THE USE OF LACTIC ACID BACTERIA TO CONTROL THE GROWTH OF FOODBORNE PATHOGENS ON FRESH-CUT FRUITS AND SPROUT VEGETABLES

A Thesis

presented to

the Faculty of California Polytechnic State University,

San Luis Obispo

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Agriculture with a Specialization in Food Science and Nutrition

by

Franca Gabriela Rossi

June 2016

COMMITTEE MEMBERSHIP

TITLE:	The Use of Lactic Acid Bacteria to Control the Growth of Foodborne Pathogens on Fresh-cut Fruits and Sprout Vegetables
AUTHOR:	Franca Gabriela Rossi
DATE SUBMITTED:	June 2016
COMMITTEE CHAIR:	Amanda Lathrop, Ph.D. Associate Professor of Food Science and Nutrition
COMMITTEE MEMBER:	Kelly Ivors, Ph.D. Associate Professor of Horticulture and Crop Sciences
COMMITTEE MEMBER:	Christopher Kitts, Ph.D. Professor and Department Chair of Biological Sciences

ABSTRACT

The Use of Lactic Acid Bacteria to Control the Growth of Foodborne Pathogens on Fresh-cut Fruits and Sprout Vegetables

Franca Gabriela Rossi

Growing consumer awareness of the health benefits associated with fruits and vegetables and demand for easy to prepare products has prompted the development of a wide variety of minimally processed fruits and vegetables. Minimally processed fruits and vegetables are often peeled, cut, or diced which compromise the produces' natural protective barriers, exposing a nutrient rich medium and providing an ideal environment for the growth of microorganisms, including foodborne pathogens. The germination conditions of sprout vegetables consisting of relatively high temperatures and humidity, low light and abundance of nutrients are also conducive to the proliferation of foodborne pathogens. Recent outbreaks and recalls indicate additional measures are needed to improve food safety and maintain the integrity of the food industry.

The objective of this research was to evaluate the efficacy of Lactic Acid Bacteria (LAB) against *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. on apple slices and alfalfa sprouts and it's influence on product quality. Apple slices inoculated with *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. (each at 10^4 CFU/g) were treated with *Lb. plantarum* alone and in combination with *Pediococcus acidophilus* and *P. pentosaceus* (LPP) (10^7 CFU/g) while alfalfa seeds were inoculated with *L. monocytogenes* and *Salmonella* spp. (each at 10^1 CFU/g and 10^3 CFU/g) and treated with LPP (10^7 CFU/g). The growth of the microorganisms on the apple slices was assessed during five and seven days of storage at 4°C and 20°C, respectively. Growth on alfalfa seeds was reported during five days of sprouting at 20°C. Populations of LAB were maintained between 7.0 log CFU/g and 8.0 log CFU/g throughout storage and sprouting on the sliced apples and alfalfa seeds, respectively.

Although LAB had no significant effect on pathogen populations on apple slices during storage at 4°C ($p > 0.05$), populations were significantly different at 20°C ($p < 0.05$). Populations of *L. monocytogenes* in the presence of *Lb. plantarum* and LPP were 1.84 log CFU/g and 2.84 log CFU/g less than the controls after five days of storage at 20°C ($p < 0.05$). Populations of *E. coli* O157:H7 in the presence of *Lb. plantarum* and LPP were 1.83 log CFU/g and 1.86 log CFU/g less than the control after one and three days of storage, respectively. Finally, populations of *Salmonella* spp. were 0.86 log CFU/g less than populations in the absence of LPP after three days of storage.

LPP had a significant effect on the growth of *L. monocytogenes* and *Salmonella* spp. on alfalfa seeds ($p < 0.05$). After five days of sprouting, populations of *L. monocytogenes* at an initial concentration of 10^1 CFU/g and 10^3 CFU/g on seeds treated with LPP were approximately 4.5 log CFU/g and 1.0 log CFU/g less than the untreated seeds, respectively. Populations of *Salmonella* spp. at an initial concentration of 10^1 CFU/g and 10^3 CFU/g were 1.0 log CFU/g less than the control.

Overall, on apple slices the combination of *Lb. plantarum* with *P. acidophilum* and *P. pentosaceus* demonstrated greater efficacy than *Lb. plantarum* alone and reduction of *L. monocytogenes* by *Lb. plantarum* and LPP was greater than *Salmonella* spp. and *E. coli* O157:H7 on apple slices and alfalfa seeds, alike. LAB had a minimal effect on the quality of the apple slices and alfalfa seeds. LAB could be an effective strategy in reducing pathogen populations at abusive temperatures and germination conditions without influencing the quality of minimally processed fruit and vegetables.

Keywords: Alfalfa sprouts, biological control, *Escherichia coli* O157:H7, fresh-cut apple slices, *Pediococcus pentosaceus, Pediococcus acidophilus;* Lactic Acid Bacteria (LAB), *Lactobacillus plantarum*; *Listeria monocytogenes, Salmonella* spp.

ACKNOWLEDGMENTS

I would like to thank my committee, Dr. Amanda Lathrop, Dr. Christopher Kitts, and Dr. Kelly Ivors, for their guidance and encouragement. They challenged me to critically analyze and assess the research enhancing my laboratory techniques and precision in experimental design. I would also like to thank my fellow colleagues, Toni de Senna and Suyapa Padilla-Antunez for their support and patience and the wonderful undergraduate students, Alvin Loi, Christopher Craddock, Nicole Neumayr, and Thei Soe, who volunteered their time and provided contagious enthusiasm and curiosity. I hope they learned just as much from me as I learned from them. Additionally, I would like to most graciously thank my family and friends for their unconditional love. Thank you for listening to my jargon, holding me up through the struggles, providing me with words of wisdom, and giving me the strength to persist. This challenging journey has truly been a testament to my faith and defined my values as a researcher. Finally, I am appreciative for the financial support provided by BiOWiSH Technologies and the California State University Agricultural Research Institute (CSU-ARI).

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER 1 LITERATURE REVIEW

1.1. Consumption of Fruits and Vegetables

Fruits and vegetables are an important component of a healthy balanced diet. They provide essential nutrients such as vitamin C, thiamine, niacin, pyridoxine, folic acid, minerals, and antioxidants for energy, cell function, growth, and maintenance (Oguntibeju, et al., 2013). Fruits and vegetables are also high in dietary fiber, which lowers cholesterol and glucose levels. Sufficient daily consumption ranges from 5 to 13 servings (2 ½ to 6 ½ cups) per day, depending on caloric needs, and is recommended by many organizations: World Health Organization (WHO), Food and Agriculture Organization (FAO), United States Department of Agriculture (USDA), and European Food Safety Authority (EFSA) to reduce the risk of chronic diseases including stroke, cancers of the lung, stomach and colon, and diabetes mellitus (USDA Food and Nutrition Service, 2008).

Growing consumer awareness of the health benefits associated with fruits and vegetables and demand for easy to prepare products has prompted the development of a wide variety of minimally processed fruits and vegetables (Produce for Better Health Foundation, 2010). Minimally processed fruits and vegetables are defined as fresh fruits and vegetables that have been processed to extend the product shelf-life while ensuring food safety and maintaining nutritional and sensory quality, which include pre-washed or pre-cut vegetables, fruit and sprouted seeds (e.g. mung beans, alfalfa) (Salunkhe et al., 1991). Production is expected to increase at an annual rate of 3.0% from 2015 to 2020, reaching $315.2 billion (IBISWorld, 2015).

1.2. Foodborne Illness Outbreaks in the United States

As production and consumption of minimally processed fruits and vegetables increases, diligence in food safety is crucial to maintaining the integrity of the food industry. In the United States the estimated annual foodborne illness attributed to foodborne pathogens is nine million individuals. Foodborne illness costs the United States food industry approximately seven billion dollars, annually (Chakrakorty and Newton, 2011). From 1998 to 2008 produce commodities

including fruits, nuts, sprout and leafy vegetables accounted for 46% of the reported foodborne illnesses. Leafy vegetables and fruits attributed to 22% (2.2. million) and 12% (1.1 million) of the annual foodborne illness outbreaks, respectively (Painter et al. 2013). From 2008 to 2015 the foodborne pathogens *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* were most frequently associated with foodborne illness attributed to leafy vegetables, fruits, and sprout vegetables in the United States (Table 1) (CDC, 2015).

Table 1. Fruit-nuts, leafy and sprout vegetables associated with L. monocytogenes, Salmonella spp. and E. coli O157:H7 from 2008- 2015 in the United States (CDC, 2015).

Year of Contamination	Foodborne Pathogen	Commodity Type	Cases/ Deaths
2015	*L. monocytogenes*	Granny Smith and Gala Apples	34/ 7
2014	*Salmonella* Newport	Clover Sprouts	5/ 0
	Salmonella Enteritidis	Cucumbers	275/ 0
	L. monocytogenes	Soy Sprouts	5/ 2
	Salmonella Enteritidis	Bean Sprouts	115/ 0
2013	*E. coli* O157:H7	Cucumbers and Ready-to-	84/ 0
	Salmonella Saintpaul	Eat Salad	33/ 0
2012	*E. coli* O157:H7	Spinach and Spring Mix	33/ 0
	Salmonella Braenderup	Mangoes	127/ 0
	Salmonella Typhimurium and Newport	Cantaloupes	27/ 0 261/ 3
2011	*E. coli* O157:H7	Romaine Lettuce	58/ 0
	L. monocytogenes	Cantaloupes	146/ 30
	Salmonella Agona	Papayas	106/ 0
	Salmonella Enteritidis	Alfalfa and Spicy Sprouts	11/ 0
2010	*E. coli* O157:H7	Shredded Romaine	31/ 0
	Salmonella spp. and Newport	Alfalfa Sprouts	140/ 0 and 44/ 0
2009	*Salmonella spp.*	Alfalfa Sprouts	235/ 0
2008	*Salmonella* Litchfield	Cantaloupes	51/ 0

1.2.1. *Escherichia coli* O157:H7

Escherichia coli is a Gram-negative, rod-shaped, facultative anaerobic bacterium predominantly found in water, soil contaminated with fecal material, and the intestinal tracts of warm-blooded organisms. Most strains of *E. coli* are non-pathogenic. However, some strains,

including Enterohemorrhagic *Escherichia coli* (EHEC) cause diarrheagenic illness (CDC, 2014). The EHEC serotypes, which include O26:H11, O103:H2, O104:H4, O145:H28, and O157:H7, are characterized by the production of several virulence factors, including both heat-labile (LT) and heat-stable (ST) toxins, as well as several colonization-factor antigens.

The serotype O157:H7 is the most prominent of the EHEC strains of *E. coli*; it accounts for approximately 75% of the EHEC infections worldwide (WHO, 2011). The estimated infective dose of *E. coli* O157:H7 is 10 to 100 organisms. Symptoms typically begin three to four days after exposure and last for two to nine days. Symptoms which include severe abdominal cramps and bloody diarrhea are classified as Hemorrhagic Colitis (HC). About 3 to 7% of HC cases progress to life threatening complications, such as Hemolytic Uremic Syndrome (HUS) and Thrombotic Thrombocytopenic Purpura (TTP), which are identified by the destruction of red blood cells, low platelet count, and acute kidney failure (U.S. Food and Drug Administration, 2012).

1.2.2. *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, rod-shaped, facultative anaerobic bacterium. *Listeria monocytogenes* is ubiquitous in the environment and is commonly found in moist environments, soil, decaying vegetation, and the intestinal tract of domestic animals. *Listeria monocytogenes* has thirteen distinct O-antigenic patterns, of which three serotypes, 1/2a, 1/2b, and 4b, are responsible for 98% of the outbreaks (FDA, 2012).

The infective dose of *L. monocytogenes* varies with the serotype, susceptibility of the host, and food matrix. Pregnant women, fetuses, newborn infants, immunocompromised, and elderly are highly susceptible. In some cases, fewer than 1,000 cells may incur symptoms, which present as a non-invasive gastrointestinal illness and a more serious invasive form, referred to as listeriosis. The non-invasive gastrointestinal illness primarily affects healthy individuals, has a relatively short incubation period of a few hours to two or three days, and includes symptoms such as fever, muscle aches, nausea and vomiting. Although the non-invasive gastrointestinal

illness may develop into listeriosis in healthy individuals, listeriosis is more of a concern in immunocompromised individuals. The incubation period ranges from three days to three months and symptoms include inflammation of vital organs (septicemia) or protective membranes covering the brain and spinal cord (meningitis) and spontaneous abortions in pregnant women. Amongst immunocompromised individuals, the case-fatality rate is between 15 to 30% (FDA, 2012).

1.2.3. *Salmonella* species (spp.)

Salmonella is a non-spore forming, Gram-negative, rod-shaped, facultative anaerobic bacterium found in exothermic and endothermic organisms, pond-water sediment, and soil contaminated with fecal material. The species of *Salmonella enterica* is most commonly associated with illness in humans.

Depending on the *S. enterica* serotype, one of two types of illness may occur – non-typhoidal salmonellosis and typhoid fever, which is caused by *S. typhi* and *S. paratyphi*. The infective dose of non-typhoidal salmonellosis, which is the disease most frequently associated with foodborne illness may be as low as one cell, depending on the serotype and the health of the host. Symptoms include nausea, vomiting, abdominal cramps, diarrhea, and fever, and may occur for four to seven days after 6 to 72 hours of exposure. Typhoid fever has an infective dose of 1,000 cells and symptoms include fever, lethargy, abdominal pains, diarrhea, and loss of appetite for two to four weeks after one to three weeks of exposure. Non-typhoidal salmonellosis and typhoid fever may also cause inflammation, resulting in blood clotting and organ failure (septicemia) and chronic diseases, such as reactive arthritis.

1.3. Contamination of Fruits and Vegetables

Escherichia coli O157:H7, *L. monocytogenes,* and *Salmonella* spp. are capable of contaminating fruits and vegetables throughout production compromising food safety. Pre and postharvest sources of contamination include soil, feces, irrigation water, water used to apply fungicides and insecticides, dust, insects, spoilage microorganisms, inadequately composted

manure, wild and domestic animals, human handling, processing equipment, rinse water, and transport vehicles. Spoilage microorganisms of the genera *Penicillium*, *Aspergillus*, *Botrytis*, and *Rhizopus* may also elevate food safety concerns by softening the structural integrity of the plant and/or fruit, thereby weakening defense against foodborne pathogens. Minimally processed fruits and vegetables are subject to washing, peeling, cutting, dicing, mixing, sanitizing, and packing (Siddiqui, et al., 2011). Unit operations, such as washing and sanitizing, do not assure the absence of foodborne pathogens. Furthermore, the unit operations of peeling, cutting, and dicing may compromise the natural protective barriers of the fruit or vegetable, exposing a nutrient-rich medium which provides an ideal environment for the growth of microorganisms (Buck et al., 2003).

1.4. Survival and Growth of Foodborne Pathogens in Fruits and Leafy Vegetables

Once the flesh of the fruit or vegetable becomes exposed, the ability of foodborne pathogens to withstand relatively acidic conditions ($pH \leq 4.6$) and refrigerated temperatures contributes to their survival and growth. *Escherichia coli* O157:H7 and *Salmonella spp.* are capable of growing at pH values as low as 4.5. *Listeria monocytogenes* and some strains of *Salmonella spp.* have the ability to grow at refrigerated temperatures (Table 2) (Ray and Bhunia, 2008). The survival and growth patterns of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella spp*. in fruits and leafy vegetables have been investigated (Abadias et al., 2012; Alegre et al., 2010; Alegre et al., 2012; Flessa et al., 2005; Huang et al. 2015; McEvoy et al., 2009; Sreedharan et al., 2015; Zhuang et al., 1995).

Table 2. Growth characteristics of E. coli O157:H7, L. monocytogenes, and Salmonella spp. (Ray & Bhunia, 2008).

Foodborne Pathogen	Optimum Temperature (^{0}C)	Minimum Temperature (^{0}C)	Maximum Temperature (^{0}C)	Lowest pH Tolerated for Growth
E. coli O157:H7	30 - 42	7 - 8	45	4.5
L. monocytogenes	30 - 37	1	44	5.0
Salmonella spp.	30 - 37	4 - 6	46	4.5

Escherichia coli O157:H7 survives and can grow in non-acidic fruits and vegetables. In fresh-cut melons (pH 6.0 to 6.7) *E. coli* O157:H7 grew by 2.0 log CFU/g at 25^{0}C after one day of storage and survived for five days at 5 ^{0}C (Abadias et al., 2012). Populations of *E. coli* O157:H7 on spinach (pH 5.5 to 6.8) increased by 2.0 log CFU/g after 12 days of storage at 7°C (Calix-Lara et al., 2012). *Escherichia coli* O157:H7 also survives in high acid fruits at ambient temperatures, but not refrigerated temperatures. *E. coli* O157:H7 populations increased on fresh cut peaches (pH 3.3 to 4.1) and apples (pH 3.3 to 4.0) by more than 2 log CFU/plug when store at 20^{0}C after 48 hours but growth was not observed at 5^{0}C. *Escherichia coli* O157:H7 decreased more than a 1 Log CFU/plug on peaches and apples after 14 days and 6 days at 5^{0}C (Alegre et al., 2012; Alegre, et al., 2010).

Growth of *L. monocytogenes* is minimal in non-acidic and acidic fruits at refrigerated temperatures. In fresh-cut cantaloupe (pH 6.1 to 6.6) after one week of storage, *L. monocytogenes* grew 0.8 and 4.2 log CFU/g at 4 and 12^{0}C, respectively (Huang et al., 2015). The microbial surrogate of L. *monocytogenes*, *L. innocua* increased more than 1 log CFU/plug in fresh-cut peaches after a storage period of 6 days at 5^{0}C (Alegre et al., 2010). In orange juice adjusted to pH 3.6, populations of *L. monocytogenes* were stable over seven days of storage at 4^{0}C. At 30^{0}C, there was a 0.5 log CFU/mL reduction in the populations of *L. monocytogenes* in the pH adjusted orange serum (Flessa et al., 2005).

Salmonella spp. has been shown to survive and grow in acidic fruits at ambient temperatures. *Salmonella spp.* is capable of multiplying at ambient temperatures in fresh-cut strawberries (pH 3.0 to 3.9) (Sreedharan et al., 2015). *Salmonella spp.* however, is unable to grow in acidic fruits at refrigerated temperatures. In strawberry purees *Salmonella spp*. was able to survive at 4^{0}C; the populations remained constant over the seven day storage period (Knudsen et al., 2001; Sreedharan, et al., 2015). Similarly, populations of *Salmonella montevideo* remained

stable on fresh-cut strawberries for nine days of storage at 5^0C, but increased 2.0 log CFU/g after storage for 96 and 22 hours at 20 and 30^0C, respectively (Zhuang et al., 1995).

1.5. Survival and Growth of Foodborne Pathogens in Sprout Vegetables

The storage and growth conditions of sprouted vegetables are ideal environments for microbial survival and growth. Foodborne pathogens are capable of surviving for months under the sprout seed storage conditions of 12 to 20^0C and 70% humidity. Foodborne pathogens proliferate under the germination conditions of relatively high temperatures and humidity, low light and abundance of nutrients proliferate during the germination and sprouting of seeds (Taormina et al., 1999). A 100,000-fold increase in populations of *E. coli* O157:H7, *L. monocytogene*s and *Salmonella* spp. during sprouting, whereby the major growth occurs during the first two days of the sprouting process, has been described. Populations of *E. coli* O157:H7 and *L. monocytogenes* increased by 6.0 log CFU/g on alfalfa sprouts during a 48 hour sprouting stage (Palmai and Buchanan, 2002; Tarmina and Beuchat, 1999). Similarly, populations of *E. coli* O157:H7 increased 7.0 log CFU/g on radish sprouts during a 72 hour sprouting stage (Xiao, et al. 2014). Populations of *S. stanley* increased 4.0 log CFU/g during a 24 hour germination period and by an additional 1.0 log CFU/g during a 72 hour sprouting stage (Jacquette, et al., 1996).

1.6. Current Intervention Strategy

Numerous sources of contamination and the ability of foodborne pathogens to survive and grow on acidic and non-acidic fruits and vegetables at refrigeration and ambient temperatures demonstrates the value of intervention strategies to improve food safety. Hypochlorite is a highly affordable, antimicrobial chemical commonly used as a sanitizer by the food industry. The two frequently used hypochlorite disinfectants are sodium and calcium hypochlorite (Penn State Extension, 2014). The active form of hypochlorite, hypochlorous acid, affects the cellular function of microbial contaminants by decreasing adenosine triphosphate (ATP) production,

denaturing deoxyribonucleic acid (DNA), inhibiting protein synthesis, and decreasing the uptake of oxygen and nutrients (CDC, 2008). Hypochlorites used at concentrations of 50 to 200 ppm, with a contact time of one to two minutes, are effective against a broad spectrum of microbial contaminates, but the antimicrobial efficacy is dependent on many factors, including wash water, pH, temperature, organic load, and the inherent properties of the produce commodity (FDA, 2005; Sikin et al., 2013). On average, the lethal activity of hypochlorite for fruits and vegetables is between 0.5 and 1.0 log CFU/g reduction of foodborne pathogens *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* (Abadias et al., 2011; Behrsing et al., 2000; Beuchat & Brackets, 1990; Keskinen & Annous, 2011; Weissinger et al., 2000). The dependency of hypochlorite on a multitude of factors to ensure efficacy and the low efficacy attained illustrates the inadequacy of hypochlorite in ensuring food safety.

1.7. Alternative Intervention Strategies

The limitations of hypochlorite underscore the importance of alternative intervention strategies. There are a variety of chemical, physical, and biological treatments that have demonstrated potential. Although many of the treatments are unable to independently achieve a 5.0 log reduction, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) recommends the use of a combination of various chemical, physical, and biological treatments to achieve the 5.0 log performance standard (FDA, 2014).

1.7.1. Chemical Intervention

Alternative chemical methods have demonstrated equivalent and even greater potential than hypochlorite. Similar to hypochlorite, efficacy is dependent on the sensitivity of the foodborne pathogen to the chemical treatment and physical characteristics of the produce. On average, the antimicrobial activity of alternative chemical methods is between 1.0 and 1.5 log CFU/g. A variety of alternative chemical treatments for produce are industrially applicable (Siddiqui et al., 2011).

1.7.1.1. Electrolyzed Water

Electrolyzed water has an effect on a broad spectrum of microorganism including foodborne pathogens. Electricity is added to water to create electrolyzed water, which positively and negatively charged ions interact with organic matter on the surface of fruits and vegetables. This interaction alters the charge of the organic matter, resulting in a repulsion of organic matter from the surface of fruits or vegetables, in addition to disrupting the outer cell membrane of microorganisms (Powitz, 2010).

Acidic electrolyzed water resulted in a 1.0 log CFU/g reduction of *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* spp. respectively in mung bean sprouts (Phua et al., 2014). Populations of *L. monocytogenes* were not significantly different on apple plugs treated with 100 mV of acidic electrolyzed water and 100 ppm of chlorine; both treatments showed a 1.0 log CFU/g reduction in *L. monocytogenes* populations. Populations *of E. coli* O157:H7 and *Salmonella spp.* were significantly different. Populations of *E. coli* O157:H7 and *Salmonella spp.* treated with acidic electrolyzed water were reduced by 2.5 and 1.5 log CFU/g, respectively in comparison to the chlorine treatment which reduced populations by 1.5 and 1.0 log CFU/g, respectively (Graca et al., 2011).

1.7.1.2. Peroxyacetic Acid

Peroxyacetic acid is composed of hydrogen peroxide and acetic acid, which breaks down into harmless products in water: acetic acid, water, oxygen, and carbon dioxide. Peroxyacetic acid is a strong oxidizer; it rapidly disrupts the outer cell membrane of microorganisms. It has been shown to be effective against foodborne pathogens (Kitis, 2004). A maximum concentration of 75 ppm is used to treat fruits and vegetables (Warburton, 2014).

Treatment of apple plugs with 40 ppm of peroxyacetic acid resulted in *E. coli* O157:H7 levels 2.3 Log CFU/g lower than apple plugs treated with 100 ppm chlorine after 6 days of storage at

10^0C. However, efficacy of peroxyacetic acid was similar to that of chlorine on apple plugs inoculated with *Salmonella* spp.; populations of *Salmonella* spp. were reduced by approximately 1.0 Log CFU/g on apple plugs by both treatments after 6 days of storage at 10^0C (Abadias et al., 2011). On mung bean sprouts there was no significant difference in populations of *L. monocytogenes* treated with 51 ppm of peroxyacetic acid and 170 ppm chlorine; both treatments reduced population levels by approximately 1.0 Log CFU/g (Neo, et al., 2013).

1.7.1.3. Organic Acids

Organic acids, such as lactic and acetic acid, are produced by Gram-positive bacteria during carbohydrate fermentation and are classified by the FDA as generally regarded as safe (GRAS) (FDA, 2012). Organic acids alter the permeability of the target cell membrane and create a highly acidic environment, which is generally unsuitable for spoilage microorganisms and foodborne pathogens. A 2% solution of lactic and acetic acid added to the surface of apples reduced the populations of *E. coli* O157:H7, *Salmonella enterica* ser. Typhimurium, and *L. monocytogenes* by approximately 1.0 and 1.5 log CFU/g, respectively (Park et al., 2011). Populations of *L. monocytogenes* decreased by 2.3 log CFU/g in minimally processed lotus sprouts treated with 2% lactic acid solution (Wang et al., 2013). In alfalfa and mung bean treated with 2% acetic acid for 24 hours a 7 to 8 log CFU/g reduction of *Salmonella* spp. was observed (Pao et al., 2008).

