figure 5.2. The treatment layout on the AP systems; C: 0 cfu/mL. L: 2 cfu/mL. H: 4 cfu/mL

5.7.3. Sample Preparation and procedures

All samples (*e.g.*, plant tank water and filter sponges for microbial analysis, physiochemical testing, IDEXX, swabs, and harvesting) were taken before fish were fed on the sampling day to avoid any effect of uneaten fish food or fish waste on microbial and physiochemical measurements. Before inoculating the systems (Day -1), samples from the plant tank and filter sponge were tested, as well as from the biofilm on the bottom of the plant tanks, to ensure the systems did not have any baseline *Listeria* or *E. coli.* After inoculation with *E. coli* TVS 354 and *Listeria innocua* 2066, samples were collected within discrete AP system compartments, including plant tanks, filters, leaves, roots, and rockwool from the 12 AP systems.

Appropriate dilutions were made for bacterial isolation and enumeration, using Petrifilm for aerobic plate count (APC) and most-probable-number (MPN) blocks. Cultural enrichment was used to increase the chance of detecting *E. coli* TVS 354 and *Listeria innocua* 2066. Tryptic soy agar with erythromycin (TSA-E) was used for the detection of *Listeria innocua* and Tryptic soy agar with rifampicin (TSA-R) for *E. coli* TVS 354 (**Figure 5.3**). Because microbial activity occurs rapidly, microbial water samples were collected on days -1, 0, 1, 3, 5, and 7 to measure inoculated bacteria growth. Microbial water samples were taken two times per week in weeks 2, 3, and 4, after which microbial activity is expected to plateau (**Figure 5.3).** In addition, swab samples were collected on day -1 and day 25 to analyze the biofilm over the course of the experiment. At the end of week 4, the growth of all plants in each tank was measured by ascertaining the wet weight in tared sterile Whirl-Pak bags. Finally, plant roots and shoots were analyzed separately for total bacteria, *E. coli TVS 354* and *Listeria innocua* 2066.

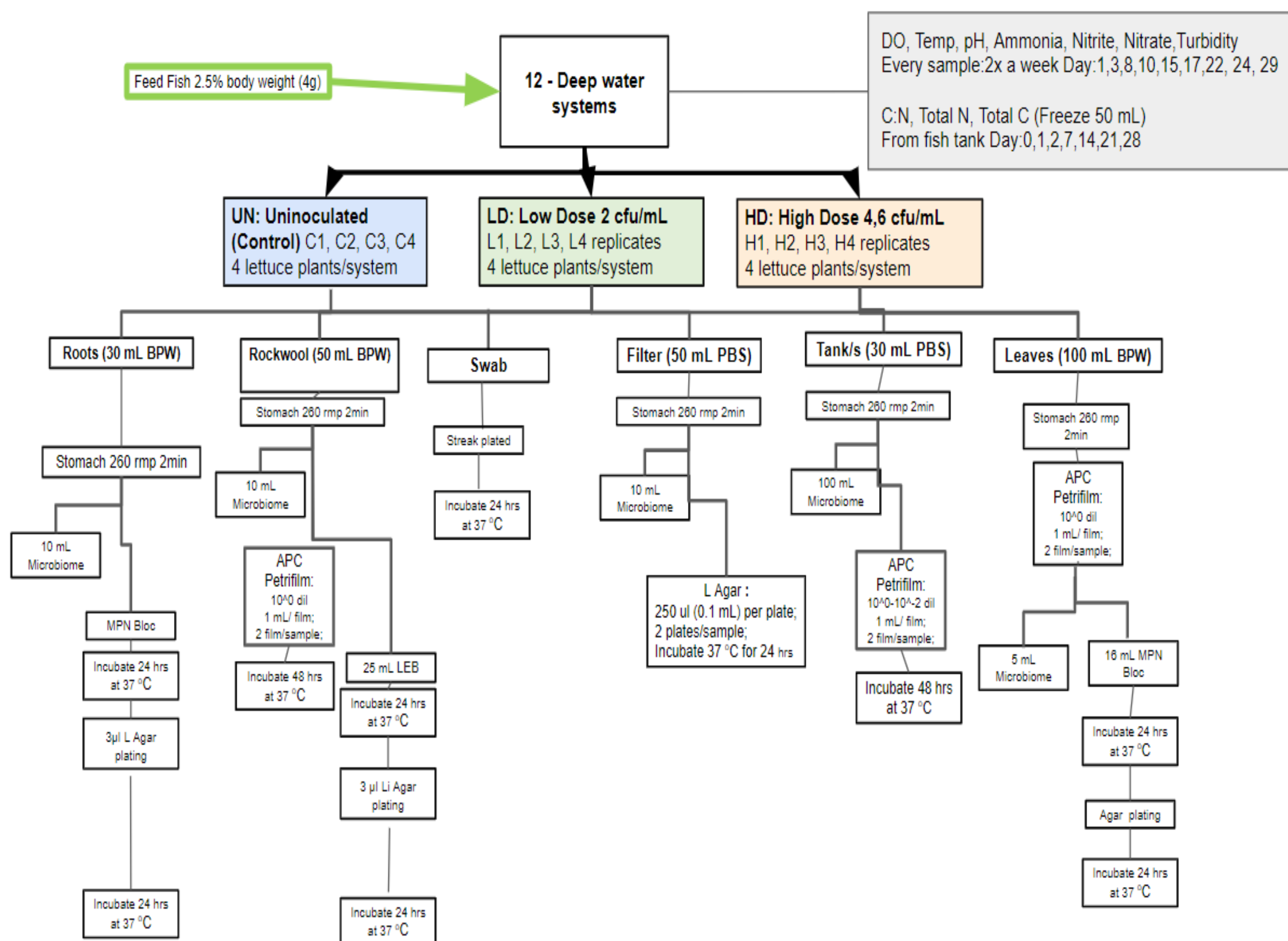

Figure 5.3. Process Flow diagram for the bench-scale study on *E. coli* TVS 354 and *Listeria innocua* 2066 survival in AP systems.

5.7.4. Plant tank procedure

Samples for MPN and APC were taken from the plant tank via a valve on the front side of the plant tank container. The valve was used to reduce splash and potential cross-contamination. For each sample, 10 mL was collected in a sterile container. For APC, 1 mL of the appropriate dilution of the sample was placed on petrifilm, and 8 mL was placed on MPN block. Plates were incubated at 37 °C for 24 hours and then APC was evaluated. MPN block was then plated on TSA-R for *E. coli* and TSA-E plate for *Listeria* and incubated at 37 °C for 24

hours and then examined for *E. coli* TVS 354 and *Listeria innocua* 2066 levels. Water samples were tested for *E. coli* TVS 354 on days 0, 1, 3, 5, 12, and 19 and for *Listeria innocua* 2066 on days 0, 1, 3, 5, 7, and 14. Finally, 200 mL was taken from each plant tank for IDEXX testing once a week also using the valve.

5.7.5. Filter sponge procedure

Each system has sponge and bioballs to promote colonization and nitrification by indigenous bacteria. The 12 sponges were tested once a week on days 1, 6, 12, and 19 for APC, *E. coli* TVS 354, and *Listeria innocua* 2066 levels. First, the sponge filter was removed, placed on a sterile bag, and stomached with 50 mL of Phosphate buffered saline (PBS). For APC, 1 mL of the solution was placed on petrifilm after appropriate dilution, and 8 mL were placed on MPN block. Plates were incubated at 37 °C for 24 hours and then APC petrifilm was examined. MPN block was then plated on TSA-R for *E. coli* and TSA-E for *Listeria innocua* 2066 and incubated at 37 °C for 24 hours, after which *E. coli* TVS 354 and *Listeria innocua* 2066 levels were evaluated.

5.7.6. Swab Testing (Baseline)

Biofilm and algae swabs were taken from various locations in the systems, including tank walls, biofilm on the bottom of the plant tanks, and inside the pipes that carry water from the plant tank to the fish tank and then streak-plated onto TSA-R for *E. coli* and TSA-E for *Listeria*. All plates were incubated at 37 °C for 24 hours and then tested for location-specific presence of *E. coli* TVS 354 and *Listeria innocua* 2066*in* the systems prior to the start of the experiment. The sampling locations represent microenvironments where those microorganisms were likely to be

present but not regularly sampled during the experiment time course. The same locations were swabbed and plated again at end of the experiment (sampled at day -1 and day 25).

5.7.7. Harvesting lower leaves

The lowest leaves on the four lettuce plants of each system were harvested and analyzed for microbial growth separately. As they often come in physical contact with the raft and rockwool, which each consistently have water on them, and carries the bacteria we are testing for. The lower leaves were harvested and tested via APC and MPN for both *E. coli* TVS 354 and *Listeria innocua* 2066 on days 21 and 16, respectively.

5.7.8. Harvest procedure

The roots, leaves, and rockwool of each of the 4 plants were separated into 2 categories, the front two plants and the back two plants. The front and back plants were collected and consolidated (the two front plants in the same bag and the two backs in the same bag). The back plants are situated by the spout of the filter and are prone to coming into direct physical contact with the water coming out of the spout. The roots, leaves, and rockwool of each plant were placed into sterile Whirl-pack bags and weighed separately. For roots, 30 mL of BPW were added to the compressed roots and stomach for 2 minutes. For leaves, 100 mL of BPW were added to the compressed leaves and stomach for 2 minutes. For rockwool, 50 mL of BPW were added to the 2 cubs and stomach for 2 minutes. After dilution, 1 mL of the solution was placed on APC petrifilm and 8 mL on MPN block. Plates were incubated at 37 °C for 24 hours and then APC petrifilm was examined. MPN blocks were then plated on TSA-R for *E. coli* and TSA-E for *Listeria* and incubated at 37 °C for 24 hours, followed by examination of *E. coli* TVS 354 and

Listeria innocua 2066 levels. *E. coli* TVS 354 was analyzed on day 25 and *Listeria innocua* 2066 on day 20.

5.7.9. Size of lettuce

The height and width of each lettuce plant were measured weekly on days -1, 4, 11, 18, and 25. The height was measured from the top of the rockwool (beginning of visible growth) to the tip of the highest leaf. The width was taken by measuring horizontally from the tip of one leaf to the tip of the leaf directly across from it. Each system included four lettuce plants, arranged in a square, spaced 5 inches apart, and numbered 1- 4 from left to right (**Figure 5.4**).

Figure 5.4. The lettuce layout on the raft in the AP system.

5.7.10. Physicochemical testing

Physicochemical parameter measurements were taken twice a week for both the plant tank and the fish tank. The physicochemical testing parameters were water and air temperature, pH, dissolved oxygen, total ammonia nitrogen, nitrites, nitrates, and turbidity. The dissolved oxygen, pH, and water temperature were taken with a HANNA HI98196 portable meter (Hanna Instruments, Woonsocket, RI), which was placed into the tank to take readings, removed,

sterilized using 70% ethyl alcohol and distilled water, and then placed into the next tank. An EC tracer pocket tester (LaMotte Company, Chestertown, MD) was used to measure turbidity in shallow cups containing 30 mL water samples taken from the tanks. Chemical testing was conducted using a Hach DR 3900 laboratory spectrophotometer (Hach Company, Loveland, CO) to measure total ammonia nitrogen, nitrite, and nitrate levels.

5.7.11. Bacterial qualitative and quantitative analysis

For *E. coli* TVS 354, colonies were detected by examining MacConkey agar plates. The appearance of a dark red colony was recorded as one colony count. For *Listeria innocua* 2066, colonies were detected by examining the TSA-E under UV light (475 nm-495 nm). The appearance of a Green Fluorescent Protein (GFP) dot was recorded as one colony count.

5.8. Results

No *E. coli* or *Listeria* were detected on plant tanks, filter sponge, biofilm and algae swab on day -1, prior to bacterial inoculation, confirming a sterile system prior to beginning the experiment.

