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MICROBIOLOGICAL AND QUALITY CHARACTERISTICS OF ALFALFA (*Medicago sativa*) AND MUNG BEAN (*Vigna radiata*) SPROUTS GROWN USING DIFFERENT WATER SOURCES AND TREATED POST-HARVEST

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Food Technology

---

by
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May 2016

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ABSTRACT

The Centers for Disease Control and Prevention has reported that approximately 48 million people become sick from the consumption of food each year in the United States. Additionally, 46% of foodborne illnesses reported between 1998 and 2008 were contributed to the consumption of fresh produce. More specifically between 1996 and 2016, 41 foodborne illnesses have been reported from the consumption of sprouts. Sprouts are most often consumed raw, thus cooking is not used to prevent contamination from pathogens. The FDA Food Safety Modernization Act of 2011 has introduced new regulations for produce and sprout producers under produce safety standards. For sprout producers, these standards include: regulation of soil additives if used, health and hygiene of workers, packaging, temperature monitoring, animal control around produce and irrigation waters, decontamination of seeds before sprouting, testing of spent irrigation water for the presence of *Salmonella* and *Escherichia coli (E. coli),* environmental testing for *Listeria monocytogenes* and prevention of releasing sprouts that test positive for pathogens. Considering these regulations, foodborne illnesses continue to occur.

The purpose of this study was to evaluate the microbiological and nutritional quality characteristics of alfalfa and mung bean sprouts grown in different water sources and treated post-harvest. Alfalfa and mung bean sprouts were selected because they are the types of sprouts most frequently consumed. Microbiological characteristics (total aerobic microorganisms, *Enterobacteriaceae*, total coliforms

and yeasts and molds) were studied on alfalfa and mung bean sprouts that were grown in either municipal tap water or aquaponics water, harvested on day 7 and treated post-harvest with tap water, chlorine or organic acid. An additional partitioning study was conducted on alfalfa sprouts inoculated on day 1 or day 4 of growth with *E. coli* to determine the presence of unrecovered *E. coli* after sterilization and internalization of *E. coli* into the sprouts. Finally, due to the lack of previous research on the quality characteristics of sprouts, particularly those grown in nutrient-rich aquaponics water, alfalfa and mung bean sprouts were analyzed to determine if the water sources influenced their nutritional quality characteristics at the time of harvest. Under conditions of this study, the data showed that the microbial load of irrigation water could influence the microbial characteristics of harvested sprouts by increasing numbers of total aerobic microorganisms, *Enterobacteriaceae,* total coliforms and yeasts and molds on sprouts. Moreover, post-harvest washes did not prove effective in reducing numbers of microorganisms more than 1 log CFU/g (90%), which alludes to the presence of biofilms on sprouts that are not affected by antimicrobial treatments. Further results of the partitioning study revealed that sprouts have the potential to internalize pathogens, particularly if contamination occurs early in growth when the pathogens have access to the seed. The results of this study also led to the conclusion that the proximate composition of sprouts is not affected by irrigation water source, however, micronutrient composition of sprouts can be influenced by the micronutrient characteristics of irrigation water. Data demonstrates that alfalfa and mung bean sprouts grown

under controlled conditions harbor large numbers of microorganisms (>9 log CFU/g) and neither irrigation water nor post-harvest antimicrobial treatments improve the microbiological or nutritional composition of treated sprouts.

DEDICATION

I would like to dedicate this dissertation to my family. Without them this opportunity would not have been possible. I am thankful for the everlasting support from my husband Ryan. His continued love and encouragement helped me make it through the hardest of days and his ability to be "Mr. Mom" when I wasn't able to made this journey possible. To my son Ben, who brought me joy and laughter throughout this project. To my parents-in-law who faithfully helped to watch Ben when I needed time to work on my research and writing. And certainly last but not least, I dedicate this dissertation to my mom and dad. You two have had faith in me since day one and knew I had it in me to finish on the days that I didn't think it was possible. You have taught me how to work hard, persevere and follow your dreams. Thank you for all of your support from far and near and for believing in me every step of the way.

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

1.1.1 Foodborne Illness

According to the Centers for Disease Control and Prevention (CDC), approximately 48 million individuals become sick from the consumption of contaminated food each year, which equates to one out of six Americans that experiences foodborne illness annually (CDC, 2011). Of the 48 million, approximately 128,000 people will become so sick that they will be hospitalized and another 3,000 individuals will die (CDC, 2011). According to the CDC, the primary pathogenic microorganisms that attribute to 91% of foodborne illness in the United States are: Norovirus, *Salmonella, Clostridium perfringens, Campylobacter spp.,* and *Staphylococcus aureus* (CDC, 2011). Approximately 88% of all foodborne illnesses resulting in hospitalization are primarily attributed to *Salmonella,* Norovirus, *Campylobacter spp., Toxoplasma gondii,* and *Escherichia coli (E. coli) O157* (CDC, 2011). *Salmonella, Toxoplasma gondii, Listeria monocytogenes,* Norovirus, and *Campylobacter spp*. are the reported causative agent for 88% of the foodborne illness related deaths each year (CDC, 2011). These pathogens are also commonly associated with foods that are consumed raw (raw agricultural commodities), such as fresh fruits and vegetables.

In 2013, Painter et al. reported that 46% of the illnesses and 23% of the deaths associated with foodborne illness outbreaks between 1998 and 2008 were from the consumption of contaminated produce, followed by meat and poultry (22% illnesses and 29% deaths), dairy and eggs (20% illnesses and 15% deaths), and fish and shellfish (6.1% illnesses and 6.4% deaths). The produce cited as the most common implicated source of the foodborne illness outbreaks was vegetables, fruits and nuts (Painter et al., 2013). In this study, vegetables were sub-categorized as fungi, leafy greens, root vegetables, sprouts and vine-stalk vegetables (Painter et al., 2013). Since this study, the CDC has reported 21 foodborne illness outbreaks from the consumption of fresh vegetables, followed by 7 from the consumption of fresh fruits and 9 from the consumption of nuts or nut butters between 2009 and 2016 (CDC, 2016).

1.1.2 Foodborne Illness and Sprouts

Sprouts, or the premature growth of a plant from a germinated seed, have the potential to produce foodborne illness outbreaks due to contamination introduced during seed production, sprout production and sprout distribution (Oregon Public Health Division et al, 2015). Johanson (2012) reported that there were 34 foodborne illness outbreaks in the United States between 1996 and 2010 related to the consumption of sprouts. These outbreaks resulted in 2,150 causes of sprout related foodborne illness, 123 hospitalizations and one death. Data from the Food and Drug Administration (FDA), shows that there were 25 sprout related foodborne

illness outbreaks linked to the commercial growers of the sprouts between 1996-2003 (FDA, 2004). In this case, these outbreaks were linked to 19 illnesses from *Salmonella* and 6 illnesses from *E. coli* O157:H7. Data from the CDC shows that there were 11 sprout related foodborne illness outbreaks between 2009 and 2016 (CDC, 2016). The varieties of sprouts implicated in these outbreaks were alfalfa (6 cases of foodborne illness outbreaks), red clover (2 cases), mung bean (1 case), and soy bean (1 case). The pathogens associated with these outbreaks included *Salmonella, E. coli* and *Listeria* monocytogenes (CDC, 2016).

Oftentimes, seeds used to produce animal feed are used for the production of sprouts (Mueller, 2008). During seed production, sources of microbiological contamination include: irrigation water, animal manure, contamination from wild animals and unsanitary hygiene of the field workers (Oregon Public Health Division et al., 2015). Contamination of the seed may also occur when seeds are transported to the storage facility, or held in storage containers that allow rodents and pests access (Mueller, 2008). Contaminated equipment can also serve as a source of pathogen transfer to seeds and sprouts. Some seeds also go through a process called scarification where the outer layer of the seed is abrasively scrubbed enough to create a rough surface that can provide an entry for microorganisms inside of the seed. Scarification is performed to increase the germination rate of the seeds, but it can make the decontamination process of the seed more difficult (Oregon Public Health Division et al., 2015).

1.1.3 Sprout Regulations

Based on the history of foodborne illness outbreaks related to sprouts and the research that has been conducted to help reduce the likelihood of future outbreaks, the Food and Drug Administration (FDA) enacted a list of recommendations in 1999 for seed producers, seed distributors and sprout producers. This guidance covers seed production; seed conditioning, storage and transportation; sprout production; seed treatment; testing for pathogens; and traceback (FDA, 1999a). The FDA (1999a) states that seeds produced for sprouts should be grown under good agricultural practices (GAPs). Furthermore, during seed conditioning, storage and transportation, seeds should be handled so that contamination is unlikely to occur (FDA, 1999a). Sprout producers as well as seed producers, conditioners and distributors are required by federal law to implement a traceback and recall system in the event of a foodborne illness outbreak that may be related to sprouts (FDA, 1999a).

The recommendation also addressed sanitation of the facility, irrigation water, equipment and hygiene of employees as mechanisms to reduce the potential for microbial contamination (FDA, 1999a). The FDA further recommended that seeds be treated with an antimicrobial agent to reduce contamination on the seeds before sprouting begins. The most common approved practice is rinsing seeds in a 20,000 ppm solution of calcium hypochlorite (FDA, 1999a). Calcium hypochlorite has shown to be the most effective treatment in reducing pathogens (approximately 4 log reduction on alfalfa seeds) without impacting the germination rate of the seeds

(FDA, 1999c). Additionally, calcium hypochlorite is commonly used in the food industry because it is chemically stable, easy to use and inexpensive. Treating seeds with 1% hydrogen peroxide or 70% ethanol has been shown to be effective in reducing pathogen levels, however germination rates were significantly decreased (FDA, 1999c). To date, no seed treatment has been found to completely eliminate pathogens that can be present on sprout seeds. Consequently, the FDA recommends that irrigation water from growing sprouts be tested for the presence of *E. coli* and *Salmonella* as these pathogens are the ones most frequently associated with sprout-related foodborne illness outbreaks (FDA, 1999c).

In 2011, The FDA Food Safety Modernization Act (FSMA) was signed as a law to improve the food safety of the American food supply. The overall effect of this law is to provide the FDA methods to prevent issues in food safety versus playing a reactive role when a problem occurs (FDA, 2011). Additionally, the FSMA provides the FDA with new roles in enforcing regulations, the addition of risk-based food safety criteria to allow a more proactive role in regulating and inspecting food manufacturers who produce foods that have a higher rate of causing foodborne illness, the ability to ensure that imported foods meet U.S. food regulations, and collaboration with state and local food regulatory authorities to create a cohesive food safety system across the country.

The FSMA has placed several mandates within the area of preventive controls. The first called “Preventive Controls for Human Food” was finalized in 2015 and large manufacturers will be required to begin complying with this law in

2016. The preventive controls for human food mandate includes the requirement that all food manufacturers implement a preventative control plan. This plan includes the evaluation of hazards (biological, chemical and physical) that can affect the safety of the food; identifying preventative controls which can significantly reduce or prevent the identified hazards; identification of how the controls will be monitored; identifying required records and monitoring activities related to the preventive controls; and identifying the actions that will be taken when a deviation occurs (FDA, 2011). The second mandate under preventive controls is the implementation of produce safety standards. This rule was finalized in 2015 and some qualified establishments must begin meeting these requirements within 2 years. These standards focus on the safe growth and harvest of fruits and vegetables, and include regulation of soil amendments, hygiene of workers, packaging, temperature control and animal control around growing produce and irrigation waters (FDA, 2011).

The produce safety rule specifically addresses the production of sprouts. Sprout producers must comply with four additional requirements that are specific to sprout production. These requirements are (1) implement measures to prevent pathogens on seeds, in addition to the current requirement of treating seeds to reduce contamination prior to sprouting; (2) the testing of irrigation water drained from the growing sprouts from each production batch; (3) the testing of the environment (growing, harvesting, packing and holding) for *Listeria monocytogenes*; and (4) taking corrective actions if any test results in a positive reading and not

releasing any sprouts into commerce with a positive result (FDA, 2015). Additionally, sprout producers have a faster compliance time compared to producers of other fruits and vegetables. Very small producers (average annual sales during previous three years of $25,000-$250,000) have three years to comply, small producers (average annual sales during previous three years of $250,000-$500,000) have two years to comply and all other producers have two years to comply (FDA, 2015).

In 2012 the Sprout Safety Alliance (SSA) was developed to further assist in improving the safety of sprouts. This alliance is a partnership between the FDA and Illinois Institute of Technology's Institute for Food Safety and Health (SSA, 2014). The mission of the alliance is to develop training materials for the sprout industry, provide tools and methods that enhance safety, act as a resource and provide training to the industry in collaboration with AFDO, USDA, professional associations and Extension services (SSA, 2014). At the time of writing this document, the SSA's training for the industry remains in the stages of development.

1.1.4 Sprouts

Sprouts can be described as an immature plant after it has emerged from the seed. They can be further characterized by having a thin hypocotyl (stem) and small immature cotyledons (leaves) that emerge in pairs. Based on the variety of sprout, they may be harvested within 1 to 8 days of planting depending on the maturity and length desired (DeEll, 2014). While all plants become a sprout as part of the natural

maturation process, plants that are harvested to be consumed as sprouts are chosen primarily for their quality characteristics. Sprouts can add a crisp element when accompanied with other foods and their flavors can range from nutty to spicy (DeEll, 2014). The most commonly consumed varieties of sprouts include alfalfa, mung bean, red clover, radish, broccoli and wheatgrass (Oregon Public Health Division et al., 2015). Sprouts are most commonly consumed raw or lightly cooked.

1.1.5 Sprout Production and Consumption

According to the USDA Vegetable Summary for 2015, fresh market vegetable and melon production for the year was 400 million hundredweight and the area for which this produce was harvested covered 1.55 million acres (USDA, 2016c). This harvested crop is estimated to be worth approximately 11.9 billion dollars (USDA, 2016c). In the USDA's 2012 Vegetables and Pulses Yearbook, sprouts were categorized as a specialty vegetable. Mung bean sprouts were listed under Miscellaneous Asian and grouped with bok choy, dikon, gobo, lobah and other Asian vegetables (USDA, 2013). Alfalfa sprouts were listed under Miscellaneous vegetables and grouped with cardoon, celery root, chicory root, Jerusalem artichoke, jicama, salsify, radicchio and tomatillos (USDA, 2013). This publication reports that in 2012, 346,000 cwt of miscellaneous Asian vegetables where shipped in the U.S. domestically and 946,000 cwt were imported (USDA, 2013). The yearbook also reports that in 2012, there were no reports of miscellaneous vegetables being

shipped domestically in the U.S., but 996,000 cwt were imported into the U.S. (USDA, 2013).

In the United States, about 80 million pounds of alfalfa seeds are produced annually (Mueller, 2008). However the majority of these seeds are produced for the growth of alfalfa hay and a very small proportion are used for producing alfalfa sprouts (Mueller, 2008). Approximately 15-20 million pounds of mung beans are produced in the United States annually and the majority of these beans are consumed as sprouts (Oregon Public Health Division et al., 2015). Additionally, approximately 75% of mung beans that are consumed are imported from China and Japan (Oregon Public Health Division et al., 2015). The International Sprout Growers Association has reported that approximately 10% of the U.S. population regularly eats sprouts (Sikin et al., 2013). This quantity of sprouts has a market value of $250 million (Silkin et al., 2013). This association has also reported that there are 475 sprout producers in the United States and collectively, produce approximately 300,000 tons of sprouts each year (Silkin et al., 2013). The 2006-2007 FoodNet Survey (N=17,372), which surveys people in the states of California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon and Tennessee, reported that 4.4% had consumed alfalfa sprouts in the past 7 days and 5.7% reported consumption of mung bean sprouts within the past 7 days (CDC, 2007). A different study which pooled survey results from NAHANES data from 1999-2006 reported that 3.02% of the U.S. population consumed sprouts (Hoelzer et al., 2012). Of the 3.02% of the population that consumed sprouts during

this time, 0.38% of the population ate the sprouts raw and 2.66% of the population consumed the sprouts heat-treated (Hoelzer et al., 2012).

Alfalfa sprouts are the most commonly consumed sprout in the United States (Oregon Public Health Division et al., 2015; Matos et al., 2002). Mature alfalfa plants are legumes and commonly used as a feed for cattle. On average, alfalfa sprouts can germinate and grow to their harvest size in 3-7 days. They are characterized as having narrow white stems and small dark green leaves, and are harvested at approximately 3.8 cm (1.5 inches) in height. Alfalfa sprouts have a mild nutty flavor and are often added to sandwiches and salads to add a crispy texture (Oregon Public Health Division et al., 2015).

Worldwide, mung bean sprouts are the most commonly consumed variety of sprout (Oregon Public Health Division et al., 2015). Like alfalfa sprouts, mung beans are legumes and are most commonly consumed in the form of a sprout. They are characterized as having a thick white stem and slender leaves that are yellow to green in color. Mung bean sprouts are harvested in 3 to 8 days after planting and approximately 1.3 to 7.6 cm (0.5 to 3 inches) in height (DeEll, 2014). Mung bean sprouts are most often used in Asian cuisines and are lightly cooked to maintain a crisp texture in the dishes in which they are used.

1.1.6 Nutritional Composition of Sprouts

Previous literature reports that sprouts are nutritionally dense in protein, vitamins and minerals because they are consumed just after emerging from the seed

and much of the nutrition of the seed and sprout has not been utilized yet in producing a mature plant (Márton et al., 2010). Research studies have shown that their nutritional value can contribute in protecting against cancer and chronic disease (Sikin et al., 2013). However, based on the Reference Amount Commonly Consumed for sprouts (10g), sprouts are not good sources of nutrients (FDA, 2013a; FDA, 2013b). In the germination phase, many changes take place within the seed in order to produce the sprout. The seed undergoes a metabolic process in which carbohydrates, proteins and fats are metabolized to provide energy for the growing plant (Masood et al., 2014). Polysaccharides are converted into oligo- and monosaccharides, fats are converted to free fatty acids and proteins become oligopeptides and free amino acids (Márton et al., 2010). Proximate composition of sprouts varies significantly depending on the variety of sprout and environmental conditions during growth such as temperature, humidity, light and time (Masood et al., 2014).

A study by Hong et al. (2009), determined that the proximate composition of freeze dried alfalfa sprouts was 10.6% water, 49.6% protein, 2.6% fat, 33.6% carbohydrate and 3.7% ash. The USDA National Nutrient Database (2016a) reports that sprouted alfalfa seeds contains 8.2 mg/100g (82 ppm) vitamin C, 32 mg/100g (320 ppm) calcium, 0.96 mg/100g (9.6 ppm) iron, 27 mg/100g (270 ppm) magnesium, 70 mg/100g (700 ppm) phosphorus, 79 mg/100g (790 ppm) potassium, 6 mg/100g (60 ppm) sodium, and 0.92 mg/100g (9.2 ppm) zinc.

A study by Dahiya et al. (2014) determined that mung bean sprouts contained 617 ppm iron, 247 ppm zinc, and 13557 ppm calcium. The protein content of mung beans can vary (approximately 19.5-31.3%) depending on the cultivar (Masood et. al., 2014). Tang et al. (2014) found that mung bean sprouts contain approximately 20-24% protein and 50-60% carbohydrate. It is important to note in this study that as the mung bean sprouts grew, the fat content decreased and protein content increased. Protein content likely increases as the sprout develops due to the natural synthesis of new proteins during the germination process (Masood et al., 2014). The ascorbic acid content in mung bean sprouts after 5 days of growth was approximately 28.50 mg/100g (285 ppm) (Masood et al., 2014). The USDA National Nutrient Database (2016b) reports that sprouted mung bean seeds contains 13.2 mg/100g (132 ppm) vitamin C, 13 mg/100g (130 ppm) calcium, 0.91 mg/100g (9.1 ppm) iron, 21 mg/100g (210 ppm) magnesium, 54 mg/100g (540 ppm) phosphorus, 149 mg/100g (1490 ppm) potassium, 6 mg/100g (60 ppm) sodium and 0.41 mg/100g (4.1 ppm) zinc.

Very little ascorbic acid (vitamin C) is found in seeds, but this rapidly increases as the seed begins to sprout (Masood et al., 2014). A review of literature by Márton et al. (2010) found that soybean sprouts have a 200-fold increase in free amino acids and vitamin C content when compared to the composition of the seed before sprouting. Vitamin C content increases as the seed germinates due to its role as an antioxidant to protect the plant's cells and to aid in the role of developing the cell walls during cell growth and division (Márton et al., 2010). For the purpose of

this study, it is important to note that cells of plants have the ability to store large amounts of l-ascorbic acid (Vitamin C), particularly in the green tissues (Hancock et al., 2001). Plant cells are also able to generate l-ascorbic acid from D-glucose (Hancock et al., 2001). According to Guo et al. (2012), mung bean sprouts have a moisture content of approximately 93.24% on days 6-9 of germination. In the same study, the mung bean sprouts had a Vitamin C content of approximately 285 mg/100g (dry weight) on day eight (Guo et al., 2012). A 104 g serving of mung bean sprouts provides approximately 21.6 mg of vitamin C on a fresh weight basis (Guo et al., 2012).