1.7.2. Physical Intervention

Consumer preference for the reduction or elimination of synthetic chemical disinfectants has resulted in the development of physical intervention methods, such as electron irradiation and ultraviolet radiation. In comparison to chemical methods, physical intervention methods have demonstrated enhanced penetration for the destruction of internalized microorganisms (Sikin et al., 2013). On average, physical intervention methods immediately reduce populations of foodborne pathogens by 1.5 to 3.0 log CFU/g. During storage; however, populations of foodborne pathogens typically increase as injured cells recover.

1.7.2.1. Electron Irradiation

Electron irradiation (E-beam) uses high-speed electrons to produce free radicals, which react, destroy, or deactivate bacterial components. Radiation dosage, expressed in kilograys (kGy), is a function of the energy of the radiation source and exposure time (Sikin et al., 2013). Low dose E-beam irradiation has proven to be effective in delaying maturation and reducing pathogenic microorganism populations in produce. Fresh-cut cantaloupe treated with 0.7 kGy electron beam irradiation showed an approximate 0.5, 1.5, and 3.0 log CFU/g reduction of *S.* Poona during storage at 5^0C for 0, 3, and 21 days, respectively (Palekar et al., 2015). A radiation dosage of 0.40 kGy on fresh baby spinach resulted in an immediate reduction of 3.7 and 3.4 log CFU/g *E. coli* O157:H7 and *Salmonella* spp., respectively (Neal et al., 2008). On blueberries, populations of *E. coli* O157:H7 were reduced by approximately 1.5 log CFU/g immediately after treatment with 0.5 kGy (Kong et al., 2014).

1.7.2.2. Ultraviolet Radiation

Ultraviolet radiation uses short-wavelength ultraviolet (UV-C) to inactivate microorganisms by disrupting nucleic acids and DNA, which prevent microorganisms from performing vital cellular functions. In clover sprouts treated with 1 kJ/ m^2 of UV-C, populations of *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* were reduced by 1.0 log CFU/g or less (Kim et al., 2009). In addition to energy dosage, efficacy of UV-C is also a function of surface topography; inactivation is greater on smoother surfaces. The greatest reduction was seen on organic apples; UV-C reduced the populations of *E. coli* O157:H7 and *L. monocytogenes* by 2.9 and 1.6 log CFU/g, respectively (Adhikari et al., 2015).

1.7.3. Biological Interventions

Biological intervention methods provide another promising strategy for minimizing the use of synthetic chemicals as a means of microbiological control of fresh produce. Biological control is the use of non-pathogenic organisms, such as bacteriophages, protective bacterial

cultures and/or their metabolites to negatively affect the viability of foodborne pathogens (Buck et al., 2003). On average, the antimicrobial activity of biological control agents is between 0.5 and 2.5 log CFU/g. Variability is highly dependent on the sensitivity of the foodborne pathogen to the biological control agent. Furthermore, analogous to chemical and physical intervention methods, the lethal activity of the biological control agent is a function of the physical composition of the produce. Unlike physical intervention strategies, biological control agents are unable to immediately reduce populations of foodborne pathogens. Biological control agents, however, are able to suppress the growth of foodborne pathogens during storage. Additionally, biological control agents do not affect the nutritional and sensory quality of the fruit or vegetable.

1.7.3.1. Bacteriocins

Bacteriocins are antimicrobial peptides produced by some species of Gram-positive bacteria, including lactic acid bacteria (LAB), with narrow to broad antimicrobial activity. Bacteriocins act on the cytoplasmic membrane, inhibiting cell wall synthesis, decreasing RNase and DNase activity, and altering the permeability of the target cell membrane. The antimicrobial activity of bacteriocins is typically limited to Gram-positive bacteria including *L. monocytogenes*, with limited efficacy against Gram-negative bacteria because the outer membrane restricts access to the cytoplasmic membrane. However, Gram-negative bacteria may become sensitive to bacteriocins if the outer membrane is disrupted (Martin et al., 2011).

Historically, bacteriocins have been used as a hurdle technology application in meat and poultry (Berry et al., 1991; Nielsen et al., 1990; Yuste et al., 1998). The application of bacteriocins in produce has recently been explored. In honeydew melon and apple slices, nisin, a broad spectrum bacteriocin produced by *Lactococcus lactis*, reduced the populations of *L.monocytogenes* by approximately 3.2 and 2.0 log CFU/g, respectively, in comparison to untreated honeydew melon and apple slices after storage for seven days at 10^0C (Leverentz, et al., 2003). Enterocin B, a narrow spectrum bacteriocin synthesized by *Enterococcus faecium*, reduced

the population of *L. monocytogenes* in alfalfa and soybean seed sprouts by 2.0 and 2.4 log CFU/g, respectively (Sikin et al., 2013).

The effectiveness of bacteriocins; however, diminishes throughout storage. Although a wash containing coagulin, a broad spectrum bacteriocin produced by *Lactobacillus paraplantarum*, reduced the populations of *L. monocytogenes* in fresh-cut lettuce by 1.6 Log CFU/g after three days of storage at 4^0C, populations of *L. monocytogenes* increased by approximately 1.4 log CFU/g after seven days of storage (Allende et al., 2007). Similarly, mung bean sprouts washed with mundticin, a bacteriocin synthesized by *Enterococcus mundtii* reduced the populations of *L. monocytogenes* by 2.0 log CFU/g but after 14 days of storage at 8^0C, populations of *L. monocytogenes* in mung bean sprouts washed with mundticin exceeded the initial inoculum level of 3.0 Log CFU/g by approximately 1.2 log CFU/g (Bennik et al., 1999). Decline in bacteriocin activity may result from proteolytic degradation of bacteriocin, reduced adherence of bactericin to the product, or gained resistance to bacteriocin (Allende et al., 2007; Bennik et al., 1999).

1.7.3.2. Bacteriophages

Bacteriophages are viruses that infect and replicate within a bacterium. Bacteriophages are classified into two distinct clades – lytic and temperate phages - based on their mode of replication. Lytic phages lyse and destroy bacterial cells immediately after replication, while temperate phages do not immediately lyse the bacterial cells. Temperate phages will integrate their genome with the bacterium genome without harming the bacterial cells. The bacteriophage will remain dormant until the conditions of the bacterial cell become unfavorable, resulting in lysis of the bacterial cell. The appeal of lytic phages is their immediate ability to lyse the bacterial cells and narrow host ranges. For example, phage IMM-0001, isolated from surface water, is specific for the enterotoxigenic *E. coli* colonization factor and does not infect common enteric bacteria including non-toxigenic *E. coli*; thereby, negating any negative impact on

beneficial, non-toxigenic *E. coli* inhabiting the intestinal microflora of warm-blooded organisms (Mahony et al., 2011). Lytic phages targeting *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica* have been isolated and characterized.

The efficacy of bacteriophages against foodborne pathogens in produce has been investigated. Populations of *S. typhimurium* and *S. enteritidis* were reduced by 3.4 and 1.9 log CFU/g, respectively on romaine lettuce treated with 10^{11} PFU/mL of phage for 30 minutes (Spricigo, et al., 2013). However, no reduction in *Salmonella* spp. was observed on fresh-cut apples treated with 10^{11} PFU/mL of bacteriophage; the acidic environment may have significantly reduced the ability of phages to suppress the growth of the foodborne pathogen (Leverentz, et al., 2001). In broccoli sprout seeds inoculated with 10^{11} PFU/mL of Phage A, a 0.5 log CFU/g reduction of *Salmonella* spp. was observed after 24 hours at 25^{0}C (Sikin et al., 2013). Physical barriers in the produce matrix may decrease the opportunity for the phage to interact with the bacterial cells; therefore, high concentrations of bacteriophage are necessary to increase the probability of phage collision and attachment to receptors on the bacterial cell walls. The development of bacteriophage insensitive mutants is another possible obstacle associated with phage treatment. Mixtures of bacteriophages specific to the foodborne pathogen would need to be employed to minimize the opportunity for the development of bacteriophage insensitive mutants (Sharma, 2013).

1.7.3.3. Protective Bacterial Cultures: Lactic Acid Bacteria

Lactic acid bacteria are Gram-positive, non-spore forming, aerotolerant anaerobic bacteria characterized by their ability to produce lactic acid (Todar, 2012). Lactic acid bacteria are ubiquitous in the environment and can tolerate high acidity and osmotic concentration of NaCl (Menconi, et al., 2014). Lactic acid bacteria are classified into two broad metabolic categories based on end products of carbohydrate fermentation: homofermentative and heterofermentative. Homofermentative lactic acid bacteria produce lactic acid from carbohydrate

fermentation and include the genera *Pediococcus, Lactococcus,* and *Streptococcus.* Heterofermentative lactic acid bacteria, which include *Leuconostoc*, *Lactobacillus*, *Oenococcus,* and *Weissella,* produce lactic, acetic, and formic acid, ethanol, and carbon dioxide from the fermentation of carbohydrates (Todar, 2012).

Lactic acid bacteria have historically been used in the preservation of foods, such as cheese, yogurt, salami and sauerkraut and are Generally Recognized as Safe (GRAS) for human consumption. The benefits of lactic acid bacteria extend beyond preservations. Studies have demonstrated that certain members of *Lactobacillus spp*. can remove carcinogens, lower cholesterol, stimulate immune response, enhance the bioavailability of nutrients, and alleviate lactose intolerance (Anas et al., 2014; Kumar et al., 2010). The recommended ingestion of lactic acid bacteria to exert a health benefit beyond inherent basic nutrition is a minimum of 7.0 log CFU/g, daily (FAO, 2002). In addition to the preservative and probiotic qualities of lactic acid bacteria, efficacy in the control of foodborne pathogens has been demonstrated. *Lactobacillus fermentum* reduced the growth of *E. coli* O157:H7 and *Salmonella* spp. on fresh-cut pineapple by approximately 1.0 and 2.0 log CFU/g after seven days of storage at 5^0C (Russo et al., 2014). The growth of *L. monocytogenes* on apples treated with *Lactobacillus rhamnosus* was reduced by approximately 1.0 log CFU/g after 28 days of storage at 5 and 10^0C (Alegre et al., 2011). *mesenteriodes* and *Weissella cibaria* reduced populations of *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* spp. on apple wounds and lettuce cuts by approximately 1.0, 3.0, and 2.0 log CFU/g after two days of storage at 20^0C, respectively (Trias et al., 2007). The growth of *L. monocytogenes* was reduced by 1.0 log CFU/g on alfalfa seeds treated with *Lactobacillus lactis* during a five day sprouting stage (Palmai and Buchanan, 2002). The antimicrobial activity of lactic acid bacteria is likely attributed to competition for nutrients and production of organic acids, hydrogen peroxide, antimicrobial enzymes and bacteriocins (Das et al., 2013).

1.7.3.3.1. *Lactobacillus plantarum*

Lactobacillus plantarum has most frequently been studied as a species of lactic acid bacteria having an inhibitory effect on *E. coli* O157:H7, *Salmonella spp*., and *L. monocytogenes* on a variety of fruits and vegetables. *Lactobacillus plantarum* is a Gram-positive, rod-shaped, facultative heterofermentative bacterium. The optimal temperature for growth is between 15 and 45^{0}C. *Lactobacillus plantarum* is acid tolerant; it can grow at pH levels as low as 3.2 (Table 3).

The aptitude of *L. plantarum* to resist acidic as well as basic and enzymatic stresses makes it an ideal protective biological culture (Anas et al., 2014). The antimicrobial activity of *L. plantarum* is most likely attributed to the production of lactic acid and peroxide radicals (Das et al., 2013). On minimally processed sliced apples, *L. plantarum* CIT3 strain significantly inhibited *E. coli* O157:H7. Populations of *E. coli* O157:H7 decreased by approximately 0.5 and 2.5 log CFU/g after two and seven days of storage at 6^{0}C. However, populations of *L. monocytogenes* remained in the presence of *L. plantarum* CIT3 strain on minimally processed apple slices after 16 days of storage at 6^{0}C (Siroli et al., 2015). In contrast, *L. plantarum* reduced the populations of *E. coli* O157:H7 and *L. monocytogenes* by approximately 0.5 and 1.0 log CFU/g on pineapple (pH 3.2 to 4.0) slices after seven days of storage at 6 ^{0}C. Differences in the effectiveness of *L. plantarum* against *E. coli* O157:H7 and *L. monocytogenes* in the different produce types emphasizes that the food matrix has an influence on the efficacy of the protective biological culture.

The effects of *L. plantarum* on the nutritional and sensory quality of produce has also been accessed. There was no significant differences in concentration of ascorbic acid, Vitamin C, total phenols, and antioxidant capacity of fresh-cut pineapple treated with *L. plantarum* after eight days of storage. Panelists indicated a reduction in the firmness of fresh-cut pineapple treated with *L. plantarum* after eight days of storage (Russo et al., 2014). The reduced firmness was also noted by panelists evaluating the texture of apples containing *Lactobacillus rhamnosa* (Roble et al., 2010). Panelists did not detect any off-flavor or odor in pineapples treated with *L. plantarum*,

indicating that *L. plantarum* does not degrade the flavor or aroma properties of the product and this was also in accordance with research conducted by Roble et al. (2010) where panelists did express a preference for apples containing *L. rhamnosa* over the control apples (Russo et al., 2014).

1.7.3.4. Protective Bacterial Cultures: *Enterobacteriaceae* and *Pseudomonas*

Gram-negative bacillus microorganisms of the family *Enterobacteriaceae* and genera *Pseudomonas* have also demonstrated efficacy in reducing populations of foodborne pathogens. Species of the family *Enterobacteriaceae* are non-spore forming, mobile facultative anaerobes. *Enterobacteriaceae* are ubiquitous in nature, commonly found in intestinal tracts of warm-blooded organisms, soil, water, and plant material. The optimal temperature for growth is between 30^0C. *Enterobacteriaceae* can grow at pH levels as low as 4.0 (Table 3). Species of the genus *Pseudomonas* are non-spore forming, mobile aerobes. *Pseudomonas* species demonstrate a diversity of metabolic functions, allowing colonization of a wide range of ecological niches. The optimal temperature for growth is 25 - 30^0C. *Pseudomonas* grows optimally at pH 7.0 to 8.0 (Table 3).

Table 3. Growth characteristics of Protective Bacterial Cultures (Ray & Bhunia, 2008).

Protective Bacterial Culture	Optimum Temperature (^{0}C)	Minimum Temperature (^{0}C)	Maximum Temperature (^{0}C)	pH
Lactobacillus plantarum	15 – 45	7 - 8	45	3.2 - 8.0
Pseudomonas spp.	25-30	0	45	7.0 - 8.0
Enterobacteriacea spp.	30	0	37	4.4 – 9.0

Species in the family *Enterobacteriaceae* and genera *Pseudomonas* have shown efficacy against foodborne pathogens at ambient temperatures. Alfalfa seeds co-inoculated with *Pseudomonas fluorescens* reduced populations of *Salmonella* spp. by 1.0 to 2.0 log CFU/g (Liao, 2008). Contrasting, alfalfa seeds soaked with *P. fluorescens* and *Enterobacter asburiae* JXI two hours reduced populations of *Salmonella spp.* by approximately 4.0 and 5.0 log CFU/g,

respectively, indicating that the method of application of the biological protective culture may impact its efficacy (Jianxiong et al., 2010; Sikin et al., 2013). On fresh-cut apples, *Pseudomonas graminis* reduced the populations of *L. monocytogenes* and *Salmonella spp.* by approximately 3.0 and 2.5 log CFU/g after five days of storage at 10^0C. The efficacy of *P. graminis;* however, decreased under stimulated commercial conditions. *Pseudomonas graminis* reduced the populations of *L. monocytogenes* and *Salmonella spp.* on fresh-cut apples by approximately 2.5 and 1.0 log CFU/g after five days of storage at 5^0C in modified atmosphere packaging. The decreased efficacy of *P. graminis* under stimulated commercial conditions was attributed to low O_2 levels reducing the growth of *P. graminis* by approximately 2.5 log CFU/g (Alegre et al., 2013). The reduced effect of *P. graminis* against *L. monocytogenes* and *Salmonella* spp. emphasizes the importance of evaluating the potential of the antagonistic strain under commercial applications.

The efficacy of *Enterobacteriacea* and *Pseudomonas* against foodborne pathogen also varies depending on the physical composition of the fruit or vegetable. On apple plugs *Enterobacteriaceae* spp. reduced populations of *E. coli* O157:H7, *Salmonella* spp. and *L. innocua* by 5.0, 4.8, and 4.5 log CFU/g, respectively after two days of storage at 20^0C.The same *Enterobacteriaceae* spp. used to suppress the growth of *E. coli* O157:H7, *Salmonella* spp. and *L. innocua* in fresh-cut apples was used on peach plugs, whose reduction values were 2.8, 2.9, and 4.4 log CFU/g, respectively after two days of storage at 20^0C. Moreover, the efficacy of *Enterobacteriaceae* spp. against *E. coli* O157:H7 was investigated at refrigeration temperature. A 0.5 and 5.0 Log CFU/g reduction of *E. coli* O157:H7 on apple and peach plugs was observed after ten days and six of storage at 5^0C, respectively (Alegre et al., 2012).

1.7.4. Commercially Available Protective Biological Cultures

The commercial availability of protective biological cultures is limited. LactiGuard™, a commercially available blend of *Lactobacillus animalis*, *Pediococcus acidilactici*, and *L. lactis*

was the only commercial product found. LactiGuard™ has gained federal approval as a Generally Recognized as Safe (GRAS) processing aid, acting as a competitive inhibitor to pathogenic organisms in fresh meat, poultry, and ready-to-eat products. LactiGuard™ has significantly suppressed the growth of *E. coli*, *Listeria*, *Salmonella*, and *Camplybacter* in fresh meat and poultry (Dow et al., 2011; Koo et al., 2012). Recent research has been conducted on the efficacy of LactiGuard™ in suppressing the growth of foodborne pathogens on produce.

Concentrations of 10^9 CFU/mL LactiGuard™ reduced the populations of *E. coli* O157:H7 on spinach by 1.4 Log CFU/g after 9 days of storage 4^0C (Brown et al., 2011). Similarly, on spinach inoculated with 10^2 CFU/ g of *E. coli* O157:H7, populations of *E. coli* O157:H7 were 1.6 Log CFU/g lower in the spinach treated with a concentration of 10^8 CFU/mL LactiGuard™ in comparison to the untreated spinach after 3 days of storage at 7^0C. The effect of LactiGuard™ on populations of *Salmonella* spp. did not occur as rapidly as it did on populations of *E. coli* O157:H7. On spinach inoculated with 10^2 CFU/ g of *Salmonella spp.*, populations of *Salmonella* spp. were 1.9 log CFU/g lower in the spinach treated with a concentration of 10^8 CFU/mL LactiGuard™ in comparison to the untreated spinach after 6 days of storage at 7^0C. LactiGuard™ was not effective at suppressing the growth of foodborne pathogens when the foodborne pathogens were inoculated at a higher concentration, 10^4 CFU/mL. The populations of *E. coli* O157:H7 and *Salmonella spp.* on the treated and untreated spinach remained constant after 6 days of storage at 7^0C (Calix-Lara et al., 2014). It; however, is highly improbable for foodborne pathogens to contaminate a fruit or vegetable at concentrations exceeding 10^2 CFU/g.

1.7.5. Combination Strategies

Researchers have investigated the efficacy of combined treatment methods in ensuring food safety. Combination strategies have demonstrated increased bactericidal effect. The combined effect of lactic acid bacteria and citric acid on *E. coli* O157:H7 and *Salmonella typhimurium* was explored. The growth of the lactic acid bacteria was not affected by the addition

of 1% citric acid, while the growth of *E. coli* O157:H7 and *S. typhimurium* were affected by the addition of lactic acid and 1% citric acid. Populations of *E. coli* O157:H7 and *S. typhimurium* were reduced by approximately 1.5 and 3.5 log CFU/mL, respectively (Seo et al., 2012). Additionally, the effectiveness of the bacteriocin enterocin AS-48, produced by *Enterococcus faecalis*, with 1.5% (w/v) lactic acid, 0.4% (w/v) polyphosphoric acid, or 100 ppm chlorine against *S. enterica* and *E. coli* O157:H7 on soybean sprouts was investigated. The combination of enterocin AS-48 and 100 ppm sodium hypochlorite resulted in an approximate 4 log CFU/mL reduction in populations of *S. enterica* after a 48 hour incubation period at 6 ^{0}C. Populations of *S. enterica* treated with enterocin AS-48 and 1.5% (w/v) lactic acid were undetectable after the 48 hour incubation period at 6 ^{0}C. The combination of enterocin AS-48 with 0.4% (w/v) polyphosphoric acid resulted in an approximate 6 log CFU/mL reduction in populations of *E. coli* O157:H7 after a 48 hour incubation period at 6^{0}C (Molinos, et al., 2008). The combined effect of lytic bacteriophage and the bacteriocin, nisin on the suppression of *L. monocytogenes* on fresh-cut honeydew melon and Red Delicious apples was also evaluated. The combination reduced *L. monocytogenes* populations by 5.7 log CFU/g on honeydew melon slices and 3.2 log CFU/g on Red Delicious apples compared to the control after seven days of storage at 10ºC (Leverentz et al., 2003).

1.7.6. Conclusion

Growing consumer awareness of the health benefits associated with fruits and vegetables and demand for easy to prepare products has prompted the development of a wide variety of minimally processed fruits and vegetables (Produce for Better Health Foundation, 2010). Production of fruits and vegetables is expected to increase at an annual rate of 3.0% from 2015 to 2020, reaching $315.2 billion (IBISWorld, 2015). As production and consumption increases, food safety concerns elevate. Food safety practices must evolve to maintain the integrity of the food industry.

A variety of chemical, physical, and biological intervention strategies have been developed and the efficacy of theses intervention methods against *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. have been investigated. Application of protective biological cultures has been most recently explored as a promising strategy in ensuring food safety and maintaining nutritional and sensory quality of the fruit or vegetable. Effectiveness is dependent on the physical composition of the produce and sensitivity of the foodborne pathogen to the protective biological culture. Therefore, antimicrobial efficacy of the protective biological culture against foodborne pathogens must be assessed on various fruits and vegetables.

CHAPTER 2 EFFECTIVENESS OF LACTIC ACID BACTERIA AGAINST *ESCHERICHIA COLI* O157:H7, *LISTERIA MONOCYTOGENES*, AND *SALMONELLA* SPP. ON APPLE SLICES AND ITS INFLUENCE ON PHYSIOCHEMICAL AND SENSORIAL QUALITY

2.1. Abstract

Minimally processed fruits are often peeled, cut, or diced which compromise the fruits' natural protective barriers, exposing a nutrient-rich medium and providing an ideal environment for the growth of microorganisms, including foodborne pathogens. Recent outbreaks and recalls associated with minimally processed fruits indicate additional measures are needed to improve product safety. The objective of this study was to evaluate the efficacy of *Lactobacillus plantarum* alone and in combination with *Lactobacillus acidophilus,* and *Pediococcus pentosaceus* (LPP) against *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. and its influence on the physiochemical and sensorial quality of minimally processed Granny Smith apple slices after five and seven days of storage at 4°C and 20°C, respectively. Apple slices inoculated with *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. (each at 10^4 CFU/g) were treated with *Lb. plantarum* or LPP (10^7 CFU/g). Populations of LAB were maintained at 7.0 log CFU/g throughout storage at 4°C and 20°C. Although LAB had no significant effect on pathogen populations during storage at 4°C ($p > 0.05$), populations were significantly different at 20°C ($p < 0.05$). The combination of *Lb. plantarum* with *P. acidophilum* and *P. pentosaceus* demonstrated greater efficacy than *Lb. plantarum* alone and reduction of *L. monocytogenes* by *Lb. plantarum* and LPP was greater than *Salmonella* spp. and *E. coli* O157:H7. LAB had a minimal effect on the physiochemical and sensorial quality of the apple slices throughout storage at 4°C and 20°C.

Keywords: Biological control, *E. coli* O157:H7, fresh-cut fruit, Lactic Acid Bacteria, *L. monocytogenes,* physiochemical and sensorial quality, and *Salmonella* spp.

2.2. Introduction

Growing consumer awareness of the health benefits associated with fruits and demand for easy to prepare products has prompted the development of a wide variety of minimally processed fruits (Produce for Better Health Foundation, 2010). Minimally processed fruits are often peeled, cut, or diced which compromise the fruits' natural protective barriers, exposing a nutrient-rich medium and providing an ideal environment for the growth of microorganisms, including foodborne pathogens. Although the high acidity of minimally processed fruits is thought to inhibit the proliferation of foodborne pathogens, survival or growth of foodborne pathogens on a variety of acidic fruits, such as apples (pH 3.3 to 4.0), oranges (pH 2.9 to 4.0), peaches (pH 3.3 to 4.1), and strawberries (pH 3.0 to 3.9), has been documented (Alegre et al., 2012; Alegre et al., 2010; Flessa et al., 2005; Sreedharan et al., 2015). From 2008 to 2015 *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* were associated with foodborne illnesses attributed to minimally processed Granny Smith and Gala apples, cantaloupes, mangos, and papaya (CDC, 2015). The recent outbreaks and recalls demonstrates the importance of control measures in ensuring food safety (Abadias et al., 2011).

