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Antimicrobial Susceptibility of Listeria monocytogenes to Bacteriophage LISTEX™ P100 in Alfalfa Sprouts (Medicago sativa)

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A Thesis by

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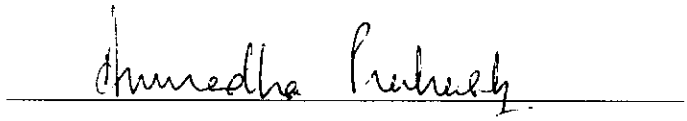

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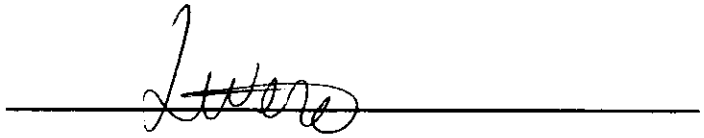

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DEDICATION

This thesis is dedicated to my family for the encouragement and support that they have provided throughout my life.

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ABSTRACT

Antimicrobial Susceptibility of *Listeria monocytogenes* to Bacteriophage LISTEX™ P100 in Alfalfa Sprouts (*Medicago sativa*)

by Tushar Prakash Sawant

The seed germination process during sprout production provides suitable environmental conditions for the growth of pathogenic bacteria, such as *Listeria monocytogenes.* A potential way to control this bacterial growth is through the use of bacteriophages, which are naturally occurring viruses that specifically attack bacterial targets and have been shown to be effective antimicrobials in some foods. Therefore, the objective of this study was to evaluate the antimicrobial susceptibility of *L. monocytogenes* to bacteriophage on alfalfa sprouts during seed germination and subsequent refrigerated storage at 4 °C. Alfalfa sprout seeds were dip-inoculated with 5.5 x 10^5 CFU/ml *L. monocytogenes* serogroups 1 and 4. This was followed by treatment with the commercial bacteriophage LISTEX™ P100 at a concentration of 5.3 x 10^7 PFU/ml. The seeds were then soaked and germinated for 80 h using the glass jar method. The concentration of *L. monocytogenes* was determined every 24 h using PALCAM agar plated in triplicate. When compared to the spiked, untreated control, treatment of sprout seeds with LISTEX™ P100 resulted in a statistically significant ($p < 0.05$) reduction of 1.6 $\log_{10}$ CFU/g *L. monocytogenes* after the initial 24 h of germination. However, the bacteriophage did not show a lasting inhibitory effect, with no statistically significant reductions in *L. monocytogenes* growth as compared to the control at subsequent time points. The bacteriophage remained stable over the entire germination and storage period. Although biocontrol of *Listeria* with bacteriophages has high potential to serve as an

alternative strategy to control foodborne illnesses, factors such as phage delivery and dose optimization in sprouts need to be further investigated.

Keywords:

Listeria monocytogenes, alfalfa sprouts, bacteriophage, LISTEX™ P100, germination

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LIST OF ABBREVIATIONS

AA – Acetic acid

ACMSF – Advisory Committee on the Microbiological Safety of Food

AEW – Acidic electrolyzed water

ANOVA – Analysis of Variance

APC – Aerobic Plate Count

BPW – Buffered Peptone Water

C – Celsius

CDC – Center for Disease Control and Prevention

CFR – Code of Federal Regulations

CFU – Colony forming unit

d - Days

DNA – Deoxyribonucleic acid

FSIS – Food Safety and Inspection Service

GRAS – Generally Recognized As Safe

h – Hour

HACCP – Hazard Analysis and Critical Control Point

ICTV - International Committee on Taxonomy of Viruses

LA – Lactic acid

LPS – *Listeria monocytogenes* + LISTEX™ P100 + alfalfa seeds

LS – *Listeria monocytogenes* + Alfalfa seeds

MA – Malic Acid

MOI – Multiplicity of Infection

NACMCF – The National Advisory Committee on Microbiological Criteria for Foods

NDB – National Nutrient Database

NRCS – Natural Resources Conservation Service.