Beyond the data listed in the USDA National Nutrient Database, there is no scientific literature that evaluates the mineral content in mung bean or alfalfa sprouts. A study by Aires et al. (2007), evaluated the mineral composition of broccoli sprouts. Aires et al. (2007) reported that broccoli sprouts had a higher mineral content (N, P, S, K, Ca, Mg, Na, Cl, Si) than mature broccoli. This study also reported that when broccoli sprouts are fertilized with N, S, K Cl, Ca, Mg, P and Na, the contents of N, S, K and Cl in the sprout will increase and Ca, Mg, P and Na will decrease (Aires et al., 2007).

1.1.7 Microbiological Characteristics of Sprouts

The sprouting process contains all of the favorable characteristics that pathogens need to grow: nutrients, pH, time, temperature, oxygen and moisture. Seeds can contain up to 10^9 CFU/g of bacteria and this level can be achieved in 2-3

days of the sprouting process (Liao, 2008). A 5 log reduction in pathogens is an industry standard for evaluating the effectiveness of antimicrobial treatments (thermal process, post-harvest washing, etc.) on food. In most cases, reducing pathogens by 5 logs is enough to significantly reduce the possibility of causing a foodborne illness if the food is consumed. However, when produce has pathogen contamination greater than 5 logs and a post-harvest treatment is used that is unable to reduce the pathogens to safe numbers, the risk for foodborne illness if the food is consumed is not reduced. For example, a study by Fett (2002) determined that unwashed alfalfa sprouts contained approximately 8 $\log_{10}$ CFU/g mesophilic aerobes, 7 $\log_{10}$ CFU/g coliforms and 3 to 4 $\log_{10}$ CFU/g yeast and molds. If a post-harvest treatment is applied to these sprouts that results in a 5 log reduction in microorganisms, enough microorganisms remain that can cause someone to become sick if consumed.

Because sprouts are known to have a high microbiological load, the sprout industry relies on washing the seeds to reduce contamination before the sprouting process begins. A study by Sikin et al. (2013) determined that depending on the variety of sprout seed, the seeds could contain approximately 10^2 to 10^6 CFU/g native microorganisms and approximately 10^2 to 10^3 CFU/g fecal coliforms. A study by Kim et al. (2012) determined that alfalfa seeds from three sprouting facilities contained approximately 10^3 to 10^4 CFU/g and 10^2 to 10^4 CFU/g total coliforms. Fett (2002), studied alfalfa sprouts and determined that the tested untreated seeds contained and average of 4.08 $\log_{10}$ CFU/g mesophilic aerobes, 3.07 $\log_{10}$ CFU/g

yeasts and molds and 1.22 log_{10} CFU/g coliforms. To remove these microorganisms, the current recommendation is to rinse the seeds in a 20,000 ppm solution of calcium hypochlorite for a contact time of 15 minutes (Fett, 2001). While this treatment is effective in reducing contamination it does not eliminate all of the pathogens (FDA, 1999b). On average, treating seeds with calcium hypochlorite will reduce contamination by 2.5 log CFU/g, but effectiveness can vary depending on the variety of seed (Sikin et al., 2013). Any pathogen that remains on the seed when sprouting begins or is introduced during sprouting can easily grow and replicate given the growing environment is optimal for pathogen growth.

Salmonella has been found to grow very quickly in the first 48 to 72 hours of sprouting (Liao, 2008). The study conducted by Liao (2008) found that the total number of *Salmonella* on alfalfa sprouts was directly correlated to the initial number of *Salmonella* present on the seeds before sprouting. Additional studies by Palmai et al. (2002) and Stewart et al. (2001) also determined that *Listeria monocytogenes* and *Escherichia coli O157:H7* reached peak growth within 48 to 72 hours of the sprouting process. Loui et al. (2008) studied alfalfa sprouts from retail outlets and found that among the samples tested they contained bacteria from the genus of *Acinetobacter, Enterobacter, Escherichia, Klebsiella, Pseudomonas, Salmonella,* and *Stenotrophomonas.* Kim et al. (2012) determined that sprouted alfalfa seeds, in which the seeds were not treated, contained approximately 7.1 to 8.7 log CFU/g aerobic microorganisms and 6.9 to 7.5 log CFU/g total coliforms. In the same study, sprouts were subsequently washed with tap water and aerobic microorganisms and

total coliforms reduced to 7.2 to 7.6 log CFU/g and 5.5 to 6.7 log CFU/g, respectively (Kim et al., 2012). The study by Fett (2002) determined that untreated alfalfa sprouts contained approximately 8 log_{10} CFU/g mesophilic aerobes, 7 log_{10} CFU/g coliforms and 3 to 4 log_{10} CFU/g yeasts and molds at the time of harvest. A study by Kim et al. (2009b) found that 45 samples of sprouts from retail markets contained more than 7 log CFU/g of both total aerobic bacteria and yeasts and molds. *Klebsiella* and *Enterobacter* are gram-negative bacteria in the family of *Enterobacteriaceae* that have been found to test positive for coliforms and fecal coliforms (Beuchant, 1998). Because of this, positive coliform tests can be a false positive for the presence of fecal coliforms. A study by Rangel-Vargas et al. (2015) tested 100 samples of alfalfa sprouts from retail supermarkets and found that all of the samples contained coliform bacteria ranging from 6.2 to 8.6 CFU/g. A study by Abadias et al. (2008) evaluated 15 samples of alfalfa and soybean sprouts from retail stores and found that they contained 7.1 to 9.2 log_{10} CFU/g aerobic microorganisms, 2.8 to 7.6 log_{10} CFU/g coliforms, and 7.2 log_{10} CFU/g *Enterobacteriaceae*. Of the 15 samples tested, 4 samples tested positive for *E. coli* (Abadias et al., 2008). Additionally, a study by Kim et al. (2012) found that coliforms increased to over 6 log CFU/g during germination even when coliform counts for the seeds were zero before the germination process began. Phua et al. (2014) studied mung bean sprouts and found that untreated sprouts contained on average 9 log CFU/g total aerobic microorganisms. A study by Tournas (2005)

found that mung bean sprouts contained approximately 1.7 x 10^7 CFU/g yeasts and molds and alfalfa sprouts contained approximately 8.1 x 10^5 CFU/g.

It is also critical to consider the internalization of pathogens into a plant. A review of literature by Deering et al. (2012) stated that the variety of plant, type of bacteria, path of contamination and age of the plant impact internalization of pathogens. Internalization of pathogens inside produce is an important factor to consider because antimicrobial treatments are unable to reach these microorganisms, and thus when they remain in the plant, can cause foodborne illness when consumed raw. A study by Warriner et al. (2003), found that when *E. coli* and *Salmonella Montevideo* were inoculated on mung bean sprouts the pathogens became internalized. In this study, the pathogens were most prevalent in the areas of the sprout's roots, but also found within the hypocotyls as well, thus showing that the pathogens are transported from the roots to the interior surfaces of the plant. Pathogens can become internalized through natural openings on the plant's surface, through the roots when lateral roots emerge causing a temporary opening in the root, and/or via contaminated water when seeds are soaked, plants are irrigated or during post-harvest washing (Warriner et al., 2003; Deering et al., 2012). A study by Ge et al. (2014), found that when mung bean sprouts were inoculated on day 1 with *Salmonella* Typhimurium via the water source, the sprouts showed internalization as early as the following day and contained more than 2 log CFU/g internal contamination at harvest on day 7. Once inside, an environment that is conducive to their ability to grow and replicate surrounds the pathogens allowing

them to increase in numbers rapidly within the boundaries of the plant (Deering et al., 2012).

Additionally, biofilms have been discovered on sprouts, particularly surrounding the roots and leaves and within the plant itself (Sikin et al., 2013; Yaron et al., 2014). Biofilms are a large population of microorganisms that inhabit a surface (in this case a plant surface) and attach themselves to each other and the surface that they cover. These microorganisms are able to affix themselves onto the surface via the production of extracellular polymeric substances (Yaron et al., 2014). To keep the biofilm intact, each biofilm contains proteins that provide structure and functionality, have the ability to retain necessary moisture and obtain nutrients (Yaron et al., 2014). The presence of biofilms acts as a protective barrier to plants from the environment including chemicals. Biofilms on food that is consumed is a food safety concern because these films often are not as affected by antimicrobial treatments as individual microorganisms (Van Houdt et al., 2010). Some research has shown that biofilms are 500 times more resistant to antimicrobial agents than individual bacteria (Fett, 2000). The efficacy of an antimicrobial agent on a biofilm is influenced by the surface environment for which the biofilm is attached, temperature, and contact time of the antimicrobial as well the bacterial resistance (Van Houdt et al., 2010). A study by Fett (2000) found biofilms on the stems, leaves and roots of alfalfa, broccoli, clover and sunflower sprouts that were purchased from grocery stores. The same study evaluated the presence of biofilms on alfalfa sprouts during growth. This study revealed that biofilms first appeared on the stem

at day 2. On day 3 biofilms were present on both the stem and leaves and by day 4 biofilms appeared on the roots (Fett, 2000). At day 4, it was estimated that the biofilms, which contained primarily mesophllic aerobes, covered approximately 29 to 59% of the alfalfa sprouts (Fett, 2000). A study by Fett et al. (2003) evaluated biofilms on mung beans sprouts from retail markets and found that these biofilms were primarily made up of cocci-shaped bacteria and yeast and located most often on the leaves.

The FDA recommends that sprout producers test spent irrigation water used to grow sprouts for *Salmonella* and *Escherichia coli* O157:H7 (FDA, 1999b). This practice was put in place because the spent irrigation water can provide a good representation of the microorganisms present in the sprouts since the water has passed over the sprouts and pooled together to provide a representative sample. However, a study by Fu et al. (2001) determined that spent irrigation water generally contains a 1 log difference in microorganisms from the sprouts themselves. Testing of the spent irrigation water is useful however, because testing of only the sprouts can potentially cause present microorganisms to be missed during the sampling process (Fu et al., 2001; FDA, 1999b).

1.1.8 Post-Harvest Washing of Sprouts

Because decontamination of sprouting seeds is not effective enough to consistently reduce pathogens to safe levels and the growing environment promotes pathogen growth, a post-harvest washing step is critical to reduce any existing

pathogens to safe levels. Currently used chemical based antimicrobial washes include chlorine, organic acids, chlorine dioxide, and ozone (Sikin et al., 2013). Chlorine is one of the most commonly used post-harvest treatments (FDA, 2014). The FDA recommends that fresh produce be washed in 50 to 200 ppm chlorine solutions for 1 to 2 minutes contact time (Sikin et al., 2013; FDA, 2014). However, chlorine washes generally result in an approximate 2 log reduction due to the decrease in available free chlorine as organic material increases in the wash water (Warriner et al., 2003; Sikin et al., 2013). In a chlorine solution, free chlorine is in the form of hypochlorous acid (HOCl) and it is the HOCl that is the active antimicrobial agent (FDA, 2014). Hypochlorous acid is dependent on pH of the solution, thus as the pH of the solution decreases the concentration of HOCl will increase (FDA, 2014). The optimal pH for antimicrobial effectiveness and reduction in equipment corrosion is 6.0-7.5 (FDA, 2014). Chlorine should also be monitored for temperature, prevalence of organic material, light and metals because as these increase, the effectiveness of the HOCl will decrease. Chlorine washes are most effective at 4°C (39°F), however, to prevent produce from absorbing the wash water and possibly further contaminating the inside of the product, it is recommended that wash water be at least 10°C higher than the temperature of the product itself (Beuchat, 1998).

Organic acids, such as lactic acid, acetic acid and citric acid, can be used as an effective antimicrobial agent and is considered by the FDA as generally regarded as safe (GRAS) (Sikin et al., 2013). They are also inexpensive and permitted to be used

as an organic antimicrobial treatment (Sikin et al., 2013). These acids, with their low pH, break down the cell membrane of the microorganisms thus inactivating them (FDA, 2014). The combination of more than one organic acid can increase the effectiveness of the antimicrobial effect, however careful consideration needs to be made as the organic acids can negatively effect the quality of sprouts causing discoloration and acidic odors (Sikin et al., 2013). A study by Akbas et al. (2007) studied the effectiveness of reducing *E. coli* on inoculated lettuce with organic acids. The study found that 0.5% solutions of lactic acid, citric acid, acetic acid and ascorbic acid each applied to the lettuce resulted in a 1.9 log_{10} CFU/g reduction, 2.1 log_{10} CFU/g reduction, 1.3 log_{10} CFU/g reduction, and 1.0 log_{10} CFU/g reduction, respectively.

Chlorine dioxide (ClO_2) is an approved antimicrobial agent for produce at a maximum concentration of 3 ppm (FDA, 2014). Chlorine dioxide has the ability to work in solutions with higher concentrations of organic matter compared to hypochlorous acid and is more effective at a neutral pH, however, produce must be rinsed with potable water, blanched, cooked or canned after treatment (FDA, 2014). A study by Kim et al. (2009a) found that broccoli sprouts that were inoculated with 7 to 8 log CFU/g of *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* and post-harvest treated with 50 ppm chlorine dioxide had an approximate reduction of 1.5 log CFU/g.

Ozone has also been studied as an antimicrobial agent for use as a post-harvest wash with produce. It has been found effective in reducing microorganisms

on shredded lettuce by 2 log/g (FDA, 2014). However, because of the oxidizing activity of ozone, some produce loses quality after treatment (FDA, 2014). Therefore, while the use of ozone is becoming wide spread in the food industry, research needs to be conducted on the produce it will be used with to determine effectiveness and quality (FDA, 2014).

Research continues on different methods of post-harvest washing that is effective in reducing pathogens to safe levels and minimizing the impact on the quality of the sprout including the use of irradiation, hydrogen peroxide and electrolyzed water. The most effective post-harvest treatment that produced a 5 log reduction of *Salmonella* and *Listeria monocytogenes* on inoculated mung bean sprouts and did not effect quality was a 268 ppm chlorous acid treatment for 10 minutes (Sikin et al., 2013). Overall, decontamination methods used on sprout seeds and harvested sprouts must take into consideration the overall effectiveness and safety of the consumer, quality of the end product, time and cost associated with the treatment(s), feasibility of use in the industry and compliance to regulations (Sikin et al., 2013).

1.1.9 Aquaponics

Sustainable agriculture is rapidly being embraced as a method of growing and producing food for farmers all across the globe. The 2011 IFIC Foundation Food & Health Survey reports that "58% of Americans have read or heard at least "a little" about sustainability in food production; and 52% say sustainability is important in

making food and beverage choices" (IFIC, 2011). Sustainable agriculture was first formally addressed in the 1990 Farm Bill and was defined as: "an integrated system of plant and animal production practices having a site-specific application that will over the long term: (1) satisfy human food and fiber needs; (2) enhance environmental quality and the natural resource base upon which the agricultural economy depends; (3) make the most efficient use of nonrenewable resources and on-farm resources and integrate, where appropriate, natural biological cycles and controls; (4) sustain the economic viability of farm operations; and (5) enhance the quality of life for farmers and society as a whole" (Gold, 1999). Farms that practice sustainable agriculture are those that work with the natural environment. For example, nature often uses waste from once source to benefit another source (Sullivan, 2003). This concept is mimicked in sustainable agriculture farm methods. One such example of this at work is in aquaponics. Aquaponics is a sustainable agriculture farm model in which fish are grown in fish tanks and produce is grown via methods of hydroponics using the nutrient rich wastewater from the fish. The benefits of this system include: re-use of the wastewater from the growing fish, production of two commodities from one system, and water and soil conservation (Diver, 2006). It is estimated that an aquaponics system uses 10% of the land needs and 5% of the water needs as traditional vegetable production (Burden et al., 2013). However, not all produce can be grown in this system. Plants that thrive in an aquaponics system are those in which their nutritional requirements can be met by the fish wastewater. This includes leafy greens (such as spinach, lettuce, and herbs),

tomatoes, peppers and cucumbers (Diver, 2006). Many fish species thrive well in the aquaponics system too, however, most commonly used are those that are food sources such as tilapia, trout, perch and bass (Diver, 2006).

Aquaponics systems are unique sustainable agriculture systems in that they are soil-less systems that have the ability to produce two commodities from the same system. Fish growing in the system produce waste that is high in ammonia (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), phosphorus and potassium (Diver, 2006). Rhizobacteria, particularly *Nitrosomonas sp.* and *Nitrobacter sp.* inhabit the system on solid surfaces including the filter system and plant roots in the form of a biofilm. The *Nitrosomonas sp.* function to convert the ammonia into nitrite and the *Nitrobacter sp.* convert the nitrite into nitrate, which acts as a fertilizer for the growing plants. This process produces acid and can lower the pH of the system (Rakocy et al., 2006). Therefore the pH of the system is often monitored to determine if an alkaline base is needed to control the pH. Aquaponics systems should have a pH of approximately 7.0 (Rakocy et al., 2006). Additional nutrients enter the system from decomposing (uneaten) fish feed and algae that may be present. Together the rhizobacteria and the plants act as a biofilter to detoxify the water to keep the fish healthy and consequently acts as a fertilizer to generate healthy produce (Diver, 2006). A study by Michalak et al. (2015), found that algae is made up of carbohydrates, proteins, lipids, minerals, polyunsaturated fats, antioxidants such as vitamins E and C, and pigments such as carotenoids and chlorophylls.

The types of crops grown in an aquaponics system are directly related to the size and stocking density of the system. Crops, such as lettuce, spinach, and herbs have a low nutritional demand and therefore thrive in a system that is not stocked heavily with fish. Conversely, crops that have a high demand for nutrients such as tomatoes, peppers and cucumbers thrive well in a system that is heavily stocked with fish (Diver, 2006).

Tilapia are the most common variety of fish used in aquaponics systems in North America (Diver, 2006). These fish are a warm-water species that adapt well to fluctuating water pH, temperature, oxygen concentration and dissolved solids (Diver, 2006).

Aquaponics systems require particular water quality parameters in order to meet the needs of the fish and plants. Water is regularly monitored for dissolved oxygen, carbon dioxide, ammonia, nitrate, nitrite, pH and chlorine (Diver, 2006). These characteristics can be impacted with the number of fish in each tank, the growth rate of the fish, the amount and frequency of fish feed added as well as environmental changes including temperature, sunlight, etc. (Diver, 2006). Fish excrete approximately 70-80% of their nitrogen intake and 60-85% of their phosphorous intake (Roosta, 2014). Aquaponics systems that utilize only fish waste for nutrients can have low levels of phosphorus, potassium, iron, manganese and sulfur and therefore supplementation of these nutrients may be required (Roosta, 2014). The study by Roosta (2014) reported the water quality characteristics of the aquaponics system used for the growth of basil. The water, before it flowed into the

grow bed, contained: 5.10 mg/L dissolved oxygen, 7.60 pH, 327 mg/L total dissolved solids, 1.02% NaCl, 34.6 NO_3-N mg/L, 1.69 mg/L Nitrite-N, 26.7 mg/L potassium, 7.98 mg/L phosphorus, 34.2 mg/L calcium, 0.21 mg/L iron, 0.37 mg/L zinc and 0.042 mg/L copper. This study also found that iron, potassium and manganese concentration of the basil decreased when grown in more aquaponics water than hydroponics water, and zinc increased as the ratio of aquaponics:hydroponics water increased (Roosta, 2014).

Another important component of an aquaponics system is the filter that separates the dissolved solids from the water. This filter can be a standalone mechanical filter in place within the system, or a gravel filter that the water passes through to naturally catch the solids before entering the grow beds with the plants (Diver, 2006). Filtering the solids from the water is necessary because high amounts of dissolved solids can rapidly decrease the concentration of dissolved oxygen in the water (Rakocy et al., 2006). These filter systems also provide additional solid surfaces for the rhizobacteria to attach and help in nitrifying the passing water.

Plants grown in aquaponics systems often grow quickly because the dissolved nutrients are available for absorption through the roots quickly and efficiently (Rakocy et al., 2006). For growth, plants require macronutrients including carbon, oxygen, hydrogen, nitrogen, potassium, calcium, magnesium, phosphorus, and sulfur. Micronutrients that are required for plant growth include: chlorine, iron, manganese, boron, zinc, copper and molybdenum (Rakocy et al.,

2006). These nutrients are derived from water, carbon dioxide, fish effluent, rhizobacteria and decaying fish feed.

The food safety concerns of aquaponics systems differ from conventional methods of growing produce except for employee hygiene and packing facility sanitation (Fox et al., 2012). For example, in aquaponics, traditional soil, which naturally contains pathogens, is not used and soil-less media is used which can be cleaned to reduce pathogens. Aquaponics systems are often elevated above the ground or set-up in greenhouses or buildings away from soil (Fox et al., 2012). Aquaponics systems are generally located and designed in a way that allows them to have relatively easy protection from contamination of outside wildlife unlike traditional soil farming. Soil farming often uses manure to enrich the soil, but if not treated adequately to kill potential pathogens can cause cross contamination to the produce that comes in contact with the soil. While an aquaponics system utilizes fish manure for nutrients, the fish do not come in contact with the edible portions of the plants and acquiring a foodborne illness from pathogens in the fish is low (Fox et al., 2012). It is not uncommon to find algae, fecal coliforms and non-pathogenic *E. coli* in aquaponics water (Fox et al., 2012). Coliforms are naturally present in aquaponics systems particularly due to the constant recirculation of the water (Fox et al., 2012; Munguia-Fragozo et al., 2015). A study that evaluated the microbial composition of lettuce grown in an aquaponics system versus conventionally in soil found that the lettuce grown in the aquaponics system had significantly less total

aerobic microorganisms, coliform and yeast and no detectable *E. coli* compared to soil grown lettuce (Munguia-Fragozo et al., 2015).