Presently, refrigeration and hypochlorite are the primary control measures against foodborne pathogens on minimally processed fruits. Low temperatures slow or inhibit microbial growth while hypochlorite affects cellular function. Although refrigeration temperature, 4°C to 7°C, has been shown to inhibit the growth of *E. coli* 0157:H7 and *Salmonella* spp., *L. monocytogenes* is capable of growing at or below refrigeration temperatures, increasing in growth by approximately 0.5 log CFU/g to 1.0 log CFU/g. Hypochlorite can reduce pathogenic growth by 0.5 log CFU/g and 1.0 log CFU/g (Beuchat & Brackets, 1990; Behrsing et al., 2000; Keskinen & Annous, 2011; Weissinger et al., 2000; Abadias et al., 2011). Antimicrobial efficacy is dependent on many factors, including wash water, pH, temperature, organic load, and the inherent properties of the produce commodity (Suslow, 1997; Sikin et al., 2013). Growth of *L. monocytogenes* at refrigeration temperatures, temperature abuse resulting in a proliferation of

pathogenic growth, and the low efficacy of hypochlorite illustrates the inefficiencies of the current control measures and the need for additional strategies to improve product safety.

The application of protective bacterial cultures could be used to reduce the growth of foodborne pathogens while maintaining product quality. Lactic acid bacteria (LAB) have demonstrated potential and have been identified as ideal protective bacterial cultures due to their historical use in the preservation of foods, such as cheese, yogurt, and sauerkraut, designation as a substance Generally Recognized as Safe (GRAS) for human consumption, and probiotic qualities (Alegre et al., 2011; Anas et al., 2014; Calix-Lara et al., 2014; Kumar et al., 2010; Russo et al., 2014; Siroli et al., 2015; Trias et al., 2007). *Leuconostoc mesenteriodes* reduced populations of *E. coli* O157:H7, *L. monocytogenes, Salmonella* spp. on apple wounds and lettuce cuts by approximately 1.0, 3.0, and 2.0 Log CFU/g after two days of storage at 20^0C, respectively (Trias et al., 2007). On fresh-cut pineapple stored at 5^0C for seven days *Lactobacillus fermentum* reduced the growth of *E. coli* O157:H7 and *L. monocytogenes* by approximately 1.0 and 2.0 Log CFU/g while *Lb. plantarum* reduced populations by approximately 0.5 log CFU/g and 1.0 log CFU/g, respectively. Additionally, *Lb. fermentum* and *Lb. plantarum* did not have a significant effect on the texture, flavor, and appearance of the pineapple slices during storage (Russo et al., 2014). The growth of *L. monocytogenes* on apples treated with *Lactobacillus rhamnosus* was reduced by approximately 1.0 Log CFU/g after 28 days of storage at 5^0C and 10^0C without significantly influencing sensory quality (Alegre et al., 2011; Roble et al., 2010). On apple slices treated with *Lactobacillus plantarum* populations of *E. coli* O157:H7 were significantly reduced by approximately 0.5 log CFU/g and 2.5 log CFU/g after two and seven storage days at 6°C, respectively; *Lb. plantarum*, however, had no significant effect on *L. monocytogenes* (Russo et al., 2014; Siroli et al., 2015). Variability in the effectiveness of the protective bacterial culture against various foodborne pathogens and in different food matrixes demonstrates the value in using a mixture of protective bacterial cultures.

The application of a mixture of protective bacterial cultures has many potential advantages, which include (i) multiple modes of action against the pathogen; (ii) potential to select organisms that affect more than one pathogen; (iii) increased consistency in performance on different products (Meyer et al., 2002). LactiGuard™, composed of three *Lactobacillus, Pediococcus,* and *Lactococcus* species, has been used on leafy vegetables. LactiGuard™ was able to significantly reduce populations of *E. coli* O157:H7 and *Salmonella* spp. on spinach by approximately 1.0 log CFU/g and 2.0 log CFU/g after three and six days of storage at 7°C, respectively (Calix-Lara, 2014). Similarly, *Salmonella* spp. on turkey breasts treated with LactiGuard™ was significantly different than the control by 1.0 log CFU/g after three days of storage at 5°C (Dow et al., 2011).

The objectives of this study were 1) to determine the efficacy of *Lb. plantarum* alone and in combination with *P. acidophilus* and *P. pentosaceus* (LPP) on the reduction of *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. on Granny Smith apples stored at 4°C and 20°C for seven and five days, respectively, 2) to assess the physiochemical quality of Granny Smith apple slices treated with lypholized *Lb. plantarum* alone and in combination with *P. acidophilus,* and *P. pentosaceus* (LCM1), and 3) to determine the consumer acceptability of Granny Smith apple slices treated with *Lb. plantarum* and LCM1 after one and seven days of storage at 4°C.

2.3. Materials and Methods

2.3.1. Antimicrobial Activity

2.3.1.1. Granny Smith Apples

Organic Granny Smith apples were obtained from a local grocery store (San Luis Obispo, CA). The apples were free from visible defects such as bruises, or cuts and were stored at 4°C in the Food Science pilot plant facility (San Luis Obispo, CA) until testing.

2.3.1.2. Foodborne Pathogen Preparation

Serotypes of *E. coli* O15:H7, *L. monocytogenes*, and *Salmonella* spp. (Table 4) were maintained on tryptic soy agar (TSA) at 4°C until inoculation. Prior to inoculation the bacterial cultures were acid adapted since microorganisms in fruits are often adapted to more acidic environmental conditions (Buchanan and Edelson, 1996; Van Schaik et al., 1999). Briefly, cultures were grown in 10 ml of tryptic soy broth (TSB) at 35°C for 18 to 20 h. A 10 µl loop of bacterial culture was transferred into 10 ml of TSB containing 0.1% glucose, and incubated at 35°C for 18 to 20 h. This was repeated and then the three serotypes of *E. coli* O15:H7, *L. monocytogenes*, and *Salmonella* spp. were combined to form a mixture and then washed twice by centrifugation (3000 rpm; 15 min) using 0.1% peptone. The cell concentrations were determined by Direct Microscope Count (DMC) using a hemocytometer and the inoculum concentration was adjusted with 0.1% peptone to achieve a target concentration of 10^4 CFU/g on the apple slice. The inoculum concentration was confirmed by plate counts.

2.3.1.3. Lactic Acid Bacteria Preparation

Lactobacillus plantarum, P. acidophilus, and *P. pentosaceus* were obtained from BiOWiSH technologies (Cincinnati, OH) (Table 4). The LAB cultures were maintained on de Man, Rogosa, and Sharpe (MRS) agar at 4°C until inoculation. Prior to inoculation *Lb. plantarum, P. acidophilus,* and *P. pentosaceus* were grown in 150 ml of MRS broth at 35°C for 18 to 24 h. *Lactobacillus plantarum* alone and in combination with *P. acidophilus,* and *P. pentosaceus* (LPP) were washed twice by centrifugation (3000 rpm; 15 min) using 15 ml of 0.1% peptone water. The cell concentrations were determined by Direct Microscope Count (DMC) using a hemocytometer and the inoculum concentration of 10^7 CFU/g on the apple slice was confirmed by plate counts.

Table 4. Foodborne pathogen and antagonist strain name, number and source.

Strain Name	Strain Number	Source[1]
E. coli 0157:H7	NFPA 4213	NFL
E. coli 0157:H7	NFPA 4217	NFL
E. coli 0157:H7	NFPA 4219	NFL
L. monocytogenes	N3-013	ILSI Na (Cornell)
L. monocytogenes	NFPA 6301	NFL
L. monocytogenes	NRRL-B33013	ARS
Salmonella spp.	ATCCBAA 1593	NFL
Salmonella spp.	NFPA 7100	NFL
Salmonella spp.	NFPA 7201	NFL
Lb. plantarum	TISTR 050	BiOWiSH
P. acidilactici	ATCC 12697	BiOWiSH
P. pentosaceus	BCC 38038	BiOWiSH

[1] ARS – Agriculture Research Services (Peoria, IL); ILSI NA – Institute of Life Sciences of North America (Cornell University, Ithaca, NY); NFL – The National Food Laboratory (Livermore, CA); BiOWiSH - BiOWiSH Technologies, Inc. (Cincinnati, OH).

2.3.1.4. Granny Smith Apple Inoculation

Prior to inoculation, the surface of the apples were washed for 15 s in running deionized water, sprayed with 5 ml of 70% ethanol and air dried for 30 min under a biological safety cabinet. The sanitized apples were sliced using a sterile stainless steel apple slicer. The weight of the apple slices was approximately 12 ± 3 g. The flesh of the apple slices was spot inoculated with 20 µL of either a pathogen mixture or 0.1% sterile peptone water. Treated apple slices were air dried at 20˚C in the biological safety cabinet for approximately 45 min. Afterwards, 200 µL of either *Lb. plantarum*, LPP, or 0.1% sterile peptone water was spot inoculated on the flesh of the apple slices. Treated apple slices were air dried at room temperature in the biological safety cabinet for approximately 1.5 h. The treated apple slices were then placed in Whirl-Pak® bags and stored at 4˚C and 20˚C for seven and five days, respectively.

2.3.1.5. Bacterial Enumeration

Samples were diluted with 0.1% peptone water, homogenized in a stomacher at 200 rpm for one minute, serially diluted, and plated on the appropriate media. The native microflora and LAB were enumerated using TSA and MRS agar, respectively. The agar plates were incubated at

35°C for 24 to 48 h. An overlay method adapted from Al-Holy et al. (2008) was used for the recovery of the stressed or injured *E.coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. Samples were pour plated with TSA and incubated at 35°C for two hours to enable to reparation of stressed and injured cells. Plates containing *E.coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. were then overlaid with the selective media, sorbitol MacConkey agar (SMAC), modified oxford agar (MOX), and xylose lysine deoxycholate agar (XLD), respectively and incubated at 35°C for 22 to 46 h. Populations of *E.coli* 0157:H7, *L. monocytogenes, Salmonella* spp., LAB, and native microflora were enumerated on Day 0, 1, 3, 5, and 7 at 4°C and Day 0, 1, 3, and 5 at 20°C. Each experiment, containing three samples for each treatment condition, was done twice.

2.3.2. Physiochemical Analysis

2.3.2.1. Lactic Acid Bacteria Preparation

Food Grade lypholized *Lb. plantarum* and a mixture of *Lb. plantarum, P. acidophilus,* and *P. pentosaceus* (LCM1) were provided by BiOWiSH Technologies, Inc. (Cincinnati, OH). The lyophilized product was stored at 4°C in the Food Science pilot plant facility until use.

2.3.2.2. Granny Smith Apple Treatment

A 1:10 dilution of the lypophilized *Lb. plantarum* and LCM1 was made using tap water. Apple slices (12 ± 3 g) were dipped in either the *Lb. plantarum* or LCM1 solution at a 3:1 LAB solution/apple slice ratio or tap water for two minutes with agitation. After treatment, apple slices were air-dried, placed in Whirl-Pak® bags and stored at 4˚C and 20˚C for 7 and 5 days, respectively. Physiochemical analysis occurred before treatment, 1, 3, 5, and 7 days after treatment at 4°C and 1, 3, and 5 days after treatment at 20°C. All analytical measurements were done in triplicate. Sensory quality testing was conducted one and seven days after treatment at 4°C.

2.3.2.3. Texture Analysis

Firmness, defined as work/deformation peak and expressed in Newton (N), was measured with a Brookfield Texture Analyzer. The established trigger, deformation peak, and speed were 1.0 g, 10.0 mm, and 1.0 mm/s, respectively. A 1.0 mm needle probe, 43.0 mm in length was used to penetrate the flesh of the apple slice. For each replicate the work and deformation peak was measured at three different points on the flesh of the apple slice.

2.3.2.4. Total Soluble Solids, Titratable Acidity, and pH

Total soluble solids (TSS) were measured with a refractometer. For pH and titratable acidity (TA), three apple slices were crushed using a stomacher. The obtained juice was used to measure pH. Then, 2 ml of juice was titrated. TA was expressed as percent of malic acid (applying the acid milliequivalent factor 0.067).

2.3.3. Sensorial Quality

2.3.3.1. Ethics Statement

Consumer panels were conducted at the Food Science and Nutrition Department at California Polytechnic University, San Luis Obispo. The University's Human Subjects Committee approved the protocol and written consent form, which participants were required to complete before participating in the acceptability testing.

2.3.3.2. Acceptability Testing

Apple slices treated with *Lb. plantarum*, LCM1, or tap water (control) were prepared as specified for physiochemical analysis and subjected to sensory analysis by a sensory panel. The sensory panel was recruited from California Polytechnic University, San Luis Obispo. The sensory panelists were frequent apple consumers; 6.90%, 65.5%, and 27.6% consumed apples daily, two to four times a week, and once a month. Of the apple consumers 75.9% consumed Granny Smith. The sensory panel was composed of 29 untrained panelists between the ages of 18 and 30 years.

Consumer acceptance tests were conducted for evaluation of aroma, taste, texture, and overall acceptance of apple slices one and seven days after treatment at 4°C. A 9-point hedonic scale was used, with 0 as 'dislike extremely' and 8 as 'like extremely'. Tasting was done in a sensorial testing room with individual booth and controlled lighting. Apple slices were served in Ziploc® sandwich bags, labeled with a four-digit code, and served in a random serving order. Panelists were provided with water for cleansing the palate between samples.

2.3.4. Statistical analysis

A total of six and three replicates for each treatment condition were used to determine the means and standard deviations for microbial populations (log CFU/g) and physiochemical quality, respectively. One way analysis of variance (ANOVA) and Tukey pairwise comparison were performed using JMP (Version 11, SAS Institute Inc., Cary, NC, USA). Significance of difference was defined at a 95% confidence interval ($\alpha = 0.05$).

2.4. Results and Discussion

2.4.1. Antimicrobial Activity

Populations *Lb. plantarum* and LPP, both in the presence or absence of the foodborne pathogens, were between 6.8 log CFU/g and 7.5 log CFU/g throughout storage at 4°C and 20°C (Table 5, Table 6). Storage stability of LAB at an approximate concentration of 10^7 CFU/g on produce has been previously reported (Calix-Lara et al., 2014; Russo et al., 2014; Siroli et al., 2015). Population levels of *Lb. plantarum* and *L. fermentum* on fresh-cut pineapple were between 6.8 log CFU/g and 8.0 log CFU/g during six days of storage at 5°C (Russo et al., 2014). Similarly, LAB on lamb's lettuce, sliced apples, and spinach stored at 7°C were between 7.6 log CFU/g and 8.0 log CFU/g, regardless of the presence of the foodborne pathogen. (Calix-Lara et al., 2014; Siroli et al., 2015).

The initial populations of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp. were 4.97 log CFU/g, 4.81 log CFU/g, and 4.77 log CFU/g, respectively (Appendix A). Pathogen

growth was not observed at 4°C. At 20°C, however, *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. increased by approximately 2.0 log CFU/g after five days of storage (Fig. 1- 6). The observed stability in populations of foodborne pathogens at refrigeration temperatures and an increase in growth at abusive temperatures were comparable to other studies. On peaches and apples stored for seven days at 5°C populations of *E. coli* O157:H7 remained stable while a 2.0 log CFU/g to 3.0 log CFU/g increase in populations of *E. coli* 0157:H7, *L. monocytogenes,* and *Salmonella* spp. was observed after two days of storage at 20°C (Alegre et al., 2012).

The antimicrobial activity of LAB against *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. on apple slices was not observed during storage at 4°C, indicating that at 4°C LAB is ineffective at reducing foodborne pathogens inoculated at an initial concentration of 10^4 CFU/g. Observed results were comparable to the literature. Russo et al. (2014) determined that populations of *Lb. plantarum* and *L. fermentum* were unsuccessful at reducing populations of *E. coli* O157:H7 and *L. monocytogenes,* inoculated at a concentration of 7.0 log CFU/g, on fresh-cut pineapple after six days of storage at 5°C. Calix-Lara et al. (2014) also observed no significant difference between populations of *E. coli* O157:H7 and *Salmonella* spp., inoculated at a concentration of 4.0 log CFU/g, in the presence or absence of the constituents of LactiGuard™ on spinach stored for 12 days at 7°C. However, a significant difference was observed between populations of *E. coli* O157:H7 and *Salmonella* spp. inoculated at a concentration of 2.0 log CFU/g in the presence of the constituents of LactiGuard™ indicating the efficacy of LAB at lower temperatures is concentration dependent.

Although a lower concentration of pathogens may be necessary to obtain an effect at 4°C, effectiveness of LAB was seen during storage at 20°C (Fig. 1 - 6). Both *Lb. plantarum* and LPP reduced populations of *L. monocytogenes* and *E. coli* while only LPP reduced populations of *Salmonella* spp. ($p < 0.05$) (Fig. 3 - 6). Populations of *L. monocytogenes* in the presence of *Lb. plantarum* and LPP were 1.84 log CFU/g and 2.84 log CFU/g less than the controls after five days of storage at 20°C ($p < 0.05$) (Appendix A). Additionally, on apple slices treated with LPP

L. monocytogenes was reduced by 1.94 log CFU/g after five days of storage (Fig 1, Fig. 2). Populations of *E. coli* O157:H7 in the presence of *Lb. plantarum* and LPP were 1.83 log CFU/g and 1.86 log CFU/g less than the control after one and three days of storage, respectively (Appendix A). The earlier observed antimicrobial activity of *Lb. plantarum* may suggest that *Lb. plantarum* alone may be more effective against *E. coli* O157:H7 than the combination of *Lb. plantarum* with *P. acidophilum* and *P. pentosaceus*. However, by day five of storage there was no difference between populations of *E. coli* O157:H7 inoculated alone and with *Lb. plantarum* ($p > 0.05$) while with LPP there was a 1.01 log CFU/g difference ($p < 0.05$) (Fig. 3, Fig. 4). Although there was no difference between populations of *Salmonella* spp. in the absence and presence of *Lb. plantarum* ($p > 0.05$), there was a difference in presence of LPP ($p < 0.05$) (Fig. 6). Populations of *Salmonella* spp. were 0.86 log CFU/g less than populations in the absence of LPP after three days of storage (Appendix A).

Overall, LPP demonstrated greater efficacy than *Lb. plantarum* alone and reduction of *L. monocytogenes* by *Lb. plantarum* and LPP was greater than *Salmonella* spp. and *E. coli* O157:H7. Higher efficacy of LPP may be related to differing modes of actions while specificity of LAB against *L. monocytogenes* may be related to the higher sensitivity of Gram positive bacteria to the production of bacteriocins and accumulation of lactic acid (Aguilar et al., 2011; Trias et al., 2008).

Table 5. Population (log CFU/g) of Lb. plantarum in the presence and absence of E. coli O157:H7, L. monocytogenes, and Salmonella spp. on Granny Smith apple slices during seven and five days of storage at 4°C and 20°C, respectively.

	Population (log CFU/g) of *Lb. plantarum*	
	Storage at 4°C	Storage at 20°C
Storage Day 0		
Lb. plantarum	7.19 ± 0.29^{Aa}	6.82 ± 0.39 ^{Aa}
Lb. plantarum/*E. coli* O157:H7	7.35 ± 0.48 ^{Aa}	7.35 ± 0.48 ^{Aa}
Lb. plantarum/*L. monocytogenes*	7.31 ± 0.66 ^{Aa}	6.70 ± 0.32 ^{Aa}
Lb. plantarum/*Salmonella* spp.	7.19 ± 0.32 ^{Aa}	6.77 ± 0.25 ^{Aa}
Storage Day 1		
Lb. plantarum	7.12 ± 0.45 ^{Aa}	7.09 ± 0.28 ^{Aa}
Lb. plantarum/*E. coli* O157:H7	7.41 ± 0.48 ^{Aa}	7.46 ± 0.49 ^{Aa}
Lb. plantarum/*L. monocytogenes*	7.19 ± 0.44 ^{Aa}	6.89 ± 0.20 ^{Aa}
Lb. plantarum/*Salmonella* spp.	7.07 ± 0.57 ^{Aa}	6.96 ± 0.34 ^{Aa}
Storage Day 3		
Lb. plantarum	6.79 ± 0.64 ^{Aa}	7.11 ± 0.47 ^{Aa}
Lb. plantarum/*E. coli* O157:H7	7.28 ± 0.59 ^{Aa}	7.52 ± 0.66 ^{Aa}
Lb. plantarum/*L. monocytogenes*	7.06 ± 0.06 ^{Aa}	6.95 ± 0.10 ^{Aa}
Lb. plantarum/*Salmonella* spp.	7.24 ± 0.69 ^{Aa}	7.13 ± 0.20 ^{Aa}
Storage Day 5		
Lb. plantarum	7.24 ± 0.44 ^{Aa}	7.21 ± 0.26 ^{Aa}
Lb. plantarum/*E. coli* O157:H7	7.48 ± 0.54 ^{Aa}	7.48 ± 0.73 ^{Aa}
Lb. plantarum/*L. monocytogenes*	6.88 ± 0.40 ^{Aa}	6.80 ± 0.13 ^{Aa}
Lb. plantarum/*Salmonella* spp.	7.35 ± 0.41 ^{Aa}	6.97 ± 0.20 ^{Aa}
Storage Day 7		
Lb. plantarum	7.29 ± 0.45 ^{Aa}	-
Lb. plantarum/*E. coli* O157:H7	7.05 ± 0.13 ^{Aa}	-
Lb. plantarum/*L. monocytogenes*	7.05 ± 0.12 ^{Aa}	-
Lb. plantarum/*Salmonella* spp.	7.23 ± 0.51 ^{Aa}	-

Reported values are means ± standard deviations (n = 6). Within the columns means with different uppercase letters between treatments on the same storage day and lowercase letters within treatments during storage are significantly different according to Tukey's test (p-value < 0.05).

Table 6. Population (log CFU/g) of LPP in the presence and absence of E. coli O157:H7, L. monocytogenes, and Salmonella spp. on Granny Smith apple slices during seven and five days of storage at 4°C and 20°C, respectively.

	Population (log CFU/g) of LPP	
	Storage at 4°C	Storage at 20°C
Storage Day 0		
LPP	7.75 ± 0.09 ^{Aa}	7.76 ± 0.09 ^{Aa}
LPP/*E. coli* O157:H7	7.87 ± 0.24 ^{Aa}	7.87 ± 0.24 ^{Aa}
LPP/*L. monocytogenes*	7.62 ± 0.59 ^{Aa}	7.69 ± 0.60 ^{Aa}
LPP/ *Salmonella* spp.	7.85 ± 0.19 ^{Aa}	7.98 ± 0.07 ^{Aa}
Storage Day 1		
LPP	7.64 ± 0.47 ^{Aa}	7.36 ± 0.14 ^{Aa}
LPP/*E. coli* O157:H7	7.80 ± 0.17 ^{Aa}	7.69 ± 0.43 ^{Aa}
LPP/*L. monocytogenes*	7.61 ± 0.34 ^{Aa}	7.36 ± 0.41 ^{Aa}
LPP/ *Salmonella* spp.	7.80 ± 0.23 ^{Aa}	7.70 ± 0.12 ^{Aa}
Storage Day 3		
LPP	7.40 ± 0.28 ^{Aa}	7.59 ± 0.19 ^{Aa}
LPP/*E. coli* O157:H7	7.67 ± 0.28 ^{Aa}	7.61 ± 0.42 ^{Aa}
LPP/*L. monocytogenes*	7.74 ± 0.39 ^{Aa}	7.68 ± 0.29 ^{Aa}
LPP/ *Salmonella* spp.	7.74 ± 0.32 ^{Aa}	7.74 ± 0.32 ^{Aa}
Storage Day 5		
LPP	7.64 ± 0.36 ^{Aa}	7.53 ± 0.19 ^{Aa}
LPP/*E. coli* O157:H7	7.78 ± 0.33 ^{Aa}	7.87 ± 0.42 ^{Aa}
LPP/*L. monocytogenes*	7.60 ± 0.18 ^{Aa}	7.69 ± 0.41 ^{Aa}
LPP/ *Salmonella* spp.	7.52 ± 0.61 ^{Aa}	7.52 ± 0.61 ^{Aa}
Storage Day 7		
LPP	7.20 ± 0.18 ^{Aa}	-
LPP/*E. coli* O157:H7	7.62 ± 0.33 ^{Aa}	-
LPP/*L. monocytogenes*	7.47 ± 0.30 ^{Aa}	-
LPP/ *Salmonella* spp.	7.37 ± 0.38 ^{Aa}	-

Reported values are means ± standard deviations (n = 6). Within the columns means with different uppercase letters between treatments on the same storage day and lowercase letters within treatments during storage are significantly different according to Tukey's test (p-value < 0.05).