5.8.1. Survival of *E. coli* TVS 354

E. coli TVS 354 was inoculated into the plant tank recirculating research aquaponic systems, in two different levels: (**1**) LD: 2 log CFU/mL, and (**2**) HD: 6 log CFU/mL. Uninoculated (UN) tanks were used as controls. Each treatment had four replicates. Since the aquaponics system is a recirculating system, the inoculated bacteria can spread through the water to different compartments within the systems. In general, applied *E. coli* TVS 354 was detected in different compartments of the systems including the plant tank, filter sponge, roots, leaves and rockwool. No *E. coli* TVS 354 was detected in any control system samples.

For plant tank, *E. coli* TVS 354 counts on day 0, immediately after inoculation, were 6.18 and 6.11 log CFU/mL for LD and HD conditions, respectively. We detected a significant reduction in *E. coli* TVS 354 populations for both LD and HD conditions, with a total reduction of 3.03 and 1.39 log CFU/mL after 24 hours, and 3.09 and 4.1 log CFU/mL after 72 hours, respectively ($p<0.05$). On day 5, *E. coli* TVS 354 was detected in only the HD conditions, albeit at very low (0.07 log CFU/mL). No *E. coli* TVS 354 was detected on days 12 and 19 post-inoculation (**Figure 5.5).** *E. coli* TVS 354 persisted in plant tanks for up to 5 days, while persisted on filters for up to 19 days, suggesting that *E. coli* TVS 354 can survive longer on a sponge-like matrix than in water.

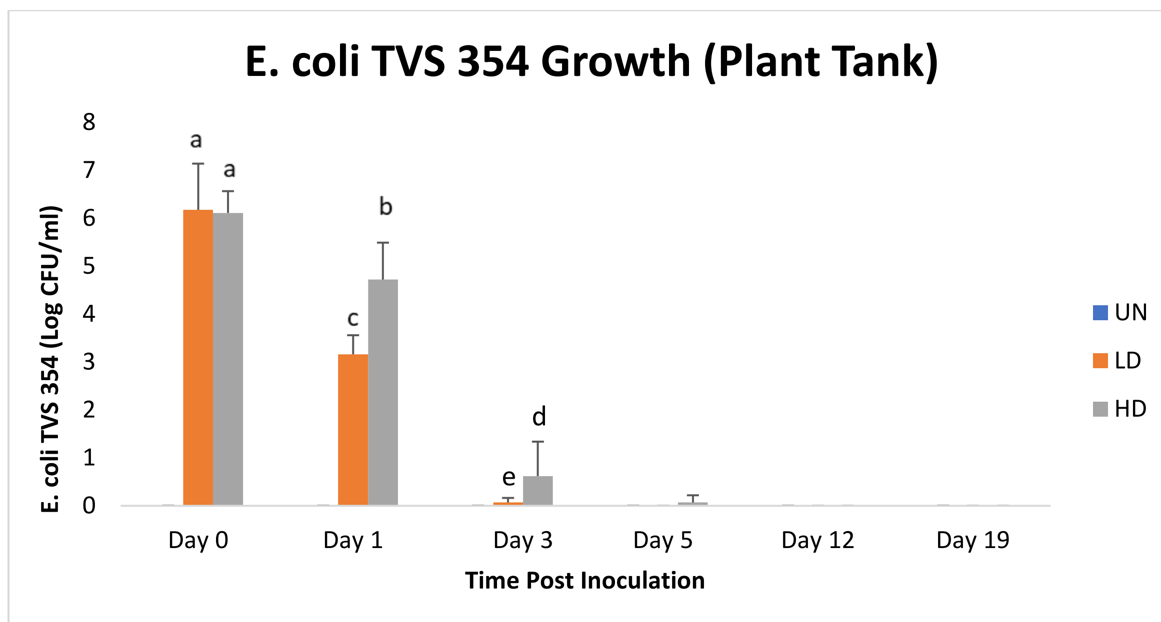

Figure 5.5. Persistence of *E. coli* TVS 354 in the plant tank of aquaponic systems. The data are presented as average counts ± standard deviation (n=3). Different lowercase letter on the top of the bar diagrams indicates significant differences (P<0.05) in between the means of all treatment conditions (LD and HD) among different times in days (ranked by LSD post-hoc test). *Note*. UN: uninoculated; LD: low-dose; HD: high-dose.

E. coli TVS 354 persisted on filter sponge with a similar trend compared to the plant tank. *E. coli* TVS 354 counts on filter sponge on day 0, immediately after inoculation, were 4.79 and

5.65 for the LD and HD conditions, respectively. A significant reduction in *E. coli* TVS 354 population was observed in both LD and HD conditions with a total reduction of 3.84 and 3.09 log CFU/mL after 24 hours, and 0.49 and 2.38 log CFU/mL after 72 hours, respectively ($p<0.05$) (**Figure 5.6**). On day 19, *E. coli* TVS 354 was detected, on filter sponges of HD conditions only, but was very low (0.05 log CFU/ mL) (**Figure 5.6)**. In general, the counts for *E. coli* TVS 354 in filter sponge were lower than plant tank samples but were detected up to 19 days post inoculation relative to 5 days post inoculation in plant tank samples. Taken together, these results suggest that *E. coli* TVS 354 can survive longer on filter (sponge) than plant tank (water).

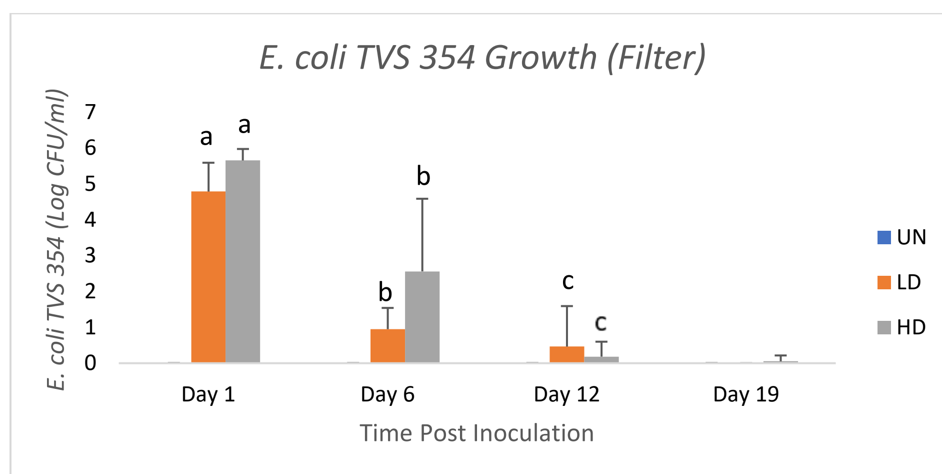

Figure 5.6. Persistence of *E. coli* TVS 354 in filter sponges of aquaponic systems. The data are presented as average counts ± standard deviation (n=3). Different lowercase letter on the top of the bar diagrams indicates significant differences ($p<0.05$) in between all the means of treatment conditions (LD and HD) among different times in days (ranked by LSD post-hoc test) . *Note*. UN: uninoculated; LD: low-dose; HD: high-dose.

On harvest day (day 25 post-inoculation), the roots, leaves and rockwool were analyzed for microbial growth. No *E. coli* TVS 354 was detected on the back and front leaves for both LD and HD conditions. *E. coli* TVS 354 was found only on the front and back of rockwool from HD conditions, displaying 0.02 and 0.2 log CFU/ mL, respectively. For the roots, *E. coli* TVS 354 was

detected only in the HD, displaying 0.32 for the front and 0.55 log CFU/ mL for the back of the root. (**Figure 5.7**). Only HD conditions were positive for *E. coli* TVS 354 after 25 days.

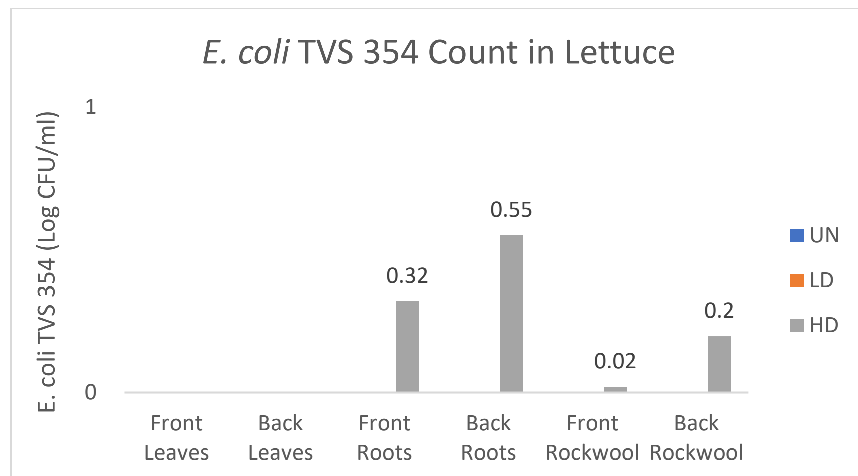

Figure 5.7. Average *E. coli* TVS 354 in lettuce plants of three different AP treatment conditions. *Note.* (UN: Uninoculated, LD: Low Dose, HD: High Dose).

5.8.2. Survival of *Listeria innocua* 2066

Listeria innocua 2066 was inoculated into the plant tanks of 12 recirculating research aquaponic systems at two different doses: (**1**) LD (2 log), and (**2**) HD (6 log CFU/mL). UN tanks were used as controls. Each treatment had four replicates.

In general, *Listeria innocua* 2066 was detected in different compartments of the systems including the plant tank, filter sponge, roots, leaves and rockwool. *Listeria innocua* 2066 was not detected in any control system. *Listeria innocua* 2066 counts in plant tanks on day 0, immediately after inoculation, were 2.93 and 3.19 log CFU/mL for LD and HD conditions, respectively. We detected a significant reduction ($p<0.05$) in *Listeria innocua* 2066 populations for both LD and HD conditions, with a total reduction of 2.44 and 1.68 log CFU/mL after 24 hours, respectively.

After 72 h, a significant reduction was detected only in the HD conditions (1.51 log CFU/mL, p<0.05). No *Listeria innocua* 2066 was detected on day 3 or day 14 post inoculation, while a small amount of *Listeria innocua* 2066 growth was detected on day 7 in the HD condition (0.02 log CFU/ mL). No significant differences were observed between treatment groups (**Figure 5.8).**

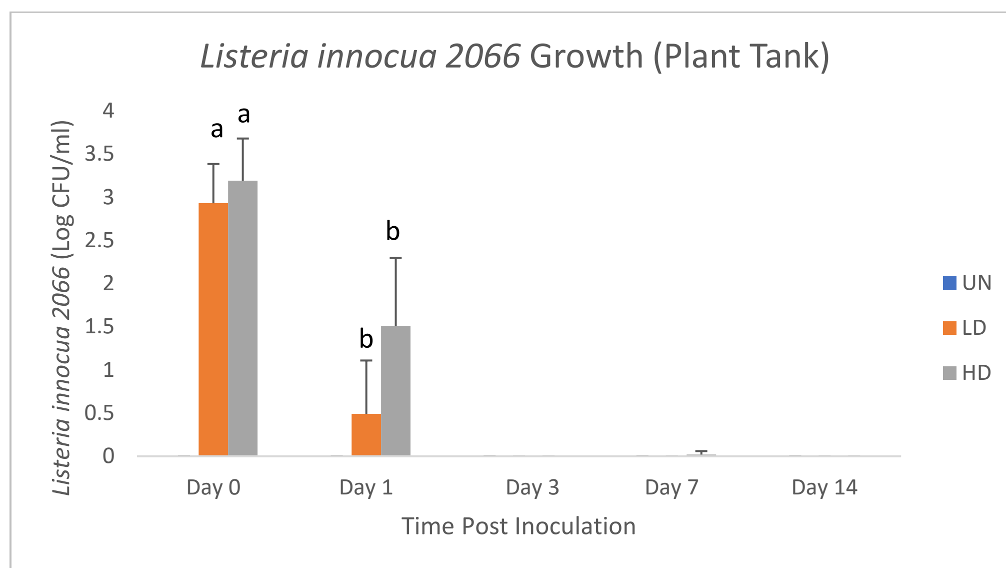

Figure 5.8. Persistence of *Listeria inoccua* 2066 in the plant tank of aquaponic systems. The data are presented as average counts ± standard deviation (n=3). Different lowercase letter on the top of the bar diagrams indicates significant differences (p<0.05) in between the treatment conditions (LD and HD) among different times in days (ranked by LSD post-hoc test). *Note.* (UN: uninoculated, LD: Low Dose, HD: High Dose) .