1.1.10 Aquaponics Production

Worldwide, approximately one hundred million tons of fish are consumed annually (Goddek et al., 2015). Fish are an excellent protein source and contribute to approximately 20% of the world's population protein needs (Goddek et al., 2015). The USDA reported that there were 71 aquaponics farms across 21 states in 2012. Of all reported aquaculture operations, 2% were aquaponics operations (Engle, 2015). Approximately 75% of the aquaponics operations had gross sales of less than $25,000, 14% had sales between $25,000 and $49,000 and 11% had sales greater than $50,000 (Engle, 2015). In the same year, the states with the most number of aquaponics operations were (in descending order): Florida, Wisconsin, Arizona, New York and Hawaii (Engle, 2015). The average size of the operations ranged from 537 gallons to 4,741 gallons (Engle, 2015). Aquaponics systems take a relatively large investment to get started, adequate energy needs and training on the part of the producer/farmer (Rakocy et al., 2006). A review of literature by Engle (2015) reported that the production of aquaponically grown vegetables can be a profitable source of income, however tilapia production is not profitable.

1.1.11 Water Conservation

The demand for water stems from the population and water needs of individuals, energy derived from water, environmental factors such as global warming and precipitation and water necessary for agriculture in terms of water needs of land and aquatic animals used for food and growing crops. Approximately 80 to 90% of the water used in the United States is used for the irrigation of agriculture crops (Schaible et al., 2012). As a whole, traditional soil based farming consumes approximately 70% of the world's fresh water, while aquaponics consumes less than 10% (Goddek et al., 2015). Aquaponics systems are in high demand in areas of the world where the climate is dry and fresh vegetables and fish are needed (Diver, 2006). Once an aquaponics system is established, only about 2% of the total water volume needs to be replaced daily to make up from evaporation losses (Munguia-Fragozo et al., 2015).

Due to the need to reduce foodborne illnesses linked to the consumption of produce including sprouts, the scientific literature that supports that produce grown in aquaponics systems harbor less pathogens than soil grown produce and the increased utilization of sustainable agriculture methods, the objective of this research study is to determine if there is a difference in the microbiological and nutritional quality characteristics of fresh produce grown hydroponically and aquaponically after post-harvest treatments with municipal tap water, chlorine and organic acids.

During plant production, irrigation water is critical because it provides essential water and is a carrier of nutrients for the growing plant. Consequently, it may influence the microbiological and nutritional quality characteristics based on the components in the irrigation water. Thus, the goal of this research was to determine if aquaponics water could influence the microbiological and nutritional quality characteristics of alfalfa and mung bean sprouts. This was accomplished in 3 objectives, which were: 1) to determine the microbiological characteristics of alfalfa and mung bean sprouts irrigated with different water sources and treated post-harvest with various rinses; 2) to study the partitioning of *Escherichia coli* contamination on alfalfa sprouts inoculated at day 1 or 4 during production; and 3) to determine the nutritional quality characteristics of alfalfa and mung bean sprouts irrigated with different water sources.

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CHAPTER TWO

MICROBIOLOGICAL CHARACTERISTICS OF ALFALFA AND MUNG BEAN SPROUTS IRRIGATED WITH DIFFERENT WATER SOURCES AND TREATED POST-HARVEST WITH VARIOUS RINSES

2.1 ABSTRACT

Fresh produce, including sprouts, have been the source of a number of foodborne illness outbreaks over the past 20 years. Sprouts are of utmost concern because they are most commonly consumed raw. In this study, the microbiological characteristics of alfalfa and mung bean sprouts were analyzed after irrigation with tap water or aquaponics water and rinsing post-harvest with tap water, chlorine or organic acids. After treatment, sprouts were tested for the recovery of total aerobic microorganisms, *Enterobacteriaceae*, total coliforms and yeasts and molds. Overall, results demonstrated that washing raw alfalfa or mung bean sprouts reduced the numbers of total aerobic microorganisms, *Enterobacteriaceae*, total coliforms, yeast and molds. The greatest reductions for all types of microorganisms that were tested occurred when organic acid was used as the post-harvest wash (2.3 to 3.6 log CFU/g lower for total aerobes; 2.2 to 4.0 log CFU/g lower for *Enterobacteriaceae*; 1.7 to 2.0 log CFU/g lower for total coliforms; and 2.5 to 3.9 log CFU/g lower for yeast and molds). A reduced efficacy for sprouts washed with 200 ppm chlorine occurred because of organic material present from the aquaponics irrigation water and an

increase in the pH (>9.0) of the chlorine wash. Recovery of total coliforms from alfalfa sprouts increased 1.4 to 1.7 log CFU/g as compared to the no wash sprouts; however similar results were not observed for mung bean sprouts. This may be related to the higher initial microbiological load on the alfalfa versus mung bean sprouts, and a surfactant effect on present biofilms releasing coliforms in the recovery diluent. Results indicate that sprouts are a variety of produce that may pose a significant risk to causing a foodborne illness when consumed raw as a result of the high microbial load.

2.2 INTRODUCTION

The Centers for Disease Control and Prevention (CDC) reports that approximately one out of 6 Americans become sick each year from a foodborne Illness, which equates to approximately 48 million people (CDC, 2011). An estimated 46% of foodborne illnesses and 23% of foodborne-illness-related deaths were attributed to the consumption of fresh produce between 1998 and 2008 (Painter et al., 2013; CDC, 2013). Between 1996 and 2016, there were 41 reported foodborne illness outbreaks that were directly related to the consumption of sprouts (Johanson, 2012; CDC, 2016). Furthermore, *Salmonella* was linked to 19 sprout-related outbreaks and *Escherichia coli (E. coli)* O157:H7 has been linked to 6 sprout-related outbreaks between 1996 and 2003 (FDA, 2004). In a report from the International Sprout Growers Association, Sikin et al. (2013) stated that about 10%

of Americans consume sprouts regularly which equates to approximately 32 million people that may be subject to sprout-related foodborne illnesses regularly.

Sprouts are most commonly consumed raw in sandwiches or on salads, or lightly cooked in dishes such as stir-fries (Oregon Public Health Division et al., 2015). Considering the large number of foodborne illnesses linked to the consumption of raw sprouts, their microbiological safety is a great concern. The 2011 Food Safety Modernization Act (FSMA) has specifically addressed the production of sprouts in order to prevent future consumption-related outbreaks. Per FSMA, sprout producers are required to follow safe practices in growing and harvesting, ensuring the safety of soil (if used), health and hygiene of workers, animal control and product temperature control (FDA, 2011). Additionally, sprout producers are required to prevent pathogens on seeds including the decontamination of seeds prior to sprouting, evaluation of spent irrigation water from each production batch (presence of *Salmonella* and *E. coli)*, establish a Listeria monitoring program in growing, harvesting, packing and holding areas of the sprout facility, and establish corrective actions that prevent any sprouts testing positive for a pathogen from being released from the facility (FDA, 2015a).

The production environment has been cited as one of the reasons that sprouts have a high incidence of foodborne illness outbreaks (Wood, 2000). The growing environment provides enough nutrients, acidity, oxygen, moisture, temperature and time for microorganisms, including pathogens, to proliferate (Wood, 2000). It is estimated that sprout seeds may contain up to 10^9 CFU/g sprout

within 2-3 days of sprout production (Liao, 2008). Because of the high levels of pathogenic microorganisms on sprout seeds, it is common practice to soak them in a 20,000 ppm solution of calcium hypochlorite for 15 minutes (Fett, 2001). While this treatment is effective in reducing numbers of pathogens on the sprout surface, it will not remove all of the pathogens from the seed (USDA, 1999). This is likely due to the action of the hypochlorous acid, which primarily prevents reattachment of bacteria after their removal by mechanical action. The National Advisory Committee on the Microbiological Criteria for Foods (NACMCF) has stated that treating sprout seeds with calcium hypochlorite will provide an average microbial reduction of 2.5 log CFU/g sprout and this may vary depending on the variety of seed and the application conditions (Sikin et al., 2013). Any pathogens remaining after antimicrobial treatment have the potential to grow during the sprouting process, thus increasing the likelihood of causing a foodborne illness if the contaminated sprouts are consumed (Sikin et al., 2013). A number of studies have found alfalfa sprouts to contain approximately 7 to 9.2 log CFU/g sprout of aerobic microorganisms, 2.8 to 8.6 log CFU/g sprout of coliforms, 7.2 log CFU/g sprout of *Enterobacteriaceae* and 3.4 to 7 log CFU/g sprout of yeasts and molds (Abadias et al., 2008; Fett, 2002; Kim et al., 2009; Kim et al., 2012; Rangel-Vargas et al., 2015; Tournas, 2005). Phua et al. (2014) and Tournas (2005) enumerated aerobic microorganisms and yeasts and molds on untreated mung bean sprouts and found that the samples contained more than 9 log CFU/g sprout and more than 7 log CFU/g sprout, respectively. Recently, research has begun to reveal that sprouts are

often contaminated with biofilms that have the potential to cover 29 to 59% of the sprout's surface (Fett, 2000; Sikin et al., 2013; Yaron et al., 2014). The presence of biofilms are of particular concern because they are much more resistant to antimicrobial treatments than individual microorganisms (Fett, 2000; Van Houdt et al, 2010).

Post-harvest washing treatments of sprouts are a common industry practice to aid in reducing microorganisms. The most frequently used post-harvest washes include chlorine, organic acids, chlorine dioxide and ozone (Sikin et al., 2013). Chlorine and organic acids have been reported to be the most common of these post-harvest washes for produce because they are typically easy to use, safe, effective and reasonably priced (FDA, 2014; Sikin et al., 2013). The FDA recommends that produce be washed in chlorinated solutions that contain 50-200 ppm chlorine with a contact time of 1 to 2 minutes to obtain microbial inhibition (FDA, 2014; Sikin et al., 2013). However, this treatment typically achieves only a 1 to 3 log reduction in counts of bacteria with variation resulting from the presence of organic material on the sprout surface (Sikin et al., 2013). Free chlorine (not bound to organic material) has the greatest efficacy against pathogens and it exists predominately as hypochlorous acid (HOCl; FDA, 2014). In solution, efficacy of HOCl is pH dependent, such that as the pH decreases, the concentration of HOCl will increase. At low pH values (less than 4.0), chlorine gas will evolve from chlorinated solutions thus reducing effectiveness against microorganisms (FDA, 2014). FDA (2014) recommends that the pH of chlorine solutions be maintained between 6.0-

7.5 to achieve optimal antimicrobial action and to reduce corrosion of equipment because it is at this pH that the HOCl concentration is at its highest level with minimal Cl_2 (g) formed (FDA, 2014). Chlorine effectiveness against microorganisms is also dependent on temperature, light, air and presence of metal ions, and these factors should be monitored in sanitizing solutions (FDA, 2014). As the pH, temperature, or concentration of organic materials, light, air or metal ions increases, the concentration of HOCl will decrease, thus reducing the effectiveness of the sanitizer (FDA, 2014). The optimal temperature of chlorine wash solutions for use on fresh produce has been reported to be 4°C (39°F); however it is recommended that the wash water should be 10°C higher than the temperature of the produce being washed to prevent adsorption of water inside the produce causing potential internal contamination (Beuchat, 1998).

Previous research reported that alfalfa sprouts treated with 500 ppm chlorine post-harvest reduced *Salmonella spp.* population by 3.4 log/g sprout, while post-harvest treatment with 2000 ppm chlorine (above acceptable levels) had undetectable levels of *Salmonella spp.* (Beuchat et al., 1997). Much of the variation in the microbiological effectiveness reported with chlorinated solutions results from the lack of control of the factors influencing HOCl concentration in the treatment solution.

Another common method of treating sprouts post-harvest is with organic acids such as citric, lactic, acetic or blends of organic and inorganic acids. Organic acids are classified by the FDA as GRAS (generally regarded as safe) and are

effective because their low pH may disrupt the cell membrane of bacteria and inhibit the bacteria's ability to survive (FDA, 2014; Sikin et al., 2013). Most organic acids are inexpensive, safe, effective, cause minimal impact on product quality, and can be used by organic farmers/producers (Sikin et al., 2013). Unfortunately, a high concentration of organic acids may also negatively impact the quality of sprouts by discoloration. The discoloration and associated acidic odor on fresh produce may occur after organic acid treatment if the concentration is high enough (low pH) (Sikin et al., 2013). Like chlorine, organic acids have been found to typically provide approximately 2 log reduction (99%) in microorganisms on fresh produce (Akbas et al., 2007).

Recently, there has been a surge in alternative methods of fresh produce production to retain organic status or to be considered 'all natural'. One such method is aquaponics, which involves growing fish in fish tanks, recirculating the fish water from the tanks once it contains fish waste, decaying fish feed, rhizobacteria and algae, to fertilize and grow produce hydroponically. The microbiological quality of produce grown in or with aquaponics water is of interest because fish that inhabit aquaponics systems and provide nutrients for the growing produce may carry pathogens that can transfer to the growing produce. Previous research has suggested that since fish are cold-blooded they do not carry pathogens, such as *E. coli* (Fox et al., 2012). However, other research has found that fish do harbor bacterial pathogens, many of which are normally present in the aquatic environment (Austin et al., 2012). Research by Gauthier (2015), reported that fish

can carry pathogens such as *Clostridium botulinum, Staphyloccus spp., Streptococcus spp., Mycobacterium spp., Aeromonas spp.* and *Enterobacteriaceae.* This report states that the potential for humans to contract a pathogenic infection from fish is low unless carried by something other than fish or contaminated on food, such as the meat of fish when it is improperly handled or not cooked thoroughly to kill any potential pathogens (Gauthier, 2015). It is not uncommon to find non-pathogenic *E. coli* and coliforms in water from aquaponics systems (Fox et al., 2012). Minimal research has been conducted to characterize the microbiological quality of produce grown in aquaponics water versus potable (tap) water that is traditionally used for irrigation.

Thus, the purpose of this study was to determine if there is a difference in the microbiological characteristics of fresh produce grown hydroponically and aquaponically and treated post-harvest.

2.3 MATERIALS AND METHODS

2.3.1 Preliminary Experiment

A preliminary experiment was conducted in which alfalfa and mung bean sprouts were irrigated with municipal tap water, aquaponics water, chlorine water (200 ppm) and organic acid (1% solution) water during growth. However, the seeds irrigated with the chlorine water had a low germination rate and of those seeds that sprouted, growth was stunted compared to the sprouts irrigated with municipal tap water or aquaponics water. The sprouts irrigated with a 1% solution

of organic acids, containing sulfuric acid, citric acid and phosphoric acid, had a 0% germination rate. For this reason, antimicrobial treatments were not used during production and rather were used post-harvest.

2.3.2 Sprout Production and Harvesting

Alfalfa and mung bean seeds were purchased from a commercial source[1] that certifies the seeds as *E. coli*-free and *Salmonella*-free. These seeds were used during each of 3 replications to grow sprouts in 16 individual growth pans (40 cm x 31.8 cm x 15.2 cm; 8 pans alfalfa sprouts and 8 pans mung bean sprouts; Figure 2.1). Pans were lined with four layers of paper towels and mung bean seeds (approximately 147 g of seeds per pan) or alfalfa seeds (approximately 32 g per pan) were spread evenly in growth pans using clean latex gloves. Four pans of mung beans and four pans of alfalfa were irrigated with room temperature tap water. The remaining pans (four pans for each variety of seed) were irrigated with fresh aquaponics water obtained from an aquaponics system located at Clemson University[2]. The aquaponics system contains three – 600 gallon tanks. Each tank holds approximately 300 – four-month-old tilapia. Water for irrigation of the sprouts was collected from a flood tank that collects the filtered water from each of the three fish tanks before it enters the growth beds containing plants. In both

[1] Johnny's Selected Seeds, Fairfield, ME, USA.
[2] Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains three-600 gallon tanks. Each tank hold approximately three hundred – 4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three tanks before it enters the grow beds containing plants.

cases, water application for growing the sprouts was static and not continuous flow. The first four days after planting, the seeds were grown in the dark with a cloth covering to omit extraneous light. On day 4, the cloth was removed and ambient lighting was introduced. Sprouts were irrigated one to two times daily until harvest as appropriate for their water needs. After seven days, mung bean and alfalfa sprouts were harvested using clean latex gloves, pooled according to treatment (type of sprout and water source) and divided into four – 25 g samples. The four samples were treated post-harvest according to the following: 1) no wash (control); 2) municipal water rinse; 3) chlorine rinse; and 4) organic acid rinse.

2.3.3 Washing

After harvest and pooling to make 25 g sample allotments, three of the four pooled sprout treatments were washed for one minute with 500 mL tap water, a chlorinated solution or an organic acid solution. The fourth treatment was a control and was not washed. To prepare the chlorine solution, the pH of the tap water was first adjusted to a pH of 6.0 using hydrochloric acid (HCl)[3]. Commercial bleach containing 8.25% sodium hypochlorite solution[4] was added to the pH 6.0 tap water until a free chlorine test[5] measured approximately 200 ppm. Chlorine was tested using the N,N diethyl-p-phenylenediamine (DPD) colorimetric method. In the

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[3] Mallinckrodt AR Select®, Paris, KY, USA.
[4] Clorox®, Oakland, CA, USA.
[5] Portable CHEMetrice Chlorine 2 SAM test kit with Vacu-vials, Chemetrics, Free/Total Chlorine I-2001, Midland, VA, USA.

presence of chlorine, DPD is oxidized producing a red color. The intensity of the color produced from this oxidation reaction is proportional to the amount of chlorine present and can be read colormetrically (530 nm) to determine chlorine concentration. The organic acid wash was prepared to contain a 1% solution of FreshFx® LP[6], which was diluted per manufacturer's guidelines using tap water. The organic acid used in this study was a blend of acids and consisted of ≤35% sulfuric acid, ≤10% citric acid and ≤5% phosphoric acid by weight.

2.3.4 Microbiology

After post-harvest washing, sprouts were drained and transferred to sterile stomacher filter bags. Two hundred twenty-five mL of sterile 0.1% peptone[7] was added to each stomacher bag and the sprouts were stomached[8] for 1 minute at 230 RPM. Serial dilutions were prepared from the rinsate using true duplicates and duplicates were plated to either aerobic plate count (APC) agar (total aerobic microorganisms), violet red bile glucose (VRBG) agar (*Enterobacteriaceae)*, dichloran rose bengal chloramphenicol (DRBC) agar[9] (yeasts and molds) or 3M Coliform Petrifilm™[10] (total coliforms). After plating, the APC and VRBG plates were inverted, and then these plates and the 3M Petrifilm™ were incubated at 37°C for 48 hours. The DRBC plates were inverted and incubated at ambient temperature for 5

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[6] Synergy Technologies, Shreveport, LA.
[7] Fisher Scientific, Pittsburgh, PA, USA.
[8] Seward, Stomacher® 400 Circulator, Worthing, West Sussex, UK.
[9] Fisher Scientific, Pittsburgh, PA, USA.
[10] 3M Microbiology, St. Paul, MN, USA.

days. After incubation, the visible colony forming units (CFU) on the plates were counted and were converted to log_{10} CFU/g sprout.

2.3.5 Irrigation Water Microbiology

Before irrigation each day, a 20 mL sample of the irrigation water (municipal tap water and aquaponics water) was collected in a sterile sample container and refrigerated (~4°C). On day 8, the tap water irrigation samples (N=8) and the aquaponics water irrigation samples (N=8) were pooled; serial dilutions were prepared from the pooled samples and were plated on the same medias as the sprout samples. The APC and VRBG plates were then inverted, and then the APC, VRBG and 3M Petrifilm™ plates were incubated at 37°C for 48 hours. The DRBC plates were inverted and incubated at ambient temperature for 5 days. After incubation, the visible CFUs on the plates were enumerated. The CFUs were then calculated to report the CFU/mL and were converted to log_{10} CFU/mL water.

2.3.6 Statistics

The entire experiment was replicated three times. Within each replication and production water treatment, four grow pans were used. Log_{10} CFU/g sprout or Log_{10} CFU/mL of irrigation water were analyzed for statistical significance using the General Linear Model procedure of SAS (version 8.2) with the main effects of the model being type of seed (alfalfa or mung bean), replication, production water (tap or aquaponics) and post-harvest treatment (no wash, tap water, chlorine or organic

acid). Means were separated using the least means square procedure with replication and the interactions of the main effects of the model serving as the error term for the model and significance reported at the $P<0.05$ level (SAS Institute, 2000).