O.D – Optical Density

OA – Oxalic acid

PA – Phytic acid

PALCAM – polymyxin acriflavine lithium chloride ceftazidimeaesculin mannitol

PFU – Plaque forming unit

PS – LISTEX™ P100 + alfalfa seeds

RNA – Ribonucleic Acid

RTE – Ready-to-Eat

S – Alfalfa seeds only

s - Seconds

TDS – Thiamine Dilauryl Sulfate

THM – Trihalomethanes

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

USFDA – United States Food and Drug Administration

UV – Ultraviolet

LOGIC MODEL

Situation	Priorities	Inputs	Outputs		Outcomes -- Impact		
			Activities	*Participation*	Short	Medium	Long
Novel strategies for the effective control of *Listeria monocytogenes* in sprouts is desired Biotechnological developments for bacteriophage use in foods to prevent pathogenic bacteria growth have been on the rise Seed sprouts have been subjected to various physical, chemical, and biological treatments as methods of decontamination Complications such as antibiotic resistance, development of undesirable organoleptic properties, and rigid consumer acceptance to chemical preservatives have resulted in low efficacy of current decontamination methods in sprouts	Identify the inoculation levels of *L. monocytogenes* and bacteriophage LISTEX™ P100 on alfalfa seeds Determine the reduction levels of *L. monocytogenes* in alfalfa seed sprouts during germination and storage Determine the stability of LISTEX™ P100 on alfalfa sprouts from seed germination to sprout storage	Advisors Industry experts Students Time, Critical thinking, Vision Chemicals Equipment Raw Material (alfalfa Seeds, bacteriophage LISTEX™ P100, *L. monocytogenes* strains)	Design a protocol for bacteria and phage inoculation Conduct research Present results and findings at conferences and presentations Publish study in scientific journal	**Participants** Advisors Phage Industry experts **Products** Presentations Scientific publications	**Knowledge** Know the effect of bacteriophage LISTEX™ P100 application to reduce *L. monocytogenes* population in alfalfa sprouts	**Actions** A natural alternative antimicrobial against *L. monocytogenes* for the benefit of public health Commercial as well as home-grown sprouters can benefit from this technology by decreasing pathogenic growth	**Conditions** *Research* Current study can form basis for further research in phage application to other foods *Social/Public Health* The consumers benefit from safer sprouts

Assumptions	**External Factors**
There is a need for alternative biocontrol strategies besides chemical (sanitizers) and physical (heat, irradiation) interventions to control *Listeria* in seed sprouts Consumer would accept and be aware of presence of phages in foods	Presence of background microbiota in alfalfa seeds may negatively affect the phage efficacy in sprouts Potential contamination sources including lab platform, refrigerator etc. may introduce variability in results A complex food matrix such as alfalfa sprouts may hinder the diffusion of phage particles to target bacteria

1 INTRODUCTION

Listeria monocytogenes is a Gram-positive, ubiquitous, facultative anaerobic bacterium that can grow at refrigerated temperatures and survive in a wide range of growth conditions such as low pH (below 4.3) and high salt levels up to 10% (USFDA 2012). *L. monocytogenes* causes a foodborne illness called listeriosis, which involves diarrhea, fever, headaches, stiffness, and nausea. In some cases, it can lead to more serious diseases like pneumonia and meningitis, as well as spontaneous abortions and still births among pregnant women (USFDA 2012). Human listeriosis is responsible for 28% of all deaths due to foodborne illness and it has the highest hospitalization rate of 92 % among food borne pathogens in United States (Scallan and others 2011). The susceptible populations for listeriosis are pregnant women, elderly and immunocompromised populations.

Since foodborne listeriosis was first reported in 1981, numerous outbreaks caused by *L. monocytogenes* have been reported in the United States (Silk and others 2013). The majority of these outbreaks have been caused by the food groups such as meat, dairy, poultry and produce (Conter and others 2009), with majority of these caused by *L. monocytogenes*. In 2013, alfalfa sprouts from Sprouters Northwest of Kent were found to be contaminated with *L. monocytogenes,* resulting in an immediate recall of over 1,953 lbs of alfalfa and other sprouts (USFDA 2013).

A potential means for controlling *L. monocytogenes* in sprouts is through the use of bacteriophages, which are naturally occurring viruses that specifically attack bacterial targets. Since 2000, bacteriophages have been applied in the food industry as biocontrol,

biopreservative, and biosanitation agents as well as for detecting pathogenic bacteria in foods (Garcia and others 2008). The advantages of bacteriophages include the following: i) they kill the bacterial target, ii) they do not cross genus boundaries as compared to chemical sanitizers such as $CaOCl_2$ and thus are specific in their action, and iii) they break down into non-toxic proteins and nucleic acids as their final product (Rossi and others 2011). In 2006, the U.S. Food and Drug Administration (FDA) approved and granted generally recognized as safe (GRAS) status to the use of the LISTEX™ P100 in raw and ready-to-eat foods as an antimicrobial agent against *L. monocytogenes* (USFDA 2007). Bacteriophages are effective at controlling pathogens such as *Salmonella*, *Escherichia coli*, and *L. monocytogenes* in a variety of food products (Hagens and Loessner 2007). The efficacy of LISTEX™ P100 was evaluated in broth and on raw salmon fillet tissues for control of *L. monocytogenes* as a function of storage temperature (Soni and Nannapaneni 2010). A 2.3 log CFU/g reduction in *L. monocytogenes* was observed with application of phage LISTEX™ P100 on raw salmon fillet tissue over a 10 d storage period at 4 °C. The potential of bacteriophages has also been studied with alfalfa sprouts where a 1-log CFU/g reduction in *Salmonella oranienburg* was observed after a 3 h phage application during sprouting (Kocharunchitt and others 2009). However, no studies have evaluated the effectiveness of LISTEX™ P100 as an antimicrobial agent against *L. monocytogenes* in alfalfa sprouts.