For filter sponge, *Listeria innocua* 2066 counts on day 0, immediately after inoculation, were 2.44 and 2.08 log CFU/mL for the LD and HD conditions, respectively. A significant reduction (p<0.05) in *Listeria innocua* 2066 populations was observed for both LD and HD treatments, displaying a total reduction of 2.44 and 1.89 log CFU/mL after 7 days, respectively. However, on day 14, a significant increase (p<0.05) in *Listeria innocua* 2066 populations was observed in the LD condition (0.15 log CFU/ mL) and a significant decrease in the HD condition (0.02 log CFU/ mL) (**Figure 5.9**). These results suggest that *Listeria innocua* 2066 growth is not

as stable as that of *E. coli* TVS 354. *Listeria innocua* 2066 appears to rapidly decrease, or become undetectable, within a week post inoculation (**Figure 5.9).**

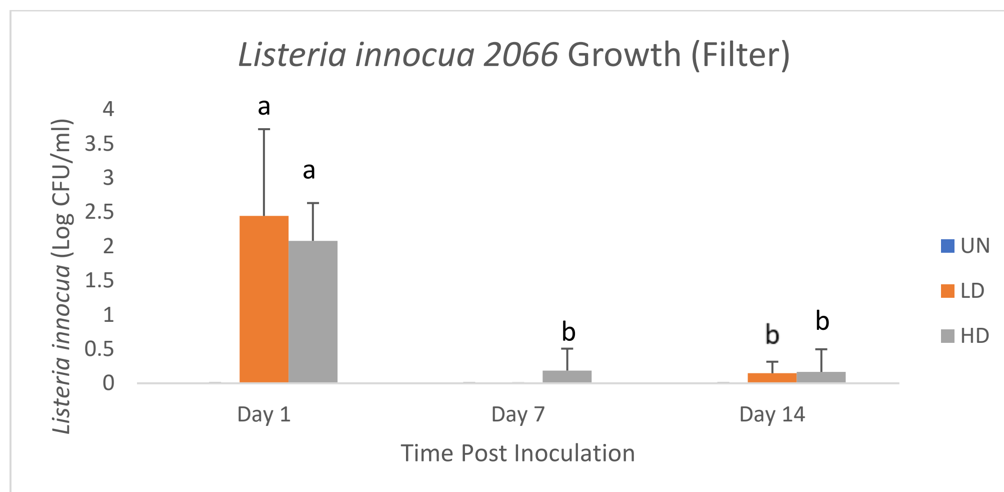

Figure 5.9. Persistence of *Listeria innocua* 2066 in filters of aquaponic systems. The data are presented as average counts ± standard deviation (n=3). Different lowercase letter on the top of the bar diagrams indicates significant differences (P<0.05) between the means of treatment condition among different times in days (ranked by LSD post-hoc analysis). *Note.* (UN: uninoculated, LD: Low Dose, HD: High Dose)

On harvest day, roots, leaves, and rockwool were analyzed for microbial growth (day 20 post-inoculation). No *Listeria innocua* 2066 was detected on the back and front leaves for both LD and HD treatment conditions. *Listeria innocua* 2066 was detected in only HD conditions on the front and back of rockwool, displaying 0.16 and 0.51 log CFU/mL, respectively. *Listeria innocua* 2066 was also detected only in the HD condition on the back of roots, displaying 0.6 log CFU/mL (**Figure 5.10**). We did not detect *Listeria innocua* 2066 in samples from front roots. In summary, we detected higher *Listeria innocua* 2066 counts than *E. coli* TVS 354 counts on the back of roots and rockwool, although *Listeria innocua* 2066 was only detected on roots and rockwool in HD conditions for ~20 days.

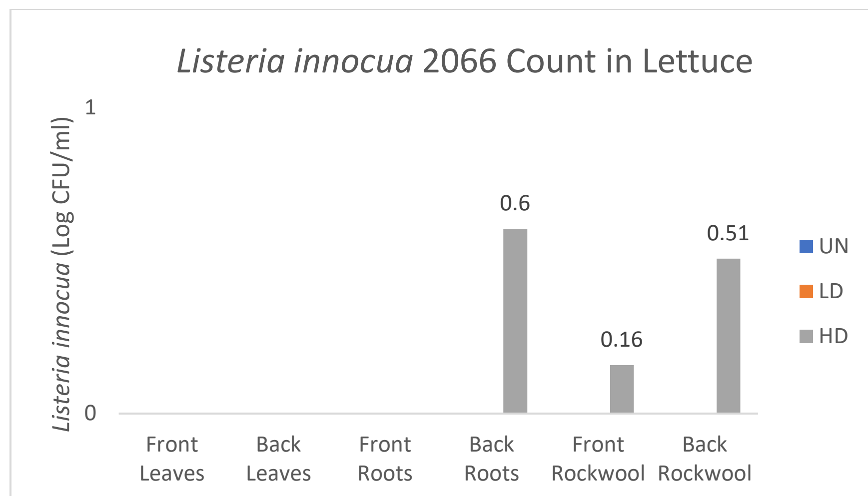

Figure **5.10.** Average *Listeria innocua 2066* counts from various lettuce plant samples. The data are presented as average counts ± standard deviation (n=3). Different lowercase letter on the top of the bar diagrams indicates significant differences (P<0.05) in between the treatments. *Note.* (UN: uninoculated, LD: Low Dose, HD: High Dose)

5.8.3. Enumeration of Aerobic Plate Count (APC)

We evaluated each treatment condition (UN, LD and HD) for general coliform counts in different compartments of the AP system including the plant tank, filter sponge, leaves, rockwool and roots. We observed a significant decrease of aerobic bacteria in the plant tanks of HD conditions at 24 hours and 14 days post inoculation (p<0.05) (**Figure 5.11**). APC for all treatments on day -1, pre-inoculation, ranged from 2.93 log CFU/mL to 3.02 log CFU/mL. On day 0, immediately after inoculation, APC ranged from 3.01 log CFU/mL to 3.04 log CFU/mL. After 24 hours, we observed a significant decrease in APC in the HD condition only, displaying a total reduction of 0.24 log CFU/mL. APC increased for all treatment conditions on day 3, followed by a significant decrease on day 14 for the HD condition only (total reduction of 0.15 log CFU/mL).

We detected a significant increase in aerobic bacteria on **filter sponges** at day 6 post-inoculation for all treatment conditions (**Figure 5.12).** Aerobic bacteria growth showed a similar trend for all conditions from day -1 to day 19, where aerobic bacteria count ranged from 5.98 to 8.50 log CFU/mL. **For roots, leaves, rockwool during harvest,** the upper leaves displayed the lowest APC for all treatments, ranging from 2.84 to 4.20 log CFU/mL, while the roots displayed the highest counts for all treatments, ranging from 5.43 to 6.18 log CFU/mL. Lettuce lower leaf samples preharvest had a significantly higher APC (5.1- 6.4 log CFU/mL) relative to harvested lettuce upper leaves (2.8 - 4.2 log CFU/mL) ($p<0.05$). However, there is no significant difference between back and front leaves for all treatments.

We did not detect differences in aerobic bacteria count between the front and back of rockwool within treatment groups. APC on rockwool ranged from 5.6 to 6.11 log CFU/mL (**Figure 5.13).** Taken together, among the AP system compartments sampled, APC was highest on the filters and lowest in the plant tank water. Low APC in the plant tank water samples relative to the other compartments in the AP system suggests that the water acts as a carrier, rather than a source, for bacteria. We next evaluated the correlation between *Listeria innocua* 2066 or *E. coli* TVS 354 and APC to determine if APC can affect the growth of bacteria in the plant tank water. We observed a weak negative correlation between APC and *Listeria innocua* 2066*,* albeit not reaching statistical significance ($r = -0.12$, $p = 0.3310$) (**Figure 5.14**) and a strong positive correlation between APC and *E. coli* TVS 354 (r =- 0.77, p<.0001) (**Figure 5.15**).

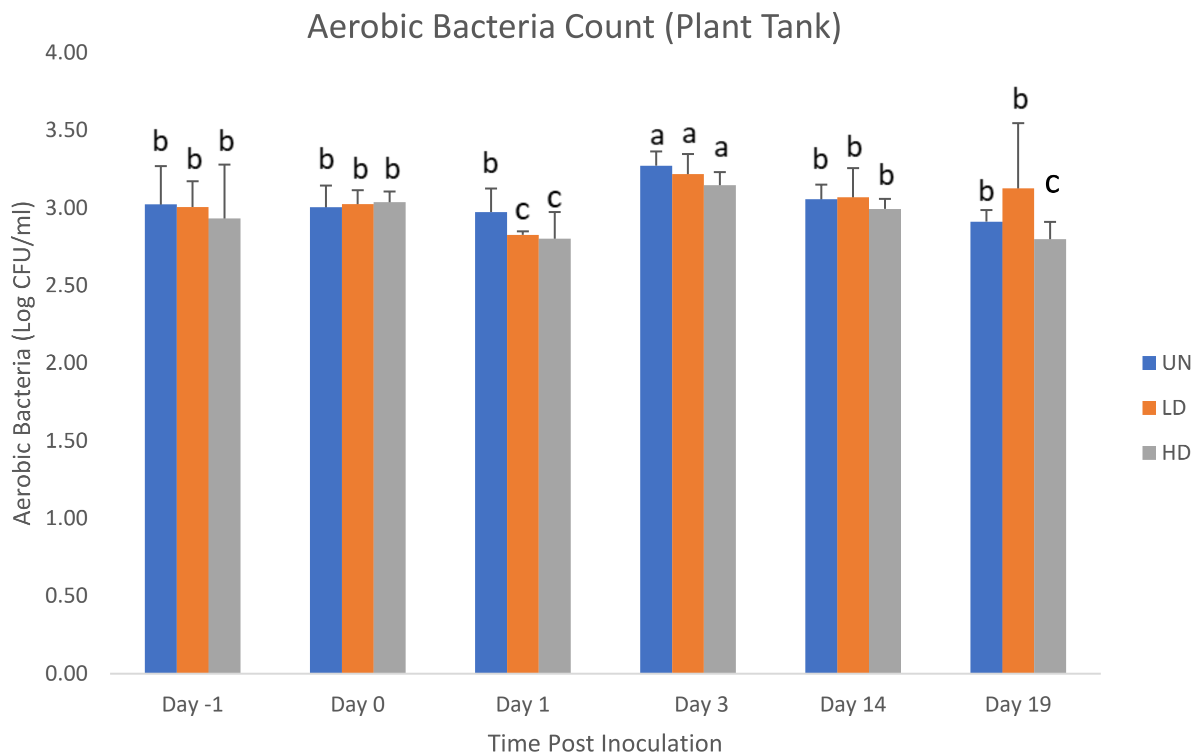

Figure 5.11. Average Aerobic Plate Count (APC) in plant tank. The data are presented as average counts ± standard deviation (n=3). Different lowercase letter on the top of the bar diagrams indicates significant differences (P<0.05) between the means of treatment conditions among different times in days (ranked by LSD post-hoc test). *Note.* (UN: uninoculated, LD: Low Dose, HD: High Dose)