2.4 RESULTS AND DISCUSSION

2.4.1 Microbiology of Production Water Sources

The tap water and aquaponics water production water sources were tested to enumerate total aerobic microorganisms, *Enterobacteriaceae*, total coliforms and yeasts and molds (Table 2.1). Approximately 1.0 $\log_{10}$ CFU/mL of total aerobic microorganisms were recovered from the tap water and 6.9 $\log_{10}$ CFU/mL of total aerobic microorganisms were recovered from the aquaponics water. The numbers of total aerobic microorganisms in the aquaponics water is not unusual considering the rhizobacteria, and other non-pathogenic microorganisms that are naturally present in aquaponics systems (Fox et al., 2012). No *Enterobacteriaceae*, total coliforms or yeasts and molds were detected in the tap water, confirming potability. The aquaponics water contained approximately 4.6 $\log_{10}$ CFU/mL of *Enterobacteriaceae*, 5.1 $\log_{10}$ CFU/mL total of coliforms and 2.4 $\log_{10}$ CFU/mL of yeasts and molds. Little research has been conducted on the microbiological characteristics of aquaponics water. A review of literature found that aquaponics water often contains algae, fecal coliforms, non-pathogenic *E. coli*, and rhizobacteria

(Fox et al., 2012). It is possible that yeast and molds may have been high in the aquaponics water due to the stagnant status during the germination process.

2.4.2 Chemistry of Irrigation Waters and Post-Harvest Washes

The average pH of municipal tap water used to irrigate the sprouts was 6.87. The aquaponics water used for irrigation had an average pH of 6.11. Both of these pH values are in the range that is optimal for pathogen growth (6.0 to 8.6; FDA, 2015b). At the time of post-harvest washing, the pH of the washes were: 6.58 for tap water, 9.15 for chlorine and 1.52 for organic acid. The chlorine wash was 200 ppm when added to water at pH 6.0. The organic acid wash was prepared to a concentration of 1%.

2.4.3 Total Aerobic Microorganisms

Alfalfa Sprouts

Alfalfa sprouts grown in municipal tap water or aquaponic water contained approximately 1 billion cells (9 log CFU/g) of total aerobic microorganisms recovered from the sprouts' surface when they were not treated post-harvest (no rinse; Table 2.2). When the sprouts were washed post-harvest in municipal water, those grown in tap water had fewer total aerobic microorganisms (7.9 $\log_{10}$ CFU/g) than the no wash sprouts, which is comparable to the findings of Kim et al. (2012), where alfalfa sprouts washed with tap water contained 7.2 to 7.6 $\log_{10}$ CFU/g of total aerobic microorganisms. No reduction in cell counts was observed from

aquaponics-produced sprouts washed with municipal water (8.8 $\log_{10}$ CFU/g sprout) when compared to alfalfa sprouts grown in aquaponics water and not rinsed post-harvest (9.0 $\log_{10}$ CFU/g). Washing sprouts post-harvest with 200 ppm chlorine reduced the number of total aerobic microorganisms by approximately 1.1 to 1.3 $\log_{10}$ CFU/g. If the pH of the chlorine wash would have been lower, the reduction might have been greater, but a reduction was observed considering the average pH of the wash was >9.0. It is likely that the pH of the chlorine solution increased as the sodium hypochlorite dissociated in water and the resulting hydroxide ions consequently increased the pH. High pH has been found to reduce bacteria in wash water used on eggs (Musgrove et al., 2008) and a similar effect likely occurred in this study. Additionally, aquaponics water is known to contain a significant quantity of organic matter from decaying fish feed, fish waste and algae (Fox et al., 2012). When organic matter is present, hypochlorous acid will consume the organic matter before providing an antimicrobial effect, therefore, reducing the effectiveness of this post-harvest treatment. The sprouts washed post-harvest with organic acid had reduced numbers of total aerobic microorganisms by 3.6 $\log_{10}$ CFU/g sprout (99.9% reduction) for both production water sources when compared to the counts recovered on the no-wash sprouts. This is likely due to the low pH of the wash solution, which averaged about 1.52.

Mung Bean Sprouts

Mung bean sprouts grown in either tap water or aquaponics water and harvested with no further treatment contained approximately 8 to 9 $\log_{10}$ CFU/g of total aerobic microorganisms (Table 2.3). When sprouts were washed post-harvest with municipal water, those grown in tap water had a reduction of approximately 0.2 log, which would have limited practical significance. Mung bean sprouts grown in aquaponics water and washed post-harvest with municipal water had a reduction of approximately 0.5 log, again, this reduction has limited practical significance. Sprouts grown in municipal water and washed post-harvest with 200 ppm chlorine had reduced numbers of total aerobic microorganisms by approximately 1.4 log as compared to the counts recovered on the no-wash sprouts. Sprouts grown in aquaponics water and washed post-harvest with 200 ppm chlorine had an approximate reduction of 0.8 log. The recovery of total aerobic microorganisms might have been lower on the sprouts grown in aquaponics water compared to those grown in municipal water due to the presence of organic material from the aquaponics water and the average pH of the chlorine greater than 9.0. Sprouts grown in municipal water and washed post-harvest with organic acid had reduced numbers of total aerobic microorganisms by approximately 99% and the sprouts grown in aquaponics water and washed post-harvest with organic acid had an approximate 98% reduction in total aerobic microorganisms when compared to no-wash sprouts. Studies by Sikin et al. (2013) and Akbas et al. (2007) reported that chlorine and organic acid washed produce generally had a 2 log reduction in

microorganisms depending on the variety of sprout and characteristics of the wash solution (pH, concentration, etc.). The results of the present study are in agreement with the findings of Sikin et al. (2013) and Akbas et al. (2007).

2.4.4 Enterobacteriaceae

Alfalfa Sprouts

Enterobacteriaceae are a family of gram-negative bacteria that includes *E. coli*, *Salmonella*, *Shigella, Klebsiella, Yersinia pestis, Proteus, Enterobacter, Serratia and Citrobacter*. Numbers of *Enterobacteriaceae* recovered from unwashed alfalfa sprouts ranged from 8.3 to 9.3 log_{10} CFU/g sprout regardless of the production water source, and counts of *Enterobacteriaceae* were not reduced when sprouts were washed post-harvest with municipal tap water (8.2 log_{10} CFU/g; Table 2.4). When sprouts were treated post-harvest with chlorine, those grown in tap water had lower numbers of *Enterobacteriaceae* (8.8 versus 7.6 log) than the unwashed sprouts. Sprouts grown in aquaponics water and washed post-harvest with chlorine had an approximate 0.4 log reduction in *Enterobacteriaceae,* and this reduction would have limited practical significance. Washing sprouts, grown in tap water and aquaponics water, post-harvest with organic acid reduced the number of *Enterobacteriaceae* by approximately 99% (3.6 to 4.0 log CFU/g) and the reduction was the same regardless of the production water source.

Mung Bean Sprouts

Numbers of *Enterobacteriaceae* recovered from unwashed mung bean sprouts grown in municipal tap water or aquaponics water was approximately 7.8 $\log_{10}$ CFU/g or 8.5 $\log_{10}$ CFU/g, respectively (Table 2.5). Counts of *Enterobacteriaceae* were not reduced when sprouts were washed post-harvest with municipal tap water regardless of production water source. Sprouts washed with 200 ppm chlorine post-harvest and grown in municipal water had a reduction of approximately 0.8 log. Sprouts grown in aquaponics water and washed post-harvest with chlorine did not have a reduction in recovered *Enterobacteriaceae* compared to the no rinse. Again, this is likely due to the high pH of the chlorine rinse (>9.0) and the presence of organic material from the aquaponics water. When sprouts were treated post-harvest with organic acid, those grown in tap water had a 99% reduction and those grown in aquaponics water had a 98% reduction in recovered *Enterobacteriaceae*.

A study by Abadias et al. (2008) enumerated approximately 7.2 $\log_{10}$ CFU/g of *Enterobacteriaceae* on alfalfa and soybean sprouts purchased at retail outlets. In the present study, alfalfa and mung bean sprouts grown in both tap water and aquaponics water and untreated post-harvest or washed with tap water had approximately 7.5 to 8.8 $\log_{10}$ CFU/g *Enterobacteriaceae.* Between both production water sources for both varieties of sprouts, the tap water rinse only reduced *Enterobacteriaceae* by about 0.4 $\log_{10}$ CFU/g. Between the chlorine and organic acid rinses, the chlorine rinse on the alfalfa and mung bean sprouts grown in aquaponics

water was least effective (0.4 and 0.0 log CFU/g reduction respectively). While the organic acid was most effective on both sprouts grown in tap and aquaponics water, alfalfa sprouts grown it tap water and aquaponics water had the most significant reduction in *Enterobacteriaceae.*

2.4.5 Total Coliforms

When total coliforms were enumerated on sprouts, it was discovered that no *E. coli* was detected on any of the samples. Bacteria that test positive for coliforms and fecal coliforms (*Klebsiella* and *Enterobacter*) are commonly found on the surface of produce from the growing environment and may colonize on the surface of the plant to utilize nutrients that the plant contains (Beuchat, 1998). Therefore, positive identification of coliforms and/or fecal coliforms is not necessarily a positive indicator of the presence of fecal coliforms (Beuchat, 1998). Total coliforms were not detectable in the tap water, however aquaponic water contained approximately 5.1 $\log_{10}$ CFU/mL total coliforms (Table 2.1). Fox et al. (2012) noted that coliforms are naturally present in aquaponics systems particularly due to the constant recirculation of the water.

Alfalfa Sprouts

Alfalfa sprouts contained approximately 6.8 to 6.9 $\log_{10}$ CFU/g of total coliforms when grown in tap or aquaponics water and not treated (no rinse) post-harvest (Table 2.6). Numbers of total coliforms recovered after washing post-

harvest with municipal water was similar to that recovered from unwashed sprouts regardless of the water source. Similarly, other research studies have been conducted in which alfalfa and soy bean sprouts, either unwashed or washed with tap water were found to contain 2.8 to 8.6 log_{10} CFU/g (Abadias et al., 2008; Fett, 2002; Kim et al., 2012; Rangel-Vargas et al., 2015). Sprouts grown in tap water and rinsed post-harvest with 200 ppm chlorine had an approximate 1.4 log_{10} CFU/g increase in recovered total coliforms compared to sprouts that were not rinsed post-harvest. Likewise, sprouts grown in aquaponics water and post-harvest rinsed with chlorine had a 1.7 log_{10} CFU/g increase in recovered total coliforms compared to counts recovered from the no-wash sprouts. Previous research has stated that free chlorine may cause a cellular injury of bacteria, which in turn causes the cell to be more sensitive to surfactants (Zaske et al., 1980). Other researchers have reported that biofilms in aqueous environments commonly contain high numbers of coliforms and these biofilms have the ability to consume high levels of free chlorine, thus reducing the effectiveness of the chlorine on the coliforms within the biofilm (Hallam et al., 2001). Considering this previous research, the increase in recovery of total coliforms on alfalfa sprouts washed post-harvest with chlorine could be due the action of surfactants naturally found on alfalfa sprouts removing injured coliform cells from biofilms that are present on the outside of the sprouts. The injured cells are then able to achieve self-repair, which can lead to their enumeration when plated on selective agar (Graham et al., 1983). Sprouts grown in

both water sources and washed post-harvest with organic acid had a 99% decrease in total coliforms compared to the no wash.

Mung Bean Sprouts

Numbers of total coliforms recovered from unwashed mung bean sprouts ranged from 4.4 to 9.6 log_{10} CFU/g sprout regardless of the production water source and counts of total coliforms were not reduced when sprouts were washed post-harvest with municipal tap water or 200 ppm chlorine (Table 2.7). Sprouts grown in tap water and rinsed with organic acid had a 2 log reduction (5.5 versus 7.5 log) when compared to numbers of total coliforms on unwashed sprouts. Sprouts grown in aquaponics water and rinsed with organic acid was not significantly different than the unwashed sprouts.

2.4.6 Yeasts and Molds

Alfalfa Sprouts

Yeast or mold was not detected in the tap water used to irrigate the alfalfa and mung bean sprouts (Table 2.1). Approximately 2.4 log_{10} CFU/mL yeast and mold was recovered from the aquaponics water used to irrigate the sprouts. Alfalfa sprouts grown in either tap water or aquaponics water and not washed post-harvest had a recovery of yeasts and molds ranging from 5.8 to 8.3 log_{10} CFU/g sprout (Table 2.8). Sprouts grown in tap water and rinsed with tap water post-harvest had approximately 0.6 log_{10} CFU/g reduction, while those grown in aquaponics water

did not have a decrease in recovery of yeasts and molds when compared to unwashed sprouts. When sprouts grown in tap water were treated post-harvest with 200 ppm chlorine, they had a 1.3 $\log_{10}$ CFU/g reduction whereas the sprouts grown in aquaponics water had a 0.3 $\log_{10}$ CFU/g reduction in yeasts and molds compared to unwashed sprouts. Sprouts from both production water sources and rinsed with organic acid both had a 99% reduction in yeasts and molds compared to sprouts there were not rinsed.

Mung Bean Sprouts

Mung bean sprouts grown in either tap water or aquaponics water and not washed post-harvest had a recovery of yeasts and molds ranging from 5.5 to 7.4 $\log_{10}$ CFU/g sprout (Table 2.9). Washing sprouts, grown in tap water, post-harvest with municipal water had a reduction of approximately 0.7 $\log_{10}$ CFU/g and those grown in aquaponics water had an approximate reduction of 0.8 $\log_{10}$ CFU/g sprout. When sprouts were treated post-harvest with chlorine, regardless of production water source, they had an approximate reduction of 0.9 $\log_{10}$ CFU/g compared to the unwashed. Numbers of yeasts and molds recovered from sprouts washed in organic acids from both production sources had a greater than 3.0 $\log_{10}$ CFU/g reduction when compared to unwashed sprouts.

Studies enumerating the recovery of yeast and mold on untreated alfalfa and mung bean sprouts determined that they contained approximately 3 to 7 $\log_{10}$ CFU/g (Fett, 2002; Kim et al., 2009; Tournas, 2005). This current study recovered

6.3 – 6.9 $\log_{10}$ CFU/g yeast and mold from alfalfa and mung bean sprouts, which is in line with previous research. A post-harvest rinse with chlorine had a significant effect ($P<0.05$) in reducing yeast and molds on alfalfa and mung bean sprouts grown in tap water (1.3 and 0.9 $\log_{10}$ CFU/g reduction) and mung bean sprouts grown in aquaponics water (0.9 $\log_{10}$ CFU/g reduction). The greatest reduction in numbers of yeasts and molds was with the use of organic acid as a post-harvest rinse, as alfalfa sprouts grown in both production waters had a 2.5 log reduction from the no rinse and mung bean sprouts grown in both production waters had more than a 3 log reduction.

2.5 CONCLUSION

Because decontamination of sprout seeds is not effective in reducing pathogens, post-harvest antimicrobial washes remain an important microbiological hurdle in the production of sprouts (Warriner et al., 2003). However, microorganisms can remain attached to sprouts surfaces, often in the form of biofilms, causing them to have the ability to remain attached to the surface, embed themselves into the surface or enter cuts/wounds, and thus decrease the effectiveness of antimicrobial washes (Matthews et al., 2014). Despite preparing a chlorine wash with water at an optimal pH and concentration, the resulting increase in pH prevented this wash from providing a more effective reduction in microorganisms. Other research has shown that pH values above 9.0 can have a microbicidal effect on bacteria in water, but this is not as great as optimal pH

chlorinated water (Musgrove et al., 2008). This demonstrates that while chlorine provides a microbicidal effect on produce during washing, it requires constant monitoring to ensure its effectiveness remains high. Additionally, because of the natural presence of organic matter in aquaponics water, chlorine washes may not be an ideal method of post-harvest treatment due to the added potential for the organic matter to interfere with the effectiveness of the wash. Overall, the combination of organic acids was the most effective method of reducing microorganisms on alfalfa and mung bean sprouts. The consistent low pH of the solution and ability to remain effective in aquaponics water contributed to its positive results. However, the researchers of this study noticed visible degradation and color changes of sprouts washed with the organic acid as well as an objectionable odor, thus reducing the overall quality. It is significant to note that while microorganisms were reduced by the organic acids in this study (despite the visual observation in the decrease of quality), the numbers of microorganisms were never reduced to a non-detectable level with any treatment. The family of *Enterobacteriaceae* has the greatest potential for causing foodborne illness because it contains pathogens (*Salmonella* and *E. coli*) that can cause humans to get sick when consumed. In this study, after the post-harvest rinse with organic acids, the sprouts still had 4.6 to 6.3 log_{10} CFU/g of sprout of *Enterobacteriaceae.* It can be concluded that there is a difference in the microbiological characteristics of fresh produce that is grown hydroponically with municipal tap water and with aquaponics water and treated post-harvest, however

the microbiological characteristics of these treatments do not necessarily produce sprouts that are safe for consumption.

Based on the results of this study, it may be concluded that sprouts are a high-risk commodity and that risk is too great to have the added potential for more microorganisms that are possible with aquaponics water.

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Table 2.1 Microorganisms[1] recovered from aquaponics and municipal water used to irrigate alfalfa and mung bean sprouts during growth

	Microorganisms[2]			
Production Water Source	Total Aerobic Microorganisms	*Enterobacteriaceae*	Total Coliforms	Yeast and Mold
Tap Water[3]	1.0 ± 0.0 (1.0-1.0)	ND[5]	ND	ND
Aquaponics Water[4]	6.9 ± 0.1 (6.6-7.0)	4.6 ± 0.1 (4.0-5.1)	5.1 ± 0.1 (4.8-5.5)	2.4 ± 0.2 (1.8-3.2)

[1]Microorganisms reported as $\log_{10}$ CFU/mL
[2]Number of samples tested between water sources was N=8.
[3]Public water system, City of Clemson, SC, USA. One sample positive on one occasion.
[4]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.
[5]ND = Not Detected
[a-b]Means ± the standard error of the mean with no common superscript within a column are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of microorganisms.

Table 2.2 Total aerobic microorganisms[1] recovered from alfalfa sprouts grown in either aquaponics or municipal water for 7 days and treated post-harvest[2]

	Post-Harvest Washing Treatments[3]			
Production Water Source	No Rinse	Municipal Water Rinse	Chlorine Rinse	Organic Acid Rinse[4]
Tap Water[5]	$9.0^{a} \pm 0.1$ (8.7-9.6)	$7.9^{by} \pm 0.2$ (7.4-8.5)	$7.7^{b} \pm 0.1$ (7.4-7.9)	$5.4^{c} \pm 0.2$ (4.7-5.8)
Aquaponics Water[6]	$9.0^{a} \pm 0.1$ (8.8-9.4)	$8.8^{ax} \pm 0.2$ (8.3-9.4)	$7.9^{b} \pm 0.3$ (6.9-8.6)	$5.4^{c} \pm 0.2$ (4.7-6.0)

[1]Total aerobic microorganisms reported as $\log_{10}$ CFU/g of alfalfa sprouts.
[2]Post-harvest treatments including: municipal tap water, chlorine (200 ppm), 1% solution of FreshFx® LP (organic acid). All treatments, except no rinse, were applied for 1 minute exposure time.
[3]N =8.
[4] FreshFx® LP(sulfuric, citric and phosphoric acids), Synergy Technologies, Shreveport, LA.
[5]Public water system, City of Clemson, SC, USA.
[6]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.
[a-c]Means ± the standard error of the mean with no common superscript within a row are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of total aerobic microorganisms.
[x-y]Means ± the standard error of the mean with no common superscripts within a column are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of total aerobic microorganisms.

Table 2.3 Total aerobic microorganisms[1] recovered from mung bean sprouts grown in either aquaponics or municipal water for 7 days and treated post-harvest[2]

	Post-Harvest Washing Treatments[3]			
Production Water Source	No Rinse	Municipal Water Rinse	Chlorine Rinse	Organic Acid Rinse[4]
Tap Water[5]	$8.0^{ay} \pm 0.1$ (7.2-8.5)	$7.8^{a} \pm 0.1$ (7.4-8.3)	$6.6^{by} \pm 0.2$ (5.9-7.0)	$4.6^{cy} \pm 0.2$ (3.9-5.10)
Aquaponics Water[6]	$8.9^{ax} \pm 0.1$ (8.4-9.5)	$8.4^{ab} \pm 0.1$ (7.9-8.6)	$8.1^{bx} \pm 0.2$ (6.9-8.5)	$6.6^{cx} \pm 0.3$ (5.6-7.6)

[1]Total aerobic microorganisms reported as $\log_{10}$ CFU/g of mung bean sprouts.
[2]Post-harvest treatments including: municipal tap water, chlorine (200 ppm), 1% solution of FreshFx® LP (organic acid). All treatments, except no rinse, were applied for 1 minute exposure time.
[3]N =8.
[4] FreshFx® LP (sulfuric, citric and phosphoric acids), Synergy Technologies, Shreveport, LA.
[5]Public water system, City of Clemson, SC, USA.
[6]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.
[a-c]Means ± the standard error of the mean with no common superscript within a row are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of total aerobic microorganisms.
[x-y]Means ± the standard error of the mean with no common superscripts within a column are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of total aerobic microorganisms.