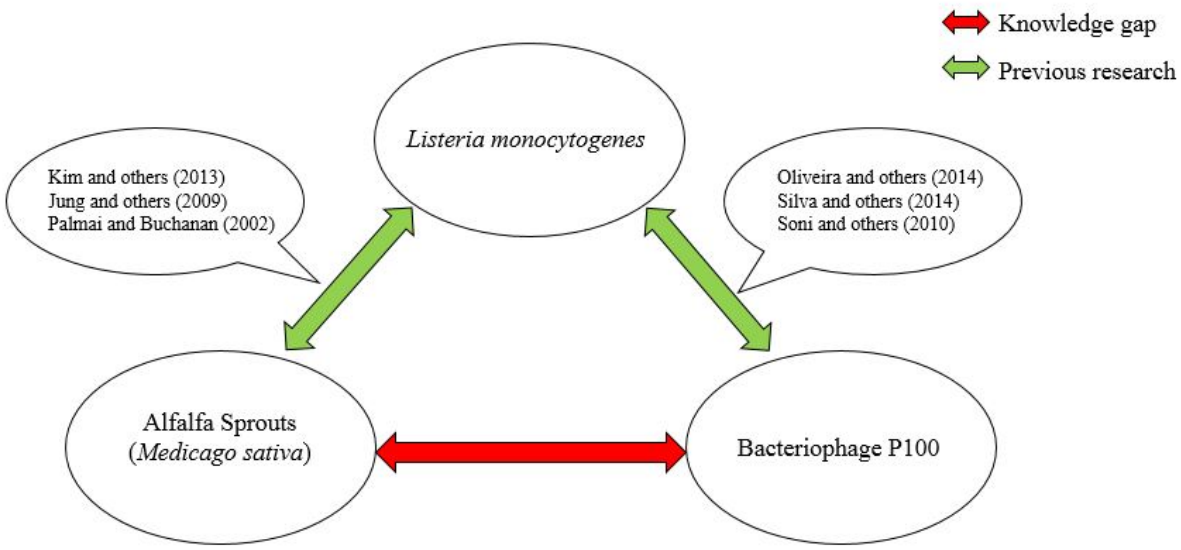


Figure 1. The gap in knowledge associated with use of bacteriophages as a biocontrol agent in alfalfa sprouts.

As seen in Figure 1, many studies have reported a relationship between *Listeria* – sprouts and *Listeria* – bacteriophage, but no study has been reported studying the relationship between *Listeria monocytogenes*, alfalfa sprouts (*Medicago sativa*) and bacteriophage LISTEX™ P100. The goal of this study was to evaluate the antimicrobial susceptibility of *Listeria monocytogenes* to the bacteriophage LISTEX™ P100 on alfalfa sprouts (*Medicago sativa*) at refrigerated temperature (4 °C).

2 LITERATURE REVIEW

2.1 *Listeria monocytogenes*

2.1.1 History of L. monocytogenes

In the late 19th century, Hayem (France, 1891) and Henle (Germany, 1893) studied patients that, in retrospect, had listeriosis and observed characteristic Gram-positive rods in their tissue section (Gray and Killinge 1966). However, their observations were not verified until 1911 when Hulphers, a Swedish scientist isolated an organism from necrotic foci in rabbit liver which he assigned as *Bacillus hepatis* and whose observed characteristics resemble most likely those of *Listeria monocytogenes* (Gellin and Broome 1989). The first major progressive step in description of the organism was achieved when Murray and others (1926) isolated the bacterium from infected rabbits and guinea pigs and named it *Bacterium monocytogenes* because of a characteristic monocytosis (Farber and Peterkin 1991). In 1927, a similar bacterium was isolated from liver of several rodents (*Iatera lobenquiae*) from Tiger River region in South Africa (Tiger river disease) and named *Listerella hepatolytica* by Pirie (1927) in the honor of Lord Joseph Lister, the father of antisepsis (Pitt and Aubin 2012; Schlech and Acheson 2000). Although several studies were reported on possible outbreaks of listeriosis, Gill (1933) first confirmed the isolation of *L. monocytogenes* from sheep suffering from circling disease (Oevermann and others 2010) and later proved the relationship of organism isolated from brain of affected animal and the disease (Gray and Killinge 1966). Listeric infection was first confirmed in humans in 1929 when Schmidt and Nyfeldt (1938) isolated the causal agent of infectious-mononucleosis from three patients and named it *Bacterium monocytogenes hominis* (Gellin and Broome 1989).