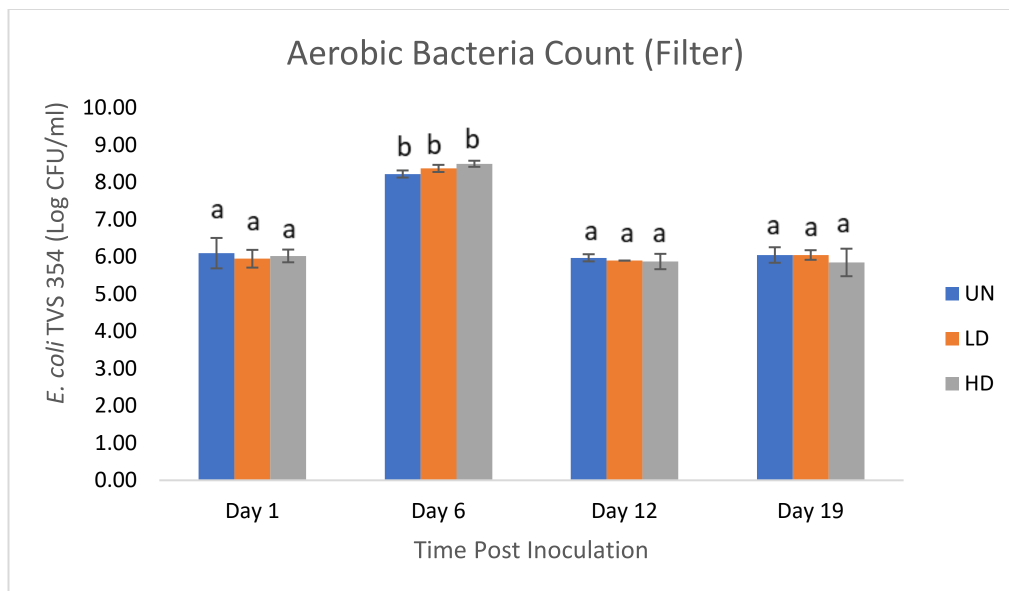

Figure 5.12. Average Aerobic Plate Count (APC) in filter sponge. The data are presented as average counts ± standard deviation (n=3). Different lowercase letter on the top of the bar diagrams indicates significant differences (P<0.05) between the means of treatment conditions among different times in days (ranked by LSD post-hoc test). *Note.* (UN: uninoculated, LD: Low Dose, HD: High Dose)

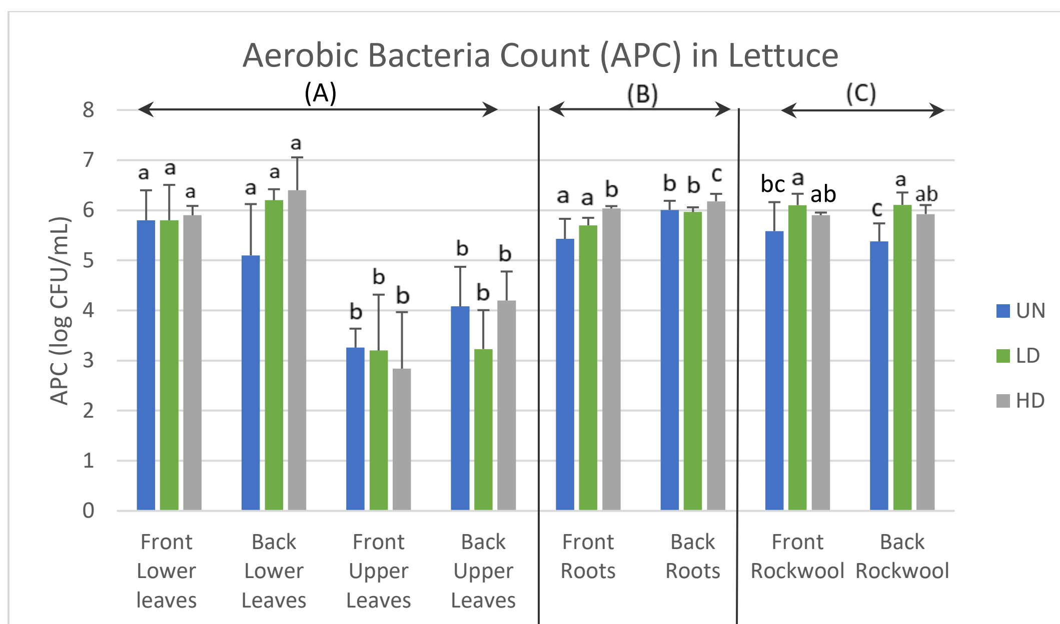

Figure 5.13. Average Aerobic Plate Count (APC) in Lettuce.: (A) Front Lower Leaves, Back Lower leaves, Front Upper Leaves and Back Upper Leaves; ((B) Front Roots, and Back Roots; (C) Front Rockwool, and Back Rockwool, the data are presented as average counts ± standard deviation (n=3). Different lowercase letter on the top of the bar diagrams indicates

significant differences (P<0.05) in between the means of treatment conditions among different times in days (ranked by LSD post-hoc test). *Note*. (UN: uninoculated, LD: Low Dose, HD: High Dose)

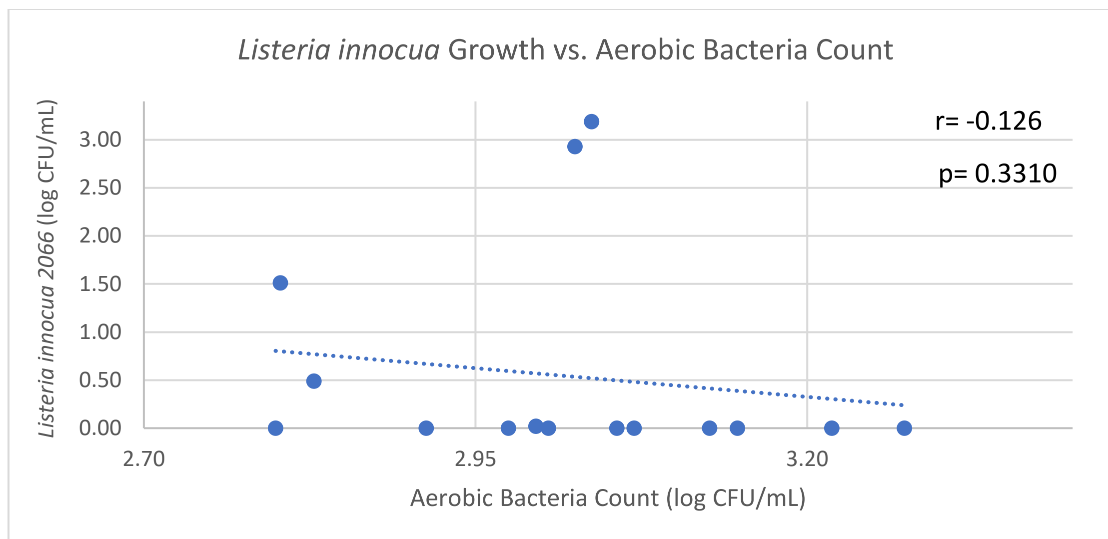

Figure **5.14.** Correlation between *Listeria innocua 2066* and Aerobic Bacteria Count (APC) in plant tank water.

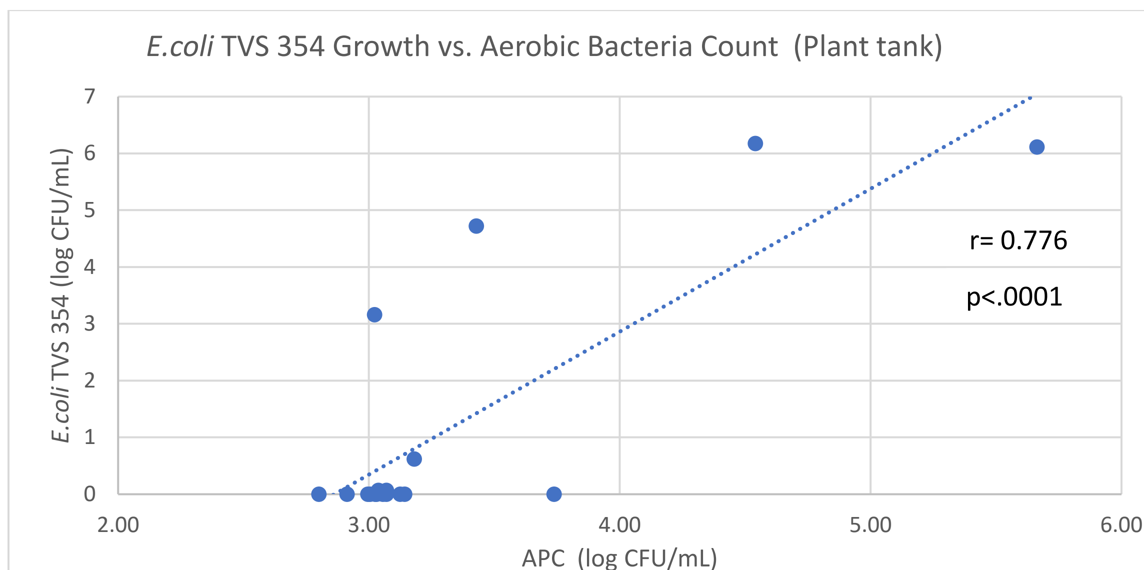

Figure 5.15. Correlation between *E. coli* TVS 354 and Aerobic Bacteria Count (APC) in plant tank water.

5.8.4. Physiochemical Parameters

5.8.4.1 Dissolved oxygen

The average dissolved oxygen (DO) for all treatments ranged from 7.71 to 9.05 mg/L (**Table 5.1.**). All samples were within the recommended level of 5-8.5 mg/L (Hager et al., 2021) except samples from day 5 from LD and HD conditions (9.01 mg/L and 8.6 mg/L, respectively), samples on day 7 from UN and LD conditions (8.69 mg/L and 9.05 mg/L, respectively) and samples on day 12 from HD conditions (8.62 mg/L). We observed a weak correlation. The effect of the dissolved oxygen on the survival of *E. coli* TVS 354 and *Listeria innocua 2066* was significant ($p<.0001$). We detected a weak positive correlation between *Listeria innocua 2066* and DO ($r = 0.28$, $p < 0.01$) (**Figure 5.16**) and between *E. coli* TVS 354 and DO ($r = 0.31$, $p < 0.01$) (**Figure 5.17**).

Dissolve oxygen, Mean (mg/L)			
Time	UN	LD	HD
Day 0	7.78±0.93	8.07±0.38	8.22±0.23
Day 5	8.19±0.19	9.01±0.38	8.6±0.56
Day 7	8.69±1.21	9.05±0.99	8.25±0.61
Day 12	8.45±0.93	7.95±0.67	8.62±0.64
Day 14	7.96±0.12	8.37±0.42	8.44±0.32
Day 19	7.71±0.11	7.75±0.19	7.81±0.08
Day 21	8.03±0.4	7.85±0.64	8±0.34

Table 5.1. Average ± standard deviation of dissolved oxygen for each treatment group and time point.

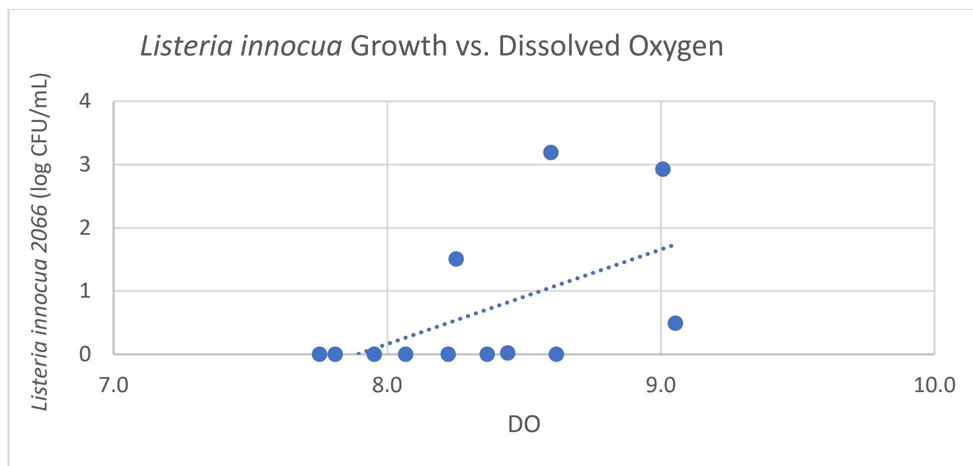

Figure 5.16. Correlation between *Listeria innocua* 2066 and dissolved oxygen in plant tank water.