Table 2.4 *Enterobacteriaceae*[1] recovered from alfalfa sprouts grown in either aquaponics or municipal water for 7 days and treated post-harvest[2]

	Post-Harvest Washing Treatments[3]			
Production Water Source	No Rinse	Municipal Water Rinse	Chlorine Rinse	Organic Acid Rinse[4]
Tap Water[5]	$8.8^{a} \pm 0.1$ (8.3-9.3)	$8.2^{ab} \pm 0.2$ (7.7-9.0)	$7.6^{by} \pm 0.1$ (7.4-8.1)	$5.2^{c} \pm 0.7$ (3.0-6.6)
Aquaponics Water[6]	$8.6^{a} \pm 0.1$ (8.4-8.9)	$8.2^{ab} \pm 0.3$ (7.3-9.0)	$8.2^{abx} \pm 0.2$ (7.6-8.9)	$4.6^{c} \pm 0.4$ (3.4-5.9)

[1]*Enterobacteriaceae* reported as $\log_{10}$ CFU/g of alfalfa sprouts.
[2]Post-harvest treatments including: municipal tap water, chlorine (200 ppm), 1% solution of FreshFx® LP (organic acid). All treatments, except no rinse, were applied for 1 minute exposure time.
[3]N =8.
[4] FreshFx® LP (sulfuric, citric and phosphoric acids), Synergy Technologies, Shreveport, LA.
[5]Public water system, City of Clemson, SC, USA.
[6]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.
[a-c]Means ± the standard error of the mean with no common superscript within a row are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of *Enterobacteriaceae*.
[x-y]Means ± the standard error of the mean with no common superscripts within a column are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of *Enterobacteriaceae*.

Table 2.5 *Enterobacteriaceae*[1] recovered from mung bean sprouts grown in either aquaponics or municipal water for 7 days and treated post-harvest[2]

	Post-Harvest Washing Treatments[3]			
Production Water Source	No Rinse	Municipal Water Rinse	Chlorine Rinse	Organic Acid Rinse[4]
Tap Water[5]	7.8^{ay} ± 0.3 (6.4-8.4)	7.5^{ab} ± 0.3 (6.0-8.0)	7.0^{by} ± 0.3 (5.7-7.9)	4.8^{cy} ± 0.3 (3.9-5.6)
Aquaponics Water[6]	8.5^{ax} ± 0.1 (8.1-9.1)	8.2^{a} ± 0.3 (7.1-8.7)	8.5^{ax} ± 0.1 (8.0-8.8)	6.3^{bx} ± 0.4 (5.0-7.4)

[1]*Enterobacteriaceae* reported as $\log_{10}$ CFU/g of mung bean sprouts.
[2]Post-harvest treatments including: municipal tap water, chlorine (200 ppm), 1% solution of FreshFx® LP (organic acid). All treatments, except no rinse, were applied for 1 minute exposure time.
[3]N =8.
[4] FreshFx® LP (sulfuric, citric and phosphoric acids), Synergy Technologies, Shreveport, LA.
[5]Public water system, City of Clemson, SC, USA.
[6]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.
[a-c]Means ± the standard error of the mean with no common superscript within a row are significantly different at P≤0.05. Numbers reported in parentheses are the range in counts of *Enterobacteriaceae*.
[x-y]Means ± the standard error of the mean with no common superscripts within a column are significantly different at P≤0.05. Numbers reported in parentheses are the range in counts of *Enterobacteriaceae*.

Table 2.6 Total coliforms[1] recovered from alfalfa sprouts grown in either aquaponics or municipal water for 7 days and treated post-harvest[2]

	Post-Harvest Washing Treatments[3]			
Production Water Source	No Rinse	Municipal Water Rinse	Chlorine Rinse	Organic Acid Rinse[4]
Tap Water[5]	$6.8^{b} \pm 0.9$ (4.4-9.5)	$7.5^{ab} \pm 1.0$ (4.4-9.4)	$8.2^{a} \pm 0.1$ (7.8-8.7)	$4.9^{c} \pm 0.6$ (3.6-6.7)
Aquaponics Water[6]	$6.9^{b} \pm 1.0$ (4.4-9.8)	$6.5^{bc} \pm 1.0$ (4.4-9.6)	$8.6^{a} \pm 0.2$ (8.3-9.2)	$5.2^{c} \pm 0.3$ (4.2-6.0)

[1]Total coliforms reported as $\log_{10}$ CFU/g of alfalfa sprouts.
[2]Post-harvest treatments including: municipal tap water, chlorine (200 ppm), 1% solution of FreshFx® LP (organic acid). All treatments, except no rinse, were applied for 1 minute exposure time.
[3]N =8.
[4] FreshFx® LP (sulfuric, citric and phosphoric acids), Synergy Technologies, Shreveport, LA.
[5]Public water system, City of Clemson, SC, USA.
[6]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.
[a-c]Means ± the standard error of the mean with no common superscript within a row are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of total coliforms.
[x-y]Means ± the standard error of the mean with no common superscripts within a column are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of total coliforms.

Table 2.7 Total coliforms[1] recovered from mung bean sprouts grown in either aquaponics or municipal water for 7 days and treated post-harvest[2]

	Post-Harvest Washing Treatments[3]			
Production Water Source	No Rinse	Municipal Water Rinse	Chlorine Rinse	Organic Acid Rinse[4]
Tap Water[5]	$7.5^{a} \pm 0.7$ (4.4-8.9)	$6.9^{a} \pm 0.8$ (4.4-8.6)	$7.4^{a} \pm 0.2$ (6.8-7.9)	$5.5^{by} \pm 0.3$ (4.8-6.4)
Aquaponics Water[6]	$6.9^{a} \pm 0.8$ (4.4-9.6)	$7.4^{a} \pm 1.0$ (4.4-9.0)	$7.4^{a} \pm 1.0$ (4.4-9.1)	$7.1^{ax} \pm 0.2$ (6.4-7.8)

[1]Total coliforms reported as $\log_{10}$ CFU/g of mung bean sprouts.

[2]Post-harvest treatments including: municipal tap water, chlorine (200 ppm), 1% solution of FreshFx® LP (organic acid). All treatments, except no rinse, were applied for 1 minute exposure time.

[3]N =8.

[4] FreshFx® LP (sulfuric, citric and phosphoric acids), Synergy Technologies, Shreveport, LA.

[5]Public water system, City of Clemson, SC, USA.

[6]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.

[a-b]Means ± the standard error of the mean with no common superscript within a row are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of total coliforms.

[x-y]Means ± the standard error of the mean with no common superscripts within a column are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of total coliforms.

Table 2.8 Yeast and mold[1] recovered from alfalfa sprouts grown in either aquaponics or municipal water for 7 days and treated post-harvest[2]

	Post-Harvest Washing Treatments[3]			
Production Water Source	No Rinse	Municipal Water Rinse	Chlorine Rinse	Organic Acid Rinse[4]
Tap Water[5]	$6.7^{ax} \pm 0.3$ (5.8-8.3)	$6.1^{b} \pm 0.3$ (5.4-7.2)	$5.4^{cy} \pm 0.2$ (4.7-6.1)	$4.2^{d} \pm 0.2$ (3.4-4.6)
Aquaponics Water[6]	$6.3^{aby} \pm 0.3$ (5.8-7.9)	$6.3^{ab} \pm 0.3$ (5.6-7.2)	$6.0^{bx} \pm 0.3$ (5.3-7.0)	$3.8^{c} \pm 0.2$ (3.2-4.4)

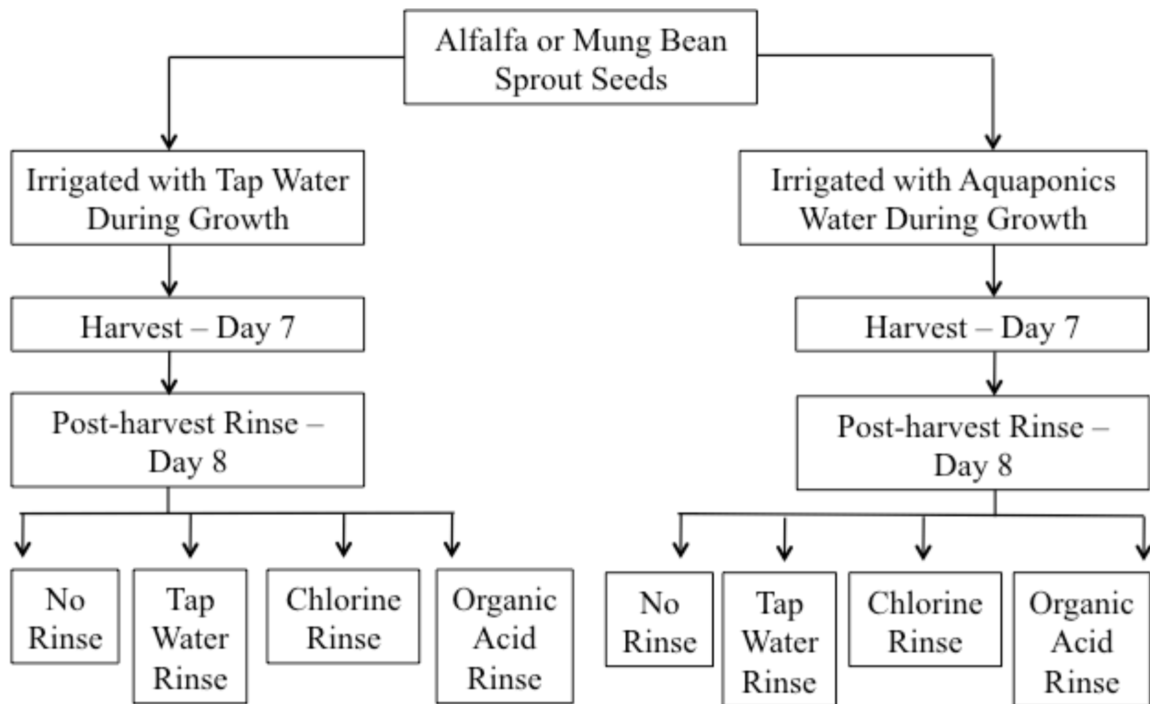
[1]Yeast and Mold reported as $\log_{10}$ CFU/g of alfalfa sprouts.
[2]Post-harvest treatments including: municipal tap water, chlorine (200 ppm), 1% solution of FreshFx® LP (organic acid). All treatments, except no rinse, were applied for 1 minute exposure time.
[3]N =8.
[4] FreshFx® LP (sulfuric, citric and phosphoric acids), Synergy Technologies, Shreveport, LA.
[5]Public water system, City of Clemson, SC, USA.
[6]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.
[a-d]Means ± the standard error of the mean with no common superscript within a row are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of yeast and mold.
[x-y]Means ± the standard error of the mean with no common superscripts within a column are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of yeast and mold.

Table 2.9 Yeast and mold[1] recovered from mung bean sprouts grown in either aquaponics or municipal water for 7 days and treated post-harvest[2]

	Post-Harvest Washing Treatments[3]			
Production Water Source	No Rinse	Municipal Water Rinse	Chlorine Rinse	Organic Acid Rinse[4]
Tap Water[5]	$6.3^{ay} \pm 0.2$ (5.5-6.9)	$5.6^{b} \pm 0.2$ (5.1-6.3)	$5.4^{by} \pm 0.3$ (4.4-6.0)	$3.2^{c} \pm 0.2$ (2.3-3.5)
Aquaponics Water[6]	$6.9^{ax} \pm 0.2$ (5.8-7.4)	$6.1^{ab} \pm 0.2$ (5.3-6.4)	$6.0^{bx} \pm 0.1$ (5.9-6.2)	$3.0^{c} \pm 0.2$ (2.7-3.7)

[1]Yeast and Mold reported as $\log_{10}$ CFU/g of mung bean sprouts.
[2]Post-harvest treatments including: municipal tap water, chlorine (200 ppm), 1% solution of FreshFx® LP (organic acid). All treatments, except no rinse, were applied for 1 minute exposure time.
[3]N =8.
[4] FreshFx® LP (sulfuric, citric and phosphoric acids), Synergy Technologies, Shreveport, LA.
[5]Public water system, City of Clemson, SC, USA.
[6]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.
[a-c]Means ± the standard error of the mean with no common superscript within a row are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of yeast and mold.
[x-y]Means ± the standard error of the mean with no common superscripts within a column are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of yeast and mold.

Figure 2.1 Experimental design to study the microbiological quality of alfalfa and mung bean sprouts irrigated with different water sources and treated post-harvest with various rinses.



CHAPTER THREE

PARTITIONING OF *ESCHERICHIA COLI* CONTAMINATION ON ALFALFA SPROUTS INOCULATED AT DAY 1 OR 4 DURING PRODUCTION

3.1 ABSTRACT

Consumption of raw sprouts may cause foodborne illness as they are commonly eaten raw and are difficult to wash post-harvest without negatively impacting quality. Minimal research has been conducted on the microbiological characteristics of alfalfa sprouts after inoculation at different times during growth. Furthermore, little is known about internalization of microorganisms in sprouts. Therefore, a partitioning study was conducted on alfalfa sprouts inoculated with *Escherichia coli (E. coli)* on day 1 or day 4 of production. In this study, alfalfa sprouts were grown for 7 days and inoculated on day 1 when the seeds were beginning to open, or day 4 when stems and leaves were developed. Sprouts were rinsed to recover surface *E. coli*, then they were sterilized with ethanol, macerated and rinsates were plated to recover remaining potential contamination on the inside. A second experiment was conducted to determine the number of exterior *E. coli* remaining after treatment with ethanol because experiment 1 did not include a separate external enumeration after alcohol treatment. The results of this study indicate that biofilms may be present due to the numbers of recovered *E. coli* after sterilization. Additionally, based on the numbers of *E. coli* recovered after

maceration, pathogens are present both on the outside and inside of the sprout. These results indicate that removal or reduction of pathogens on sprouts can be difficult due the presence of biofilms and internalized pathogens that are not affected when treated with antimicrobial treatments.

3.2 INTRODUCTION

Sprouts are commonly consumed raw on salads and sandwiches (Oregon Public Health Division et al., 2015). A report by the International Sprout Growers Association stated that 10% of Americans eat sprouts on a regular basis (Sikin et al., 2013). Unfortunately, the consumption of raw sprouts has been identified as the causative agent for 34 foodborne illness outbreaks in the United States between 1996 and 2010, which resulted in a total of 2,150 illnesses, 123 hospitalizations and one death (Johanson, 2012).

Contamination of the sprout can begin when the seeds are being grown in the field because many of the seeds are ultimately used for the production of animal feed, and thus not treated as if they will be utilized for human consumption (Mueller, 2008). Further contamination of the seed may occur if seeds undergo a scarification process whereby the surface of the seed is abrasively rubbed to increase germination rates (Oregon Public Health Division et al., 2015). The scarification process creates a rough outer layer on the seed that potentially opens small pores allowing access to the interior portions of the seed by pathogenic microorganisms (Oregon Public Health Division et al., 2015). Seeds can also

undergo a polishing process to increase germination by which the seeds are uniformly polished to reduce the outer coat of the seed without causing a rough surface where microorganisms can become unreached by antimicrobial treatments (Holliday et al., 2001). A study by Holliday et al. (2001) analyzed alfalfa seeds from two suppliers in which seeds were either scarified, polished or control (not scarified or polished). Results of this study were inconclusive as to the effectiveness of antimicrobial agents on seeds that are scarified or polished. Scarified seeds from one producer showed less effectiveness to various chemical antimicrobial treatments, while scarified and polished seeds from a different producer did not have a significant difference in recovered microorganisms from the control when treated with the same antimicrobial treatments (Holliday et al., 2001).

In 1999, the Food and Drug Administration (FDA) published a guidance document for the sprout industry that provided recommendations for seed production, conditioning, storage, transportation, seed treatment, sprout production, pathogen testing and traceback methods to reduce the incidence of foodborne illnesses associated with consumption of sprouts (FDA, 1999). Furthermore, the 2011 Food Safety Modernization Act (FSMA) mandated the produce safety standards in 2015 in a Produce Safety Rule (FDA, 2011). These required standards include environmental control of areas FDA deems to provide sources of microbiological contamination. The Produce Safety Rule provides requirements to all produce growers that cover the safe growing and harvest of produce, application of soil amendments, health and hygiene of workers, packaging,

temperature control of produce and control of animals around growing produce and water sources used for irrigation (FDA, 2011). This regulation also specifically requires that sprout producers treat seeds to reduce pathogen contamination, test the spent irrigation agricultural water for *Salmonella* and *E. coli*, test the sprouting environment for *Listeria monocytogenes* and prevent sprout samples that test positive for pathogens from entering the food supply (FDA, 2015). Within this regulation, growers must establish a microbiological quality profile of their agricultural water – water directly applied to produce as irrigation or in herbicide/pesticide treatment. This establishes a 'baseline' of contamination in the water. The irrigation water standards for produce (not sprouts) allows for a geometric mean (average) of 126 or less CFU generic *E. coli*/100 mL water and a statistical threshold of 410 CFU or less of generic *E. coli*/100 mL water (FDA, 2015).

A study by Liao (2008) determined that sprout seeds could contain a maximum of 10^9 CFU/g sprout of bacteria. It is for this reason and other similar studies that the FDA recommends before sprouting that seeds be soaked in a solution of 20,000 ppm calcium hypochlorite for 15 minutes to reduce microorganisms (Fett, 2001). This process has been found to reduce the load of microorganisms by approximately 2.5 log CFU/g sprout depending on the variety of seed (Sikin et al., 2013). The most common post-harvest washes that are applied to grown sprouts are chlorine and organic acids, however, both antimicrobials independently reduce microorganisms by approximately 2 logs (Akbas et al., 2007; FDA, 2014; Sikin et al., 2013; Warriner et al., 2003). Pathogens that remain on the

seeds have the potential to further contaminate the sprout as it grows and develops (Sikin et al., 2013). The sprout growing process provides all of the requirements that microorganisms need to grow and replicate including optimal nutrients, acidity, moisture, oxygen, temperature and time. Studies have shown that during the growing period, alfalfa sprouts have the potential to contain 7 to 9 log CFU/g sprout of aerobic microorganisms and more than 7 log CFU/g sprout *Enterobacteriaceae* (Abadias et al., 2008; Fett, 2002; Kim et al., 2009; Kim et al., 2012).

Microorganisms that remain on sprouts after post-harvest washing may be adhered to the sprout in the form of a biofilm. Biofilms are large populations of microorganisms that have the ability to attach to each other and the surface that they cover (Yaron et al., 2014). Biofilms are generally made up of 15% microorganisms and 85% matrix that assist in carrying water, nutrients and oxygen to the individual cells (Agle, 2007). As a biofilm develops, microorganisms have the ability to detach from the conglomerate of cells if there are not enough nutrients available (Agle, 2007). However, once the biofilm reaches maturity it will become permanently attached by a series of bonds including dipole-dipole, hydrogen, ionic and covalent bonds and hydrophobic interactions between cells (Agle, 2007). At this point biofilms cannot be removed via rinsing, and only physical disruption, such as scraping is effective in removing a mature biofilm (Agle, 2007). At maturity, biofilms become stronger and more stable by the secretion of exopolymeric substances (Agle, 2007). These substances contain polysaccharides, proteins, nucleic acids and phospholipids that in conjunction prevent adsorption of

antimicrobial agents into the cells of the microorganisms (Agle, 2007). On plants, biofilms act as a protective barrier for the plant's cells from the environment and chemicals such as antimicrobials (Van Houdt et al., 2010). Fett (2000) stated that biofilms are 500 times more resistant to antimicrobial washes than individual, unattached microorganisms. This effect is due to the slow growth of cells within a biofilm compared to the more rapid growth of single cells (Agle, 2007). Since cells grow more slowly when part of a biofilm, if an antimicrobial reaches the cell, the uptake is very slow rendering it ineffective when contact time is not enough to degrade the cell(s) (Agle, 2007). The effectiveness of an antimicrobial wash on a biofilm is also dependent on the surface characteristics of the plant, temperature, contact time and bacterial resistance (Van Houdt et al., 2010). Biofilms can contain one species of microorganisms or several species. In nature, biofilms generally contain more than one species of microorganism (Agle, 2007). Multispecies biofilms have the ability to become thicker than single species biofilms and more stable, thus rendering them even less effective to antimicrobial agents than single species biofilms (Agle, 2007). Biofilms have been observed on the stems and leaves of sprout samples purchased from retail outlets (Fett, 2000). This shows that despite the regulation of sprout growing conditions and adherence to these regulations, sprouts continue to enter the market containing biofilms, which could pose a foodborne illness threat when consumed.

Additionally, pathogens have the potential to enter the interior portions of sprouts at several points during the growth and distribution process (Deering et al.,

2012). Most often, internalization of microorganisms occurs from contamination on the seed and/or irrigation water and level of contamination depends upon the type of plant, bacterial strain, source of contamination and age of the plant (Deering et al., 2012). Pathogens most commonly become internalized through the natural openings in the plant such as pores in roots or leaf stomata, cuts and bruises on the surfaces and/or adsorption through osmosis while soaking seeds, irrigating and washing (Deering et al., 2012). The vascular system within the root structure does contain protective border cells to prevent or inhibit bacterial entry; however, bacteria may still enter the roots when lateral roots emerge from the plant and thus cause a temporary opening in the root surface (Warriner et al., 2003).