Burn (1936) reported *L. monocytogenes* as a causal agent of meningitis in three newborn infants and an adult. In 1940, Seeliger (1988) named the organism as *Listeria monocytogenes*.

2.1.2 Outbreaks associated with L. monocytogenes

All 13 reported serovars of *Listeria monocytogenes* are capable of causing listeriosis, however, serovars 1/2a, 1/2b and 4b cause most of the cases (Rocourt and Bille 1997; Swaminathan and Gerner-Smidt 2007). According to FDA's Risk & Safety Assessment, the recognition of *L. monocytogenes* as a food borne pathogen started in the early 1980's due to serious illness outbreaks associated with consumption of contaminated dairy products and coleslaw (USFDA 2003). The first scientifically proven listeriosis outbreak was observed in Maritime Provinces, Canada in 1980-81 where 41 cases of infection were caused by consumption of contaminated coleslaw resulting in 14 deaths (Schlech and Acheson 2000; Schlech and others 1983).

There have been several incidences of *L. monocytogenes* outbreaks associated with foods. Los Angeles County, CA, observed a large-scale listeriosis outbreak due to contaminated Mexican-style soft cheese consumption in 1985. Out of a total 142 cases, the outbreak led to 48 deaths which included 20 fetuses, 10 neonates and 18 non-pregnant adults (USFDA 2012). Several reported incidences of growth of *L. monocytogenes* in a wide variety of foods such as meat, dairy products, seafood, and vegetables indicate a high survival capability of the pathogen in foods (Farber and Peterkin 1991). A multistate listeriosis outbreak caused by contaminated hot dog consumption resulted in six deaths and two miscarriages in 1998-99 (USFDA 2012). A similar outbreak occurred due to

contaminated turkey deli meat consumption in Northeastern U.S. in 2002, resulting in 46 cases of illness, 7 deaths and 3 miscarriages. The outbreak strain was serotype 4b in both the outbreaks (CDC 2002). In 2008, production of contaminated deli meat by a Maple Leaf Foods plant in Toronto, Canada led to 57 cases and 22 deaths due to human listeriosis caused by serotype 1/2a (USFDA 2012).

In 2011, a multistate outbreak of listeriosis due to whole cantaloupes from Jensen Farms in Colorado, resulted in 33 deaths to (CDC (2011a). Human listeriosis was linked to Imported Frescolina Marte Brand Ricotta Salata Cheese distributed by Forever Cheese, Inc. which resulted in 20 hospitalization cases and 4 deaths (CDC 2013b). In 2013, another cheese-related listeriosis outbreak was reported from Les Frères, Petit Frère, and Petit Frère with Truffles cheeses made by Crave Brothers Farmstead Cheese Company, Waterloo, WI (CDC 2013c). The FDA ordered a recall of alfalfa, broccoli sandwich sprouts, clover and spicy sprouts from Sprouters Northwest Kent, WA, for potential *L. monocytogenes* contamination in January 2013 (USFDA 2013). Surveillance sampling of products from retail locations by the Washington State Department of Agriculture (WSDA) helped in restricting the spread of disease (USFDA 2013).

Table 1. Foodborne outbreaks in United States associated with *L. monocytogenes* in different food groups (USFDA 2003).

Geographical location, and year	**Implicated food**	**Consequences**	**Citations**
Oklahoma and Texas, 2015	Ice cream	Total cases - 8, Hospitalization - 8, Deaths - 3	CDC (2015a)