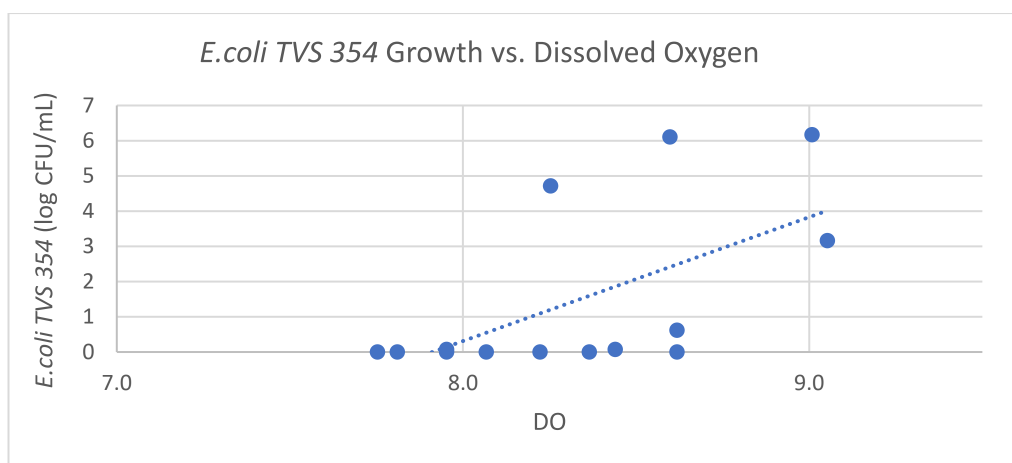

Figure 5.17. Correlation between *E. coli* TVS 354 and dissolved oxygen in plant tank water.

5.8.4.2. pH

The optimal pH level for aquaponics water and bacteria ranges between 6-8.5 mg/L. In this study, the average pH for all treatments ranged from 5.17 to 7.72 mg/L (**Table 5.2**). All samples of the three treatments were within the recommended level except those taken on days 5 and 7 for all treatments, which displayed pH values from 5.21 to 5.83 mg/L. The effect of the pH on the survival

of *E. coli* TVS 354, *Listeria innocua* 2066 *and* aerobic bacteria APC was significant (p<.0001). A modest negative correlation was observed between the *Listeria innocua* 2066 and pH (r = -0.52, p <.0001) (**Figure 5.18**). A strong negative correlation was also observed between *E. coli* TVS 354 and pH (r = -0.67, p<.0001) (**Figure 5.19**). A modest negative correlation was observed between APC and pH (r = -0.40, p <.0001) (**Figure 5.20**).

pH, Mean			
Time	UN	LD	HD
Day 0	6.25±0.15	6.31±0.06	6.17±0.29
Day 5	5.52±0.56	5.69±0.41	5.69±0.51
Day 7	5.83±0.8	5.17±0.12	5.21±0.1
Day 12	7.36±0.13	7.55±0.01	7.61±0.03
Day 14	7.52±0.09	7.67±0.01	7.72±0.03
Day 19	6.95±0.13	7.21±0.04	7.34±0.04
Day 21	7.02±0.17	7.39±0.06	7.54±0.05

Table 5.2. Average pH ± standard deviation for all treatment conditions and time points

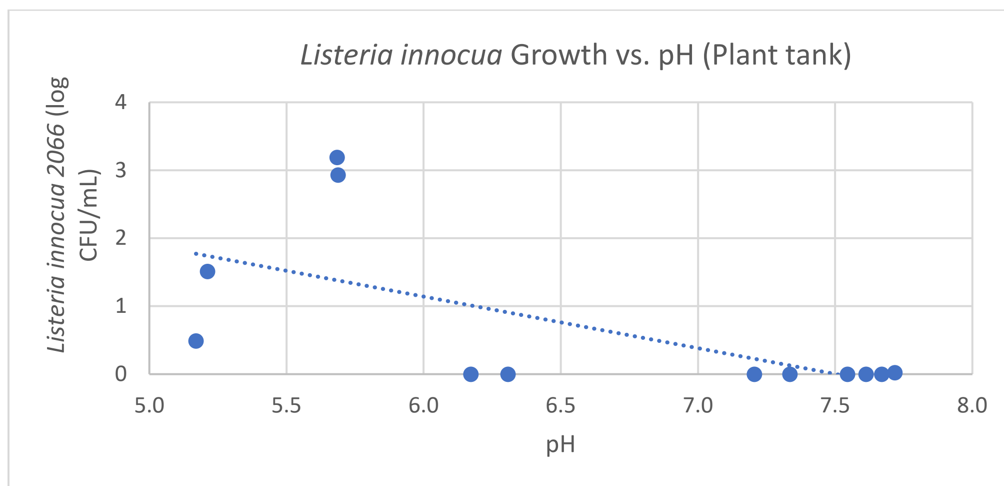

Figure 5.18. Correlation between *Listeria innocua* 2066 growth and pH.

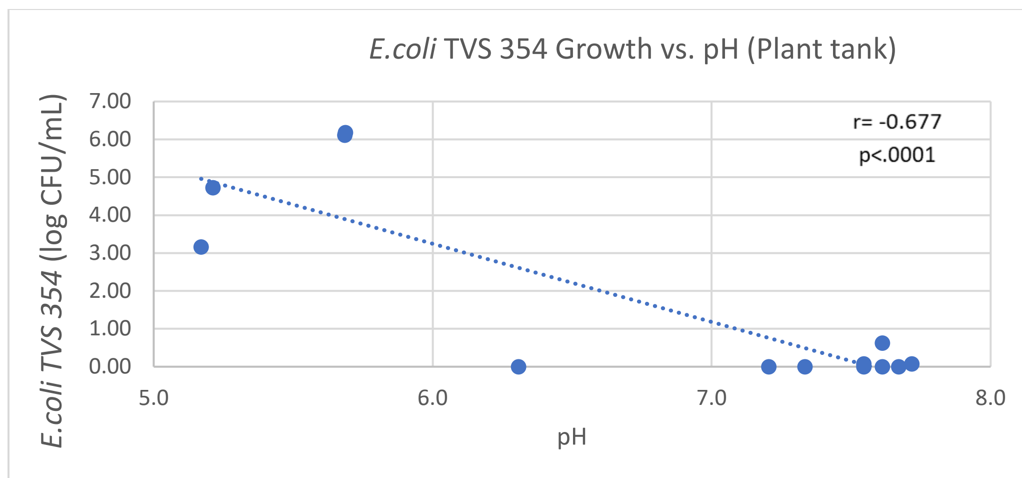

Figure 5.19. Correlation between *E. coli* TVS 354 growth and pH.

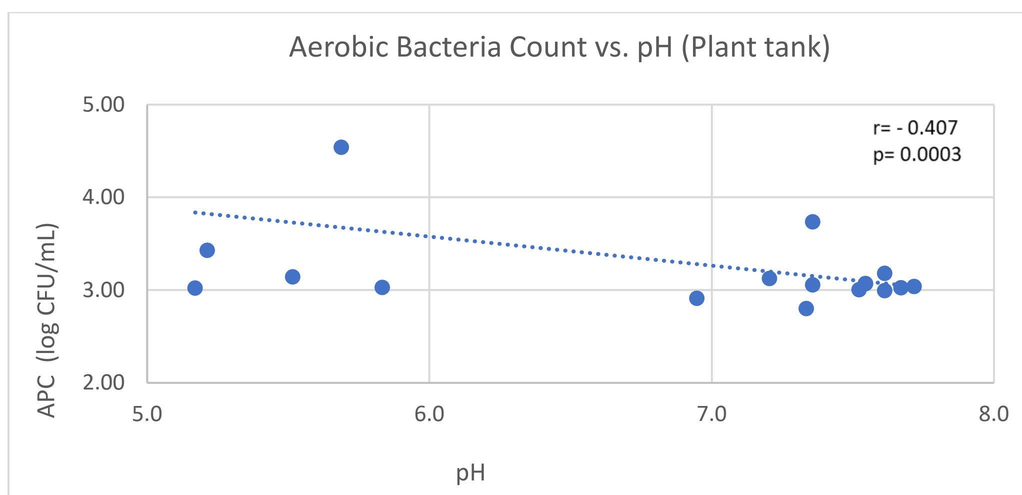

Figure 5.20. Correlation between aerobic bacteria count and pH.

5.8.4.3. Temperature

The average water temperature was recorded twice a week for all treatments. Temperate across treatments ranged from 23 °C to 25.48 °C, within the optimal range of 18 °C to 30 °C (**Table 5.3**). The highest temperature was recorded on day 15 in the UL, LD and HD conditions, displaying temperatures of 25.33 °C, 25.48 °C, and 25.28 °C, respectively. Temperature did not appear to have an impact on *Listeria innocua* 2066*, E. coli* TVS 354 and APC counts (r <1).

Water temperature, Mean (°C)			
Time	UN	LD	HD
Day 0	23.63±0.21	23.7±0.08	23.55±0.4
Day 5	23.83±0.33	24.05±0.24	23.78±0.38
Day 7	23.88±0.1	23.98±0.15	23.88±0.19
Day 12	24±0.22	24.03±0.15	23.9±0.22
Day 14	23.6±0.08	23±0.27	23.4±0.22
Day 19	25.33±0.13	25.48±0.05	25.28±0.15
Day 21	23.4±0.22	23.5±0.08	23.15±0.25

Table 5.3. Average water temperature ± standard deviation for all treatment conditions and time points.

5.8.4.4. Total Ammonia-Nitrogen

The average total ammonia nitrogen, nitrite, and nitrate for the different treatments are provided in **Table 5.4**. All samples displayed ammonia levels within the recommended levels <1, except samples from the UN treatment on day 0 (1.21 mg/L). All samples from all treatments displayed nitrite levels within the recommended levels. Nitrate levels exceeded recommended levels on days 5, 7, 12 and 18 for all treatment conditions, ranging from 153.68 to 188.38 mg/L. Ammonia, nitrite, and nitrate levels did not appear to have an impact on *Listeria innocua* 2066, *E. coli* TVS 354 and APC counts (r <1).

Total Ammonia-Nitrogen									
Time	Ammonia (mg/L)			Nitrite (mg/L)			Nitrate (mg/L)		
	UN	LD	HD	UN	LD	HD	UN	LD	HD
Day 0	1.21±0.87	0.93±0.83	0.78±0.39	0.01±0	0.02±0.01	0.01±0	120.63±48.43	108.65±40.53	145±112.74
Day 5	0.75±0.63	0.55±0.42	0.49±0.09	0.01±0.01	0.07±0.12	0.04±0.03	173.95±33.71	160.05±22.63	153.68±29.45
Day 7	0.58±0.53	0.59±0.28	0.49±0.13	0.01±0	0.02±0.01	0.03±0.01	188.38±45.36	181.08±34.4	166.55±24.23
Day 12	0.46±0.38	0.35±0.16	0.23±0.16	0.01±0	0.05±0.07	0.09±0.09	187.83±42.21	165.53±22.98	163.28±28.56
Day 14	0.29±0.17	0.44±0.19	0.49±0.23	0.02±0.01	0.09±0.1	0.02±0.01	122.53±12.53	109.1±15.12	96.38±21.02
Day 19	0.33±0.07	0.3±0.04	0.31±0.06	0.04±0.03	0.03±0.04	0.02±0.01	175.15±30.82	167.73±26.44	163.6±21.51
Day 21	0.25±0.06	0.21±0.02	0.23±0.04	0.01±0.01	0.01±0.01	0.02±0.01	128.33±31.42	124.28±22.57	115.95±8.87

Table 5.4. Average total ammonia nitrogen, nitrite, and nitrate levels ± standard deviation for all treatment conditions and time points.

5.8.4.5. Turbidity

Turbidity (TDS) in water could be a sign of nutrient imbalance, algal growth, or sediment buildup in aquaponics systems. The optimal TDS level for water in aquaponic systems is below 1000mg/L. The average TDS for all treatments ranged from 753 to 865 mg/L (**Table 5.5**). All samples of the three treatments were within the recommended level. TDS did not appear to have an impact on *Listeria innocua* 2066, *E. coli* TVS 354 and APC counts (r <1).