Once inside the plant, pathogens move passively with the flow of water via osmosis, and they (namely *Salmonella* and *E. coli*) survive by obtaining the nutrients and moisture from the plant (Deering et al., 2012). Microorganisms are also capable of replicating to high levels given time and the elements that they need to survive in the plant system (Deering et al., 2012). Whether they are present on the outside or inside of a plant, pathogens typically grow to detectable levels within 48 hours after the sprouting process has begun (USDA, 1999). Pathogens are most commonly found on the roots of sprouts and the seed coat (Wood, 2000). Since the entire sprout is consumed raw, the internalization of pathogens is a concern because antimicrobial treatments cannot reach these pathogens and therefore can cause someone to get sick when consumed.

Previous research on the internalization of pathogens in food systems (animal, plant, or other) has most commonly been performed using bacterial cells that are genetically labeled for ease of identification (Ma et al., 2011). One of the most common methods for labeling bacteria is through the incorporation of a green fluorescent protein (GFP; Ma et al., 2011). GFP originates from a species of jellyfish and can be genetically inserted into the genetic material of pathogens, which then appears green when shown under a black light (396 nm; Ma et al., 2011). Enumeration of internal pathogens is most often performed by initially sterilizing the surface of the plant followed by tissue maceration and recovery techniques (Deering et al., 2012).

Ge et al. (2014) studied the internalization of pathogens in mung bean sprouts that were inoculated with irrigation water containing 9 log CFU/mL *Salmonella* Typhimurium. Samples were either inoculated on one day of production or each day of production. Sprouts were treated post-harvest with UV light and a chlorine wash. All inoculated sprouts demonstrated internalization of the *Salmonella* the day after inoculation, and day 7 (harvest) all inoculated sprouts contained 2.0 to 5.1 log CFU/g sprout of internalized *Salmonella*. This study shows that pathogens have the capability to enter alfalfa sprouts through the root system, and once inside they are given an optimal environment for them to grow and thrive (Ge et al., 2014). To date, the research by Ge et al. (2014) is the only published report documenting the internalization of pathogens within alfalfa sprouts in relation to the day of contamination. Although Ge et al. (2014) documented

internalization of *Salmonella* in alfalfa sprouts; these researchers did not examine internalization of other pathogens.

Thus, the purpose of this study was to determine the internal microbiological characteristics of alfalfa sprouts inoculated with non-pathogenic *E. coli* on day 1 or day 4 of growth. These days of inoculation were selected because on day 1, alfalfa seeds are beginning to open and the sprout may be beginning to emerge from the seed; and on day 4, the stems and leaves are fully developed. Inoculation of sprouts on day 1 or day 4 will provide insight into the numbers of *E. coli* recovered from alfalfa sprouts at different stages of growth.

3.3 MATERIALS AND METHODS

3.3.1 Sprout Production and Harvesting

Alfalfa seeds were purchased from a commercial source[11] that certifies the seeds as *E. coli*-free and *Salmonella*-free. These seeds were used during each of 3 replications to grow sprouts in 6 individual growth pans (40 cm x 31.8 cm x 15.2 cm; Figure 3.1). Each pan was lined with four layers of paper towels prior to growing the alfalfa sprouts. Two growth pans were assigned to each of the following treatments: no inoculation; day 1 inoculation; and day 4 inoculation. Sprouts were inoculated on either day 1 or day 4 in order to determine how the internalization of the inoculum affected the sprout when introduced on day 1 when the seed was just beginning to open and on day 4 as this was the point when the

[11] Johnny's Selected Seeds, Fairfield, ME, USA.

sprout stems and leaves were present. Alfalfa seeds (approximately 32 g per pan) were spread evenly in 6 pans using clean latex gloves. The first four days after planting, the seeds were grown in the dark with a cloth covering to omit extraneous light. On day 4, the cloth was removed and ambient lighting was introduced. Sprouts were irrigated one to two times daily with tap water until harvest as appropriate for their water needs. The only exception to this watering scheme was on the day of inoculation when 20 mL of the prepared inoculum was mixed with 30 mL of tap water and evenly sprinkled on the pan being inoculated. After seven days, two (duplicate) - 25 g samples of alfalfa sprouts were harvested from each of the 6 growth pans with clean latex gloves. Each pan produced approximately 125 to 150 g of mature alfalfa sprouts.

3.3.2 E. coli Inoculation Preparation

An inoculum of fluorescent green *E. coli*[12] was prepared to contain approximately 10^7 to 10^8 cells per mL. The inoculum was prepared 48 hours prior to use for each replication by adding 10 uL of fluorescent green *E. coli* inoculum to each of six sterile test tubes containing 10 mL of sterile tryptic soy broth[13] and 1.0 mg of ampicillin[14]. The solutions were mixed and incubated at 37°C for 48 hours. The solutions were vortexed two times per day during incubation. On the day of the

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[12] Non-pathogenic *Escherichia coli* ampicillin-resistant strain JM109 labeled with jellyfish green fluorescent protein. Maintained and cultured according to Jiang et al. (2002).
[13] Fisher Scientific, Pittsburgh, PA, USA.
[14] Ampicillin Sodium Salt, Fisher Scientific, Pittsburgh, PA, USA.

inoculation, the solutions were transferred to sterile centrifuge tubes and centrifuged[15] at 4,000 rpm (1,935 x g) for 10 minutes. After centrifugation, the tryptic soy broth was removed with a pipette. Ten milliliters of a sterile 0.1% peptone solution was added to each centrifuge tube, the tubes were then mixed and centrifuged at 4,000 rpm (1,935 x g) for 10 minutes to wash the bacterial pellet. The bacterial pellet was washed twice each time before use. After the second wash, 10 mL of 0.1% peptone was added, and the solution was then divided into two sterile centrifuge tubes. The optical density[16] of both inoculums were read at 600 nm to estimate concentration and the dilution adjusted with peptone until the absorbance was approximately 0.45 (10^7 to 10^8 cells per mL). Serial dilutions of the inoculum were prepared and plated in duplicate on APC agar with ampicillin to confirm the inoculum concentration. Plates of inoculum were inverted and incubated at 37°C for 48 hours before the colony forming units (CFU) were counted under a black light to enumerate the fluorescent green CFUs.

3.3.3 E. coli Inoculation Application

Two pans of alfalfa seeds were inoculated on day one (day after planting) and two separate pans were inoculated on day four. The remaining two pans were not inoculated and were labeled as the control. This was repeated for a total of three replications. On the day of each inoculation, 20 mL of inoculum was combined with

[15] Beckman Coulter Avanti J-26S XPI Centrifuge, Chaska, MN, USA.
[16] Thermo Scientific Genesis 10S UV-VIS Spectrophotometer, Brookfield, WI, USA.

30 mL of tap water and this mixture evenly sprinkled onto approximately 31.5 g of alfalfa seeds. Alfalfa sprouts were harvested after 7 days of growth.

3.3.4 Microbiology

At harvest, two – 25 g samples of sprouts were collected from each pan, placed into individual sterile filter stomacher bags with 225 mL of sterile 0.1% peptone, and stomached[17] for 1 minute at 230 RPM. Serial dilutions of the rinsate were then prepared and plated on APC agar that contained 200 ppm ampicillin. The plates were inverted and incubated at 37°C for 48 hours. After incubation the plates were counted under a black light to enumerate the fluorescent green CFUs. The CFUs were then calculated to report the CFU/g and were converted to $\log_{10}$ CFU/g sprout.

To enumerate the *E. coli* that may remain on the outside and inside of the sprouts, the 25 g samples used previously were recovered from the peptone. Each sample was sprayed with > 95% ethanol[18] and allowed to air dry (approximately ½ hour) under a biological laminar flow hood. After drying, sprouts were then placed into clean blender jars with 100 mL of sterile 0.1% peptone and blended on low speed for 5 seconds. Serial dilutions were then prepared of the mixture and the dilutions were plated on APC agar containing 200 ppm ampicillin. The plates were inverted and incubated at 37°C for 48 hours. After incubation the plates were

[17] Seward, Stomacher® 400 Circulator, Worthing, West Sussex, UK.
[18] Pharmco-AAPER, Brookfield, CT, USA.

counted under a black light to enumerate the fluorescent green CFUs. The CFUs were then calculated to report the CFU/g and were converted to $\log_{10}$ CFU/g sprout.

3.3.5 Spent Irrigation Water Microbiology

At the time of harvest, residual water from each growth pan was transferred to sterile sample cups. Serial dilutions of the irrigation water were prepared and plated on APC agar containing 200 ppm ampicillin. The plates were inverted and incubated at 37°C for 48 hours. After incubation the plates were counted under a black light to enumerate the fluorescent green CFUs. The CFUs were then calculated to report the CFU/mL and were converted to the average $\log_{10}$ CFU/mL water.

3.3.6 Recovery of E. coli After Sterilization

To test the effectiveness of the ethanol on sterilizing the outside of the alfalfa sprouts, an independent trial was performed (Figure 3.2). Alfalfa sprouts were grown using the same methods as previously stated with inoculation again performed on day 1, day 4 or non-inoculated (control), again using *E. coli*. At harvest, samples of alfalfa sprouts were collected and sprayed with > 95% ethanol. The sprouts were then allowed to air dry for approximately ½ hour under a biological laminar flow hood. Sprouts were then placed in sterile filter stomacher bags with 225 mL sterile 0.1% peptone and stomached at 230 RPM for 1 minute. Serial dilutions of the rinsate were prepared in duplicate and plated on APC agar with 200 ppm ampicillin. The same sprouts were then tested to enumerate the *E.*

coli that had become internalized plus any residual exterior bacteria. Samples were recovered from the peptone after the first exterior rinse, placed in clean blender jars with 100 mL 0.1% sterile peptone, blended on low speed for 5 seconds and transferred to sterile filter bags. Serial dilutions of the mixture were prepared in duplicate and plated on APC agar with 200 ppm ampicillin. All plates were inverted and incubated at 37°C for 48 hours. After incubation the plates were read under a black light to enumerate the fluorescent green CFUs. The CFUs were then calculated to report the average $\log_{10}$ CFU/g sprout for each treatment.

3.3.7 Statistics

Data were statistically analyzed using the General Linear Model procedure of SAS (version 8.2), with the main effects of the model being treatment (inoculation day 1, day 4, no inoculation) and replication. Means were separated using least mean square with significance of $P < 0.05$ (SAS Institute, 2000).

3.4 RESULTS AND DISCUSSION

Approximately 4.6 log CFU/g sprout of *E. coli* were recovered from the exterior of sprouts inoculated on d1 (Table 3.1). When inoculated on d4, approximately 3.5 log CFU/g sprout were recovered from the exterior of the sprouts, and this recovery was lower than that of the d1 inoculated sprouts ($P<0.05$). After treatment with ethanol, counts of *E. coli* recovered from macerated sprouts were 0.4 to 0.5 log units lower than those recovered from the exterior rinse;

however the overall pattern of higher counts with an earlier inoculation (day 1) remained.

On the day of harvest (day 7), irrigation water remaining in the growth pans was tested for the recovery of *E. coli*. Approximately 5.2 log CFU/mL of *E. coli* was recovered from the irrigation water of sprouts inoculated on d1, while approximately 1.5 log less (5.2 versus 3.7 log CFU/mL) was recovered from irrigation water of sprouts inoculated on d4. In each case, water contained higher than or equal to the counts of *E. coli* observed on the outside of sprouts. These results are different from those found by Fu et al. (2001) in which spent irrigation water used to water sprouts generally had a 1 log decrease in pathogens compared to the number of pathogens on the exterior surface of the sprouts. This inconsistency demonstrates that using spent irrigation water to quantify the presence of pathogens on sprouts may not be a reliable method of determination. Testing spent irrigation water could be an effective positive or negative indicator of microorganisms, but should not be held reliable for continued accuracy.

Data in Table 3.1, shows that either ethanol may not be removing all of the surface *E.* coli or nearly all of the bacteria are becoming internalized. Thus, a second experiment was conducted to determine the number of *E. coli* recovered on the outside after treatment with ethanol. In this experiment sprouts were inoculated d1 or d4 and grown for 7 days (Table 3.2). *E. coli* was then recovered from the outside of the sprouts after ethanol treatment. These same sprouts were then macerated and plated to recover the remaining bacterial cells. Approximately 4.3 log CFU/g

sprout of *E. coli* were recovered from the exterior of sprouts inoculated on d1 grown and treated with ethanol. When inoculated on d4, grown and treated with ethanol, approximately 3.5 log CFU/g sprout were recovered from the exterior of the sprouts, and this recovery was lower than that observed on sprouts inoculated on d1 ($P<0.05$). After maceration, counts of *E. coli* recovered from sprouts inoculated on d1 were approximately 0.1 log higher than those recovered from the exterior rinse. Macerated sprouts inoculated on d4 had a 0.4 log decrease in recovered *E. coli* from the exterior rinse. This suggests that the maceration although mild may have resulted in cell damage. This data provides evidence that *E. coli* remains on the outside of the sprout after sterilization with ethanol. Sterilization may have been improved with some degree of agitation to disrupt biofilms.

To ensure that the GFP on the *E. coli* remained stable throughout the progress of the study, the first two inoculations prepared were re-plated in duplicate on APC agar with 200 ppm ampicillin every 48 hours and incubated at 37°C. Prior to re-plating, each plate was observed under a black light for the presence of the fluorescent green *E. coli*. After confirmation of the GFP producing *E. coli*, approximately 10 ul of *E. coli* was transferred and streaked on a new plate. A study by Ma et al. (2011) showed that *E. coli* that is labeled with GFP is stable through multiple generations. Ma et al. (2011) found that the protein remained stable through approximately 20-50 generations depending on the bacteria strain. In the present study, E. coli was found to retain its fluorescence for 10 generations.

3.5 CONCLUSION

Under the conditions of this study, early contamination (day 1 of production) resulted in higher post-harvest numbers of pathogens than sprouts inoculated on day 4. Furthermore, treatment with ethanol did not kill all surface pathogens (approximately 0.3 to 0.7 log reduction). Additionally, the possible presence of biofilms on alfalfa sprouts makes the enumeration of internal microorganisms difficult because it is unclear as to whether or not the biofilm regenerated. The study results reveal that due to potential biofilms, sterilization of the outside of the sprouts cannot be achieved without some additional hurdle (agitation, heat, or other). Recovery patterns of *E. coli* allude to the presence of internalized *E. coli*, however estimating the internalization could not be effectively calculated in this study. Therefore, considering the ineffectiveness of the ethanol at reducing levels of *E. coli* to safe numbers and the likely presence of internalized *E. coli* that cannot be treated with a post-harvest antimicrobial treatment, it can be concluded that sprouts no matter when contamination occurs, can contain enough pathogens in the form of biofilms or internalized, to make someone sick if the sprouts are consumed raw.

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Table 3.1 Fluorescent green *E. coli*[1] recovered from inoculated alfalfa sprouts after 7 days of growth and their water source

	E. coli Recovery Location[2]		
Day of Inoculation	Average Outside Sprout[3]	Average Macerated Sprout[4]	Average Spent Irrigation Water
Control	ND[5]	ND	ND
Day 1	$4.6^{y} \pm 0.1$ (4.0-5.3)	$4.2^{y} \pm 0.1$ (3.5-5.1)	$5.2^{y} \pm 0.1$ (4.6-5.9)
Day 4	$3.5^{z} \pm 0.2$ (3.0-4.2)	$3.0^{z} \pm 0.2$ (2.6-3.2)	$3.7^{z} \pm 0.3$ (2.0-4.9)

[1]*E. coli* associated with sprouts reported as $\log_{10}$ CFU/g. *E. coli* associated with water reported as $\log_{10}$ CFU/mL

[2]Number of samples tested among inoculations was n=6.

[3]Recovery from sprout samples before sterilization with ethanol.

[4]Macerated sprout is the $\log_{10}$ CFU/g of *E. coli* recovered from sprout samples that were sterilized with >95% ethanol on the outside, blended and the mixture plated for enumeration.

[5]ND= not detected

[y-z]Means ± the standard error of the mean with no common superscript within a column are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of *E. coli*.

Table 3.2 Fluorescent green *E. coli*[1] recovered from inoculated alfalfa sprouts after 7 days of growth to validate effectiveness of ethanol sanitizer applied after harvest

	E. coli Recovery Location[2]	
Day of Inoculation	Average Outside Sprout[3]	Average Macerated Sprout[4]
Control	ND[5]	ND
Day 1	$4.3^{y} \pm 0.3$ (3.6-4.9)	$4.4^{y} \pm 0.1$ (4.2-4.5)
Day 4	$3.5^{z} \pm 0.2$ (3.0-3.9)	$3.1^{z} \pm 0.1$ (2.6-3.7)

[1]*E. coli* associated with sprouts reported as $\log_{10}$ CFU/g.
[2]Number of samples tested among control and day 1 inoculations was N=2. Number of samples tested among day 4 inoculations was N=4.
[3]Recovery from sprout samples that were sanitized with >95% ethanol.
[4]Macerated sprout is the $\log_{10}$ CFU/g of *E. coli* recovered from sprout samples that were blended and the mixture plated for enumeration.
[5]ND = not detected
$^{y\text{-}z}$Means ± the standard error of the mean with no common superscript within a column are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range in counts of *E. coli*.

Figure 3.1 Experimental design to study the partitioning of *E. coli* contamination on alfalfa sprouts inoculated at day 1 or 4 during production.

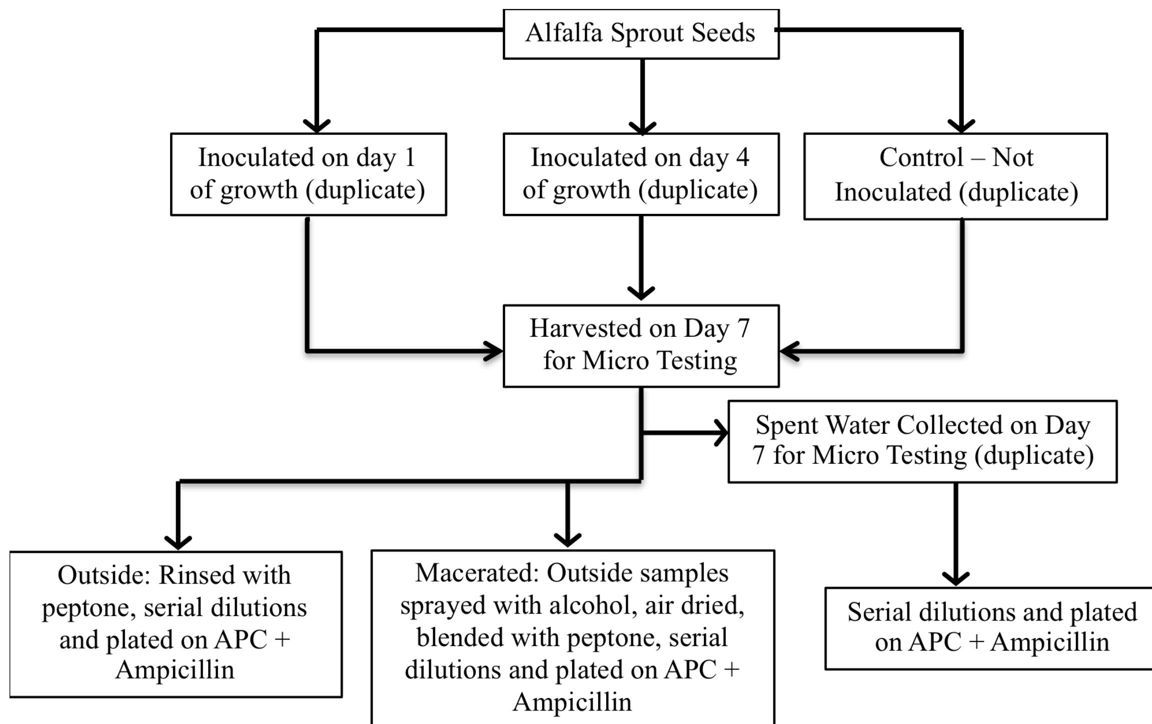
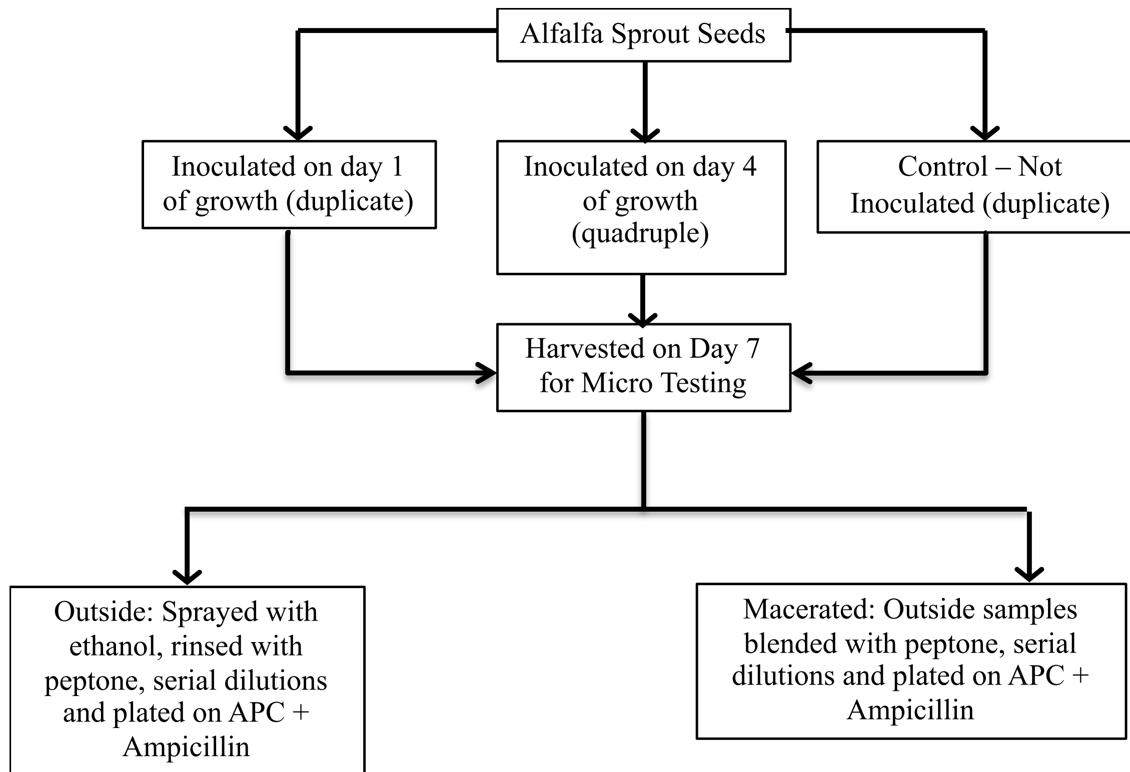


Figure 3.2 Experimental design to evaluate the effectiveness of ethanol sanitizer applied to the outside of alfalfa sprouts inoculated with *E. coli* on day 1 or 4 during production.