Bakersfield, California, 2015	Caramel apples	Total cases - 35, Hospitalization - 34, Deaths - 7 (1 fetal loss)	CDC (2015b)
Chicago, 2014	Mung bean sprouts	Total cases - 5, Hospitalization - 5, Deaths - 2	CDC (2014b)
Oasis Brands Inc., 2014	Quesito casero (unaged soft cheese from pasteurized milk)	Total cases - 5, Hospitalization - 4	CDC (2014c)
Multistate, 2012	Frescolina Marte brand ricotta salata cheese	Total cases - 22, Hospitalization - 20, Deaths - 2 (1 fetal loss)	CDC (CDC 2013b)
Delaware, 2013	Cheese	Total cases - 8, Hospitalization - 7, Deaths - 1	CDC (CDC 2014a)
Waterloo, WI, 2013	Farmstead cheeses	Total cases - 6, Hospitalization - 6, Deaths - 1 (1 miscarriage)	CDC (CDC 2013c)
Jensen farms, Colorado, 2011	Cantaloupes	Total cases - 147, Deaths - 33 (1 miscarriage)	CDC (2011a)
Louisiana, 2010	Hog head cheese	Total cases - 14, Hospitalization - 7, Deaths - 2	CDC (2011b)
Massachusetts, 2007	Pasteurized Milk	Total cases - 5, Hospitalization - 5, Deaths - 3 (1 stillbirth)	CDC (2008)
Northeastern United States, 2002	Turkey deli meat	Total cases - 63, Deaths - 7 (3 stillbirths)	CDC (2002)
Los Angeles, CA, 2001	Delicatessen meat	Total cases - 28	Frye and others (2002)
North Carolina, 2000-2001	Mexican style soft cheese	Total cases - 12, Stillbirths - 5, Premature deliveries - 3, Infected newborns - 2	CDC (2001)
Multistate, 2000	Deli turkey meat	Total cases-29, Deaths-7	Hurd and others (2000)
Multistate, 1998-1999	Meat frankfurters	Total cases - 108, Deaths - 14 (4 stillbirths)	Mead and others (2006)
Multistate, 1998	Hot dogs and other meat products	Total cases - 40, Deaths - 4 (1 fetal loss)	CDC (1998)

	Deli meats, Hot dogs	Total cases-101, Deaths-21	CDC (1999)
Illinois, 1994	Chocolate milk	Total cases-45, Hospitalization-5	Dalton and others (1997)
Connecticut, 1989	Shrimps	Total cases - 10, Fetal loss - 1	Riedo and others (1994)
Pennsylvania, 1986-1987	Salami, Brie cheese, Ice cream	Total cases-36, Deaths-16	Schwartz and others (1989)
California, 1986-1987	Raw eggs	Total cases-2	Schwartz and others (1988)
Los Angeles County, CA, 1985	Mexican-style soft cheese	Total cases - 145, Deaths - 48 (20 fetal loss, 18 non-pregnant adults, 10 neonates)	Linnan and others (1988)
Massachusetts, 1983	Pasteurized milk	Total cases - 32, Deaths-14	Fleming and others (1985)
Massachusetts, 1979	Raw vegetables or cheese	Total cases - 20, Deaths-3	Ryser and Marth (2007)

Table 2. Foodborne outbreaks outside United States associated with *L. monocytogenes* in different food groups (USFDA 2003).

Geographical location, and year	**Implicated food**	**Consequences**	**Citations**
Gipuzkoa, Spain, 2014	Specific food identified	Total cases - 27, Deaths - 6 (2 miscarriages, 1 stillbirth)	Pérez-Trallero and others (2014)

Switzerland, 2013	Ready-to-eat salads	Total cases - 32, Deaths - 4	Stephan and others (2015)
Europe, 2009	Quargel cheese	Total cases - 27, Deaths - 7	Fretz and others (2010)
France, 1999-2000	Pigs tongue in aspic	Total cases-26, Deaths-7	Dorozynski (2000)
Finland, 1998-1999	Butter	Total cases-25, Deaths-6	Lyytikäinen and others (2000)
Northern Italy, 1997	Tuna/corn salad	Total cases-1566, Hospitalization-292, Deaths-0	Aureli and others (2000)
Ontario, 1996	Imitation crab meat	Hospitalization - 2	Farber and others (2000)
France, 1995	Soft ripened cheese	Total cases-33, Deaths-4	Goulet and others (1995)
Northern Italy, 1993	Rice salad	Total cases-18, Hospitalization-4	Salamina and others (1996)
France, 1993	Rillettes	Total cases-38, Deaths-11	Goulet and others (1998)
France, 1992	Pork tongue in jelly	Total cases-280, Deaths-63	Jacquet and others (1995)
Australia, 1990	Pate and meat spreads	Total cases-11, Deaths-6	McLauchlin (1997)
Denmark, 1989-1990	Semi-soft cheese (blue)	Total cases-23, Deaths-0	Jensen and others (1994)
Austria, 1986	Unpasteurized milk and organic vegetables	Total cases-28, Deaths-5	Allerberger and Guggenbichler (1988)
Switzerland, 1983-1987	Vacherin Mont d'Or cheese	Total cases-122, Deaths-31	Büla and others (1995)