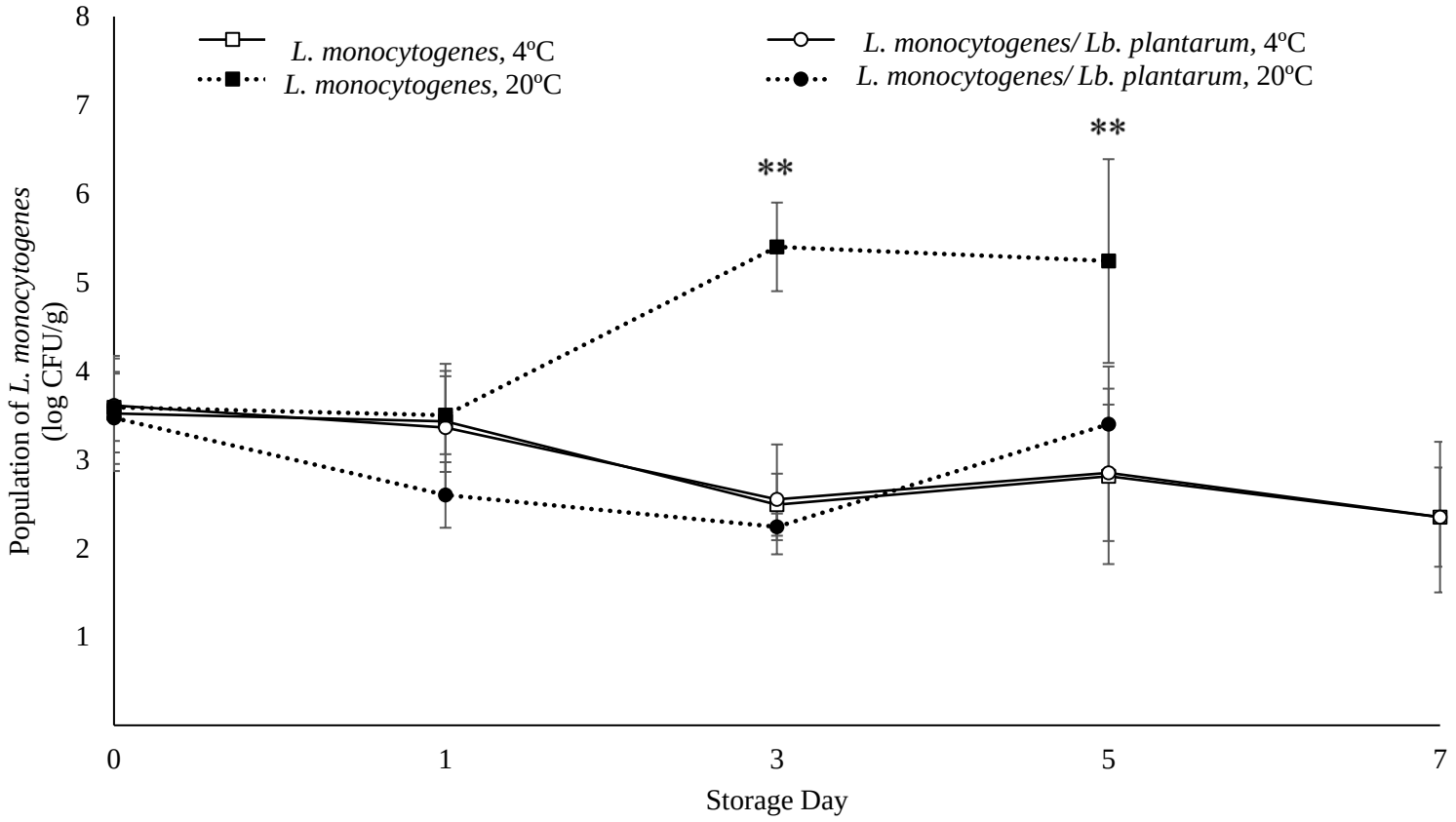

Figure 1. Mean population (log CFU/g) of L. monocytogenes in the presence and absence of Lb. plantarum at 4°C and 20°C during seven and five days of storage, respectively (n =6).Error bars represent standard deviation of the mean. ** Values between treatments on the same storage day at 20°C are significantly different according to Tukey's test (p-value < 0.05).

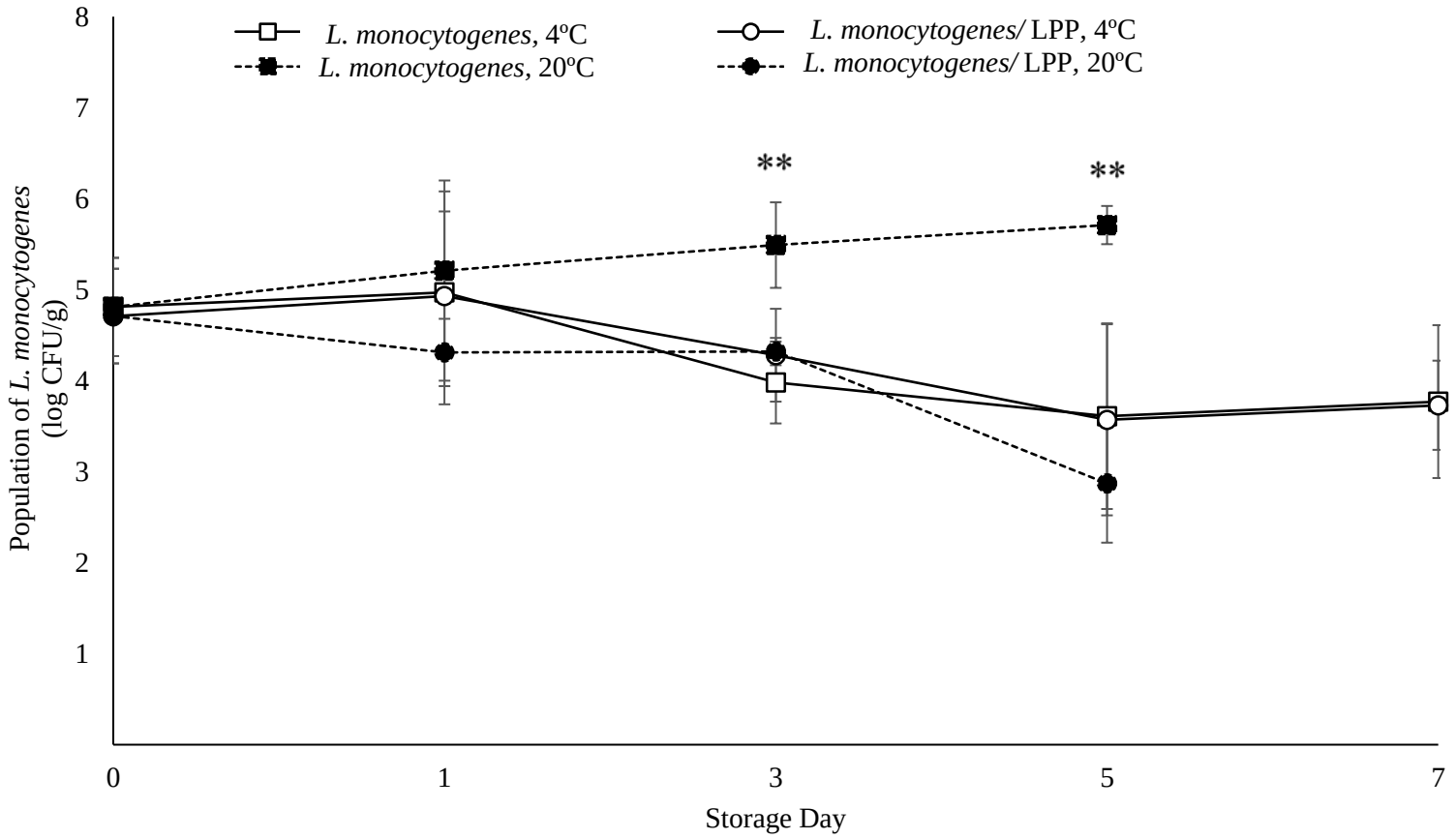

Figure 2. Mean population (log CFU/g) of L. monocytogenes in the presence and absence of LPP at 4°C and 20°C during seven and five days of storage, respectively (n =6). Error bars represent standard deviation of the mean. ** Values between treatments on the same storage day at 20°C are significantly different according to Tukey's test (p-value < 0.05).

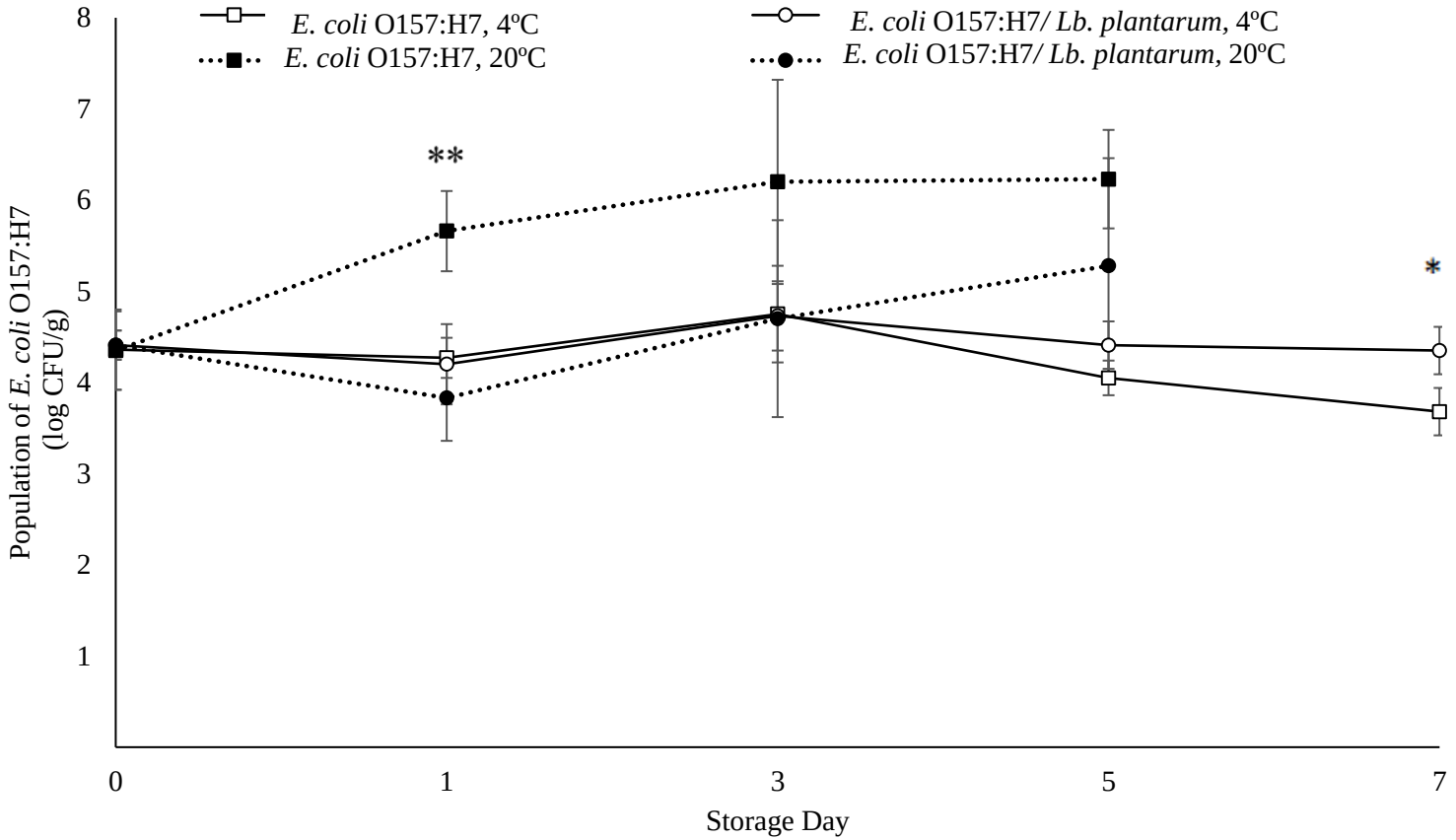

Figure 3. Mean population (log CFU/g) of E. coli O157:H7 inoculated in the presence and absence of Lb. plantarum at 4°C and 20°C during seven and five days of storage, respectively (n =6).Error bars represent standard deviation of the mean. * Single and ** double stars indicates significant differences between treatments on the same storage day at 4°C and 20°C, respectively (p-value < 0.05).

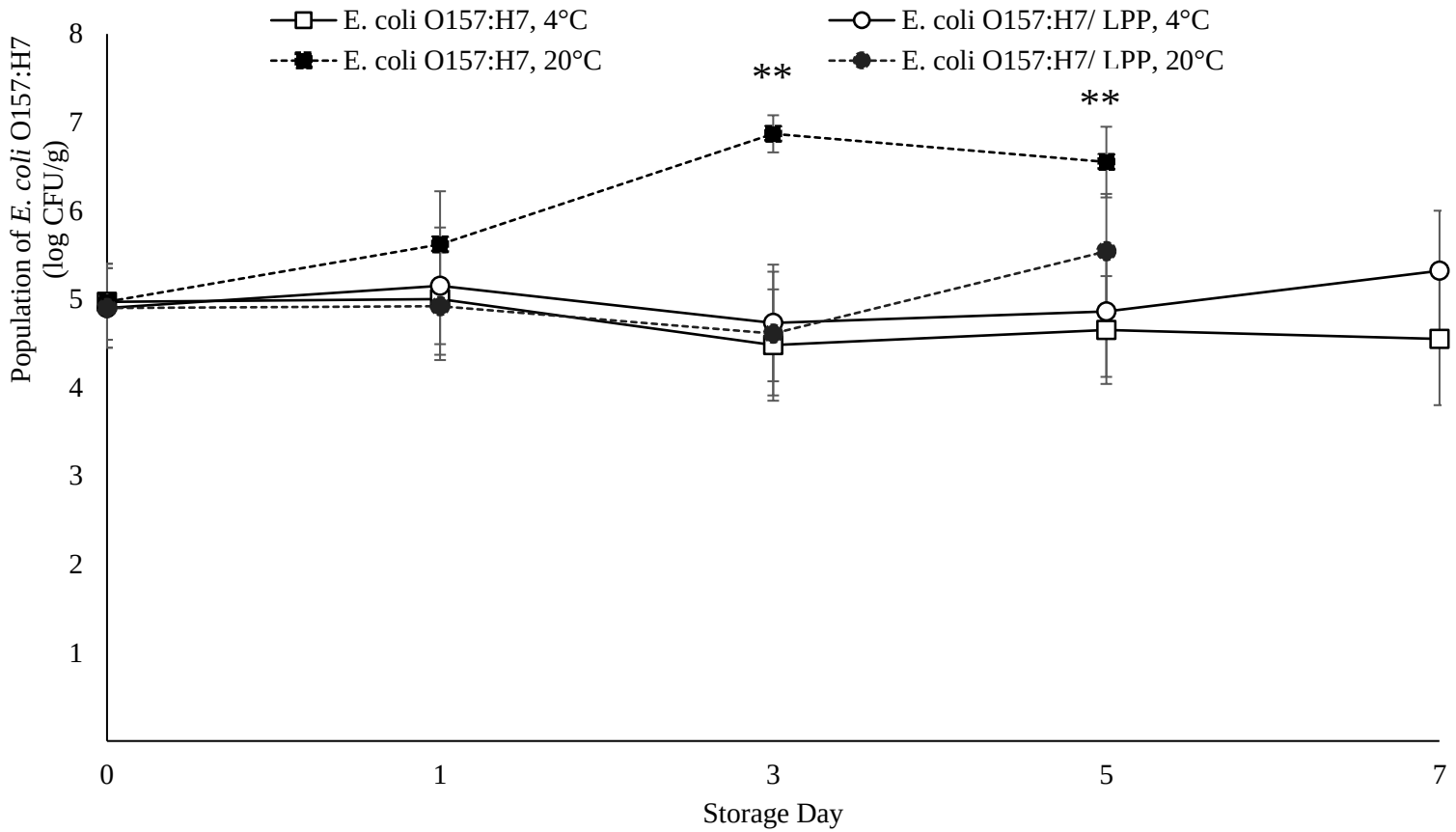

Figure 4. Mean population (log CFU/g) of E. coli O157:H7 in the presence and absence of LPP at 4°C and 20°C during five and seven days of storage, respectively (n =6). Error bars represent standard deviation of the mean. *Single and **double stars indicates significant differences between treatments on the same storage day at 4°C and 20°C, respectively (p-value < 0.05).

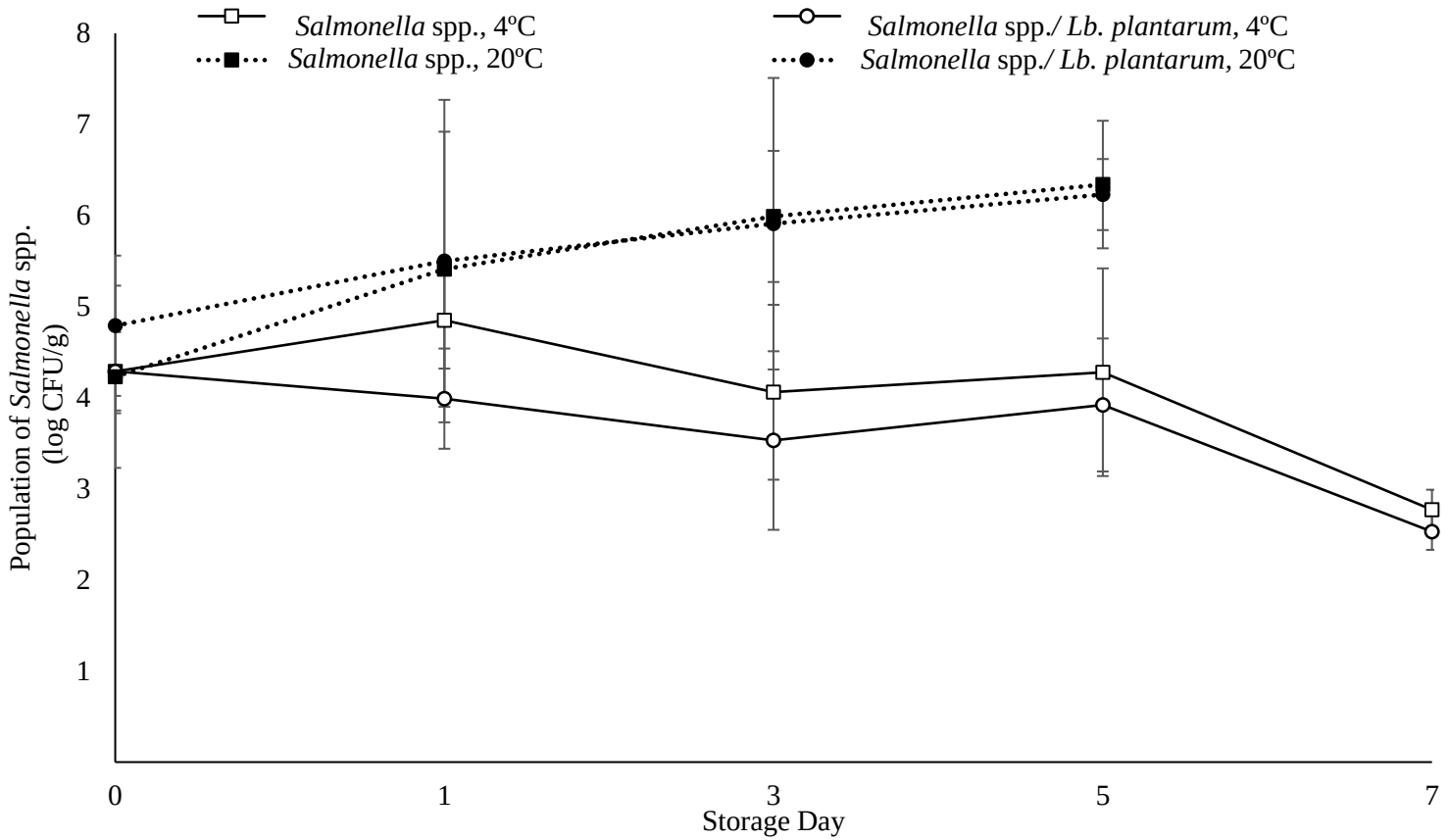

Figure 5. Mean population (log CFU/g) of Salmonella spp.in the presence and absence of Lb. plantarum at 4°C and 20°C during seven and five days of storage, respectively (n =6). Error bars represent standard deviation of the mean.

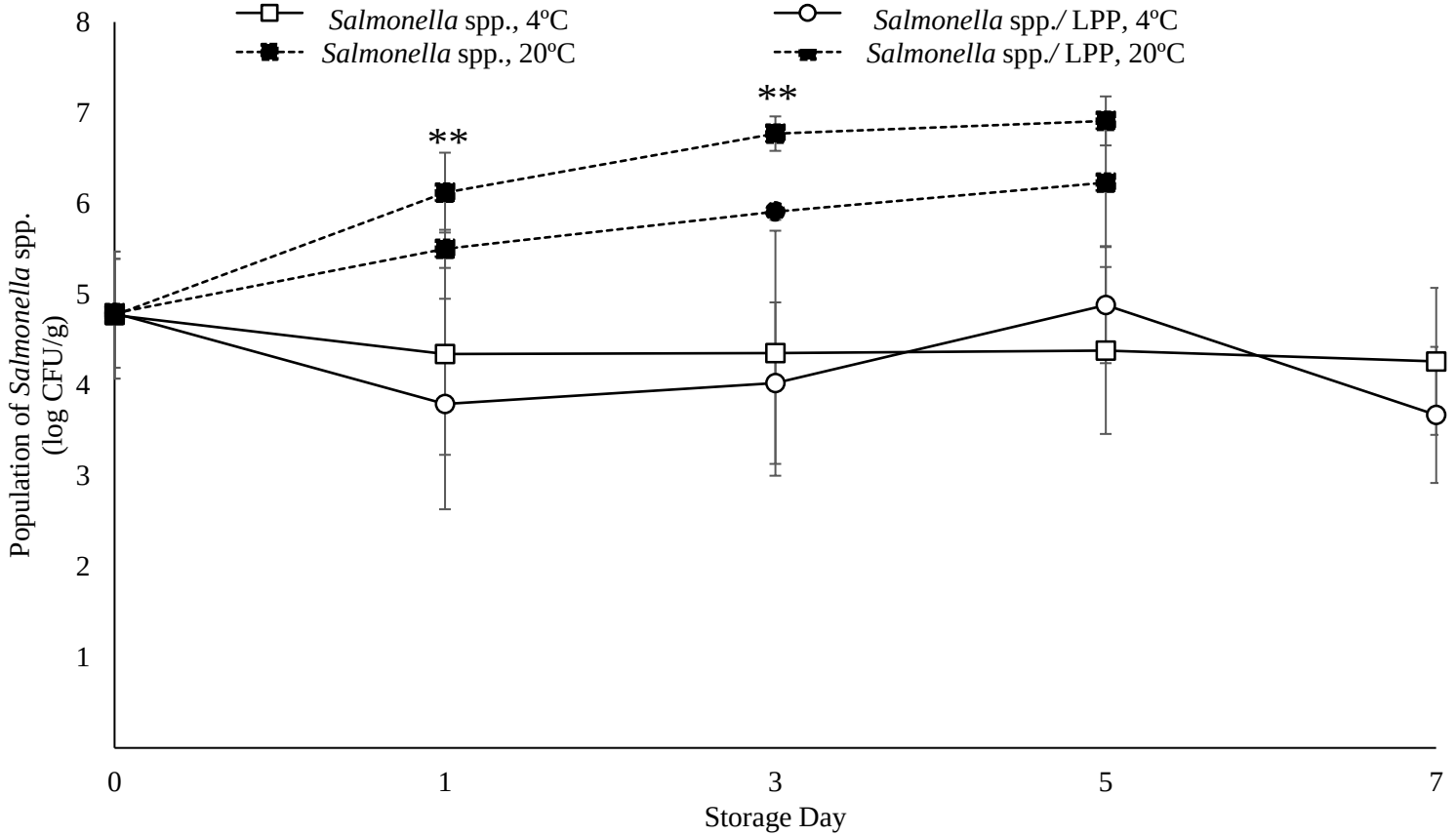

Figure 6. Mean population (log CFU/g) of Salmonella spp.in the presence and absence of LPP at 4°C and 20°C during seven and five days of storage, respectively (n =6). Error bars represent standard deviation of the mean. ** Values between treatments on the same storage day at 20°C are significantly different according to Tukey's test (p-value < 0.05).

2.4.2. Physiochemical Quality

Before treatment the apple slices had a firmness of 1.3 N and the juice extracted from the apple slices had a solid soluble content of 12.1, pH of 3.40, and titratable acidity of 0.36%. The lactic acid bacteria had a minimal effect on the physiochemical quality of the apple slices throughout storage, however, some differences were observed in solid soluble content, juice pH, and titratable acidity (Table 2.4; Table 2.5). The titratable acidity and the °Brix of apple slices treated with LCM1 was significantly less than the untreated and treated apple slices with *Lb. plantarum* after five days of storage at 4°C ($p < 0.05$). The pH and the °Brix of the apple slices treated with *Lb. plantarum* were significantly less than the untreated and treated apple slices with LCM1 after seven days of storage at 4°C ($p < 0.05$) (Table 7). The pH of the apple slices treated with *Lb. plantarum* and LCM1 were significantly less than the untreated apple slices after one day of storage at 20°C ($p < 0.05$) (Table 8).