CHAPTER FOUR

QUALITY CHARACTERISTICS OF ALFALFA AND MUNG BEAN SPROUTS IRRIGATED WITH DIFFERENT WATER SOURCES

4.1 ABSTRACT

A study was conducted to determine the nutritional quality characteristics of alfalfa and mung bean sprouts grown in municipal or aquaponics water for 7 days. Sprouts were analyzed for total moisture, ash, protein, carbohydrate, fat, vitamin C and mineral composition (phosphorus, potassium, calcium, magnesium, zinc, copper, manganese, iron, sulfur, sodium and nitrate-nitrogen). Results were reported on a dry weight basis. Total moisture of alfalfa sprouts was approximately 39 to 40%, and protein was about 48 to 49%. Fat, carbohydrate and ash for alfalfa sprouts was 5 to 6%, 39% and 5 to 6%, respectively. Mung beans contained a similar amount of moisture (41%) and ash (4 to 6%), but had lower protein (29 to 30%), and fat (~1%), and higher carbohydrate (57 to 58%) than alfalfa sprouts. Micronutrients were also evaluated and were found to be significantly affected by the water source during production, for both alfalfa and mung bean sprouts. In both alfalfa and mung bean sprouts irrigation with aquaponic water resulted in lower levels of vitamin C and copper, but higher levels of calcium and nitrate-nitrogen than those sprouts irrigated with municipal water. Additionally, alfalfa sprouts had a higher content of phosphorus, potassium, and magnesium when grown in

aquaponics water. Statistical analysis of the data demonstrated that water source during production did not alter the proximate composition (macronutrients) of alfalfa or mung bean sprouts. However, micronutrient composition was affected in both alfalfa and mung bean sprouts. While vitamin C content increased in both alfalfa and mung bean sprouts grown in tap water, patterns of mineral content between alfalfa and mung bean sprouts were significantly different. Therefore, based on the results of this study, there is evidence that the source of irrigation water does not influence the macronutrients of sprouts, but does influence the micronutrient composition.

4.2 INTRODUCTION

Sprouts, which are the immature plant that emerges from the seed with thin stems and two small immature leaves, are typically harvested between 1 and 8 days depending on the variety, maturity and size desired (DeEll, 2014). The International Sprout Growers Association has indicated that approximately 10% of Americans eat sprouts regularly, and most consume their sprouts raw on salads and sandwiches to add a crisp texture, or lightly cooked in dishes such as stir-fries (Sikin et al., 2013). Plants consumed as sprouts are primarily chosen for their flavor, which can vary from nutty to spicy (DeEll, 2014). The most popular varieties of sprouts include alfalfa, mung bean, red clover, radish, broccoli and wheatgrass (Oregon Public Health Division et al., 2015).

In the U.S., alfalfa sprouts are the most commonly sprout consumed, but mung bean sprouts are the most commonly consumed sprouts worldwide (Oregon Public Health Division et al., 2015). Both alfalfa and mung beans are categorized as a legume, which in the category of food also includes peas, beans, lentils and peanuts (Higdon et al., 2009). Legumes are agriculturally beneficial because of their ability to generate and deposit nitrogen into the soil (USDA, 1998). This process occurs as the legume germinates and matures. Once roots develop, nitrogen specific rhizobacteria infiltrate the plant's roots, multiply, and develop nodules within the roots that are high in nitrogen (USDA, 1998). Nodules on legumes subsequently decay and release nitrogen into the surrounding soil (USDA, 1998). This characteristic also results in high protein concentration in legumes from the excess nitrogen produced during the plant's growth (USDA, 1998).

Typically, most plants require carbon, oxygen, hydrogen, nitrogen, potassium, calcium, magnesium, phosphorus, sulfur, chlorine, iron, manganese, boron, zinc, copper and molybdenum for optimal growth (Rakocy et al., 2006). In traditional farming, these nutrients are provided by the soil, added fertilizers, water and natural bacteria of the soil (Rakocy et al., 2006). In an aquaponics system, nutrients may be obtained from the water which contains, carbon dioxide, fish waste, rhizobacteria, algae and decaying fish feed (Rakocy et al., 2006).

Plant seeds contain most of the nutrients that a plant needs to germinate and mature. Because sprouts are harvested just after germination, the developing plant has not utilized much of these nutrients, and thus the concentration of these

nutrients remains higher than the mature plant of the same variety (Márton et al., 2010). Research has reported that sprouts also contain significant levels of phytochemicals that may help in protecting humans against cancer and chronic diseases (Sikin et al., 2013).

During germination, the natural metabolic process of the seed utilizes carbohydrates, proteins and fats for energy to generate the new plant (Masood et al., 2014). In this process, polysaccharides are broken down to oligo- and monosaccharides; fats are broken into free fatty acids, and proteins are broken down to oligopeptides and free amino acids (Márton et al., 2010). The growing environment (temperature, humidity, light and growing time) as well as the variety of sprout can result in variation in the proximate composition of the sprout (Masood et al., 2014).

According to the current Recommended Dietary Allowance (RDA), protein requirements are 46 and 56 g/d for adult women and adult men, respectively (IOM, 2005). The RDA for fiber is 25 g/d for women ages 19-50 and 38 g/d for men ages 19-50 (IOM, 2005). According to the RDA for vitamin C, adult women (>19 years) should consume 75 mg/d and adult men (>19 years) should consume 90 mg/d (IOM, 2000). The RDA for iron is 18 mg/d for women between the ages of 19 and 50 and 8 mg/d for men over 19 years (IOM, 2001). Men and women between the ages of 19 and 70 should consume 1,000 mg/d calcium (IOM, 2011). For individuals that choose to be vegetarians, legumes may serve as an alternative protein source. Depending on calorie needs (1,000 – 3,200 calories per day) the 2015-2020 Dietary

Guidelines for Americans recommends that vegetarians consume 1 to 6 cups of legumes per week (USDHHS et al., 2015).

Previous literature states that sprouts provide a good source of protein, vitamins and minerals (Oregon Public Health Division et al., 2015). However, the FDA defines a good source to be a food that contains 10-19% of the daily value per RACC (Reference Amount Commonly Consumed; FDA, 2013a). The RACC for sprouts is 10 g (FDA, 2013b). The USDA National Nutrient Database (2016a) stated that sprouted alfalfa seeds contains 3.99 g/100g protein, 8.2 mg/100g (82 ppm) vitamin C, 32 mg/100g (320 ppm) calcium, 0.96 mg/100g (9.6 ppm) iron, 27 mg/100g (270 ppm) magnesium, 70 mg/100g (700 ppm) phosphorus, 79 mg/100g (790 ppm) potassium, 6 mg/100g (60 ppm) sodium, and 0.92 mg/100g (9.2 ppm) zinc. Similarly, sprouted mung bean seeds contains 3.04 g/100g protein, 13.2 mg/100g (132 ppm) vitamin C, 13 mg/100g (130 ppm) calcium, 0.91 mg/100g (9.1 ppm) iron, 21 mg/100g (210 ppm) magnesium, 54 mg/100g (540 ppm) phosphorus, 149 mg/100g (1490 ppm) potassium, 6 mg/100g (60 ppm) sodium and 0.41 mg/100g (4.1 ppm) zinc (USDA, 2016b). Based on this data and the definition of a good source, sprouts are not good sources of protein, vitamins and minerals.

In 2009, Hong et al. reported that the proximate composition of freeze dried alfalfa sprouts was 10.6% water, 49.6% protein, 2.6% fat, 33.6% carbohydrate and 3.7% ash. Dahiya et al. (2014) reported that mung bean sprouts contained 617 ppm iron, 247 ppm zinc, and 13557 ppm calcium. Another study found that protein

levels in mung beans can vary from approximately 19.5 to 31.3% (Masood et al., 2014). Since total solids and total moisture comprise the composition of foods, and proteins makes up a portion of the total solids, the variation reported by Masood et al. (2014) may explain previous discrepancies of data. These same researchers found that as the mung bean sprouts grew, the fat content decreased and protein content increased (Masood et al., 2014). The protein content likely increases as the sprout grows because of the production of new proteins during growth and development (Masood et al., 2014).

Seeds typically contain little to no ascorbic acid (vitamin C), however, the content of vitamin C in the sprout will increase rapidly as the seed begins to grow and plant cells are developed (Masood et al., 2014). For example, soybean sprouts have been found to have a 200-fold increase in vitamin C content compared to the composition of the seed before sprouting (Márton et al., 2010). Masood et al. (2014) determined that mung beans, which sprouted after 120 hours (5 days) of growth, contained approximately 28.50 mg/100 g (285 ppm) ascorbic acid. Vitamin C is required in plants for proper growth and development, action as a co-factor for several essential enzymes and antioxidant properties (Zhang, 2013). Vitamin C also functions to protect plants from stress caused by lack of water, salt, extreme temperature fluctuations, heavy metals, insects, pathogens and disease (Zhang, 2013). The level of vitamin C in a plant can be directly related to the stress on the plant (Zhang, 2013). During photosynthesis, vitamin C is an essential cofactor in reactions that are catalyzed by copper and iron that assist in the development of the

cell wall (Zhang, 2013). However, vitamin C plays a critical role in plants by acting as an antioxidant. Vitamin C is a strong reducer, which has the ability to be oxidized into a radical state or oxidized twice to dehydroascorbate, which is stable (Zhang, 2013). Radicals are detrimental to plants and animals as they can cause damage to nucleic acids, proteins and lipids and are often formed during times of stress (Zhang, 2013). When radicals are present, vitamin C reacts with them to transfer an electron and therefore stop the damaging interactions caused within the plant (or animal; Zhang, 2013). Vitamin C also protects plants when excess heavy metals are present, as excess metals can generate free radicals (Zhang, 2013). Vitamin C is produced in plant cells and is oxidized by ascorbate oxidase into dehydroascorbic acid (Lee et al., 2000). Ascorbate oxidase contains copper and is found primarily in the plant tissues that grow quickly in early development (Zhang, 2013). It is reported that ascorbate oxidase will increase in a plant when stress levels are high (Lee et al., 2000). Zhang (2013) reported that plants grown hydroponically, with low levels of nutrients in the irrigation water, are shown to increase vitamin C levels. Additionally, previous research has stated that when nitrogen is added to growing plants, vitamin C levels in the plant will decrease (Lee et al., 2000; Zhang, 2013). Levels of vitamin C have also been shown to decrease when plants are infected with pathogenic bacteria due to altered metabolism of vitamin C caused by exopolysaccharides produced from the bacteria and an increase in hydrogen peroxide production in plant cells that require vitamin C to oxidize for the safety of the plant (Zhang, 20130).

With the exception of the USDA National Nutrient Database, there is no known scientific literature that reports the mineral composition of mung bean or alfalfa sprouts. A review of literature by Aires et al. (2007) found that the mineral content (N, P, S, K, Ca, Mg, Na, Cl, Si) of broccoli sprouts was higher than that of mature broccoli; and when fertilized the N, S, K and Cl content will rise and the Ca, Mg, P and Na content will decrease. A similar finding may occur in alfalfa and mung bean sprouts.

Considering the limited research regarding the nutritional quality characteristics of sprouts, particularly of those, which are grown in an aquaponics system, this study was conducted to determine if there is a difference in the nutritional quality characteristics of fresh sprouts grown hydroponically and aquaponically.

4.3 MATERIALS AND METHODS

4.3.1 Sprout Production and Harvesting

Alfalfa and mung bean seeds were purchased from a commercial source[19]. During each of three replications, 8 growth pans (40 cm x 31.8 cm x 15.2 cm) were lined with four layers of paper towels and were used to grow either alfalfa or mung bean sprouts (Figure 4.1). Mung bean seeds (approximately 147 g of seeds per pan) or alfalfa seeds (approximately 32 g of seeds per pan) were spread evenly in 8 pans. Two pans of mung beans and two pans of alfalfa were irrigated with tap water. The

[19] Johnny's Selected Seeds, Fairfield, ME, USA.

remaining pans of alfalfa and mung bean seeds were irrigated with fresh aquaponics water obtained from an aquaponics system located at Clemson University[20]. The first four days of growth the seeds were grown in the dark with a cloth covering to omit extraneous light. On day 4, the cloth was removed and introduced to light. Sprouts were irrigated one to two times daily until harvest as appropriate for their water needs with either the designated tap or aquaponics water. Clean latex gloves were used to handle seeds and water to reduce the possibility of contamination. After seven days, mung bean and alfalfa sprouts were harvested using clean latex gloves, pooled according to treatment (type of sprout and water source) and divided into samples for quality analysis.

4.3.2 Sample Preparation

After harvest, samples were divided for further compositional analysis. Approximately 25 g of each sample was retained for moisture and ash analysis. Approximately 80-100 g alfalfa sprout and 100-150 g mung bean sprout samples were dried using the convection oven drying method (105°C for 18-24 hours), placed in labeled sample bags and frozen at -80°C until further analysis. Prior to analyses, samples were thawed by drying at 105°C for 30 minutes to remove any moisture that may have developed during frozen storage. Samples were tested to

[20] Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.

determine mineral, nitrate-nitrogen, protein and fat content. The remaining (fresh) sprout samples were placed in labeled sample bags and frozen at -80°C for use in Vitamin C determination.

4.3.3 Total Moisture

The percent moisture of the sprout samples was analyzed using the AOAC 925.10 convection oven drying method (AOAC, 1990). Briefly, this method involved weighing approximately 15-20 g alfalfa sprouts and approximately 17-19 g mung bean sprouts into clean, labeled, preweighed crucibles. The weight of each sample (crucible and wet sprout sample) was first recorded, and then placed into a convection drying oven set at 105°C for 18-24 hours. After removal from the oven, samples were cooled to room temperature in desiccators, reweighed (crucible and dried sprout sample) and the weight recorded. The percent moisture was calculated using the following formula:

$$\%\ \text{Moisture} = \frac{\text{g wt of wet sample} - \text{dry weight}}{\text{g wt of wet sample}} \times 100$$

4.3.4 Ash

Ash was determined using the AOAC 900.02 method (AOAC, 1990). After weighing crucibles containing dried sprouts for moisture determination, samples were placed into a muffle furnace and set to 550°C. After 18-24 hours, crucibles were removed and cooled to room temperature in desiccators. Samples were then removed and their weights (crucible and ash sprout sample) recorded. Weights of

the ash sample and dry sample were adjusted to remove the weight of the crucible and the percent ash was then calculated using the following formula:

$$\% \text{ Ash} = \frac{\text{g wt of ash sample}}{\text{g wt of dry sample}} \times 100$$

4.3.5 Compositional Analyses

Clemson University's Agricultural Services Laboratory[21] conducted the analysis for the determination of mineral composition. Sprout samples were analyzed for phosphorus, potassium, calcium, magnesium, zinc, copper, manganese, iron, sulfur and sodium content using an inductive coupled plasma method. To accomplish this, a wet ashing procedure was used with nitric acid (HNO_3) and 30% hydrogen peroxide (H_2O_2). For this method, approximately 0.5 g of each dried sprout sample was placed into 100 mL digestion tubes, and 5 mL of HNO_3 was then added to each sample to predigest the sample for 30 minutes. The digestion tubes were then heated on a heating block at 125°C for 1.5 hours, cooled, and 3 mL of 30% H_2O_2 was slowly added to each sample. Samples were then placed back on the digestion block, heated at 125°C for 1 hour, cooled to room temperature, and an additional 3 mL of 30% H_2O_2 was added to each sample. Samples were heated at 125°C for an additional hour, and then the temperature was increased to 200°C for 1 hour to complete the drying of the samples. After removal from the heating block, 10 mL of 1:10 HNO_3 was added to each sample followed by cooling for 15 minutes. Cooled samples were diluted to 50 mL with deionized water, capped and shaken

[21] Clemson, SC, USA.

vigorously to mix. Aliquots of each prepared sample was transferred to inductively coupled plasma (ICP) tubes and read by mass spectrometry[22] using a laterally diffused metal oxide semiconductor technology.

4.3.6 Nitrate-Nitrogen

Clemson University's Agricultural Services Laboratory conducted the analysis for the determination of nitrate (NO_3) nitrogen (N) content using a specific ion electrode method. An extraction solution was prepared by combining 173.2 g aluminum sulfate ($Al_2(SO_4)_3$), 12.8 g boric acid (H_3BO_3), 0.7218 g potassium nitrate (KNO_3) and 25.2 g sulfamic acid (NH_2SO_3H) with 3.0 L deionized water. This extraction solution was then diluted to 10.0 L with deionized water and the pH adjusted to 3.0 using approximately 15 mL sodium hydroxide (NaOH). Two g of each dried sprout sample was weighed, placed into a 100 mL beaker and 50 mL of extraction solution was added to each beaker. After 30 minutes, the calibrated electrode was used to measure NO_3-N in each sample. Each reading was multiplied by 10 to account for the dilution and recorded as ppm.

4.3.7 Fat

Clemson University's Agricultural Services Laboratory conducted the analysis for the determination of percent fat using the Soxhlet method (AOAC 920.39; Min et al., 2010). Approximately 1.0 g of sample was placed into prelabeled,

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[22] Spectro ARCOS ICP-OES Analyzer, Model FHS12, Mahwah, NJ, USA.

weighed filter bags and the weight (filter bag and sprout sample) was recorded (weight 1). Each filter bag was heat sealed and placed in a drying oven for 3 hours at 105°C. After drying, bags were cooled in desiccant pouches and then their weights recorded (weight 2). Filter bags containing samples were then placed in the extraction chamber with ether for 5 to 10 minutes. In the chamber, the ether solvent soaked into the sample and siphoned fat into the boiling flask. Once the extraction was complete, samples were placed in the drying oven for 30 minutes at 105°C. After drying, each sample bag was weighed and recorded (weight 3) to determine fat content by weight loss of the sample (Min et al., 2010). The percent fat was then calculated using the following formula:

$$\% \text{ Fat} = \frac{(\text{weight 2} - \text{weight 3})}{\text{weight 1}} \times 100$$

4.3.8 Protein

Clemson University's Agricultural Services Laboratory conducted the analysis for the determination of total nitrogen using the Dumas combustion method[23]. The nitrogen combustion analyzer (Dumas) was calibrated using three samples of ethylenediaminetetraacetic acid (EDTA), which contains a standard 9.57% nitrogen. After calibration, approximately 0.1 g of each dried sprout sample was weighed into an aluminum foil tube. Samples were placed into the analyzer, heated to combustion at 850°C and the instrument's thermal conductivity detector

[23] LECO FP528 Nitrogen Combustion Analyzer, St. Joseph, MI, USA.

measured the nitrogen. Protein content was then calculated by multiplying each sample's nitrogen level by 6.25 conversion factor.

4.3.9 Carbohydrate

Total carbohydrate content of each sprout sample was calculated by adding the percent protein, fat and ash of each sample and subtracting this value from 100%.

4.3.10 Vitamin C

Vitamin C content of the samples was conduced by a private laboratory[24], using the HPLC (AOAC 967.22) method of determination. This method measures the presence of the fluorescent quinoxaline compound that is produced by the oxidation of ascorbic acid to dehydroascorbic acid by the addition of an o-phenylenediamine reagent (Pegg et al., 2010).

4.3.11 Statistics

Data were statistically analyzed using the General Linear Model procedure of SAS (version 8.2), with the main effects of the model being treatment, replication and type of sprout. Means were separated using the least mean square option with significance of $P < 0.05$ (SAS Institute, 2000).

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[24] Merieux NutriSciences, Silliker, Illinois Laboratory, Crete, IL, USA.