Canada, 1981	Raw vegetables	Total cases-41, Deaths-17	Schlech (1996)
New Zealand, 1980	Raw seafood (finfish and mollusks)	Total cases-22, Deaths-6	Becroft and others (1984)
Australia, 1978-1979	Raw vegetables	Total cases-12, Deaths-0	Le Souef and Walters (1982)

2.1.3 Taxonomy of L. monocytogenes

L. monocytogenes belongs to the Kingdom Bacteria, Division Firmicutes, Class Bacilli, Order Bacillales, Family Listeriaceae, Genus *Listeria*, species *monocytogenes*. Besides *L. monocytogenes*, other species within the genus include *L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. grayi* (Rocourt and Buchrieser 2007), *L. marthii* (Graves and others 2010), *L. rocourtii* (Leclercq and others 2010), *L. weihenstephanensis* (Halter and others 2013), *L. fleischmannii* (Bertsch and others 2013), *L. floridensis, L. aquatic, L. cornellensis, L. riparia, L. grandensis* (den Bakker and others 2014), *L. booriae*, and *L. newyorkensis* (Weller and others 2015). Among these species, *L. monocytogenes* is responsible for most of the clinical infections, whereas *L. ivanovii* has been found to be typically associated with abortion in ruminants.

Since 2000, alfalfa sprouts have been involved in most of the sprout-related outbreaks mainly because of their availability and larger consumption as compared to other sprouts (Yang and others 2013). The major bacterial pathogens associated with these outbreaks include *Salmonella spp.*, *Escherichia coli* O157, and *L. monocytogenes*. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) has reported 21 sprout-related outbreaks and 1500 cases since 1995, with *Salmonella* being

the causal agent in most of the outbreaks, although cases involving *Bacillus cereus*, *L. monocytogenes* and *E. coli* O157: H7 have also been reported. In 2013, *Listeria* contamination in alfalfa sprouts from Sprouters Northwest Kent was reported by the FDA (2013).

2.1.4 Emergence of Listeria monocytogenes as a pathogen

The increasing consumer trend for convenience has prompted retailers in offering ready-to-eat and ready-to-cook food products that require minimal processing (Davis and Stewart 2002; Stewart and others 2006). Consequently, the levels of exposure of food to *L. monocytogenes* have also increased (ACMSF 2009). Incidences of listeriosis have been on the rise mainly due to increased average life span of immunocompromised or elderly individuals due to advancement in medical technologies, globalization of food attracting consumption of ethnic foods, negligence of food production and processing facilities with regards to food safety, and changing consumer habits towards consumption of convenience foods (Rocourt and Bille 1997). Another factor contributing to the emergence *Listeria* as one of the leading causes of death due to food borne illnesses is the ability of the pathogen to survive under low-temperature conditions (Likotrafiti and others 2013). Refrigeration or freezing is one of the most widely used food preservation methods, which not only restricts microbial growth by delaying the cellular metabolic reactions but also preserves the taste, texture and nutritional value of food (Delgado and Sun 2001). In foods such as seed sprouts, the environment created during germination provides suitable conditions for pathogens such as *Listeria* to grow and proliferate (Palmai and Buchanan 2002). The humid conditions during seed sprouting can promote internalization of bacteria in the sprouts (Yang and others 2013).

2.1.5 Characteristics of L. monocytogenes

Listeria monocytogenes is a small, Gram-positive, ubiquitous, non-sporulating, rod-shaped, facultative anaerobic bacterium responsible for food borne illness, listeriosis (Low and Donachie 1997; Mishra and others 2013). *Listeria* colonies exhibit a blue/green color under obliquely transmitted light (Low and Donachie 1997). This food borne pathogen is able to grow at refrigerated temperatures and survive in a wide range of environmental conditions such as low pH and high salt levels (Boziaris and Nychas 2006; Odriscoll and others 1997). *L. monocytogenes* possesses peritrichous flagella and has a characteristic temperature-driven motility, only between 20 – 25 °C (Peel and others 1988). The saprophytic nature of *L. monocytogenes* surviving on plants, soil samples and wild animal feces was confirmed by Weis and Seeliger (1975). Additionally, *L. monocytogenes* has also been found in mud, sewage, silage, milk, and slaughterhouse wastes worldwide (Gray and Killinge 1966). Although found mostly in soils, *L. monocytogenes* is transmitted from the environment by animals and through contaminated foods during processing. Thus, it can be considered as an environmental bacterium (Rebagliati and others 2009). When *L. monocytogenes* cultures are grown on solid media, they exhibit a characteristic sour, buttermilk-like odor (Gray and Killinge 1966).