Table 7. Physiochemical quality (°Brix, pH, rupture load force, and titratable acidity) of Granny Smith apple slices treated with *Lb. plantarum* or LCM1 and stored for 7 days at 4°C.

	°Brix	pH	Rupture load force (N)	Titratable acidity (%)
Storage Day 0				
	12.1 ± 0.14	3.40 ± 0.11	1.3 ± 0.26	0.36 ± 0.02
Storage Day 1				
Control	11.9 ± 0.06^{a}	3.56 ± 0.03^{a}	1.0 ± 0.20^{a}	0.33± 0.02^{a}
Lb. plantarum	11.3 ± 0.06^{b}	3.53 ± 0.03^{a}	0.7 ± 0.01^{a}	0.29± 0.03^{a}
LCM1	11.9 ± 0.00^{a}	3.56 ± 0.04^{a}	1.0 ± 0.15^{a}	0.34 ± 0.02^{a}
Storage Day 3				
Control	11.6 ± 0.21^{a}	3.34 ± 0.01^{a}	1.3 ± 0.55^{a}	0.31 ± 0.02^{a}
Lb. plantarum	11.5 ± 0.49^{a}	3.35 ± 0.02^{a}	1.0 ± 0.45^{a}	0.33 ± 0.02^{a}
LCM1	11.6 ± 0.47^{a}	3.35 ± 0.03^{a}	0.7 ± 0.23^{a}	0.33 ± 0.02^{a}
Storage Day 5				
Control	15.4 ± 2.23^{a}	3.34 ± 0.02^{a}	0.9 ± 0.00^{a}	0.32 ± 0.00^{a}
Lb. plantarum	12.5 ± 0.69^{ab}	3.34 ± 0.03^{a}	0.9 ± 0.17^{a}	0.35± 0.03^{a}
LCM1	11.3 ± 0.76^{b}	3.36 ± 0.03^{a}	0.7 ± 0.20^{a}	0.25 ± 0.02^{b}
Storage Day 7				
Control	11.2 ± 0.23^{a}	3.52 ± 0.03^{a}	0.9 ± 0.30^{a}	0.29 ± 0.00^{a}
Lb. plantarum	10.7 ± 0.23^{b}	3.43 ± 0.02^{b}	0.9 ± 0.26^{a}	0.30 ± 0.00^{a}
LCM1	10.9 ± 0.15^{ab}	3.52 ± 0.05^{ab}	1.0 ± 0.21^{a}	0.30 ± 0.02^{a}

Reported values are means ± standard deviations (n = 3). Within the columns means with different letters on the same storage day are significantly different according to Tukey's test (p value < 0.05).

Table 8. Physiochemical quality (○Brix, pH, rupture load force, and titratable acidity) of Granny Smith apple slices treated with *Lb. plantarum* or LCM1 and stored for 5 days at 20◦C.

	◦Brix	pH	Rupture load force (N)	Titratable acidity (%)
Storage Day 0				
	12.1 ± 0.14	3.40 ± 0.11	1.3 ± 0.26	0.36 ± 0.02
Storage Day 1				
Control	11.9 ± 0.15[a]	3.66 ± 0.01[a]	1.0 ± 0.20[a]	0.27± 0.02[a]
Lb. plantarum	11.6 ± 0.49[a]	3.55 ± 0.03[b]	0.7 ± 0.00[a]	0.29± 0.09[a]
LCM1	10.9 ± 0.95[a]	3.53 ± 0.02[b]	1.0 ± 0.15[a]	0.36 ± 0.02[a]
Storage Day 3				
Control	11.2 ± 0.26[a]	3.35 ± 0.06[a]	1.1 ± 0.25[a]	0.31 ± 0.02[a]
Lb. plantarum	11.3 ± 1.10[a]	3.38 ± 0.03[a]	1.2 ± 0.40[a]	0.33 ± 0.03[a]
LCM1	9.7 ± 0.46[a]	3.31 ± 0.02[a]	0.7 ± 0.06[a]	0.33 ± 0.00[a]
Storage Day 5				
Control	10.8 ± 0.69[a]	3.41 ± 0.02[a]	1.2 ± 0.31[a]	0.27 ± 0.02[a]
Lb. plantarum	11.3 ± 0.41[a]	3.39 ± 0.01[ab]	0.9 ± 0.14[a]	0.26± 0.04[a]
LCM1	9.6 ± 0.53[a]	3.36 ± 0.01[a]	1.0 ± 0.17[a]	0.30 ± 0.02[a]

Reported values are means ± standard deviations (n = 3). Within the columns means with different letters on the same storage day are significantly different according to Tukey's test (p value < 0.05).

2.4.3. Sensorial Quality

Panelists were able to detect subtle difference in the aroma and texture of the apple slices treated with *Lb. plantarum* and LCM1 after one day of storage at 4°C (Table 9). Aroma of the apple slices treated with *Lb. plantarum* was significantly less liked than the untreated and treated apple slices with LCM1 ($p < 0.05$). Acceptability of the texture of the apple slices treated with LCM1 was significantly less than the untreated and treated apple slices with *Lb. plantarum* ($p < 0.05$). Overall acceptability of the untreated apple slices was greater than the treated.

Panelists, however, did not indicate a significant difference in the acceptability of the aroma, taste, and texture of the untreated and treated apple slices after seven days of storage at 4°C ($p > 0.05$), signifying that panelists may not notice differences in the sensorial quality of the apple slices as the

storage time increases (Table 2.6). The acceptability of the taste, texture, and overall acceptability of the untreated and treated apple slices; however, was low. The low acceptability values may be attributed to the oxidation of the cut flesh. An anti-oxidizing agent, such as ascorbic acid, was not applied to the flesh apple slices; therefore, the oxidation of the cut flesh may have resulted in what many panelists termed as 'mushy' texture. Overall, panelists did observe a significant reduction in the texture of the apple slices after seven days of storage when compared to the initial values ($p < 0.05$). The result is comparable to the study accessing the effects of *Lb. plantarum* on the sensory qualities of fresh-cut pineapple (Russo et al., 2014).

Table 9. Sensory Evaluation of fresh-cut Granny Smith apples treated with Lb. plantarum or LCM1 after one and seven days of storage at 4°C.

	Aroma	Taste	Texture	Overall Acceptability
Storage Day 1				
Control	4.86 ± 1.46^{a}	5.00 ± 1.58^{a}	5.10 ± 1.93^{a}	5.46 ± 1.45^{a}
Lb. plantarum	3.48 ± 1.72^{b}	3.93 ± 2.07^{ab}	4.66 ± 2.33^{a}	3.97 ± 1.88^{b}
LCM1	4.93 ± 1.22^{a}	4.14 ± 1.98^{ab}	2.72 ± 2.17^{b}	3.71 ± 1.90^{b}
Storage Day 7				
Control	4.52 ± 1.05^{a}	4.28 ± 1.71^{ab}	2.62 ± 1.57^{b}	3.38 ± 1.47^{b}
Lb. plantarum	4.79 ± 1.47^{a}	3.41 ± 1.85^{b}	2.24 ± 1.75^{b}	3.10 ± 1.82^{b}
LCM1	4.86 ± 1.55^{a}	3.82 ± 2.14^{ab}	2.68 ± 1.91^{b}	3.59 ± 2.03^{b}

Reported values are means ± standard deviations (n = 29). Within the columns means with different letters on the same storage day are significantly different according to Tukey's test (p value < 0.05).

2.5. Conclusion

Populations of *Lb. plantarum* and LPP were maintained at approximately 7.0 log CFU/g on the apple slices throughout storage at 4°C and 20°C. Although *Lb plantarum* and LPP were not effective against *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp. on apple slices stored at 4°C, efficacy was observed at 20°C. LAB had a minimal effect on the soluble solids content, pH, firmness, and titratable acidity of the apple slices throughout storage at 4°C and 20°C. Although panelists were able to detect a difference in the taste and firmness of the apple slices treated with LAB after one day of storage

at 4°C no noticeable differences in the sensory quality was indicated after seven days of storage. In conclusion LAB could be an effective strategy in reducing pathogen populations at abusive temperatures without influencing the quality of the product.

CHAPTER 3 USE OF LACTIC ACID BACTERIA TO CONTROL THE GROWTH OF *LISTERIA MONOCYTOGENES* AND *SALMONELLA* SPP. ON ALFALFA SPROUTS

3.1. Abstract

The germination conditions of sprout vegetables consisting of relatively high temperatures and humidity, low light and abundance of nutrients are ideal for pathogen survival and growth. The continual occurrence of outbreaks and recalls associated with sprout vegetables indicate additional measures are needed to improve product safety. The objective of this study was to evaluate the efficacy of a mixture of *Lactobacillus plantarum, Lactobacillus acidophilus,* and *Pediococcus pentosaceus* (LPP) against *L. monocytogenes* and *Salmonella* spp. on alfalfa sprouts during five days of sprouting at 20°C and its influence sprout quality. Alfalfa seeds were inoculated with *L. monocytogenes* or *Salmonella* spp. (each at 10^1 CFU/g and 10^3 CFU/g) and LPP (10^7 CFU/g). Populations of LPP were maintained at 7.5 log CFU/g – 8.0 log CFU/g throughout sprouting. LPP had a significant effect on the growth of *L. monocytogenes* and *Salmonella* spp. ($p < 0.05$). After five days of sprouting, populations of *L. monocytogenes* at an initial concentration of 10^1 CFU/g and 10^3 CFU/g on seeds treated with LPP were approximately 4.5 log CFU/g and 1.0 log CFU/g less than the untreated seeds, respectively. Populations of *Salmonella* spp. at an initial concentration of 10^1 CFU/g and 10^3 CFU/g were 1.0 log CFU/g less than the control. LPP did not compromise the yield, seedling length, or pH of the sprouts.

Key Words: Alfalfa sprouts, biological control, Lactic Acid Bacteria, *L. monocytogenes, Salmonella* spp.

3.2. Introduction

Sprout vegetables, including alfalfa, mung bean, clover, and radish, have been associated with several foodborne outbreaks caused by *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp. (Painter et al., 2013). Contaminated seeds have been identified as the primary source of the foodborne outbreaks. The storage and growth conditions of sprouts are ideal environments for pathogen survival and growth. Pathogens are capable of surviving for month under the sprout seed storage conditions of 12°C to 20^0C and 70% humidity and proliferate under the germination conditions of relatively high temperatures and humidity, low light and abundance of nutrients (Taormina and Beuchat., 1999). A 100,000-fold increase in populations of *E. coli* O157:H7, *L. monocytogene*s and *Salmonella* spp. during sprouting, whereby the major growth occurs during the first two days of the sprouting process, has been described (Jacquette et al., 1996; Palmai and Buchanan, 2002; Taormina and Beuchat, 1999; Xiao, et al., 2014). Studies have also shown internalization of pathogens and the presence of biofilms in sprouts (Fransisca et al., 2011; Itoh et al., 1998). Thus, treatment of seeds before exposure to ideal sprouting conditions for pathogenic growth has been recognized as the most effective food safety approach.

Presently, 20,000 ppm of calcium hypochlorite is the recommended disinfection treatment for the reduction of pathogens on seeds (FDA, 2004). The antimicrobial efficacy is dependent on many factors, including wash water, pH, temperature, organic load, and the inherent properties of the seed surface coating (FDA, 2004; Holliday et al., 2001; Sikin et al., 2013; Suslow, 1997). On average, the lethal activity of calcium hypochlorite for sprout vegetables is a 3.0 log CFU/g reduction of pathogens (Taormina and Beuchat, 1999; Weissinger et al., 2000). However, during sprouting injured or surviving pathogens exponential increase, rendering the disinfection treatment ineffective (Lang et al., 2000). The low efficacy of calcium hypochlorite attained during sprouting illustrates the limited capability of calcium hypochlorite alone in ensuring food safety and the need for additional measures to improve product safety.

Currently, no alternative chemical or physical intervention strategies, such as electrolyzed water, peroxyacetic acid, and ultraviolet radiation, is known to have demonstrated a suppression in pathogen growth during sprouting (Kim et al., 2009; Neo et al., 2013; Phua et al., 2014; Wang et al., 2013). The application of protective bacterial cultures could be used to influence the growth and survival of multiple foodborne pathogens on sprouts. Several Gram-negative and Gram-positive bacteria in the family *Enterobacteriaceae* and genera *Pseudomonas, Lactococcus, Pedioccoccus,* and *Lactobacillus* have been identified as ideal protective bacterial cultures. The identified cultures are known to antagonize foodborne pathogens by several mechanisms, including competition for nutrients and production of organic acids, hydrogen peroxide, antimicrobial enzymes and bacteriocins (Anas et al., 2014; Das et al., 2013; Kumar et al., 2010)

Efficacy of protective bacterial cultures in controlling foodborne pathogens on sprout vegetables during sprouting has only recently been explored, with the majority of the research focusing on antagonistic Gram-negative bacteria (Liao et al., 2008; Palmai and Buchanan, 2002; Ye et al., 2010). On mung bean seeds treated with *Enterobacter asburiae* populations of *Salmonella* spp. were approximately 5.5 log CFU/g less than the untreated seeds after four days of sprouting at 25°C (Ye et al., 2010). Moreover, populations of *Salmonella* spp. on alfalfa seeds treated with *Psedomonas fluorescens* were 2.0 log CFU/g less the controls after four days of sprouting at 25°C (Liao et al., 2008). Populations *L. monocytogenes* on seeds treated with *Lactococcus lactis* were approximately 1.0 log CFU/g different than the controls after 24 h of sprouting (Palmai and Buchanan, 2002).

To date, no research has explored the efficacy of a mixture of protective bacterial cultures against foodborne pathogens on sprout vegetables during sprouting. Effectiveness has, however, been documented in leafy vegetables. LactiGuard™, composed of three *Lactobacillus, Pediococcus,* and *Lactococcus* species, was able to significantly reduce populations of *E. coli* O157:H7 and *Salmonella* spp. on spinach by approximately 1.0 log CFU/g and 2.0 log CFU/g after three and six days of storage at 7°C, respectively (Calix-Lara, 2014). As opposed to using a single protective bacterial culture, the proposed

advantages associated with a mixture cultures, include (i) multiple modes of action against the pathogen; (ii) potential to select organisms that affect more than one pathogen; (iii) increased consistency in performance on different produce commodities (Meyer et al., 2002).

The objectives of this study were 1) to determine efficacy of a mixture of *Lb. plantarum*, *Pediococcus acidophilusm* and *P. pentosaceus* (LPP) on the growth of *L. monocytogenes* and *Salmonella* spp. on growing alfalfa sprouts and 2) to assess the quality of alfalfa sprouts treated with LPP.

3.3. Materials and Methods

3.3.1. Alfalfa Seeds

Alfalfa seeds were donated by the International Specialty Supply (Cookeville, TN). Alfalfa seeds were stored at 4°C in the Food Safety Laboratory (San Luis Obispo, CA) until testing.

3.3.2. Foodborne Pathogen Preparation

Three serotypes of *L. monocytogenes* and *Salmonella* spp. (Table 10) were maintained on tryptic soy agar (TSA) at 4°C until inoculation. Inoculum was prepared using the Almond Board of California guidelines for seed inoculum preparation (Almond Board of California, 2014). Briefly, cells from isolated colonies were transferred into 10 ml of tryptic soy broth (TSB) at 35°C for 18 to 20 h. A 10 µl loop of bacterial culture was then transferred into 10 ml of TSB, incubated at 35°C for 18 to 20 h. Finally, 1 ml of bacterial culture was spread over TSA plates and incubated at 35°C for 22 to 26 h. Approximately, 6 ml of 0.1% peptone was added to each plate and the bacterial lawn was loosened using a sterile spreader. A sterile pipette was used to collect the cells into a sterile Falcon tube. The three serotypes of *L. monocytogenes* and *Salmonella* spp. were combined to form a mixture and the inoculum level was determined by direct microscope counts (DMC) using a hemocytometer and confirmed by plate counts. To achieve a concentration of 10^1 CFU/g and 10^3 CFU/g on the alfalfa seed the inoculum was adjusted using 0.1% peptone

Table 10. Foodborne pathogen strain name, number and source.

Strain Name	Strain Number	Source[1]
L. monocytogenes	FSL N1-227	ILSI Na (Cornell)
L. monocytogenes	NFPA 6301	NFL
L. monocytogenes	NFPA 6310	ARS
S. enterica	NFPA 7100	NFL
S. enteriditis	NFPA 7201	NFL
S. orangienburg	ATCC BAA 1045	NFL

[1] ARS – Agriculture Research Services (Peoria, IL); ILSI NA – Institute of Life Sciences of North America (Cornell University, Ithaca, NY); NFL – National Food Laboratory (Livermore, CA).

3.3.3. Lactic Acid Bacteria Preparation

Lb. plantarum, *P. acidophilus*, and *P. pentosaceus* were obtained from BiOWiSH technologies (Cincinnati, OH) (Table 11). The protective bacterial cultures were maintained on de Man, Rogosa, and Sharpe agar (MRS) at 4°C until inoculation. Inoculum was prepared similar to the preparation of the foodborne pathogens. A slight modification was done; instead of using TSB and TSA, MRS broth and agar were used. Inoculum level was determined by direct microscope counts (DMC) using a hemocytometer and confirmed plate counts. Inoculum was approximately 10^8 CFU/ml.

Table 11. Antagonist strain name, number and source.

Strain Name	Strain Number	Source[1]
Lb. plantarum	TISTR 050	BiOWiSH
P. acidilactici	ATCC 12697	BiOWiSH
P. pentosaceus	BCC 38038	BiOWiSH

[1] BiOWiSH Technologies, Inc. (Cincinnati, OH).

3.3.4. Alfalfa Seed Inoculation

Prior to inoculation, the surface of the alfalfa seeds were sanitized with 2,000 ppm calcium hypochlorite for 15 min, rinsed with sterile deionized water, air dried for three hours at 20°C in the biological safety cabinet, and stored overnight at 4°C. One hundred grams of the sanitized alfalfa seeds were placed into sterile Whirl-Pak® bags and 6.25 ml of either *L. monocytogenes* or *Salmonella* spp. at a

targeted concentration of 10^1 CFU/g or 10^3 CFU/g on the seed or 0.1% peptone water was added. Contents were mixed by vigorously shaking the bag for one minute. Seeds were then air dried in the bag with the seal open at 20°C in the biological safety cabinet. After 45 min, inoculated seeds were treated with 6.25 ml of either LPP or 0.1% peptone water. Contents were once again mixed and allowed to dry for 45 min.

3.3.5. Alfalfa Seed Soak and Irrigation

Approximately, 2 g of treated alfalfa seeds were placed in a Petri dish (150 x 15 mm). Seeds were soaked in 20 ml of sterile deionized water for 20 min. Solution was decanted and seeds were sprayed with 5 ml of sterile deionized water. Petri dishes were stored in an incubator at 20°C for five days. Each day, seeds were sprayed with 5 ml of sterile deionized water.

3.3.6. Bacterial Enumeration

Approximately, 2 g of alfalfa seeds or sprouts were diluted with 0.1% peptone water, homogenized in a stomacher at 200 rpm for one min, serially diluted, and plated on the appropriate media. *L. monocytogenes*, *Salmonella* spp., LAB, and native microflora were enumerated using modified oxford agar (MOX), xylose lysine deoxycholate agar (XLD), MRS, and TSA respectively. The agar plates were incubated at 35°C for 24 to 48 h. Populations of *L. monocytogenes*, *Salmonella* spp., LAB, and native microflora were enumerated in triplicate on sprouting day 0, 1, 3, and 5. The experiment was conducted twice.

3.3.7. Sprout Maturity and Quality

The length of the radical, yield, and pH of the alfalfa sprouts untreated and treated with LPP were determined on the final sprouting day. The radicals of ten sprouted sprouts from each treatment sample were measured by extending the length of the radical. All sprouts were weighed to determine yield. A 1:1 dilution of alfalfa sprouts and deionized water was used to evaluate the pH.

3.3.8. Statistical Analysis

Means and standard deviations for enumerated bacterial populations (log CFU/ml) and quality parameters were determined using JMP (Version 11, SAS Institute Inc., Cary, NC, USA). One way analysis of variance (ANOVA) and Tukey pairwise comparison were used to determine if there is a difference between the enumerated bacterial populations and quality parameters at a 95% confidence interval ($\alpha = 0.05$).

3.4. Results and Discussion

3.4.1. Survival and Growth of LPP

Population levels of LPP were maintained between 7.5 log CFU/g and 8.0 log CFU/g throughout sprouting and populations of LPP in the presence or absence of the foodborne pathogens were similar ($p > 0.05$) (Table 12, Table 13). Maintenance in populations of LPP on alfalfa sprouts during five days of sprouting, regardless of whether foodborne pathogens were present, was consistent with the results from the previous chapter evaluating the survival of *Lb. plantarum* and LPP on apple slices (Chapter 2.4.1.) and the literature (Calix-Lara et al., 2014; Russo et al., 2014; Siroli et al., 2015). LAB constituents in LactiGuard™ on spinach stored at 7°C were maintained between 7.6 log CFU/g and 8.0 log CFU/g both in the presence and absence of *E. coli* O157:H7 and *Salmonella* spp. (Calix-Lara et al., 2014). Stability of LPP at a concentration of 10^7 CFU/g was essential, as other studies evaluating the effectiveness of biological control on sprouts recommended densities greater than 10^4 CFU/g to substantially reduce populations of foodborne pathogens (Liao, 2007; Palamai and Buchanan, 2002) Additionally, preliminary research showed that populations of *L. monocytogenes* in the presence of LPP at a concentration of 10^4 CFU/g were not different than the untreated seeds ($p > 0.05$) while populations in the presence of LPP at a concentration of 10^7 CFU/g were different ($p < 0.05$) (Appendix C).

Although populations of LPP were inoculated at a higher concentration relative to the initial levels of the native microflora (10^2 CFU/g), the native microflora was present at an equivalent concentration to LPP after three days of sprouting, indicating the native microflora is not affected by the

presence of LPP nor does it influence the survival of LPP (Table 14). Palmai and Buchanan (2002) similarly observed that the native microflora reached a maximum concentration of 10^7 CFU/g after three days of sprouting even though the seeds were inoculated with a higher concentration of *Lact. lactis* (10^5 CFU/g) relative to the initial concentration of the native microflora (10^3 CFU/g).

Table 12. Population of LPP (log CFU/g) in the presence or absence of L. monocytogenes at an initial concentration of 101 CFU/g and 103 CFU/g on alfalfa seeds during five days of sprouting at 20°C.

Pathogen	Pathogen Concentration (CFU/g)	LPP (log CFU/g)			
		Sprouting Day 0	Sprouting Day 1	Sprouting Day 3	Sprouting Day 5
Not present	-	7.49 ± 0.09^{Aa}	7.95 ± 0.22^{Ab}	7.80 ± 0.18^{Aab}	7.71 ± 0.15^{Aab}
L. monocytogenes	10^1	7.64 ± 0.11^{Aa}	7.96 ± 0.18^{Aa}	8.00 ± 0.12^{Aa}	7.80 ± 0.17^{Aa}
L. monocytogenes	10^3	7.63 ± 0.18^{Aa}	8.00 ± 0.19^{Aa}	7.87 ± 0.14^{Aa}	7.86 ± 0.17^{Aa}

Reported values are means ± standard deviations (n = 6). Means followed by different uppercase letters within columns and lowercase letters within rows are significantly different according to Tukey's test (p-value < 0.05).

Table 13. Population of LPP (log CFU/g) in the presence or absence of Salmonella spp. at an initial concentration of 101 CFU/g and 103 CFU/g on alfalfa seeds during five days of sprouting at 20°C.

Pathogen	Pathogen Concentration (CFU/g)	LPP (log CFU/g)			
		Sprouting Day 0	Sprouting Day 1	Sprouting Day 3	Sprouting Day 5
Not present	-	7.49 ± 0.09^{Aa}	7.95 ± 0.22^{Ab}	7.80 ± 0.18^{Aab}	7.71 ± 0.15^{Aab}
Salmonella spp.	10^1	8.15 ± 0.46^{Ba}	7.97 ± 0.11^{Aa}	8.02 ± 0.09^{Aa}	7.75 ± 0.11^{Aa}
Salmonella spp.	10^3	7.80 ± 0.13^{ABa}	8.09 ± 0.09^{Aab}	8.21 ± 0.15^{Ab}	7.57 ± 0.20^{Aab}

Reported values are means ± standard deviations (n = 6). Means followed by different uppercase letters within columns and lowercase letters within rows are significantly different according to Tukey's test (p-value < 0.05).

Table 14. Population of native microflora (log CFU/g) in the presence or absence of LPP on alfalfa sprouts during five days of sprouts at 20°C.