Turbidity, Mean (mg/L)			
Time	UN	LD	HD
Day 0	798.75±92.22	753±68.47	767.25±126.97
Day 5	842.5±125.74	778±54.64	779.5±71.99
Day 7	844±191.14	828±50.35	832.75±93.31
Day 12	804.75±169.92	802.75±66.64	787±77.44
Day 14	865.25±126.54	813.25±62.48	799.25±82.29
Day 19	851±143.28	851.5±53.83	832±93.48
Day 21	828.25±170.2	779±47.69	773.75±82.28

Table 5.5. Average Total Turbidity ± standard deviation for all treatment conditions and time points.

5.8.5. Lettuce Growing Phases

When lettuce was harvested (25 days post inoculation for *E. coli* TVS 354, 20 days for *Listeria innocua* 2066), the average weight of front and back leaves was 65.2 g and 61.5, respectively. For roots, the average weight of front and back roots was 3.9 g and 3.8, respectively. For rockwool, the average weight of front and back rockwool was 35.3 g and 33.2, respectively.

Figure 5.21. shows the average width and height of the lettuce growing over one aquaponic production cycle.

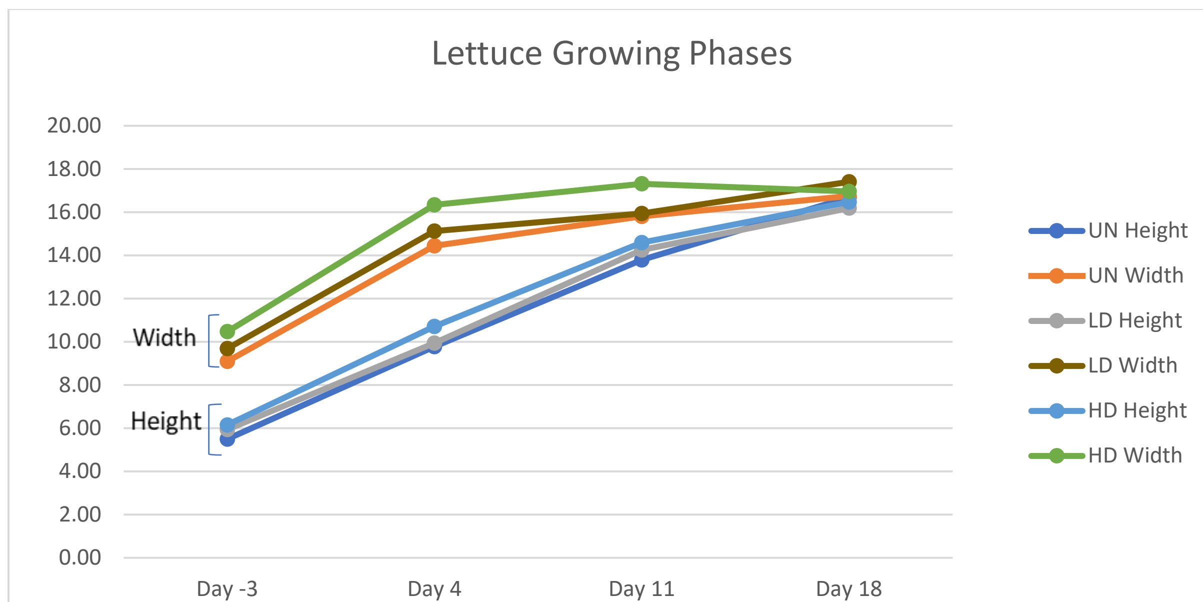

Figure 5.21. Average Lettuce growing phases for all treatment conditions and time points.

5.9. Discussion

This study provides an assessment on the behavior and dynamics of pathogens, as modeled by surrogate bacterial strains, in conditions that simulate the production of lettuce pre- and post-harvest processing. *E. coli* TVS 354 and *Listeria innocua* 2066 were used as pathogen surrogates in this study.

On day -1 (before inoculation) no *E. coli* TVS 354 or *Listeria innocua* 2066 was detected on plant tanks, filter sponges, biofilm, and algae swabs, confirming sterility of the AP systems at baseline and accuracy of bacterial surrogate inoculations. No *E. coli* TVS 354 or *Listeria innocua* 2066 were detected in samples collected from the control (UN) systems. The GAPS and polycarbonate sheets placed between each AP system sufficed to prevent cross-contamination. In general, we observed a rapid decline of *E. coli* TVS 354 *and Listeria innocua* 2066 in plant tank water and filter sponges over the experimental time course. These results are consistent with previous findings by Moyne et al., (2011), who evaluated the survival of low-dose *E. coli* (5 log

CFU/mL) on lettuce plants. After 2 days post-inoculation, they did not recover *E. coli* from the AP systems, but bacteria were detected by selective enrichment up to 14 days post-inoculation. Contrary to this finding, *E. coli* was previously detected for up to 77 and 177 days in lettuce and parsley, respectively (Islam et al., 2004).

In this study, time had a significant impact on survival of *E. coli* TVS 354 and *Listeria innocua* 2066 in the bench-scale aquaponics systems. We observed a modest negative correlation between sampling time and *E. coli* TVS 354 ($r = -0.48$, $p < 0.0001$) and *Listeria innocua* 2066 ($r = -0.45$, $p = 0.0002$) counts. Targeted bacteria populations decreased as holding time increased. The reduction in *Listeria innocua* 2066 and *E. coli* TVS 354 was statistically significant at 24 and 72 hours ($p < 0.05$) for both LD and HD conditions, displaying a total reduction of 1-4 logs. *E. coli* TVS 354 and *Listeria* were detected up to 5 and 7 days in plant tank water, respectively. Our study suggests that *E. coli* TVS 354 and *Listeria innocua* 2066 can survive longer on filter (sponge-like matrix) than in plant tank water and can persist on a sponge-like matrix for 14 and 19 days, respectively. The persistence of bacteria on filter sponge confirms that water acts as a carrier, rather than a source, for most microorganisms in the system and suggests that the sponge-like matrix is a microenvironment favoring bacterial multiplication (Riser et al., 1984). During harvest day (25 days post inoculation for *E. coli* TVS 354, 20 days for *Listeria innocua* 2066), no *E. coli* TVS 354 *or Listeria innocua* 2066 *was* detected on back or front leaves. *E. coli* TVS 354 and *Listeria innocua* 2066 were both detected on roots and rockwool, with higher counts on the backs compared to the fronts. Because the back plants are situated by the out spout of the filter, they may be more prone to coming into physical contact with water coming out of the out spout.

General coliform counts in different compartments of the AP system including the plant tank, filter sponge, leaves, rockwool and roots were evaluated for each treatment condition (UN, LD, and HD). The filter sponge displayed the highest APC counts for all treatments, ranging from 5.98 to 8.50 log CFU/mL while the plant tank water displayed the lowest APC for all treatments, ranging from 2.93 to 3.02 log CFU/mL. Low APC in the plant tank water samples relative to the other compartments in the AP system suggests that the water acts as a carrier, rather than a source, for bacteria. Lettuce lower leaf samples preharvest had a significantly higher APC (5.1-6.4 log CFU/mL) relative to harvested lettuce upper leaves (2.8 - 4.2 log CFU/mL) ($p < 0.05$), indicating a likelihood of transfer from rockwool and water since the lower leaves can come into contact with them.

pH level has been identified as an important factor influencing bacterial survival. Our results showed that pH levels had a significant impact on *E. coli* TVS 354 and *Listeria innocua* 2066 survival. A negative correlation was observed between the survival of *Listeria inccoua* and pH ($r = -0.524$, $p < 0.0001$). Samples from plant tank water on days 5 and 7 for all treatments displayed pH values from 5.17 to 5.83 mg/L, which could explain the decline of the *E. coli* and *Listeria innocua* 2066 population that persists in pH ranges of 6–8.5 (Somerville et al., 2014). Dissolved oxygen levels have been identified as a critical factor influencing aerobic bacteria survival, such as *E. coli*. In this study, the impact of dissolved oxygen on *E. coli* TVS 354 and *Listeria innocua* 2066 survival was significant ($p<.0001$). A weak positive correlation was observed between *E. coli* TVS 354 counts and DO ($r= 0.31$, $P< 0.01$). All water samples from plant tanks were within the recommended levels of DO except samples on day 5 from the LD and HD treatments (9.01 mg/L and 8.6 mg/L), on day 7 from UN and LD treatments (8.69 mg/L and 9.05 mg/L) and on day 12 from HD treatment (8.62 mg/L). *Listeria innocua* 2066 is an

anaerobic bacterium that can survive without oxygen (Chalmers, 2004), which was supported in our study by a weak correlation between *Listeria innocua* 2066 and dissolved oxygen ($r = 0.283$, $p = 0.0263$). Ammonia, nitrite, nitrate, and turbidity did not have a significant impact on *Listeria innocua* 2066, *E. coli* TVS 354 and APC counts.

Other major components of water quality that were not assessed in this study include algae and parasites. Algae and parasites were both detected by microscope during the experiment and might contribute to the observed decline in bacterial populations. Aquaponics is an ecosystem comprised mainly of fish, nitrifying bacteria, and plants. However, over time, other organisms may contribute to this, including parasites, other bacteria and small organisms. Some of these organisms are helpful, such as earthworms, and facilitate the decomposition of fish waste. Other organisms are benign, such as various crustaceans that live in biofilters, and neither help nor harm the system. Parasites, pests, and other bacteria may be detrimental, but almost impossible to avoid in a non-sterile aquaponics system environment. The best management practice to prevent these threats from becoming dangerous infestations is to grow healthy, stress-free fish and plants by ensuring highly aerobic conditions with access to all essential nutrients. This practice promotes optimal health and prevents infection among organisms grown in aquaponic systems (Somerville et al., 2014).

5.10. Microbial Contamination Pathways

There are various ways of microbial contamination in aquaponic system. Figure 5.22 shows different pathways of microbial contamination that farmers must take into consideration to prevent food contamination. These are animal manure, the use of contaminated water, use of contaminated fish food, personal hygiene. First, albeit animal manure is a great source of nutrients for crops and an efficient method of recycling waste, raw compost may also be a possible cause of human

pathogens and one of the main sources of microbial water pollution. Animal feces are a potential source of zoonotic pathogens, namely, *E. coli* O157:H7, *Salmonella spp*. and *Listeria spp*. and several others (Angelakis, et. al., 2014). Second, aside from animal manure, water may likewise be a carrier for pathogenic strains of bacteria, such as *E. coli*, *Salmonella* spp., *Vibrio cholerae*, *Shigella* spp., and numerous other viruses. Even small amounts of contamination with some of these organisms can result in foodborne illness in humans. Therefore, it is important to note that the quality of water, when and how it is used, and the characteristics of the crop highly affect the probability of water to contaminate produce. Generally, the water quality that is in direct contact with the edible portion of the plant or the produce shall need to be of better quality compared to areas where there is insignificant contact (Chalmers, 2004).

Lastly, another potential source of microbial contamination is personal hygiene. Since human hands can have contact with fresh plants or produce, failure to sanitize hands increases the likelihood of spreading diseases and viruses. Not only hands, but also harvesting or agricultural tools can also spread pathogens to products, which is why it is similarly important to maintain cleanliness within the vicinity of aquaponics systems and the apparatuses being used (Barnhart, et. al., 2015).