2.1.6 Pathogenesis of L. monocytogenes

Human listeriosis is a potentially fatal infection caused by *L. monocytogenes* (Allerberger and Wagner 2010). Most of the listeriosis patients undergo gastrointestinal symptoms as the gastrointestinal tract is the site of entry for the pathogen into the host

(Gellin and Broome 1989; Vazquez-Boland and others 2001). *L. monocytogenes* is able to invade host cells and its movement in the host cell cytoplasm is mediated by the polymerization of actin filaments to form a long actin tail (Ryser and Marth 1999). In cases of gastroenteritis caused by *L. monocytogenes*, the infection begins about 20 h after the pathogen enters the host through contaminated foods, whereas the incubation period for the invasive form of illness is longer, usually estimated to vary from 3 days to few months (Vazquez-Boland and others 2001). There have been no major studies reporting the minimal infection dose but the various listeriosis case studies imply a high dose of 10^6 cells (Vazquez-Boland and others 2001). More than 90 % of listeriosis outbreaks are associated with 3 out of 12 serovars of *L. monocytogenes* (1/2a, 1/2b, 4b) (Vazquez-Boland and others 2001). Besides the strain, host susceptibility including health status of the individual affects the pathogenicity of *L. monocytogenes* (Vazquez-Boland and others 2001). As a result, immunocompromised individuals are at a greater risk for listeriosis.

Host macrophages that reside in the intestinal epithelium ingest *L. monocytogenes*. Subsequently, the pathogens rapidly translocate within the organs depending upon the number of pathogens present and presence of the virulence factor, hemolysin (Ryser and Marth 1999; Vazquez-Boland and others 2001). After the bacteria cross the intestinal lining, they are carried by lymph or blood to the spleen and liver primarily. During the multiplication process in the liver, the macrophages kill the ingested bacteria after the first 6 h of infection but the surviving *Listeria* cells start to grow and increase within 2-5 days. Besides the liver, the pathogen may also penetrate the placental barrier to cause abortion and stillbirth and nerve tissue resulting in meningitis (Vazquez-Boland and others 2001).

2.1.7 Listeria biofilm formation is a growing concern in food industry

The ability of *Listeria* to survive for long periods under harsh conditions in equipment and environments of food industries is related to the formation of biofilms (da Silva and De Martinis 2013). Biofilms are communities of microorganisms that are attached to a surface and are enclosed in a matrix (Gandhi and Chikindas 2007). *Listeria* forms biofilms on surfaces such as plastic, polypropylene, rubber, stainless steel and glass (Bonsaglia and others 2014). This biofilm formation is observed at different temperatures, including refrigeration which is one of the most common preservation methods (Bonsaglia and others 2014). This formation of biofilms is a great concern to the food industry, as bacteria from these biofilms can be transferred to food products (Gandhi and Chikindas 2007). Cleaning and sanitization of equipment with acetic acid, sodium hypochlorite, quaternary ammonium, and lactic acid can control biofilm formation to a certain extent. Besides sanitation, maintaining physical conditions such as exposure time, temperature and applying mechanical removal or chemical intervention strategies can also restrict biofilm formation (da Silva and De Martinis 2013).

2.1.8 Listeriosis – A fatal foodborne illness caused by L. monocytogenes

L. monocytogenes causes a food borne illness called listeriosis, which involves diarrhea, fever, headaches, stiffness, and nausea, and can lead to more serious diseases like pneumonia and meningitis, as well as spontaneous abortions and still births among pregnant women (Todd and Notermans 2011). The susceptible populations for listeriosis are pregnant women, elderly and immunocompromised populations. According to CDC estimates, *L. monocytogenes* causes 1600 cases and 260 deaths due to listeriosis annually

with an average annual incidence rate of 0.29 case per 100,000 population in the United States for 2009-2010 (Scallan and others 2011; CDC 2011c). Human listeriosis is responsible for 28% of all deaths due to foodborne illness and it has the highest hospitalization rate of 92 % (Figure 2) among food borne pathogens in United States (Scallan and others 2011). Although the hospitalizations caused by *L. monocytogenes* accounted for only 3.8 % of total food borne illnesses, the death rate was 28 % of total food borne deaths (Crim and others 2014). This makes *L. monocytogenes* one of the leading causes of death from food borne illnesses in the United States (USFDA 2012). The infective dose depends on strain, host susceptibility and substrate involved in the dose-response relationship (USFDA 2012). As the dose-response relationship varies depending on various factors, similarly the incubation period for *L. monocytogenes* varies from a few hours to 2-3 days to a few months in cases of serious invasions (USFDA 2012). A typical listeriosis infection has a 15-30 % case fatality rate, which can reach higher levels under severe conditions such as septicemia (case fatality rate 50 %), listerial meningitis (case fatality rate 70 %) and perinatal/neonatal infections (case fatality rate > 80 %) (USFDA 2012).