	Native Microflora (log CFU/g)			
Treatment	Sprouting Day 0	Sprouting Day 1	Sprouting Day 3	Sprouting Day 5
No LPP	2.57 ± 0.27^{Aa}	6.80 ± 0.52^{Ab}	8.31 ± 0.53^{Ac}	7.73 ± 0.23^{Ac}
LPP	2.73 ± 0.40^{Aa}	6.81 ± 0.28^{Ab}	7.83 ± 0.44^{Ac}	7.84 ± 0.24^{Ac}

Reported values are means ± standard deviations (n = 6). Means followed by different uppercase letters within columns and lowercase letters within rows are significantly different according to Tukey's test (p-value < 0.05).

3.4.2. Antagonistic Effect of LPP

The initial populations of *L. monocytogenes* on alfalfa seeds not treated with LPP were 1.58 log CFU/g and 2.99 log CFU/g while the initial populations of *Salmonella* spp. were 0.71 log CFU/g and 2.68 log CFU/g (Appendix B). On untreated seeds, pathogen populations increased rapidly during the first three days of sprouting, reaching a maximum population of 7.0 log CFU/g; populations were then maintained. Regardless of the initial concentration, populations of *L. monocytogenes* and *Salmonella* spp. were similar after the one day of sprouting (Fig. 7, Fig. 8). The observed bacterial growth was consistent with studies assessing the growth of *L. monocytogenes, E. coli* O157:H7, and *Salmonella* spp. on alfalfa sprouts (Charkowski et al., 2002; Liao, 2007; Palmai and Buchanan, 2002; Stewart et al., 2001).

LPP inhibited the growth of *L. monocytogenes.* However, the degree of inhibition was dependent on the initial pathogen concentration. On seeds treated with LPP *L. monocytogenes* at an initial concentration of 10^1 CFU/g was approximately 4.5 log CFU/g less than in the untreated seeds after five days of sprouting (Fig. 7). Although populations of *L. monocytogenes* at an initial concentration of 10^3 CFU/g was approximately 3.5 log CFU/g less than the control after three days of sprouting, populations of *L. monocytogenes* increased and only a 1.0 log CFU/g difference was seen on the final day of sprouting ($p < 0.05$) (Fig. 7). In an attempt to ensure the maintained reduction of the pathogen at an initial concentration of 10^3 CFU/g growing alfalfa sprouts were sprayed with LPP on days three, four, and five of sprouting. Populations of *L. monocytogenes*, however, were similar, regardless the spray treatment ($p > 0.05$) (Appendix D).

Unlike *L. monocytogenes*, the antimicrobial activity of LPP was not dependent on the initial inoculum concentration of *Salmonella* spp. Additionally, only minor inhibition was observed on a few of the sprouting days. Populations of *Salmonella* spp. at both the initial concentrations of 10^1 CFU/g and 10^3 CFU/g were approximately 1.0 log CFU/g less than the control after one day of sprouting and populations at an initial concentration of 10^3 CFU/g were 0.7 log CFU/g less after five days of sprouting ($p < 0.05$) (Appendix B). Furthermore, on the seeds treated with LPP the growth pattern of populations of *Salmonella* spp. were similar to the untreated seeds; there was an initial rapid increase in populations *Salmonella* spp. followed by a stability in growth (Fig 8).

Overall, the effect of LPP on *L. monocytogenes* was greater than *Salmonella* spp. Higher specificity of LAB against *L. monocytogenes* may be related to the higher sensitivity of Gram-positive bacteria to the production of bacteriocins and accumulation of lactic acid (Aguilar et al., 2011; Trias et al., 2008). Previous studies, however, have shown a 2.0 log CFU/g to 4.0 log CFU/g reduction of *Salmonella* spp. by Gram-negative bacteria, such as *Enterobacter asburiae* and *Pseudomonas fluorenscens*, on growing sprout vegetables, indicating that species of the family *Enterobacteriaceae* and genera *Pseudomonas* may be more ideal protective bacterial cultures against Gram-negative pathogenic bacteria (Jianxiong et al., 2010; Liao, 2008; Ye et al., 2010). The specific inhibitory mode of LPP is not known. However, the assessment on the effects of the inoculum preparation of LPP seems to indicate that antimicrobial activity is a combination of competition for nutrients and production of either organic acids, hydrogen peroxide, antimicrobial enzymes or bacteriocins (Appendix D).

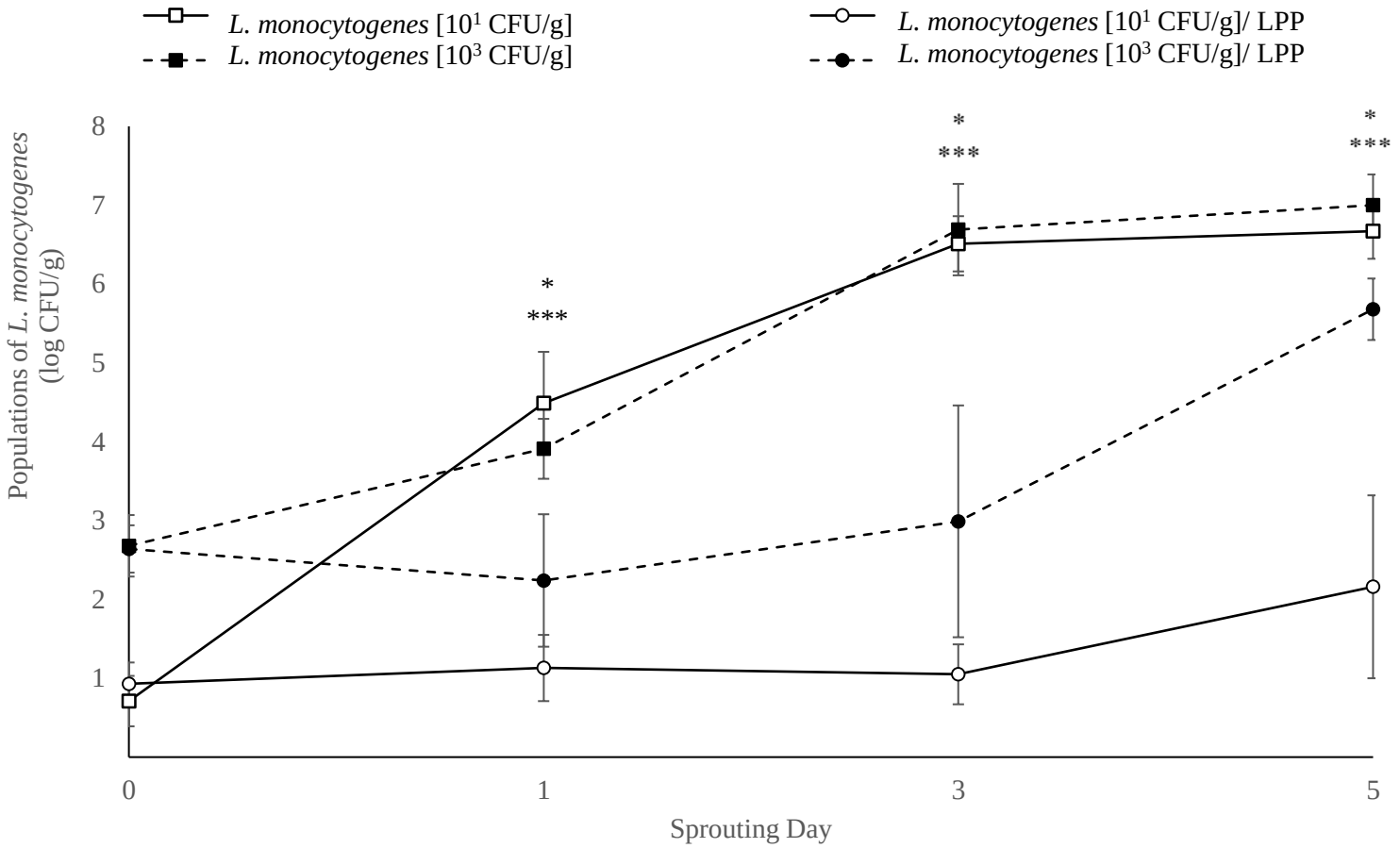

Figure 7. Mean population of L. monocytogenes (log CFU/g) at an initial concentration of 101 CFU/g and 103 CFU/g on untreated and treated alfalfa seeds with LPP during five days of sprouting at 20°C. Error bars represent standard deviation of the mean (n = 6). *Single and ***triplicate stars indicates a significant difference between pathogen at an initial concentration of 10^1 CFU/g and 10^3 CFU/g in the presence and absence of LPP, respectively.

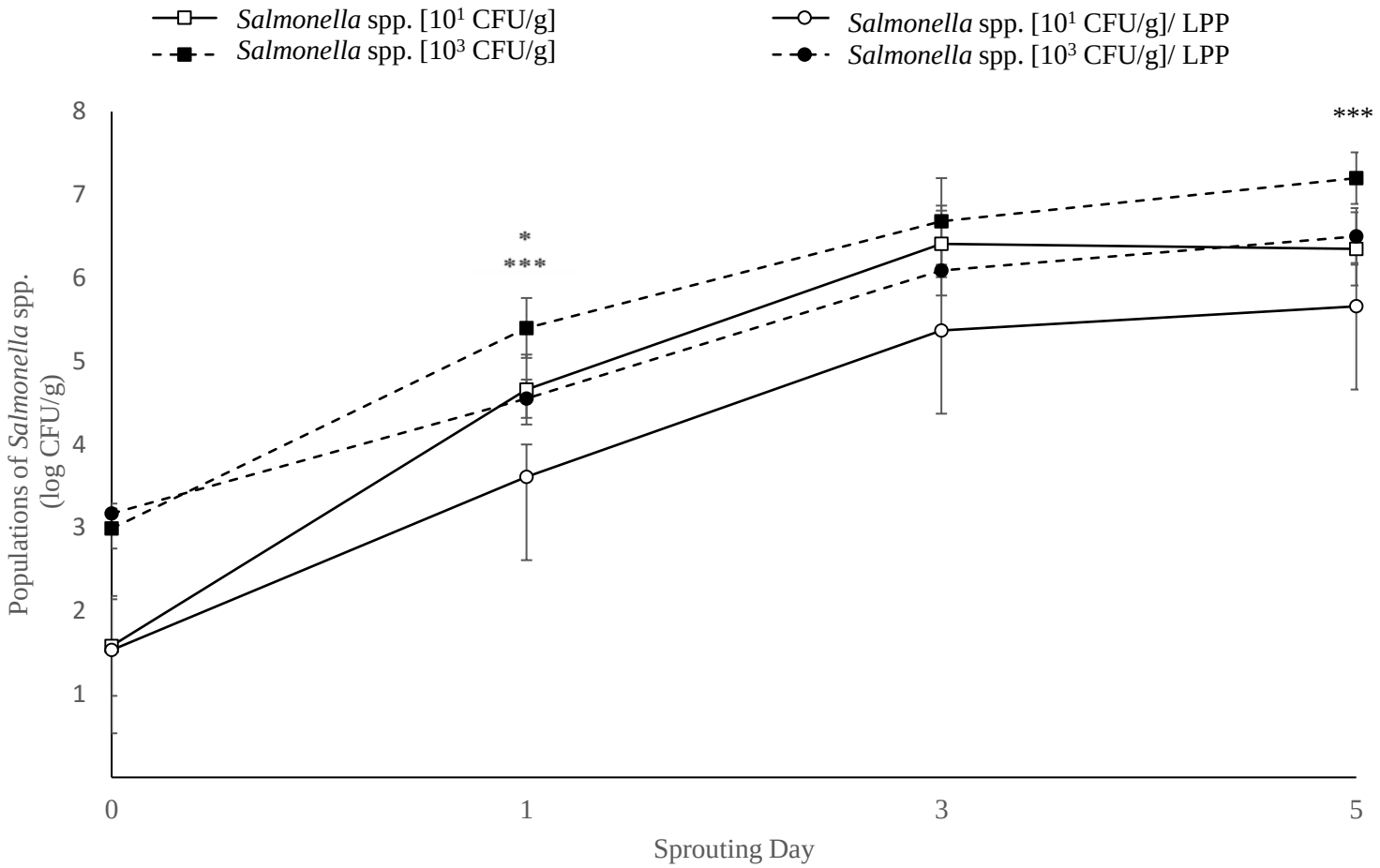

Figure 8. Mean population of Salmonella spp. (log CFU/g) at an initial concentration of 101 CFU/g and 103 CFU/g on untreated and treated alfalfa seeds with LPP during five days of sprouting at 20°C. Error bars represent standard deviation of the mean (n = 6). *Single and ***triplicate stars indicates a significant difference between pathogen at an initial concentration of 10^1 CFU/g and 10^3 CFU/g in the presence and absence of LPP, respectively.

3.4.3. Sprout Maturity and Quality

There was no difference in the yield and pH of alfalfa sprouts treated with LPP in comparison to the control ($p > 0.05$). There was difference in the seedling length of the alfalfa sprouts treated with LPP in comparison to the control ($p < 0.05$). Although the seedling length of alfalfa seeds treated with LPP were 0.8 cm less than the control, the length exceeded the desired length of 2.6 to 3.8 cm (Suslow and Cantwell, 2002) (Table 15).

Table 15. The yield (g), seedling length (cm) and pH of alfalfa sprouts treated with LPP after five days of sprouting at 20°C.

Treatment	Yield (g)	Seedling length (cm)	pH
No treatment	18.3 ± 2.01^{A}	5.1 ± 0.36^{A}	5.45 ± 0.13^{A}
LPP	16.9 ± 1.05^{A}	4.3 ± 0.66^{B}	5.42 ± 0.34^{A}

Reported values are means ± standard deviations (n = 6). Means followed by different uppercase letters within columns are significantly different according to Tukey's test (p-value < 0.05).

3.5. Conclusion

LPP demonstrated greater lethality against *L. monocytogenes* in comparison to *Salmonella* spp. LPP did not impact sprout maturity or quality. Application has potential as an effective intervention to enhance safety of sprouts.

CHAPTER 4 FUTURE RESEARCH

Future research assessing the effectiveness of LAB against foodborne pathogens on minimally processed fruits should be done with a lower inoculum concentration of the foodborne pathogen on the fruit (10^2 CFU/g) since efficacy at refrigerator temperatures may be concentration dependent. Research on sprout vegetables should also include effect of LAB on the growth of *E. coli* O157:H7. Additionally, the use of a mixture of LAB in combination with an antagonist Gram-negative bacteria, such as *Pseudomonas fluorescens*, should be investigated. The antagonistic Gram negative bacteria may have a stronger inhibitory effect against the Gram negative foodborne pathogens. Finally, future research should characterize and quantify the antimicrobial compounds produced by the antagonistic organisms. Identification can optimize product formulation and application.

REFERENCES

Abadias, M., I. Alegre, M. Oliveira, R. Altisent, and I. Vinas. 2012. Growth potential of *Escherichia coli* O157:H7 on fresh-cut fruits (melon and pineapple) and vegetables (carrot and escarole) stored at different conditions. *Food Control.* 27(1): 37-44.

Abadias, M., I. Alegre, J. Usall, R. Torres, and I. Vinas. 2011. Evaluation of alternative sanitizers to chlorine disinfection for reducing foodborne pathogens in fresh-cut apples. *Postharvest Biology and Technology*. 59(3): 289-297.

Adhikari, A., R. Syamaladevi, K. Killinger, and S. Sablani. 2015. Ultraviolet-C light inactivation of *Escherichia coli* O157:H7. *International Journal of Food Microbiology.* 210(1): 136-142.

Aguilar, C., C. Vanegas, and B. Klotz. 2011. Antagonistic effect of *Lactobacillus* strains against *Escherichia coli* and *Listeria monocytogenes* in milk. *Journal of Dairy Research.* 78(2): 136-143.

Alegre, I., M. Abadias, M. Anguera, J. Usall, and I. Vinas. 2010. Fate of *Escherichia coli* O157:H7, *Salmonella* and *Listeria innocua* on minimally-processed peaches under different storage conditions. *Food Microbiology*. 27(7): 862-868.

Alegre, I., I. Vinas, J. Usall, M. Anguera, M. Figge, and M. Abadias. 2012. An *Enterobacteriaceae* species isolated from apples controls foodborne pathogens on fresh-cut apples and peaches. *Postharvest Biology and Technology*. 74: 188-124.

Alegre, I., I. Vinas, J. Usall, N. Teixido, M. Figge, and M. Abadias. 2013. Control of foodborne pathogens on fresh-cut fruit by a novel strain of *Pseudomonas ginis*. *Food Microbiology.* 34(2): 390-399.

Allende, A., B. Martinez, V. Selma, M. Gil, J. Suarez, and A. Rodriguez. 2007. Growth and bacteriocin production by lactic acid bacteria in vegetable broth and their effectiveness at reducing *Listeria monocytogenes* in vitro and in fresh-cut lettuce. *Food Microbiology,* 24(7-8): 759-766.

Anas, M., K. Ahmed, and K. Mebrouk. 2014. Study of the antimicrobial and probiotic effect of *Lactobacillus plantarum* isolated from raw goat's milk from the region of Western Algeria. *International Journal of Sciences: Basic and Applied Research (IJSBAR).* 13(1): 18-27.

Anon. 1962. pH values of food products. *Food Engineering.* 34(3): 98-99.

Behrsing, J., S. Winkler, P. Franz, and R. Premier. 2000. Efficacy of chlorine for inactivation of *Escherichia coli* on vegetables. *Postharvest Biology Technology.* 19: 187-192.

Bennik, M., W. Overbeek, E. Smid, and L. Gorris. 1999. Biopreservation in modified atmosphere stored mungbean sprouts: the use of vegetable-associated bacteriocinogenic lactic acid bacteria to control the growth of Listeria monocytogenes. *Letters in Applied Microbiology.* 28: 226-232.

Berry, E, R. Hutkins, and R. Mandigo. 1991. The use of bacteriocin-producing *Pediococcus acidilactici* to control post-processing Listeria monocytogenes contamination of Frankfurters. *Journal of Food Protection*. 9: 662-742.

Beuchat, L. and R. Brackets. 1990. Survival and growth of *Listeria monocytogenes* on lettuce as influenced by shredding, chlorine treatment, modified atmosphere packaging and temperature. *Journal of Food Science.* 55(3): 755-758.

Bhunia, A. 2008. Foodborne Microbial Pathogens: Mechanisms and Pathogenesis. Springer, New York City.

Brown, A., J. Brooks, E. Karunasena, A. Echeverry, A. Laury, and M. Brashears. 2011. Inhibition of *Escherichia coli* O157:H7 and *Clostridium sporogenes* in spinach packaged in modified atmospheres after treatment combined with chlorine and Lactic Acid Bacteria. *Journal of Food Science.* 76(6): 427-432.

Buck, J., R. Walcott, and L. Beuchat. 2003. Recent Trends in Microbiological Safety of Fruits and Vegetables. Available at: http://www.apsnet.org/publications/apsnetfeatures/Pages/microsafety.aspx. Accessed 8 May 2016.

Calix-Lara, T., M. Rajendran, S. Talcott, S. Smith, R. Miller, A. Castillo, J. Sturino, and M. Taylor. 2014. Inhibition of *Escherichia coli* O157:H7 and *Salmonella enterica* on spinach and identification of antimicrobial substances produced by a commercial Lactic Acid Bacteria food safety intervention. *Food Microbiology.* 38: 192-200.

CDC. 2014. *Escherichia coli*. Available at: http://www.cdc.gov/ecoli/general/index.html# Accessed 8 May 2016.

CDC. 2015. *List of Selected Multistate Foodborne Outbreak Investigations.* Available at: http://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html Accessed 8 May 2016.

Charkowski, A., J. Barak, C. Sarreal, R. Mandrell. Differences in growth of *Salmonella enterica* and *Escherichia coli* O157:H7 on alfalfa sprouts.

Das, J., D. Mishra, P. Ray, P. Tripathy, T. Beuria, N. Singh, and M. Suar. 2013. In vitro evaluation of anti-infective activity of a *Lactobacillus plantarum* strain against *Salmonella enterica* serovar Enteritidis. *Gut Pathogens.* 5(11).

Dow, A., C. Alvarado, and M. Brashears. 2011. Reduction of inoculated *Salmonella* cocktail in ground turkey and turkey breasts using *Lactobacillus*-based intervention. *Poultry Science.* 90(4): 876-879.

FDA. 2002. Guidelines for the Evaluation of Probiotics in Food. Available at: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf. Accessed 8 May 2016.

FDA. 2004. Growing sprouts in retail food establishment. Available at: http://www.fda.gov/Food/GuidanceRegulation/RetailFoodProtection/ucm078758.htm Accessed 8 May 2016.

FDA. 2012a. More in Causes of Foodborne Illness: Bad Bug Book. Available at: http://www.fda.gov/Food/FoodborneIllnessContaminants/CausesOfIllnessBadBugBook/ Accessed 8 May 2016.

FDA. 2012b. Direct food substances affirmed as generally recognized as safe, listing of specific substances affirmed as GRAS, lactic acid. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?cfrpart=184 Accessed 8 May 2016.

Flessa, S., D. Lusk, and L. Harris. 2005. Survival of *Listeria monocytogenes* on fresh and frozen strawberries. *International Journal of Food Microbiology*. 101(3): 255-262.

Fransisca L., B. Zhou, H. Park, and H. Feng. 2011. The effect of calcinated calcium and chlorine treatments on *Escherichia coli* O157:H7 87-23 population reduction in radish sprouts. *Journal of Food Science.* 76(6): 404-412.

Gomez-Lopez, V. (ed.). 2012. Decontamination of fresh and minimally processed produce, 4th Ed. John Wiley & Sons, Oxford.

Graca, A., M. Abadias, M. Salazar, and C. Nunes. 2011. The use of electrolyzed water as a disinfectant for minimally processed apples. *Postharvest Biology and Technology.* 61(2-3): 172-177.

Herman, K., A. Hall, and L. Gould. 2015. Outbreaks attributed to fresh leafy vegetables, United States, 1973-2012. *Epidemiological Infections*. 143(14): 1-11.

Hoffmann, S., M. Batz, J. Morris, and J. Glenn. 2012. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. *Journal of Food Protection. 7*: 1292-1302.

Holliday, S., A. Scouten, and L. Beuchat. 2001. Efficacy of chemical treatments in eliminating *Salmonella* and *Escherichia coli* O157:H7 on scarified and polished alfalfa seeds. *Journal of Food Protection.* 64(10): 1489-1495.

Huang, J., Y. Luo, and X. Nou. 2015. Growth of *Salmonella enterica* and *Listeria monocytogenes* on fresh-cut cantaloupe under different temperature abuse scenarios. *Journal of Food Protection.* 78(6): 1125-1131.

IBISWorld. 2015. *IBISWorld Industry Report Global Fruit & Vegetables Processing.* Available at: http://www.ibisworld.com/industry/global/global-fruit-vegetables-processing.html. Accessed 8 May 2016.

Itoh, Y., Y. Sugita-Konishi, F. Kasuga, M. Iwaki, Y. Hara-Kudo, N. Saito, Y. Noguchi, H. Konuma, and S. Kumagai. Enterohemorrgagic *Escherichia coli* O157:H7 present in radish sprouts. *Applied and Environmental Microbiology.* 64(4): 1532-1535.

Jaquette, C., L. Beuchat, and B. Mahon. 1996. Efficacy of chlorine and heat treatment in killing *Salmonella stanley* inoculated onto alfalfa seeds and growth and survival of pathogen during sprouting and storage. *Applied and Environmental Microbiology.* 62(7): 2212-2215.

Jianxiong Y., M. Kostrzynska, K. Dunfield, and K. Warriner. 2010. Control of *Salmonella* on sprouting mung bean and alfalfa seeds using a biocontrol preparation based on antagonistic bacteria and lytic bacteriophages. *Journal of Food Protection*. 73(1): 9-17.

Kechagia, M., D. Basoulis, S. Konstantopoulou, D. Dimitriadi, K. Gyftopoulou, N. Skarmoutsou, and E. Fakiri. 2013. Health benefits of probiotics: a review. *International Scholarly Research Notices (ISRN) Nutrition*. 2013: 1-7.

Keskinen, L. and B. Annous. 2011. Efficacy of adding detergents to sanitizer solutions for inactivation of *Escherichia coli* O157:H7 on Romaine lettuce. *International Journal of Food Microbiology.* 147(3): 157-161.

Kim, Y., M. Kim, and K. Bin Song. 2009. Combined treatment of fumaric acid with aqueous chlorine dioxide or UV-C irradiation to inactivate *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes* inoculated on alfalfa and clover sprouts. *Food Science and Technology.* 42(10): 1654-1658.

Kitis, M. 2004. Disinfection of wastewater with peracetic acid: a review. *Environmental International.* 30(1): 47-55.

Knudsen, D., S. Yamamoto, and L. Harris. 2001. Survival of *Salmonella* spp. and *Escherichia coli* O157:H7 on fresh and frozen strawberries. *Journal of Food Protection.* 64(10): 1483-1488.

Kong, Q., A. Wu, W. Qi, R. Qi, J. Carter, R. Rasooly, and X. He. 2014. Effects of electron-beam irradiation on blueberries inoculated with *Escherichia coli* and their nutritional quality and shelf life. *Postharvest Biology and Technology.* 95: 28-35.