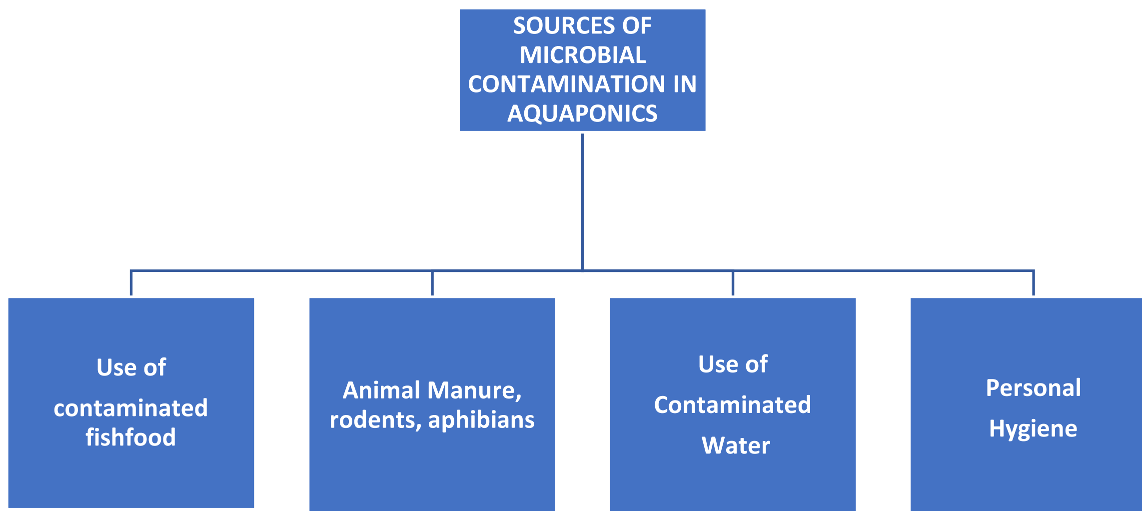

Figure 5.222. Microbial contamination pathway

5.11. How to prevent contamination

1. The reputational and financial impact of a product recall can be devastating for a company. (Bellemans, 2016). Controlling and monitoring the sources of contamination (animal manure, water quality and human sanitation) can be prevented by following good practices on aquaponics farms (Castro, L, et. al., 2009). Human sanitation – humans are the one who handles the production. It is important for personnel to sanitize all the equipment used in the process of production, hands must be washed with liquid soap and potable water then dried with a clean paper towel and is not allowed to touch anything before touching the product.
2. Harvesting produce safely – sanitizing all the harvesting tools and the area used must be away from a toilet and must maintained dry. The harvesting personnel must wash hands

and cover it with clean disposal gloves and touch only the produce when harvesting excluding its root system or growing cup because it might contain other pathogens.

3. Managing warm-blooded animals – animals are prohibited in the production area. Production area must be netted to prevent animal-to-plant contact. The production tank must be off the ground to limit access to animals or pests.
4. Water sources for fish and produce – potable water must be sampled and tested from pathogens before using it in the aquaponics system.
5. Zoonoses prevention – personnel must wear appropriate personal protective equipment, be healthy and wound-free, and must sanitize hands before handling the products. It is important to report and separate the sick fish immediately to prevent the spread of diseases.
6. Disposing of systems wastewater - the wastewater can be used to irrigate and fertilize soiled crops and plants. Disposing the wastewater back into the main water sources (stream, river, etc.) is prohibited because it might contain small fishes or other aquatic life forms (Castro, L, et. al., 2009).

The USDA provides audits for aquaponic operations that meet good agricultural practice (GAP) standards to verify that fruits and vegetables are produced, packed, handled, and stored to minimize microbial food safety risks (USDA 2022). The requirements for aquaponic operations include: (1) plants must be housed in a separate tank from fish; (2) water moving between fish and plant tanks must be filtered; (3) water moving between the plant and fish tank, that contacts the edible portion of the plant, must be sanitized; (4) water must be tested monthly, after sanitation; (5) water must have no detectable generic E. coli (in 100mL of water), and; (6) cross-contamination between fish and plant tanks must be minimized. Cross-contamination can be

prevented by segregating and sanitizing tools used for each tank, having different employees working with fish and crops, handwashing/sanitization between fish and crop activities, and requiring personal protective equipment (USDA 2022). Results from this project can directly inform pre-harvest and post-harvest produce safety risk management strategies and will be useful to the USDA-Agricultural Marketing Service GAPS certification program for policy making.

5.12. Conclusion

In this study, we report on the growth and dynamics of *E. coli* TVS 354 and *Listeria innocua* 2066 within the lettuce plant (*Lactuca sativa* var. Truchas) during production and harvest over one aquaponic production cycle. We further evaluate the relationship between key physiochemical parameters and bacterial survival and persistence. The results of this study demonstrate that both low- and high-dose *E. coli* TVS 354 and *Listeria innocua* 2066 can survive during production and after in the bench-scale aquaponics system. Field-based trials, under real-world conditions, will be needed to corroborate and strengthen these findings. Follow-up experiments/surveys will be necessary to evaluate the public health concern related to these findings. Future studies should focus on evaluating the efficacy of root disinfection in the packaged product, elimination of root and grow-cube from retail products, and of cleaning/sanitization procedures for tanks, systems and contact surfaces. This research contributes to understanding potential food safety risks and factors associated with bacterial growth and persistence, including physicochemical factors, and other environmental conditions in aquaponics systems. Results from this project can directly inform pre-harvest and post-harvest produce safety risk management strategies, and useful to the USDA-Agricultural Marketing Service GAPS certification program.

Appendix A (Experimental Design)

Table A.1. Project Experimental design showing each block with treatments and sampling.

Blocks*	**Treat-ments**	**Water samples**	**Plant tank**	**Filter**	**Plant samples**			**Fish samples**
		Temp, pH, DO, TDS, NH3 NO2, NO3, Tr	Microbial assay	Microbial assay	Leaves	Roots	rockwool	Feces
1	Control	2x a week	Days -1, 0, 1, 3, 5, 7, 12, 14, 19, 21	Days 1, 6, 12, 19	Harvest day	Harvest day	Harvest day	IN
	E. coli							
	Listeria							
2	Control	2x a week	Days -1, 0, 1, 3, 5, 7, 12, 14, 19, 21	Days 1, 6, 12, 19	Harvest day	Harvest day	Harvest day	IN
	E. coli							
	Listeria							
3	Control	2x a week	Days -1, 0, 1, 3, 5, 7, 12, 14, 19, 21	Days 1, 6, 12, 19	Harvest day	Harvest day	Harvest day	IN
	E. coli							
	Listeria							
4	Control	2x a week	Days -1, 0, 1, 3, 5, 7, 12, 14, 19, 21	Days 1, 6, 12, 19	Harvest day	Harvest day	Harvest day	IN
	E. coli							
	Listeria							

*The systems were positioned randomly within each block

A.2. Breakdown of Animal Use

Experiment (Year)	1	2	3
Control Group (Fish)	40		
E. coli: Treatment 1 **(Fish)**	40		
***Listeria*: Treatment 2 (Fish)**	40		
Pre-experiential Euthanized Fish	0 (Zero)		
Estimated Fish Replaced*	4	0	0
Total Fish	124	0	0
Pain Category			

*Unforeseen circumstances could lead to fish fatality with no method of prevention

A.3. Planned Animal Use

The planned use of animal numbers over the 2yr approval period per species Note: This information is requested to provide an estimate for resource planning purposes only. It is understood that actual use per year may vary.

Include as appropriate:

Year 1	
Species 1	124 goldfish will be used in 12 Aquaponics systems all year-round. Fish will be acclimated for 14 days before the 6-week experiment will start. Therefore, fish will be maintained for up to 10 weeks.
Species 2	
Species 3	

Appendix B (study 1)

B.1. Sample collection scheme

A strict procedure was used in order to obtain samples. Samples were collected in a sterile manner using gloves. Each sample was labeled with a unique name and number. A total of 130 samples from ten systems, each with two lettuce plant (Table B.1) were collected. Twenty root samples were collected from the net cup, 20 lettuce cores, 20 hydroton samples, and 40 water samples were collected from the fish tank and the plant tank. The biofilter sponges collected from each system. Also, we collected 10 fish feces samples from 4 fish per unit.

Table B.1. Location, number, and method used to assay each type of sample.

Type of Sample	**Analytical Method**	**# of Units per tank**	**Unit Type**	# of Samples unit	**# of Tanks**	**# of Samples**	**Samples code**
Roots	Roots sample in 30 mL PBS, stomach 260 rmp 1min, MPN block, incubate 24hr at 37ºC, 3µL streak on MacR plating, incubate 24hrs at 37 ºC, examine	1	lettuce	2	10	20	4 samples from control, A2, A3. 8 samples from Inoculated 1,000 cfu/mL, B1, B2, B3, B4. 8 samples from Inoculated 1,0000 cfu/mL, C1, C2, C3, C4
Lettuce Core	Core sample in 20 mL PBS, stomach 260 rmp 1min, APC Petrifilm, incubate 48hr at 37ºC, examine. Then enrich with 25 mL TSBR, incubate 24hrs at 37 ºC, 3µL streak	2	core	1	10	20	4 samples from control, A2, A3. 8 samples from Inoculated 1,000 cfu/mL, B1, B2, B3, B4. 8 samples from Inoculated 1,0000 cfu/mL, C1, C2, C3, C4

	on MacR plating, incubate 24hrs at 37 ^{0}C, examine						
Hydroton	Hydroton sample in 25 mL in PBS, stomach 260 rmp 1min. Then enrich with 25 mL TSBR, incubate 24hrs at 37 ^{o}C, 3μL streak on MacR plating, incubate 24hrs at 37 ^{o}C, examine	2	lettuce	1	10	20	4 samples from control, A2, A3. 8 samples from Inoculated 1,000 cfu/mL, B1, B2, B3, B4. 8 samples from Inoculated 1,0000 cfu/mL, C1, C2, C3, C4
Plant Tank Water	water sample from plant tank in 30 mL PBS, stomach 260 rmp 1min, APC Petrifilm, incubate 48hr at 37°C, examine.	1	tank	2	10	20	4 samples from control, A2, A3. 8 samples from Inoculated 1,000 cfu/mL, B1, B2, B3, B4. 8 samples from Inoculated 1,0000 cfu/mL, C1, C2, C3, C4
FishTank Water	water sample from fish tank in 30 mL PBS, stomach 260 rmp 1min, APC Petrifilm, incubate 48hrs at 37°C, examine	1	tank	2	10	20	4 samples from control, A2, A3. 8 samples from Inoculated 1,000 cfu/mL, B1, B2, B3, B4. 8 samples from Inoculated 1,0000 cfu/mL, C1, C2, C3, C4
BioFilter	sponge sample in 50 mL PBS, stomach 260 rmp 1min. MACR: 250	1	sponge	1	10	10	2 samples from control, A2, A3. 4 samples from Inoculated 1,000

	3μL (0.1 mL) per plate; 2 plates/sample, incubate 37ºC for 24hrs., examine						cfu/mL, B1, B2, B3, B4. 4 samples from Inoculated 1,0000 cfu/mL, C1, C2, C3, C4
Fish feces	Fish feces sample from 4 fish per tank in (30 mL PBS), MPN block, incubate 24hr at 37ºC, 3μL streak on MacR plating, incubate 24hrs at 37 ºC, examine. also, enrich sample in 25 mL TSBR, incubate 24hrs at 37 ºC, 3μL streak on MacR plating, incubate 24hrs at 37 ºC, examine	1	Fish from 4 fish	2	10	20	4 samples from control, A2, A3. 8 samples from Inoculated 1,000 cfu/mL, B1, B2, B3, B4. 8 samples from Inoculated 1,0000 cfu/mL, C1, C2, C3, C4

Appendix C (Study 2)

Table C.1. Average ± standard deviation of *Listeria innocua* 2066 in aquaponic effluent from the fish tank, fish tank replenished, plant tank, and plant tank replenished.

Sampling day	***Listeria innocua* 2066 count, Mean log CFU/mL**			
	Fish tank	Fish tank- R	Plant tank	Plant tank- R
Day 0	5.57 ± 0.06	6.5 ± 0.10	5.93 ± 0.42	6.1± 0.44
Day 1	1.8 ± 0.79	3.43 ± 0.06	2.33 ± 1.10	3.23 ± 0.57
Day 2	1.67 ± 1.04	1.7 ± 0.66	1.43 ± 0.29	2.5 ± 1.21
Day 3	2.23 ± 0.49	2.0 ± 0.66	1.67 ± 0.57	1.23 ± 0.40
Day 7	1.03 ± 1.26	1.03 ± 0.64	1.13 ± 0.93	1.03 ± 0.29

Table C.2. Average ± standard deviation of aerobic plate count (APC) in aquaponic effluent from the fish tank, fish tank replenished, plant tank, and plant tank replenished.