4.4 RESULTS AND DISCUSSION

Proximate composition, vitamin C and mineral content were measured in alfalfa and mung bean sprouts grown in municipal tap water and aquaponics water for 7 days (Table 4.1 and 4.2). Source of water (municipal or aquaponic) had no effect on the proximate composition (protein, fat, carbohydrate and moisture) of alfalfa and mung bean sprouts, except for ash, which was higher (1.3% and 1.7%, respectively) in both sprouts grown in aquaponics water. Minerals not affected by water source (municipal or aquaponics water) in alfalfa sprouts were zinc, manganese, iron, sulfur and sodium. Furthermore, water sources did not affect phosphorus, potassium, magnesium, zinc, manganese, iron, sulfur or sodium content in mung beans (Table 4.2).

For alfalfa sprouts, moisture ranged from 39-41%, protein ranged from 48-49%, fat ranged from 5-6% and carbohydrate was 39% regardless of the irrigation water source. The higher ash content (6.1% versus 4.8%) observed with aquaponics water may be due to the high levels of minerals found in aquaponics water, such as phosphorus and nitrogen, which contribute to ash content (Roosta et al., 013).

The proximate composition analysis of alfalfa sprouts observed in this study were similar to those found by Hong et al. (2009). Alfalfa sprouts grown in aquaponics water (13.8 ppm, Vitamin C) had a 48% decrease in vitamin C content compared to sprouts grown in tap water (26.5 ppm). Based on the reports of Zhang (2013) and Lee et al. (2000) this decrease is likely due to the bacterial stress placed on the aquaponics water grown sprouts due to the natural presence of bacteria in

aquaponics systems. In this situation, vitamin C in aquaponically grown sprouts is being utilized to oxidize free radicals in order to protect itself. Additionally, as previous research has reported, plants that are fertilized with nitrogen (such as the high nitrogen content naturally found in aquaponics water) causes a decrease in vitamin C content (Zhang, 2013; Lee et al., 2000).

Copper was the only mineral that had a decrease (41%) in content in the sprouts grown in aquaponics water. This may be caused by copper adsorption from the rhizobacteria and algae that are naturally present in aquaponics systems and also require copper for proper growth and development (Yruela, 2009). Furthermore, copper is found in ascorbate oxidase the enzyme that increases during plant stress to reduce vitamin C to dehydroascorbic acid (Zhang, 2013). Considering the reduction of vitamin C levels in aquapnically grown sprouts compared to those grown in municipal tap water may reveal this relationship between vitamin C and copper in stressed sprouts. Alfalfa sprouts grown in aquaponics water had a 5% increase in phosphorous, 18% increase in potassium, 34% increase in calcium, 16% increase in magnesium and 91% increase in nitrate-nitrogen compared to alfalfa sprouts grown in municipal water. Compared to the USDA National Nutrient Database (2016a) for alfalfa sprouts, vitamin C had significantly higher values, and mineral content significantly lower values than the results of the present study. This difference may be due to the unknown age of the alfalfa sprouts analyzed for the USDA database. Based on the research by Márton et al. (2010) and Aires et al. (2007), in which vitamin C content may increase and mineral content may decrease

as the plant ages, sprouts analyzed for the USDA database were possibly harvested past 7 days. Therefore, younger plants or sprouts may be more nutrient dense compared to those that are harvested at a more mature stage of growth.

Production water source (municipal water and aquaponics water) did not affect the proximate composition (moisture, protein, fat and carbohydrate) of mung bean sprouts (Table 4.2). The protein content of mung bean sprouts tested during the present study was similar to that reported by Masood et al. (2014; 29-30% versus 20-31%). Ash content of mung beans grown in aquaponics water was 1.7% higher than those grown in municipal water. The vitamin C content was 56% lower in the aquaponically grown mung bean sprouts, which is likely the same cause as the vitamin C decrease in the aquaponically grown alfalfa sprouts.

Minerals that were not significantly different between treatments included: phosphorus, potassium, magnesium, zinc, manganese, iron, sulfur and sodium. Similar to the alfalfa sprouts, there was a decrease in copper (20%) in the mung bean sprouts grown in aquaponics water. There was a 21% increase in calcium in the mung bean sprouts grown in aquaponics water. Mung beans grown in aquaponics water had a significantly higher concentration of nitrate-nitrogen than those grown in municipal water (244 ppm versus 81 ppm). This difference is likely due to the nitrate and nitrogen produced in the aquaponics system from the rhizobacteria (Roosta et al., 2013). The vitamin and mineral composition for mung bean sprouts reported in the USDA nutrient database was higher for vitamin C while the mineral content was lower than the values observed in the present study. The

USDA National Nutrient Database (2016b) does not report the age of the mung bean sprouts at the time of analysis, which may be different from 7 days and thus could explain the differences. Márton et al. (2010) reported that soybean sprouts have a 200-fold increase in vitamin C when comparing the seed versus the sprout, and Aires et al. (2007) reported that broccoli sprouts have a higher mineral content than mature broccoli. These reports could explain the variable composition of mung bean sprouts depending on the maturity of the sprouts at harvest.

An independent researcher analyzed the tap water and aquaponics water collected during the present study and used for irrigating the alfalfa and mung bean sprouts. This researcher found that the aquaponics water contained approximately 1.14 mg/L nitrogen and 4.40 mg/L phosphorus, and the tap water contained 0.06 mg/L nitrogen and 0.27 mg/L phosphorus. The alfalfa sprouts grown in aquaponics water showed a 5% increase in phosphorus and 91% increase in nitrate-nitrogen content. The mung bean sprouts grown in aquaponics water had a 67% increase in nitrate-nitrogen content. The higher content of these elements in the aquaponics water may have influenced the increase in the harvested sprout. However, the mung bean sprouts did not have a significant difference of phosphorus.

The alfalfa and mung bean sprouts did not show similar mineral results between treatments. The alfalfa sprouts showed that when grown in aquaponics water, phosphorus, potassium, calcium, magnesium and nitrate-nitrogen increased, however in mung bean sprouts grown in aquaponics water, only calcium and nitrate-nitrogen increased. This result reveals that the variety of sprout and

composition of the irrigation water does impact the composition of the sprout (Masood et al., 2014).

4.5 CONCLUSION

Based on the results of this study, it can be concluded that there is a difference in the quality characteristics of fresh grown produce grown hydroponically and aquaponically. Proximate composition is not impacted significantly between treatments, however evidence of this study reveals that vitamin and mineral content is affected based on the variety of produce and the composition of the irrigation water when all other growing characteristics (temperature, light, growing media, volume of water, etc.) remains the same.

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Table 4.1 Proximate composition, vitamin and mineral analyses[1] of alfalfa sprouts grown in municipal water[2] and aquaponics water[3] for 7 days.

	Alfalfa Sprouts In Municipal Water	Alfalfa Sprouts In Aquaponics Water
Moisture (%)	40.6 ± 1.5 (33.1-48.3)	39.4 ± 1.9 (29.5-45.3)
Protein[4] (%)	47.8 ± 0.8 (45.6-48.8)	49.1 ± 2.2 (43.1-53.8)
Fat (%)	5.6 ± 0.9 (3.8-7.8)	5.0 ± 0.5 (4.1-5.9)
Carbohydrate[5] (%)	38.7 ± 1.5 (35.5-42.7)	38.8 ± 2.4 (34.9-45.6)
Ash (%)	4.8[b] ± 0.1 (4.4-5.1)	6.1[a] ± 0.6 (3.3-8.3)
Vitamin C (ppm)	26.5[a] ± 1.8 (23.0-30.0)	13.8[b] ± 3.8 (10.0-25.0)
Phosphorus (ppm)	8707.8[b] ± 65.1 (8581.0-8884.4)	9169.1[a] ± 318.4 (8231.70=9649.6)
Potassium (ppm)	12041.9[b] ± 508.6 (11447.0-13562.4)	14712.1[a] ± 807.6 (12481.5-16315.7)
Calcium (ppm)	1963.8[b] ± 101.5 (1737.7-2231.2)	2968.1[a] ± 215.9 (2468.7-3523.8)
Magnesium (ppm)	2226.8[b] ± 12.0 (2195.0-2252.1)	2647.3[a] ± 95.4 (2524.5-2931.8)
Zinc (ppm)	87.0 ± 0.8 (84.8-88.3)	87.5 ± 2.5 (84.0-95.0)
Copper (ppm)	18.9[a] ± 1.5 (16.2-22.8)	11.2[b] ± 1.2 (9.6-14.9)
Manganese (ppm)	22.2 ± 1.9 (19.4-27.7)	54.4 ± 22.5 (20.9-117.1)
Iron (ppm)	115.9 ± 2.2 (111.8-122.1)	380.7 ± 262.2 (109.5-1167.1)
Sulfur (ppm)	3178.6 ± 79.5 (2945.5-3304.2)	3405.5 ± 99.7 (3134.4-3610.9)
Sodium (ppm)	534.9 ± 59.5 (403.4-679.0)	1944.9 ± 1292.5 (454.8-5813.3)
Nitrate Nitrogen (ppm)	86.8[b] ± 10.0 (61.0-110.0)	989.3[a] ± 346.2 (501.0-1980.0)

[1]Proximate composition and mineral analyses reported on a dry weight basis, with the exception of moisture data.

[2]Public water system, City of Clemson, SC, USA.

[3]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.

[4]%Protein = %N x 6.25 (16% Nitrogen in Protein)

[5]Calculated by difference. CHO = 100 – (% Protein + % Fat + % Ash)

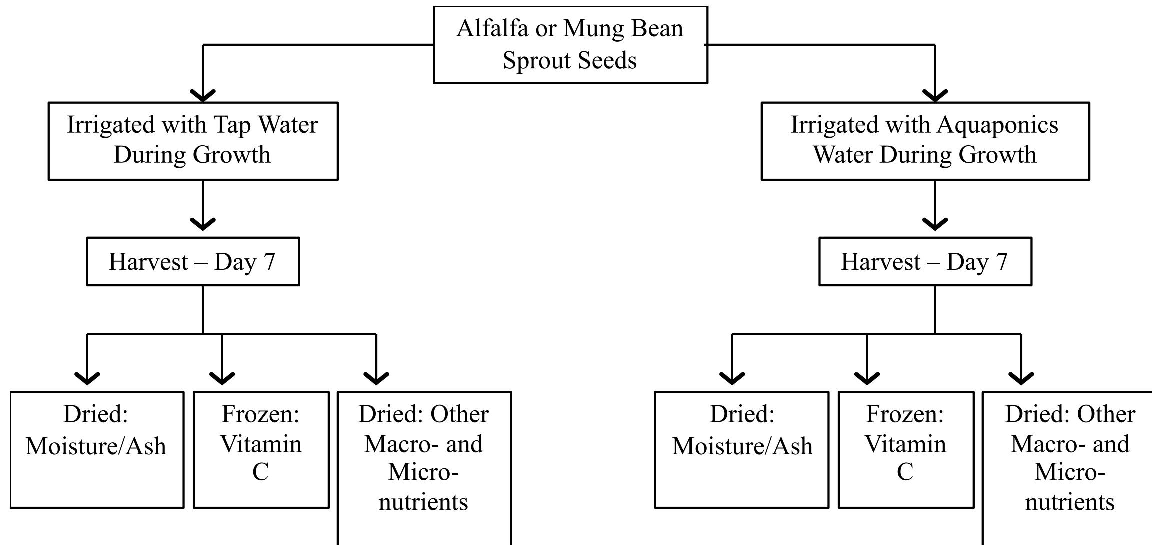
[a-b]Means ± the standard error of the mean with no common superscript within a row are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range.

Table 4.2 Proximate composition, vitamin and mineral analyses[1] of mung bean sprouts grown in municipal water[2] and aquaponics water[3] for 7 days.

	Mung Bean Sprouts In Municipal Water	Mung Bean Sprouts In Aquaponics Water
Moisture (%)	41.3 ± 1.9 (30.0-50.2)	41.2 ± 1.5 (34.6-47.5)
Protein[4] (%)	29.5 ± 0.2 (29.4-30.0)	29.4 ± 0.7 (28.1-31.3)
Fat (%)	1.1 ± 0.0 (1.0-1.2)	0.9 ± 0.1 (0.8-1.0)
Carbohydrate[5] (%)	57.6 ± 0.1 (57.2-57.8)	56.7 ± 0.7 (54.7-58.1)
Ash (%)	4.1[b] ± 0.1 (3.6-4.5)	5.8[a] ± 0.5 (4.5-8.3)
Vitamin C (ppm)	61.5[a] ± 5.3 (48.0-73.0)	26.8[b] ± 6.8 (10.0-41.0)
Phosphorus (ppm)	4006.4 ± 155.3 (3720.0-4311.7)	4230.0 ± 93.1 (4081.9-4500.9)
Potassium (ppm)	12257.3 ± 356.83 (11586.40-12903.3)	13442.8 ± 401.6 (12860.7-14569.1)
Calcium (ppm)	1020.4[b] ± 88.6 (759.2-1152.3)	1293.0[a] ± 28.2 (1239.3-1371.4)
Magnesium (ppm)	1568.5 ± 74.5 (1379.9-1695.2)	1659.0 ± 34.9 (1581.3-1743.8)
Zinc (ppm)	30.2 ± 1.0 (28.0-32.5)	30.1 ± 1.1 (27.2-32.5)
Copper (ppm)	13.1[a] ± 0.3 (12.3-13.7)	10.5[b] ± 0.3 (9.9-11.2)
Manganese (ppm)	14.1 ± 0.4 (13.0-14.8)	17.3 ± 1.4 (15.1-21.2)
Iron (ppm)	67.4 ± 3.4 (59.7-74.7)	88.6 ± 21.3 (61.0-152.0)
Sulfur (ppm)	2331.3 ± 29.7 (2272.5-2383.5)	2389.5 ± 35.2 (2284.1-2429.7)
Sodium (ppm)	128.5 ± 20.1 (90.7-163.6)	683.1 ± 493.5 (151.8-2161.6)
Nitrate Nitrogen (ppm)	80.8[b] ± 12.1 (48.0-105.0)	244.3[a] ± 100.3 (114.0-539.0)

[1]Proximate composition and mineral analyses reported on a dry weight basis, with the exception of moisture data.
[2]Public water system, City of Clemson, SC, USA.
[3]Aquaponics water, Clemson University, Clemson, SC, USA. Aquaponics system contains 3-600 gallon tanks. Each tank holds approximately 300-4 month old tilapia. Water was collected from a tank that collects the filtered water from each of the three fish tanks before it enters the grow beds containing plants.
[4]%Protein = %N x 6.25 (16% Nitrogen in Protein)
[5]Calculated by difference. CHO = 100 – (% Protein + % Fat + % Ash)
[a-b]Means ± the standard error of the mean with no common superscript within a row are significantly different at $P \leq 0.05$. Numbers reported in parentheses are the range.

Figure 4.1 Experimental design to study the nutritional quality characteristics of alfalfa and mung bean sprouts irrigated with different water sources.



CHAPTER FIVE

CONCLUSION

5.1 CONCLUSION

The Produce for Better Health Foundation (PBH, 2015) reported that consumption of fresh vegetables and fruit decreased by 7% and 2%, respectively over the past 10 years. This decrease is attributed to consumers demand for convenience foods and limiting side dishes with meals, which often incorporates fruits and vegetables (PBH, 2015). However, this consumption pattern is expected to change over the next 5 years as a result of increased availability of fresh fruits and vegetables from more area farmer markets, trends to consume local foods, and trends to incorporating more minimally processed vegetables and fruits into diets (PBH, 2015).

Another reason for reduced consumption of fruits and vegetables is the high incidence of foodborne illnesses frequently associated with their consumption. The CDC (2016) reports 23 foodborne illness outbreaks over the last decade (2006-2016) from the consumption of fresh vegetables and 7 outbreaks from the consumption of fresh fruits. Of these outbreaks, 10 (33%) were linked to the consumption of fresh sprouts (CDC, 2016). This is greater than any other single commodity and it explains the Food and Drug Administration's decision to specifically target sprouts in new regulatory standards.

The produce safety standards (Produce Safety Rule) of the Food Safety Modernization Act have been recently mandated (September 2015 final rule) to specifically target the prevention of foodborne illnesses related to the consumption of fresh vegetables and fruits. While the produce safety standards are just beginning to be regulated within the food industry, preventing foodborne illnesses from consumption of fresh produce is a concern for all growers of fresh produce regardless of the implementation timeline for their operation. Considering the number of outbreaks that have been linked to sprouts, the current research outlined in this body of work focused on the microbiological and quality characteristics of sprouts with the intent to provide more information and understanding to the prevention of foodborne illnesses caused by consuming fresh sprouts.

This research study evaluated the production of alfalfa and mung bean sprouts in municipal tap water and aquaponics water, the microbial quality of these sprouts treated post-harvest with various washing schemes (tap water, chlorine and organic acids) and nutritional quality characteristics of sprouts grown in both types of production water sources. Additionally, a partitioning study was conducted on alfalfa sprouts that were inoculated with *Escherichia coli (E. coli)* on day 1 or day 4 of production to determine the level of internalization of bacteria. Under the conditions of this study, alfalfa sprouts and mung bean sprouts were tested for the recovery of total aerobic microorganisms, *Enterobacteriaceae*, total coliforms and yeasts and molds after 7 days of growth. Data showed that aquaponics water naturally contains significant numbers of microorganisms, but recovery of total

aerobic organisms and *Enterobacteriaceae* on alfalfa sprouts, and total coliforms on both alfalfa and mung bean sprouts grown in both production water systems were not significantly different (P>0.05). Mung bean sprouts grown in aquaponics water did have a higher level of total aerobic microorganisms, *Enterobacteriaceae*, and yeasts and molds than those grown in municipal tap water. Therefore, it can be concluded that the microbial quality of irrigation water will impact the microbial quality of the sprout at the time of harvest. This is further evidenced by the importance that FDA places on agricultural water covered in the Produce Safety Rule.

Additionally, the post-harvest washing treatments (tap water, chlorine and organic acids) were not effective at reducing the numbers of microorganisms to a level considered safe for consumption of a raw agricultural commodity, particularly considering the high risk groups in the U.S. population (young children, elderly and those with a compromised immune system). The sprouts washed in organic acids contained the least amount of microorganisms; however, the numbers still ranged from 3.0 – 7.1 $\log_{10}$ CFU/g sprout, which are high enough to cause a foodborne illness when consumed raw. The number of pathogenic cells that cause someone to become ill when consumed (infective dose) is variable based on the infecting pathogen and characteristics (age, health, medications, nutritional status, immune system, etc.) of the person consuming the infected food (FDA, 2012). In the case of *Salmonella*, the infective dose can be as low as one cell and as low as 10-100 cells of *E. coli* O157:H7 (FDA, 2012). Furthermore, data shows that the quality of sprouts is

reduced along with the microbiological counts when washed post-harvest with organic acids.

The partitioning study demonstrated that bacteria may reside inside sprouts which prevents post-harvest treatments from coming in contact with the internalized microorganism and effectively reducing their numbers. Additionally data shows the presence of biofilms on the sprouts, which also makes the use of post-harvest washes ineffective, and biofilm regeneration may occur easily once a baseline film is established.

Evaluating the nutritional quality characteristics of sprouts grown in tap water and aquaponics water showed that proximate composition is not significantly ($P>0.05$) affected by the production water source; conversely vitamin and mineral content can be influenced depending upon the levels of nutrients in the production water. However, despite their nutritional composition, based on the RACC for sprouts (10 g), sprouts do not provide a good source of nutrients and may not be worth the while considering their significant risk for causing a foodborne illness when consumed raw. Risk of foodborne illness can be reduced when sprouts are cooked, however, much of the sensory qualities that sprouts are consumed for (crisp texture) will be lost.

The results of this study show that sprouts harbor a significant level of microorganisms no matter the source of their production water. Using potable water (municipal) still resulted in microbiological contamination. Furthermore, due to the presence of biofilms and internalized microorganisms post-harvest

processing (washes) are not effective at reducing microorganisms, on raw sprouts and thus, thermal treatments may be the only single solution to reducing contamination.

Data from the present study demonstrate that it is critical for sprout producers to incorporate a hurdle effect to reduce numbers of microorganisms to safe levels throughout the growing and distribution process if the sprouts are intended to be consumed raw. These hurdles include: decontamination of the sprout seed, testing spent irrigation water and sprouts for presence of pathogens, following Good Agricultural Practices (GAPs), and the use of multiple chemical based post-harvest washes. Because sprouts are most often consumed raw and biofilms are difficult to remove from sprouts because they cannot withstand physical scrubbing, it is recommended that sprouts not be consumed by young children, elderly or anyone else who may have a compromised immune system. Additionally this research has shown that no matter the nutrient content of irrigation water, sprouts are not a good source of nutrition based on the quantities commonly eaten.

Further research should be conducted to determine if other post-harvest treatments are effective in reducing pathogens on sprouts without compromising quality. Additionally more research should be conducted on preventing and eliminating biofilms on sprouts in addition to preventing internalization of pathogens. Overall, future research on sprouts should concentrate on methods that sprout producers can effectively use to prevent pathogen contamination on sprouts

so that consumers can safely eat them for their culinary appeal without the concern of acquiring a foodborne illness.

5.2 References

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