Koo, O., M. Eggleton, C. O'Bryan, P. Crandall, and S. Ricke. 2012. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes* on frankfurters formulated with and without lactate/diacetate. *Meat Science*. 92(4): 533-537.

Kumar, M., A. Kumar, R. Nagpal, D. Mohania, P. Behare, V. Verma, P. Kumar, P. Aggarwal, C. Henry, S. Jain, and H. Yadav. 2010. Cancer-preventing attributes of probiotics: an update. *International Journal of Food Science and Nutrition.* 61(5): 473-496.

Lang, M., B. Ingham, and S. Ingham. 2000. Efficacy of novel organic acid and hypochlorite treatments for eliminating *Escherichia coli* O157:H7 from alfalfa seeds prior to sprouting. *International Journal of Food Microbiology.* 58(1-2): 73-82.

Leverentz, B., W. Conway, Z. Alavidze, W. Janisiewicz, Y. Fuchs, M. Camp, E. Chighladze, and A. Sulakvelidze. 2001. Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: a model study. *Journal of Food Protection.* 64(8): 1116-1121.

Leverentz, B., W. Conway, M. Camp, W. Janisiewicz, T. Abuladze, M. Yang, R. Saftner, and A. Sulakvelidze. 2003. Biocontrol of *Listeria monocytogenes* on fresh-cut product by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology.* 69(8): 4519-4526.

Liao, C. 2008. Growth of Salmonella on sprouting alfalfa seeds as affected by the inoculum size, native microbial load and *Pseudomonas fluorescens* 2-79. *Letters in Applied Microbiology*. 46(2): 232-236.

Mahmoud, B. 2010. The effects of X-ray radiation of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica*, and *Shigella flexneri* inoculated on whole Roma tomatoes. *Food Microbiology*. 27(8): 1057-1063.

Mahony, J., O. Auliffe, R. Ross, and D. van Sinderen. 2011. Bacteriophages as biocontrol agents of food pathogens. *Current Opinion in Biotechnology.* 22: 157-163.

Martin-Visscher, L., S. Yoganathan, C. Sit, C. Lohans, and J. Vederas. 2011. The activity of bacteriocins from *Carnobacterium maltaromaticum* UAL307 against g-negative bacteria in combination with EDTA treatment. *Federation of European Microbiological Society (FEMS) Microbiology Letters.* 317(2): 152-159.

McEvoy, J., Y. Luo, W. Conway, B. Zhou, and H. Feng. 2009. Potential of *Escherichia coli* O157:H7 to grow on field-cored lettuce as impacted by postharvest storage time and temperature. *International Journal of Food Microbiology.* 128(3): 506-509.

Menconi, A., G. Kallapura, J. Latorre, M. Morgan, N. Pumford, B. Hargis, and G. Tellez. 2014. Identification and characterization of Lactic Acid Bacteria in a commercial probiotic culture. *Bioscience of Microbiota, Food and Health.* 33(1): 25-30.

Meyer, S. and D. Roberts. 2002. Combinations of biocontrol agents for management of plant-parasitic nematodes and soilborne plant-pathogenic fungi. *Journal of Nematology.* 34(1): 1-8.

Molinos, A., H. Abriouel, R. Lopez, E. Valdivia, N. Omar, and A, Galvez. 2008. Combined physico-chemical treatments based on enterocin AS-48 for inactivation of G-negative bacteria in soybean sprouts. *Food and Chemical Toxicology.* 46(8): 2912-2921.

Neal, J., Cabrera-Diaz, M. Marquez-Gonzalez, J. Maxim, and A. Castillo. 2008. Reduction of *Escherichia coli* O157:H7 and *Salmonella* on baby Spinach, using electron beam radiation. *Journal of Food Protection.* 71(12): 2415-2420.

Neo, S., P. Lim, L. Phua, G. Khoo, S. Kim, S. Lee, and H. Yuk. 2013. Efficacy of chlorine and peroxyacetic acid on reduction of natural microflora, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. on mung bean sprouts. *Food Microbiology.* 36(2): 475-480.

Nielson, J., J. Dickson, and J. Crouse. 1990. Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. *Applied Environmental Microbiology*. 56 (7): 2142-2145.

Oguntibeju, O., Truter, E., & Esterhuyse, A. (2013). The Role of Fruit and Vegetable Consumption in Human Health and Disease Prevention. In O. Oguntibeju, *Diabetes Mellitus - Insights and Perspectives.*

Painter, J., R. Howkstra, T. Ayers, R. Tauxe, C. Braden, F. Angulo, and P. Griffin. 2013. Attribution of foodborne illnesses, hospitalizations, and death to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases.* 19(3).

Palmai, M. and R. Buchanan. 2002. Growth of *Listeria monocytogenes* during germination of alfalfa sprouts. *Food Microbiology.* 19: 195-200.

Palekar, M., T. Taylor, J. Maxim, and A. Castillo. 2015. Reduction of *Salmonella enterica* serotype Poona and background microbiota on fresh-cut cantaloupe by electron beam irradiation. *International Journal of Food Microbiology.* 202: 66-72.

Pao, S., S. Rolph, E. Westbrook, and H. Shen. 2008. Eliminating *Salmonella enterica* in alfalfa and mung bean sprouts by organic acid in hot water immersions. *Journal of Food Processing Preservation.* 32: 335-342.

Park, S., M. Choi, J. Park, K. Park, M. Chung, S. Ryu, and D. Kang. 2011. Use of organic acids to inactivate *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on organic fresh apples and lettuce. *Journal of Food Science.* 76(6).

Phua, L., S. Neo, G. Khoo, and H. Yuk. 2014. Comparison of the efficacy of various sanitizers and hot water treatment in inactivating inoculated foodborne pathogens and natural microflora on mung bean sprouts. *Food Control.* 42: 270-276.

Powitz, R. 2010. Activated and electrolyzed water: a brief review of a new generation of cleaners and sanitizing agents. Available at: http://www.foodsafetymagazine.com/magazine-archive1/augustseptember-2010/activated-and-electrolyzed-water-a-brief-review-of-a-new-generation-of-cleaners-and-sanitizing-agents/ Accessed 8 May 2016.

Produce for Better Health Foundation. 2010. State of the Plate 2010 Study on America's Consumption of Fruits & Vegetables. Available at: http://www.pdhfoundation.org Accessed 8 May 2016.

Ray, B., and A. Bhunia. 2008. Fundamental Food Microbiology, 4th Ed. CRC Press, Boca Rato.

Roble, C., M. Auty, N. Brunton, R. Gormley, and F. Butler. 2010. Evaluation of fresh-cut apple slices enriched with probiotic bacteria. *Innovation Food Science and Emerging Technologies*. 11(1): 203-209.

Russo, P., M. Valeria de Chiara, A. Vernile, M. Amodio, M. Arena, V. Capozzi, S. Massa, and G. Spano. 2014. Fresh-cut pineapple as a new carrier of probiotic lactic acid bacteria. *BioMed Research International*. 2014: 1-9.

Salunkhe, D., H. Bolin, and N. Reddy. 1991. Minimal processing. In D. Salunkhe, H. Bolin, and N. Reddy. Storage processing, and nutritional quality of fruit and vegetables. Boca Raton CRC Press, Boca Raton.

Seo, S., D. Jung, X. Wang, D. Seo, M. Lee, B. Lee, and C. Choi. 2012. Combined effect of lactic acid bacteria and citric acid on *Escherichia coli* O157:H7 and *Salmonella* Typhimurium. *Food Science Biotechnology.* 22(4): 1171-1174.

Sharma, M. 2013. Potential interventions against enteric bacterial pathogens on produce. *Bacteriophage.* 3(2).

Siddiqui, M., I. Chakraborty, J. Ayala-Zavala, and R. Dhua. 2011. Advances in minimal processing of fruits and vegetables: a review. *Journal of Scientific & Industrial Research.* 70: 823-834.

Sikin, A., C. Zoellner, and S. Rizvi. 2013. Current intervention strategies for the microbial safety of sprouts. *Journal of Food Protection.* 76(12): 2099-2123.

Siroli, L., F. Patrignani, D. Serrazanetti, G. Tabanelli, C. Montanari, F. Gardini, and R. Lanciotti. 2015. Lactic acid bacteria and natural antimicrobials to improve the safety and shelf-life of minimally processed sliced apples and lamb's lettuce. *Food Microbiology.* 47: 74-84.

Spricigo, D., C. Bardina, P. Cortes, and M. Llagostera. 2013. Use of a bacteriophage cocktail to control *Salmonella* in food and the food industry. *International Journal of Food Microbiology.* 165(2): 169-174.

Sreedharan, A., O. Tokarskyy, S. Sargent, and K. Schneider. 2015. Survival of *Salmonella* spp. on the surface-inoculated forced-air cooled and hydrocooled intact strawberries, and in strawberry puree. *Food Control.* 51: 244-250.

Stewart, D., K. Reineke, J. Ulaszek, t. Fu, and M. Tortorello. 2001. Growth of *Escherichia coli* O157:H7 during sprouting of alfalfa seeds. *Letters in Applied Microbiology.* 33(2): 95-99.

Suslow, T. 1997. Postharvest chlorination: basic properties and key points for effective disinfection. Available at: http://anrcataLog.ucdavis.edu/pdf/8003.pdf. Accessed 8 May 2016.

Suslow, T. and M. Cantwell. 2002. Sprouts, Seed: Recommendations for maintaining postharvest quality. Available at: http://postharvest.ucdavis.edu/pfvegetable/SproutsSeed/. Accessed 8 May 2016.

Taormina, P. and L. Beuchat. 1999. Behavior of enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa sprouts during the sprouting process as influenced by treatments with various chemicals. *Journal of Food Protection.* 62(8): 850-856.

Todar, K. 2012. *Lactic Acid Bacteria*. Available at: http://textbookofbacterioLogy.net/lactics.html. Accessed 8 May 2016.

Trias, R., L. Baneras, E. Badosa, and E. Montesinos. 2008. Bioprotection of Golden Delicious apples and Iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. *International Journal of Food Microbiology*. 123(1-2): 50-60.

USDA. 2008. Increasing Fruit and Vegetable Consumption through the USDA Nutrition and Assistance Progs. Available at: www.fns.usda.gov. Accessed 8 May 2016.

Wang, C., S. Wang, T. Chang, L. Shi, H. Yang, Y. Shao, W. Feny, and M. Cui. 2013. Efficacy of lactic acid in reducing foodborne pathogens in minimally processed lotus sprouts. *Food Control.* 30(2): 721-726.

Warburton, R. 2014. Peracetic acid in the fresh food industry. Available at: http://www.foodsafetymagazine.com/signature-series/peracetic-acid-in-the-fresh-food-industry/. Accessed 8 May 2016.

Weissinger, W., W. Chantarapanont, and L. Beuchat. 2000. Survival and growth of *Salmonella* Baildon in shredded lettuce and diced tomatoes, and effectiveness of chlorinated water as sanitizer. *International Journal of Food Microbiology.* 62: 123-131.

WHO 2011. Enterohemorrhagic *Escherichia coli* (EHEC). Available at: http://www.who.int/mediacentre/factsheets/fs125/en/. Accessed 8 May 2016.

Xiao, Z., X. Nou, and Y. Luo. 2014. Comparison of the growth of *Escherichia coli* O157:h7 and O104:H4 during sprouting and microgreen production from contaminated radish seeds. *Food Microbiology.* 44: 60-63.

Ye, J., M. Kostrzyska, K. Dunfield, and K. Warrineri. 2010. Control of *Salmonella* on sprouting mung bean and alfalfa seeds by using a biocontrol preparation based on antagonistic bacteria and lytic bacteriophages. *Journal of Food Protection.* 73(1): 9-17.

Yuste, J., M. Mor-Mur, M. Capellas, B. Guamis, and R. Pla. 1998. Microbiological quality of mechanically recovered poultry meat treated with high hydrostatic pressure and nisin. *Food Microbiology*. 15(4): 401-414.

Zhuang, R., L. Beuchat, and F. Angulo. 1995. Fate of *Salmonella montevideo* on and in raw tomatoes as affected by temperature and treatment with chlorine. *Applied and Environmental Microbiology.* 61(6): 2127-2131.

APPENDIX A. ANTIMICROBIAL ACTIVITY OF LAB AGAINST FOODBORNE PATHOGENS ON APPLE SLICE

Table 16. Population (log CFU/g) of E. coli O157:H7, L. monocytogenes, and Salmonella spp. in the presence and absence of LPP on Granny Smith apple slices during seven and five days of storage at 4°C and 20°C, respectively.

	Population (log CFU/g) of pathogen					
	E. coli O157:H7		*L. monocytogenes*		*Salmonella* spp.	
	Storage at 4°C	Storage at 20°C	Storage at 4°C	Storage at 20°C	Storage at 4°C	Storage at 20°C
Storage Day 0						
Pathogen	4.97 ± 0.43^{Aa}	4.97 ± 0.43^{Aa}	4.81 ± 0.54^{Aa}	4.81 ± 0.54^{Aa}	4.77 ± 0.70^{Aa}	4.77 ± 0.70^{Aa}
Pathogen/LPP	4.90 ± 0.45^{Aa}	4.90 ± 0.45^{Aa}	4.71 ± 0.52^{Aab}	4.71 ± 0.52^{Aab}	4.79 ± 0.60^{Aa}	4.79 ± 0.60^{Aa}
Storage Day 1						
Pathogen	5.00 ± 0.63^{Aa}	5.62 ± 0.60^{Ab}	4.97 ± 1.23^{Aa}	5.21 ± 0.87^{Aa}	4.34 ± 1.11^{Aa}	6.12 ± 0.44^{Ab}
Pathogen/LPP	5.15 ± 0.66^{Aa}	4.92 ± 0.61^{Aa}	4.93 ± 0.93^{Aa}	4.31 ± 0.37^{Ab}	3.79 ± 1.16^{Aa}	5.50 ± 0.21^{Bab}
Storage Day 3						
Pathogen	4.48 ± 0.63^{Aa}	6.87 ± 0.21^{Ab}	3.98 ± 0.45^{Aa}	5.49 ± 0.47^{Aa}	4.35 ± 1.35^{Aa}	6.77 ± 0.19^{Aab}
Pathogen/LPP	4.73 ± 0.66^{Aa}	4.61 ± 0.70^{Ba}	4.28 ± 0.51^{Aab}	4.32 ± 0.15^{Bb}	4.02 ± 0.89^{Aa}	5.91 ± 0.09^{Bb}
Storage Day 5						
Pathogen	4.65 ± 0.61^{Aa}	6.55 ± 0.40^{Ab}	3.61 ± 1.02^{Aa}	5.71 ± 0.21^{Aa}	4.38 ± 0.92^{Aa}	6.91 ± 0.27^{Ac}
Pathogen/LPP	4.86 ± 0.74^{Aa}	5.54 ± 0.65^{Aa}	3.57 ± 1.05^{Ab}	2.87 ± 0.65^{Bc}	4.88 ± 0.64^{Aa}	6.23 ± 0.70^{Ab}
Storage Day 7						
Pathogen	4.55 ± 0.75^{Aa}	-	3.77 ± 0.84^{Aa}	-	4.26 ± 0.81^{Aa}	-
Pathogen/LPP	4.82 ± 0.68^{Aa}	-	3.73 ± 0.49^{Aab}	-	3.67 ± 0.75^{Aa}	-

Reported values are means ± standard deviations (n = 6). Within the columns means with different uppercase letters between treatments on the same storage day and lowercase letters within treatments during storage are significantly different according to Tukey's test (p-value < 0.05).

Table 17. Population (log CFU/g) of E. coli O157:H7, L. monocytogenes, and Salmonella spp. in the presence and absence of Lb. plantarum on Granny Smith apple slices during seven and five days of storage at 4°C and 20°C, respectively.

	Population (log CFU/g) of pathogen					
	E. coli O157:H7		*L. monocytogenes*		*Salmonella* spp.	
	Storage at 4°C	Storage at 20°C	Storage at 4°C	Storage at 20°C	Storage at 4°C	Storage at 20°C
Storage Day 0						
Pathogen	4.36 ± 0.44^{Aab}	4.35 ± 0.43^{Aa}	3.52 ± 0.65^{Aa}	3.59 ± 0.72^{Aa}	4.29 ± 0.46^{Aa}	4.23 ± 1.00^{Aa}
Pathogen/*Lb. plantarum*	4.41 ± 0.16^{Aa}	4.41 ± 0.16^{Aa}	3.61 ± 0.53^{Aa}	3.47 ± 0.38^{Aa}	4.29 ± 0.43^{Aa}	4.63 ± 0.77^{Aa}
Storage Day 1						
Pathogen	4.26 ± 0.22^{Aab}	5.66 ± 0.44^{Aab}	3.43 ± 0.57^{Aab}	3.50 ± 1.01^{Aa}	4.85 ± 0.53^{Aa}	5.41 ± 1.77^{Aab}
Pathogen/*Lb. plantarum*	4.20 ± 0.44^{Aa}	3.83 ± 0.47^{Ba}	3.36 ± 0.72^{Aa}	2.60 ± 0.44^{Aa}	3.99 ± 0.55^{Aa}	4.47 ± 0.22^{Aa}
Storage Day 3						
Pathogen	4.75 ± 0.53^{Ab}	6.21 ± 1.12^{Ab}	2.49 ± 0.35^{Aab}	5.39 ± 0.26^{Ab}	4.06 ± 0.96^{Aa}	5.99 ± 0.72^{Ab}
Pathogen/*Lb. plantarum*	4.73 ± 0.38^{Aa}	4.70 ± 1.07^{Aa}	2.55 ± 0.62^{Aa}	2.24 ± 0.50^{Ba}	3.53 ± 0.98^{Aa}	5.39 ± 1.60^{Aab}
Storage Day 5						
Pathogen	4.05 ± 0.19^{Aa}	6.23 ± 0.54^{Ab}	2.91 ± 0.99^{Aab}	5.24 ± 1.15^{Ab}	4.28 ± 1.14^{Aab}	6.24 ± 0.69^{Ab}
Pathogen/*Lb. plantarum*	4.41 ± 0.26^{Aa}	5.28 ± 1.18^{Aa}	2.85 ± 0.77^{Aa}	3.40 ± 1.14^{Ba}	3.92 ± 0.73^{Aab}	6.25 ± 0.69^{Ab}
Storage Day 7						
Pathogen	3.68 ± 0.26^{Aa}	-	2.35 ± 0.56^{Ab}	-	2.77 ± 0.22^{Ab}	-
Pathogen/*Lb. plantarum*	4.35 ± 0.26^{Ba}	-	2.35 ± 0.85^{Aa}	-	2.53 ± 0.20^{Ab}	-

Reported values are means ± standard deviations (n = 6). Means with different uppercase letters between treatments on the same storage day and lowercase letters within treatments during storage are significantly different according to Tukey's test (p-value < 0.05).

APPENDIX B. ANTIMICROBIAL ACTIVITY OF LPP AGAINST FOODBORNE PATHOGENS ON ALFALFA SPROUTS

Table 18. Population of L. monocytogenes (log CFU/g) at an initial concentration of 10^1 CFU/g and 10^3 CFU/g in the absence or presence of LPP on alfalfa sprouts during five days of sprouting at 20°C.

Concentration of Pathogen (log CFU/g)	LPP	*L. monocytogenes* (log CFU/g)			
		Sprouting Day 0	Sprouting Day 1	Sprouting Day 3	Sprouting Day 5
10^1	Absent	0.71 ± 0.32^{Aa}	4.49 ± 0.65^{Cb}	6.51 ± 0.35^{Cc}	6.67 ± 0.35^{Cc}
10^1	Present	0.93 ± 0.27^{Aa}	1.13 ± 0.42^{Aa}	1.05 ± 0.38^{Aa}	2.16 ± 1.16^{Aa}
10^3	Absent	2.68 ± 0.39^{Ba}	3.91 ± 0.38^{Cab}	6.69 ± 0.58^{Cc}	7.00 ± 0.39^{Cc}
10^3	Present	2.64 ± 0.30^{Ba}	2.24 ± 0.84^{Ba}	2.99 ± 1.47^{Ba}	5.68 ± 0.39^{Bb}

Reported values are means ± standard deviations (n = 6). Means followed by different uppercase letters within columns and lowercase letters within rows are significantly different according to Tukey's test (p-value < 0.05).

Table 19. Population of Salmonella spp. (log CFU/g) at an initial concentration of 10^1 CFU/g and 10^3 CFU/g in the absence or presence of LPP on alfalfa sprouts during five days of sprouting at 20°C.

Concentration of Pathogen (log CFU/g)	LPP	*Salmonella* spp. (log CFU/g)			
		Sprouting Day 0	Sprouting Day 1	Sprouting Day 3	Sprouting Day 5
10^1	Absent	1.58 ± 0.60^{Aa}	4.66 ± 0.42^{Bb}	6.41 ± 0.40^{ABc}	6.35 ± 0.44^{Ac}
10^1	Present	1.53 ± 0.61^{Aa}	3.61 ± 0.39^{Ab}	5.37 ± 1.50^{Ac}	5.66 ± 0.52^{Ac}
10^3	Absent	2.99 ± 0.24^{Ba}	5.40 ± 0.36^{Cb}	6.68 ± 0.52^{Bc}	7.20 ± 0.20^{Bc}
10^3	Present	3.17 ± 0.12^{Ba}	4.55 ± 0.23^{Bb}	6.09 ± 0.30^{ABc}	6.50 ± 0.34^{Ac}

Reported values are means ± standard deviations (n = 6). Means followed by different uppercase letters within columns and lowercase letters within rows are significantly different according to Tukey's test (p-value < 0.05).

APPENDIX C. EVALUATION OF THE EFFICACY OF LPP AT A CONCENTRATION OF 10^4 CFU/G AND 10^7 CFU/G ON THE REDUCTION OF *L. MONOCYTOGENES* ON ALFALFA SPROUTS DURING SPROUTING

C.1. Objective

The objectives of the pilot study were 1) to determine the survival and growth of LPP at a concentration of 10^4 CFU/g and 10^7 CFU/g on alfalfa sprouts during sprouting 2) to determine efficacy of LPP at a concentration of 10^4 CFU/g and 10^7 CFU/g on the reduction of *L. monocytogenes* on alfalfa sprouts during sprouting, and 3) to assess the quality of alfalfa sprouts treated with LPP at a concentration of 10^4 CFU/g and 10^7 CFU/g.

C.2. Materials and Methods

The materials and methods used for the pilot study were analogous to the materials and methods used in the study on the 'effectiveness of Lactic Acid Bacteria against *Listeria monocytogenes* and *Salmonella* spp. on sprouting alfalfa sprouts and its influence on sprout maturity and quality'(Chapter 3.3). Modifications included the inoculum levels of *L. monocytogenes* (10^3 CFU/g) and LPP (10^4 CFU/g and10^7 CFU/g) and sample size (n =3).

C.3. Results

C.3.1. Survival and growth of LPP

Populations of LPP at a concentration of 10^4 CFU/g grew approximately 2.0 log CFU/g and 1.0 log CFU/g after one and three days of sprouting, respectively, reaching a maximum concentration of 6.8 log CFU/g. Populations of LPP at a concentration of 10^7 CFU/g were maintained throughout sprouting. Minimal differences were observed between populations of LPP in the presence or absence of *L. monocytogenes;* indicating that the pathogen has little to no effect on the survival and growth of the antagonist (Table 1).