Sampling day	**APC, Mean log CFU/mL**			
	Fish tank	Fish tank- R	Plant tank	Plant tank- R
Day 0	4.32 ± 0.7	4.27 ± 0.04	4.16 ± 0.16	4.30 ± 0.04
Day 1	4.94 ± 0.18	4.90 ± 0.23	5.17 ± 0.09	4.89 ± 0.14
Day 2	4.90 ± 0.28	5.03 ± 0.19	5.09 ± 0.40	4.99 ± 0.26
Day 3	5.12 ± 0.17	4.80 ± 0.08	4.92 ± 0.17	5.15 ± 0.12
Day 7	4.92 ± 0.29	4.49 ± 0.44	4.61 ± 0.22	4.70 ± 0.30

Appendix D (Study3)

Table D.1. Average ± standard deviation of *Listeria innocua* 2066 and Aerobic bacteria count in plant tank for all treatment conditions and time points.

Sampling Day	*Listeria* count, Mean log CFU/mL Plant Tank			APC, Mean log CFU/mL Plant Tank		
	UN	LD	HD	UN	LD	HD
Day -1	0	0	0	3.02 ± 0.25	3.01 ± 0.16	2.93 ± 0.35
Day 0	0	2.93 ± 0.45	3.19 ± 0.49	3.01 ± 0.14	3.03 ± 0.09	3.04 ± 0.07
Day 1	0	0.49 ± 0.62	1.51 ± 0.79	2.97 ± 0.15	2.82 ± 0.02	2.80 ± 0.17
Day 3	0	0	0	3.27 ± 0.09	3.22 ± 0.13	3.15 ± 0.08
Day 7	0	0	0.02 ± 0.04	3.06 ± 0.09	3.07 ± 0.19	3 ± 0.06
Day 14	0	0	0	2.91 ± 0.07	3.13 ± 0.42	2.8 ± 0.11

Table D.2. Average ± standard deviation of *Listeria innocua* 2066 and Aerobic bacteria count in filter sponge for all treatment conditions and time points.

Sampling Day	*Listeria* count, Mean log CFU/mL (Filter sponge)			APC, Mean log CFU/mL (Filter sponge)		
	UN	LD	HD	UN	LD	HD
Day 1	0	2.44 ±1.27	2.08 ± 0.55	8.24 ± 0.1	8.35 ± 0.11	8.53 ± 0.09
Day 7	0	0	0.19 ± 0.32	5.98 ± 0.07	5.90 ± 0.03	5.88 ± 0.21
Day 14	0	0.15 ± 0.17	0.17 ± 0.33	6.05 ± 0.21	6.05 ± 0.16	5.85 ± 0.38

Table D.3. Average ± standard deviation of *E. coli* TVS 354 and Aerobic bacteria count in plant tank for all treatment conditions and time points.

Sampling Day	*E. coli* counts, Mean log CFU/mL (Plant Tank)			APC, Mean log CFU/mL (Plant Tank)		
	UN	LD	HD	UN	LD	HD
Day -1	0	0	0	3.02 ± 0.25	3.01 ± 0.16	2.93 ± 0.35
Day 0	0	6.18 ± 0.96	6.11 ± 0.45	3.14 ± 0.12	4.54 ± 0.12	5.66 ± 0.33
Day 1	0	3.16 ± 0.40	4.72 ± 0.77	3.03 ± 0.22	3.02 ± 0.14	3.43 ± 0.29
Day 3	0	0.07 ± 0.09	0.62 ± 0.72	3.74 ± 0.06	3.07 ± 0.02	3.18 ± 0.28
Day 12	0	0	0	3.06 ± 0.09	3.07 ± 0.19	3 ± 0.06
Day 19	0	0	0	2.91 ± 0.07	3.13 ± 0.42	2.8 ± 0.11

Table D.4. Average ± standard deviation of *E. coli* TVS 354 and Aerobic bacteria count in filter sponge for all treatment conditions and time points.

Sampling Day	*E. coli* TVS 354 counts, Mean log CFU/mL (Filter Sponge)			APC, Mean log CFU/mL (Filter Sponge)		
	UN	LD	HD	UN	LD	HD
Day 1	0	4.79 ± 0.80	5.65 ± 0.32	6.1 ± 0.41	5.95 ± 0.24	6.03 ± 0.17
Day 6	0	0.95 ± 0.59	2.56 ± 2.03	8.23 ± 0.10	8.38 ± 0.10	8.5 ± 0.08
Day 12	0	0.46 ± 1.13	0.18 ± 0.42	5.98 ± 0.10	5.90 ± 0	5.88 ± 0.21
Day 19	0	0	0.05 ± 0.16	6.05 ± 0.21	6.05 ± 0.13	5.85 ± 0.37

D.5. Day procedure for study 3

Aseptic technique and proper personal protective equipment, including sterile gloves, were used throughout all procedures.

Day -1 (pre-inoculation). Samples from the plant tank were plated on APC petri film, as well as drop plated on TSA-R and TSA-E plates to evaluate baseline *Listeria innocua* 2066 or *E. coli* TVS 354 levels in the tank water. The sponge filter was then removed and stomached with 50 mL of distilled water. That water sample was then plated on an APC petrifilm and drop-plated on TSA-R and TSA-E plates. A swab was taken of the biofilm on the bottom of the plant tanks then spread plated on TSA-R and TSA-E plates. Two hundred milliliters of plant tank water were collected for IDEXX analysis. Finally, the height and width of each lettuce plant was measured and recorded. All agar plates and petrifilms were inoculated for 24 hours at 37 °C.

Day 0. Physiochemical analysis was conducted on both plant and fish tanks. Agar plates and petrifilm from day -1 were counted and recorded, before the designated tanks were inoculated with *E. Coli* TVS 354 (low-dose in tanks L1, L2, L3, and L4; high-dose in tanks H1, H2, H3, and H4). After one hour, samples were taken from the plant tank and plated on APC petrifilm and

incubated at 37 °C for 24 hours. Samples from the plant tank were also put into a MPN block, enriched in TSB-R and TSB-E or LEB-E media and incubated at 37 °C for 24 hours.

Day 1. The APC plates from day 0 were counted. Samples from the plant tank were then plated on APC petrifilm and put into an MPN block, then enriched in TSB-R and LEB-E media before being placed in the incubator for 24 hours at 37ºC. Samples from the sponge filter were then plated on APC petrifilm. Samples from the sponge filter were put into an MPN block, enriched in TSB-R and TSA-E media then placed in the incubator at 37 °C for 24 hours. Plant tank samples from the incubated MPN blocks were plated on TSA-E and TSA-R plates and incubated at 37 °C for 24 hours. Two hundred milliliters of plant tank water were collected for IDEXX analysis.

Day 2. Plant tank APC petrifilm from Day 1 were counted as well as the Sponge Filter APC films and then incubated on TSA-E and TSA-R plates. Sponge filter samples from the day 1 incubated MPN blocks were plated on TSA-E and TSA-R plates and incubated at 37 °C for 24 hours.

Day 3. TSA-E and TSA-R plates from Day 1 were counted. Samples from the plant tank were plated on APC petrifilm. Samples from the plant tank were put into an MPN block and enriched in TSB-R and TSA-E. Two hundred milliliters of plant tank water were collected for IDEXX analysis.

Day 4. The height and width of the lettuce plants were taken. On day 3, plant tank APC films were counted. On day 3, plant tank samples from the incubated MPN blocks were plated on TSA-E and TSA-R plates and incubated at 37 °C for 24 hours.

Day 5. The system is inoculated with *Listeria innocua* 2066. Plant Tank samples from Day 3, the incubated TSA-E and TSA-R plates are counted. Samples from the Plant tank were then plated on APC petri film and put into an MPN block, then given TSB-R and TSA-E enrichments, and both were then put in an incubator for 24 hours at 37 °C. Two hundred milliliters of plant tank water were collected for IDEXX analysis. Physicochemical analysis was conducted on both the plant and fish tanks.

Day 6. Plant tank APC films from day 5 were counted. Plant tank samples from the day 5 incubated MPN blocks were plated on TSA-E and TSA-R plates and incubated at 37ºC for 24 hours. Samples from the Plant tank were then plated on APC petrifilm as well as put into an MPN block with TSB-R and TSA-E enrichments. These were incubated for 24 hours at 37 ºC. Samples from the sponge filter were then plated on APC petrifilm, and put into an MPN block, then given TSB-R and TSA-E enrichments. These were incubated for 24 hours at 37 °C.

Day 7. Plant tank and sponge filter APC films from day 6 were counted. Plant Tank samples from day 5 incubated TSA-E and TSA-R plates were counted. Plant tank samples from the day 6 incubated MPN blocks were plated on TSA-E and TSA-R plates and incubated at 37 °C for 24 hours. Sponge filter samples from the day 6 incubated MPN blocks were plated on TSA-E and

TSA-R plates and incubated at 37 °C for 24 hours. Physicochemical analysis was conducted on both the plant and fish tanks.

Day 8. Plant tank and sponge filter samples from day 6 incubated TSA-E and TSA-R plates were counted. Samples from the plant tanks were put into an MPN block and enriched in TSA-E.

Day 9. Plant tank samples from the day 8 incubated MPN blocks were plated on TSA-R plates and incubated at 37 °C for 24 hours.

Day 10. Plant tank samples from day 8 incubated TSA-E and TSA-R plates were counted, and samples from the plant tank were then plated on APC petri film.

Day 11. The height and width of the lettuce plants were taken. Plant tank APC films from day 10 were counted.

Day 12. Samples from the plant tanks were plated on APC petrifilm as well as put into an MPN block, then given TSB-R and LEB-E enrichments. Samples from the sponge filter were then plated on APC petrifilm, and samples from the sponge filter were put into an MPN block and given TSB-R and TSA-E enrichments. The films and MPN blocks were incubated for 24 hours at 37°C. Two hundred milliliters of plant tank water were collected for IDEXX analysis. Physicochemical analysis was conducted on both the plant and fish tank samples.

Day 13. Plant tank and sponge filter APC films from day 12 were counted. Plant tank samples from the day 12 incubated MPN blocks were plated on TSA-E and TSA-R plates and incubated at 37 °C for 24 hours. Sponge Filter samples from the day 12 incubated MPN blocks were plated on TSA-E and TSA-R plates and incubated at 37 °C for 24 hours.

Day 14. Plant tank and sponge filter samples from day 12 incubated TSA-E and TSA-R plates were counted. Physicochemical analysis was conducted on both the plant and fish tank.

Day 18. The height and width of the lettuce plants were measured.

Day 19. Samples from the plant tank were plated on APC petrifilm. Samples from the plant tank were put into an MPN block, then given TSB-R and TSA-E enrichments. Samples from the sponge filter were then plated on APC petrifilm. Samples from the sponge filter were put into an MPN block, then given TSB-R and TSA-E enrichments. Two hundred milliliters of plant tank water were collected for IDEXX analysis. Physicochemical analysis was conducted on both the plant and fish tanks.

Day 20. Plant tank and sponge filter APC films from day 19 were counted. Plant tank samples from the day 19 incubated MPN blocks were plated on TSA-E and TSA-R plates and incubated at 37 °C for 24 hours. Sponge filter samples from the day 19 incubated MPN blocks were plated on TSA-E and TSA-R plates and incubated at 37 °C for 24 hours.

Day 21. Plant tank and filter sponge samples from day 19 incubated TSA-E and TSA-R plates were counted. The lower leaves (4 lowest) of the lettuce plants are harvested and samples were stomached, APC plated, and also put into an MPN block with TSB-R and TSA-E enrichments. Physicochemical analysis was conducted on both the plant and fish tanks.

Day 22. Lower leaves APC films from day 21 were counted. Lower leaves samples from the day 21 incubated MPN blocks were plated on TSA-E and TSA-R plates and incubated at 37 °C for 24 hours.

Day 25 (Harvest Day). Each plant was carefully removed from the rafts and placed onto a sterilized metal tray. Using sterilized tools, the leaves were removed and placed into a stomacher bag with 100 mL of BPW, the roots were removed and placed into a stomacher bag with 30 mL of BPW and the rockwool were removed and placed into a stomacher bag with 50 mL of BPW. After being stomached, the leaves, roots, and rockwool samples were plated on APC petrifilm. The leaves, roots, and rockwool samples were put into an MPN block, then given TSB-R and TSA-E enrichment.

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