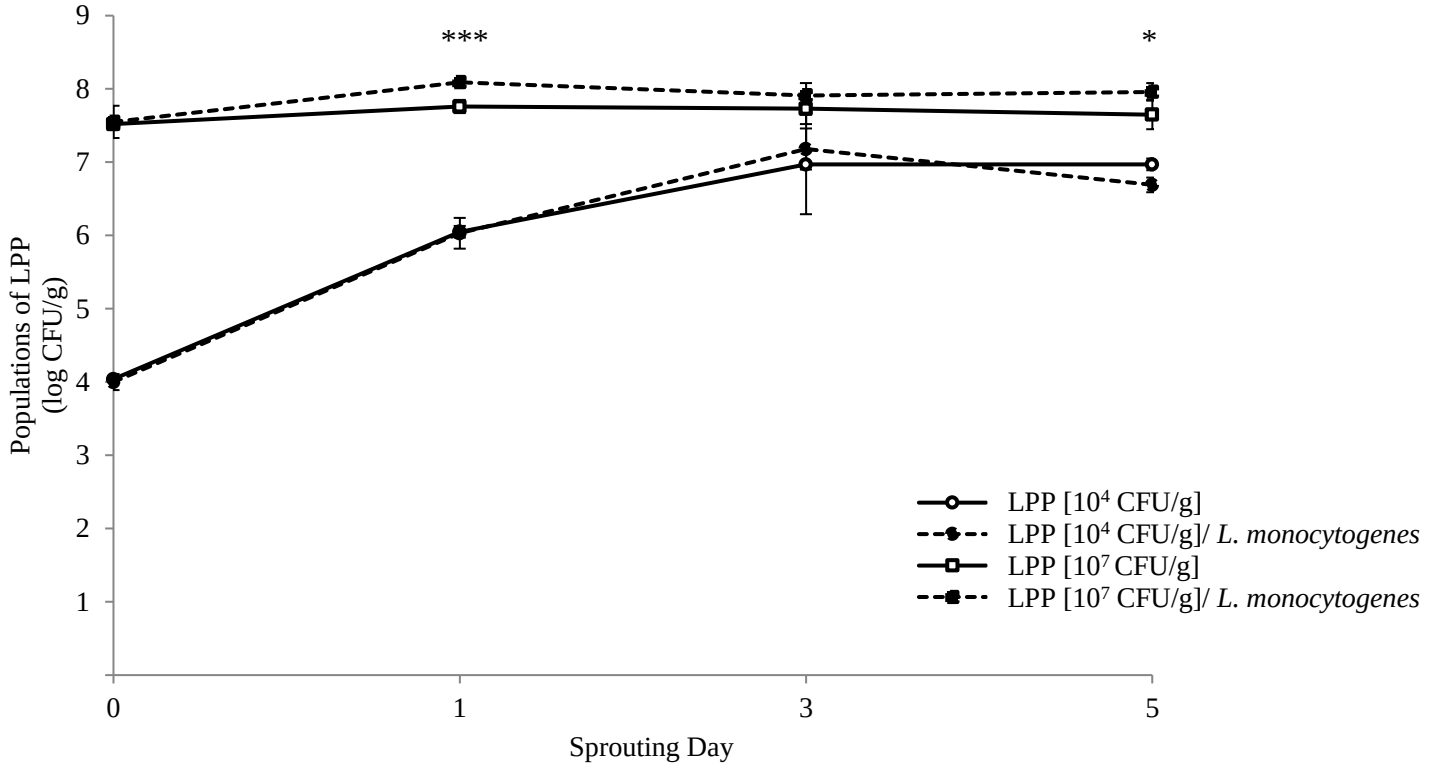

Figure 9. Mean populations of LPP (log CFU/g) at an initial concentration of 10^4 CFU/g and 10^7 CFU/g in the presence or absence of L. monocytogenes on alfalfa seeds during five days of sprouting at 20°C. *Single and ***triplicate stars indicates a significant difference between LPP at an initial concentration of 10^4 CFU/g and 10^7 CFU/g in the presence and absence of *L. monocytogenes*, respectively

C.3.2. Antagonistic effect LPP

The initial population of *L. monocytogenes* was approximately 10^3 CFU/g. Pathogen populations increased during sprouting by 4.0 log CFU/g, reaching a maximum concentration of 10^7 CFU/g after five days of sprouting. When LPP was applied to the seed at an initial concentration of 10^4 CFU/g populations of *L. monocytogenes* were not different from the control ($p > 0.05$). A difference in *L. monocytogenes*, however, was seen when LPP was applied at an initial concentration of 10^7 CFU/g. During one and three days of sprouting populations of *L. monocytogenes* in the presence of LPP at a concentration of 10^7 CFU/g were 3.0 log CFU/g, 5.0 log CFU/g, and 1.0 log CFU/g less than the controls ($p < 0.05$). However, on day five populations of *L. monocytogenes* increased by approximately 5.0 log CFU/g and populations were only a 1.0 log CFU/g less than the control ($p < 0.05$) (Figure 1).

C.3.3. Sprout Maturity and Quality

There was no difference in the yield, seeding length, and pH of the alfalfa sprouts treated with LPP at a concentration of 10^4 CFU/g and 10^7 CFU/g ($p < 0.05$). On average, the yield, seeding length, and pH of the alfalfa sprouts were 18.8 g, 4.2 cm, and 5.6, respectively (Table 2).

Table 20. The yield (g), seedling length (cm) and pH of alfalfa sprouts treated with LPP at an initial inoculum level of 104 CFU/g or 107 CFU/g after five days of sprouting at 20°C.

Treatment	Yield (g)	Seedling length (cm)	pH
No LPP	19.0 ± 2.06^{A}	4.3 ± 0.66^{A}	5.73 ± 0.17^{A}
LPP [10^4 CFU/g]	18.7 ± 1.24^{A}	4.1 ± 0.12^{A}	5.83 ± 0.21^{A}
LPP [10^7 CFU/g]	18.6 ± 1.99^{A}	4.0 ± 0.67^{A}	5.52 ± 0.35^{A}

Reported values are means ± standard deviations (n = 3). Means followed by different uppercase letters within columns are significantly different according to Tukey's test (p-value < 0.05).

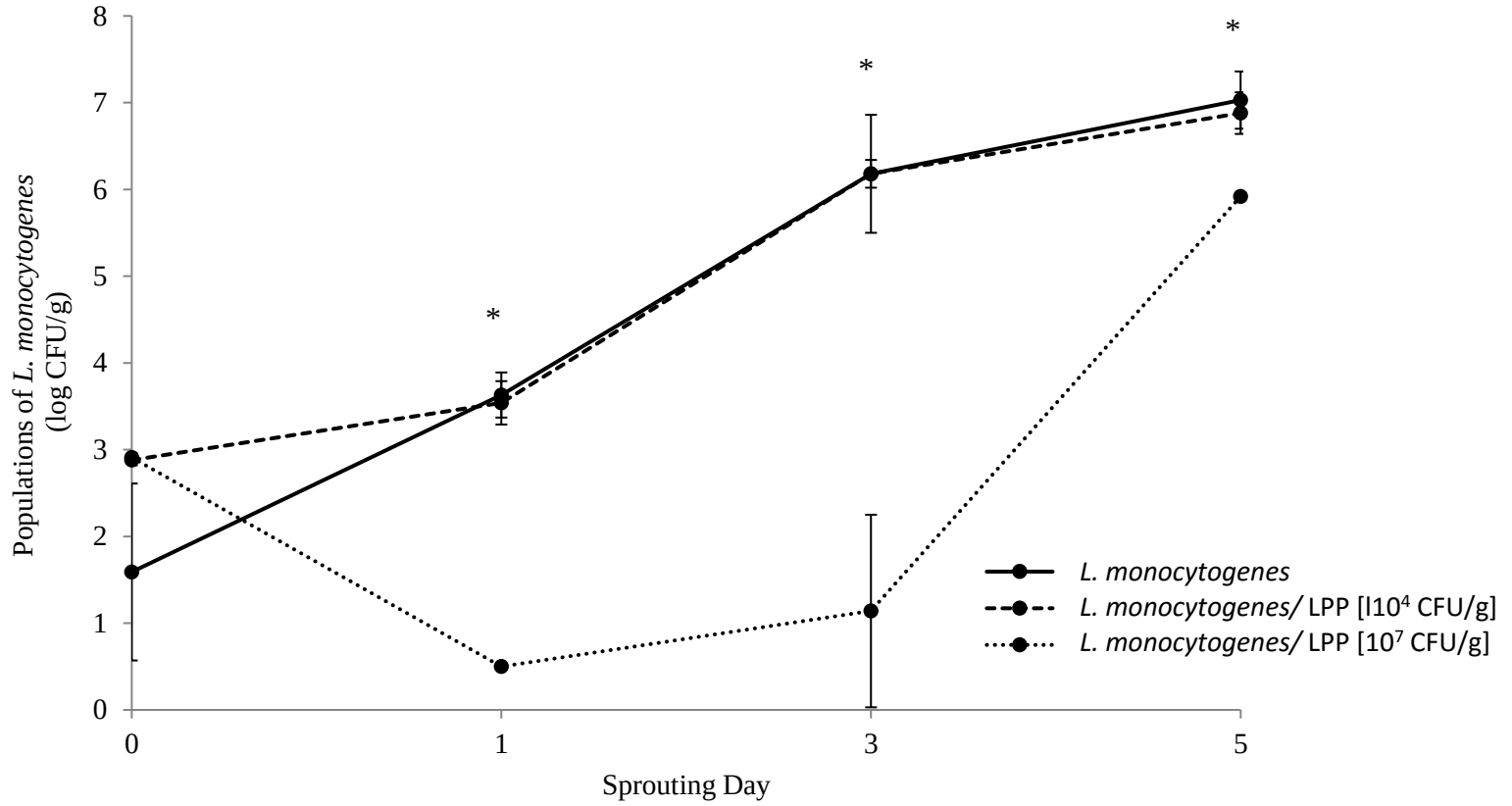

Figure 10. Mean populations (log CFU/g) of L. monocytogenes in the presence or absence of LPP at an initial concentration of 10^4 CFU/g and 10^7 CFU/g on alfalfa seeds during five days of sprouting at 20°C. **Single star indicates a significant difference between *L. monocytogenes* in the presence and absence of LPP at an initial concentration of 10^4 CFU/g.

C.4. Conclusion

LPP could be a promising strategy against foodborne pathogens on growing sprout vegetables; however, a high concentration of the antagonist must be used. LPP does not comprise sprout maturity and quality.

APPENDIX D. EVALUATION OF PREPARATION OF LPP AND SPRAY APPLICATION ON THE REDUCTION OF *L. MONOCYTOGENES* ON ALFALFA SPROUTS DURING SPROUTING

D.1. Objective

The objectives of the pilot study were 1) to determine the survival and growth LPP on alfalfa sprouts during sprouting 2) to determine the efficacy of LPP (LPP.A), a combination of LPP and its metabolites (LPP.B), and its metabolites alone (LPP.C) on the reduction of *L. monocytogenes*, 3) to assess the quality of alfalfa sprouts treated with LPP.A, LPP.B, and LPP.C., 4) to determine the efficacy of spraying the alfalfa sprouts with LPP.A. LPP.B, and LPP.C after three days of sprouting on the reduction of *L. monocytogenes*

D.2. Materials and Methods

The materials and methods used for the pilot study were analogous to the materials and methods used in the study on the 'effectiveness of Lactic Acid Bacteria against *Listeria monocytogenes* and *Salmonella* spp. on alfalfa sprouts and its influence on sprout maturity and quality'(Chapter 3.3). Modifications included Lactic Acid Bacteria preparation, spraying application, inoculum level of *L. monocytogenes* (10^1 CFU/g), and sample size (n=3). Specifics pertaining to Lactic Acid Bacteria preparation and spraying application are specified.

D.2.1. Lactic Acid Bacteria Preparation

Lb. plantarum, P. acidophilus, and *P. pentosaceus* were obtained from BiOWiSH technologies (Cincinnati, OH) (Table 3.2). The protective bacterial cultures were maintained on de Man, Rogosa, and Sharpe agar (MRS) at 4°C until inoculation. Inoculum was prepared using the Almond Board of California guidelines for seed inoculum preparation (Almond Board of California, 2014). Briefly, cells from isolated colonies were transferred into 10 ml of MRS broth at 35°C for 18 to 20 h. A 10 µL loop of bacterial culture was then transferred into 10 ml of MRS broth, incubated at 35°C for 18 to 20 h. Finally, 1ml of bacterial culture was spread over MRS plates and incubated at 35°C for 22 to 26 h. Approximately, 6 ml of 0.1% peptone was added to each plate and the bacterial lawn was loosened using a sterile spreader. A sterile pipette was used to collect 18 ml of cells, 6 ml of each LAB species, into two sterile Falcon tubes labeled LPP.A

and LPP.B, representing LPP harvested, unwashed (pH 4.26) and washed (pH 6.01), respectively. The cells in the sterile Falcon tube labeled as LPP.B were centrifuged at 3000 rpm for 15 min and the supernatant was poured into a sterile Falcon tube labeled LPP.C (pH 4.29). The pellet was cleaned twice by centrifugation using 18 ml of 0.1% peptone. Inoculum of LPP.A and LPP.B were approximately 10^7 CFU/ml.

D.2.2. Spray Application

After three days of sprouting, alfalfa seeds inoculated with *L. monocytogenes* and coinoculated with LLP.A, LPP.B, LPP.C were sprayed for two days with either 5 ml of sterile deionized water (Treatment A) or LLP.A, LPP.B, or LPP.C (Treatment B).

D.3. Results

D.3.1. Survival and growth of LPP

Populations of LPP were maintained between 7.5 log CFU/g and 8.0 log CFU/g during sprouting. No significant difference was observed between populations of LPP in the presence or absence of *L. monocytogenes* (Table D.1). LPP was not influenced by the native flora present on the alfalfa sprouts. Populations of LPP were maintained as populations of the native flora increased. The native flora grew approximately 4.0 log CFU/g and 2.0 log CFU/g after one and three days of sprouting, reaching a maximum concentration of 10^8 CFU/g (Table D.2).

D.3.2. Antagonistic effect LPP and its metabolites

LPP.A significantly reduced populations of *L. monocytogenes* during sprouting. LPP.A had the greatest effect on the growth of *L. monocytogenes*; populations of *L. monocytogenes* were approximately 3.0 log CFU/g and 4.2 log CFU/g less than the control after one and five days of sprouting, respectively ($p < 0.05$). Populations of *L. monocytogenes* on seeds treated with LPP.B were also less than the control by approximately 3.0 log CFU/g and 2.0 log CFU/g after one and five days of sprouting, respectively ($p < 0.05$). LPP.C had the lowest effect on the growth of *L.*

monocytogenes. Populations were 1.0 log CFU/g less than the control throughout sprouting ($p < 0.05$) (Figure D.1).

Table 21. Populations of LPP.A or LPP.B in the absence or presence of L. monocytogenes on alfalfa seeds during five days of sprouting at 20°C.

Treatment	Pathogen	Population (log CFU/g) of LPP			
		Sprouting Day 0	Sprouting Day 1	Sprouting Day 3	Sprouting Day 5
LPP.A	Absent	7.46 ± 0.11^{Aa}	8.13 ± 0.11^{Ab}	7.87 ± 0.15^{Abc}	7.76 ± 0.08^{Ac}
LPP.A	Present	7.61 ± 0.14^{Aa}	8.07 ± 0.10^{Ab}	8.09 ± 0.08^{Ab}	7.77 ± 0.22^{Aab}
LPP.B	Absent	7.58 ± 0.17^{Aa}	8.18 ± 0.06^{Aa}	7.82 ± 0.13^{Aa}	7.45 ± 0.59^{Aa}
LPP.B	Present	7.53 ± 0.15^{Aa}	8.02 ± 0.21^{Ab}	7.93 ± 0.11^{Aab}	7.90 ± 0.02^{Aab}

Reported values are means ± standard deviations (n = 3). Means followed by different uppercase letters within columns and lowercase letters within rows are significantly different according to Tukey's test (p-value < 0.05).

Table 22. Populations of native microflora in the absence or presence of LPP.A and LPP.B on alfalfa seeds during five days of sprouting at 20°C.

Treatment	Population (log CFU/g) of Native Microflora			
	Sprouting Day 0	Sprouting Day 1	Sprouting Day 3	Sprouting Day 5
No Treatment	2.46 ± 0.03^{Aa}	6.41 ± 0.04^{Ab}	8.60 ± 0.21^{Ac}	7.80 ± 0.33^{Ac}
LPP.A	2.40 ± 0.01^{Aa}	6.58 ± 0.21^{Ab}	7.53 ± 0.46^{Bc}	7.84 ± 0.38^{Ac}
LPP.B	2.65 ± 0.43^{Aa}	6.27 ± 0.51^{Ab}	7.95 ± 0.45^{Ac}	7.57 ± 0.22^{Ac}

Reported values are means ± standard deviations (n = 3). Means followed by different uppercase letters within columns and lowercase letters within rows are significantly different according to Tukey's test (p-value < 0.05).

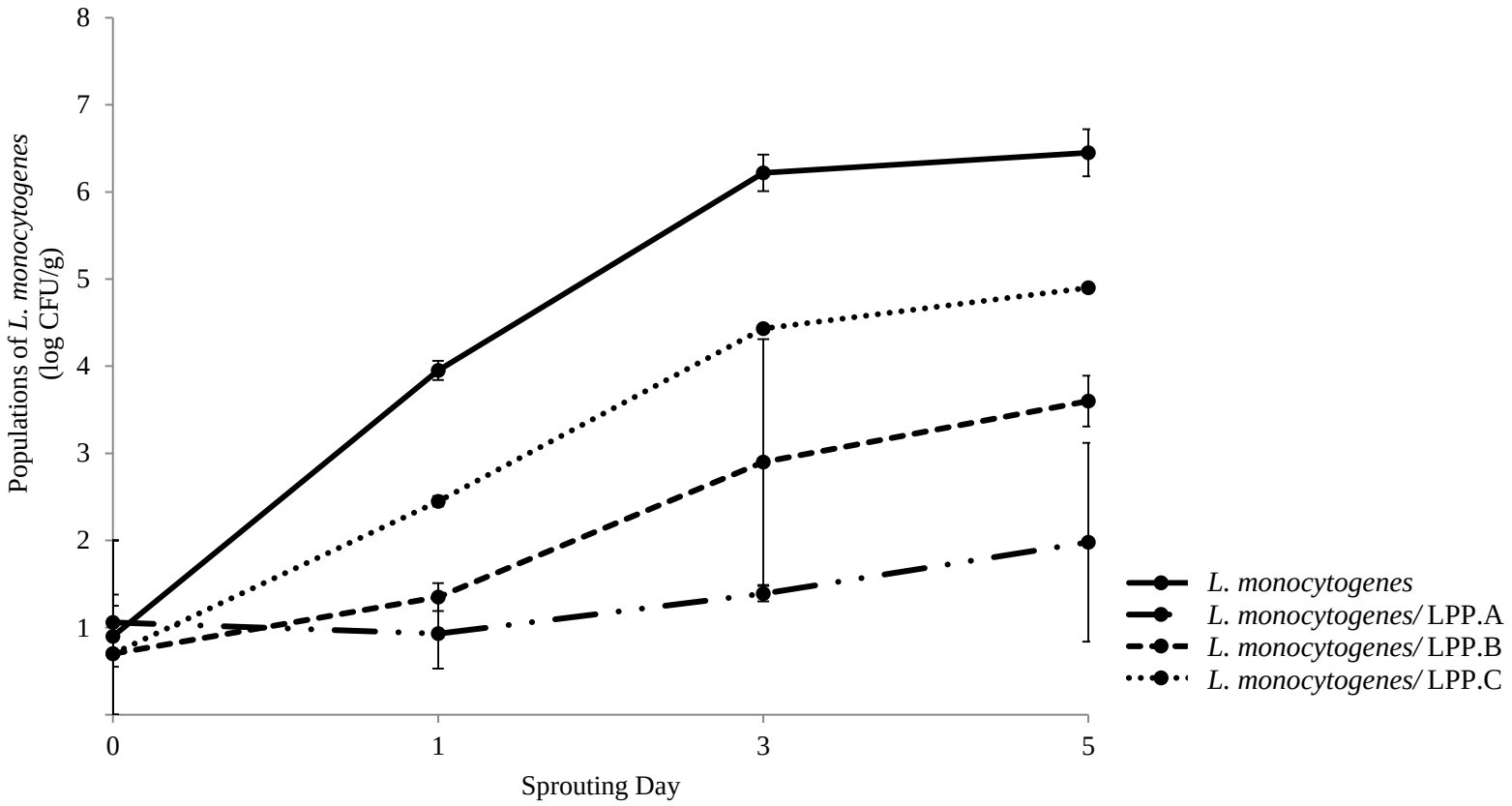

Figure 11. Mean populations (log CFU/g) of L. monocytogenes in the absence or presence of LLP.A LPP.B, or LPP.C on alfalfa seeds during five days of sprouting at 20°C.

.

D.3.3. Spray Application

The spray application did not improve efficacy of LPP against *L. monocytogenes* during the final days of sprouting.There was no differences between populations of *L. monocytogenes* on alfalfa seeds treated with with LPP.A, LPP.B, or LPP.C and sprayed with either 5 ml of sterile deionized water or LLP.A, LPP.B, or LPP.C ($p > 0.05$) (Figure D.2).

D.3.4. Sprout Maturity and Quality

There was no observable difference in the percent germination of the alfalfa sprouts treated with LPP.A, LPP.B, and LPP.C. There was a significant difference in the yield, seedling length, and pH of the treated alfalfa sprouts in comparison to the untreated control ($p < 0.05$). The yields of LPP.A and LPP.B were approximately 2.9 g and 3.9 g less than the untreated control. The yield, and pH of LPP.C was 7.0 g and 0.80 less than the untreated control (Table 3).

Table 23. The yield (g), seedling length (cm) and pH of alfalfa sprouts treated with LPP.A, LPP.B, and LPP.C after five days of sprouting at 20°C.

Treatment	Yield (g)	Seedling length (cm)	pH
Control	19.6 ± 0.67^{A}	5.0 ± 0.36^{AB}	5.52 ± 0.09^{A}
LPP.A	16.5 ± 1.11^{B}	3.7 ± 0.66^{B}	5.63 ± 0.05^{A}
LPP.B	15.7 ± 0.84^{B}	5.2 ± 0.38^{A}	5.47 ± 0.09^{A}
LPP.C	12.6 ± 0.64^{C}	3.9 ± 0.58^{B}	4.70 ± 0.06^{B}

Reported values are means ± standard deviations (n = 3). Means followed by different uppercase letters within columns are significantly different according to Tukey's test (p-value < 0.05).

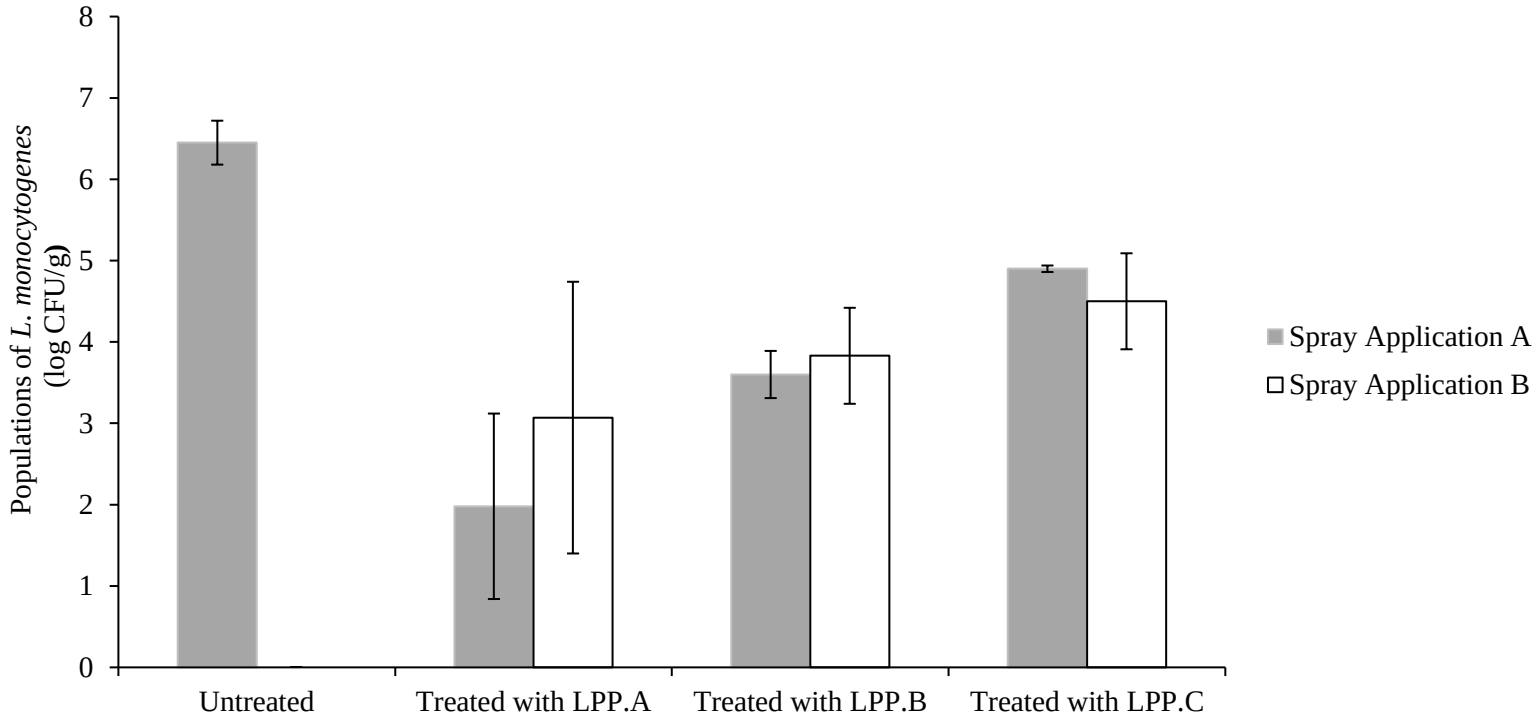

Figure 12. Mean populations (log CFU/g) of L. monocytogenes after five days of sprouting at 20°C. Alfalfa seeds were untreated or treated with LPP.A, LPP.B or LPP.C and sprayed with either 5 ml of deionized water (Spray Application A) or LLP.A, LPP.B, or LPP.C (Spray Application B) on the final three days of sprouting.

D.4. Conclusion

In comparison to LPP.B and LPP.C, LPP.A had the greatest effect on the growth of *L. monocytogenes*, indicating that a combination of the lactic acid bacteria and its metabolites are beneficial in achieving a more potent lethal activity against foodborne pathogens on growing sprouts. Application of LPP on the final days of sprouting does not increase the potency of the treatment. LPP does not have an effect on sprout maturity and quality.