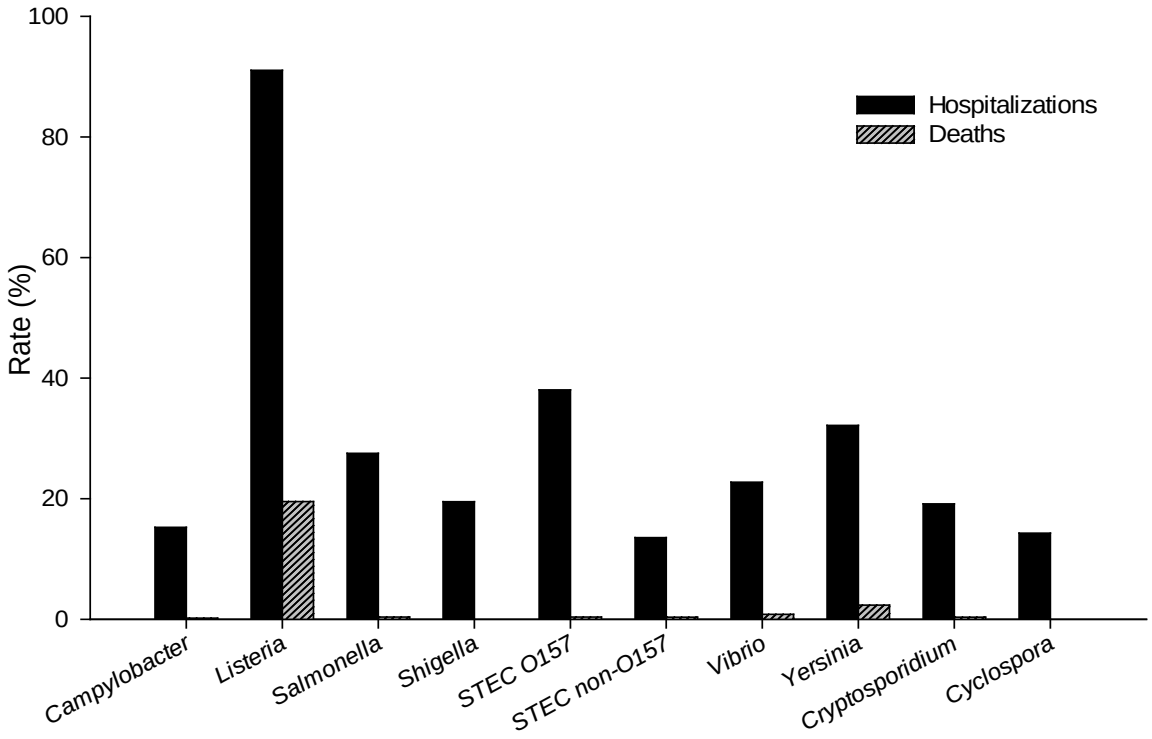


Figure 2. High hospitalization and case-fatality rates of *Listeria monocytogenes* when compared to other common food borne pathogens (adapted from Crim and others (2014)).

2.1.9 Listeria in foods

Since food borne listeriosis was first reported in 1981, numerous food borne outbreaks caused by *L. monocytogenes* have been reported worldwide (Zhang and others 2007), including a recent outbreak associated with alfalfa sprouts in 2013 (USFDA 2013). The most common vehicles for listeriosis have been found to be contaminated dairy products especially mozzarella, queso fresco cheese, unwashed raw vegetables, sprouts, meat, seafood, sausages, hot dogs and poultry products (Conter and others 2009;

Messi and others 2000; Leggett and others 2012; Kozak and others 1996; Soylemez and others 2001; Jung and others 1992). Besides occurrence of *L. monocytogenes* in food products, the pathogen has also been found in sewage sludge (De Luca and others 1998), soil (Vivant and others 2013), animal feces (Burow and others 1996) and slaughter houses (Wendlandt and Bergann 1994). Some of the food involved with most outbreaks associated with *Listeria* are summarized in Figure 3. Additional studies reporting on *L. monocytogenes* isolation are given in Table 3.

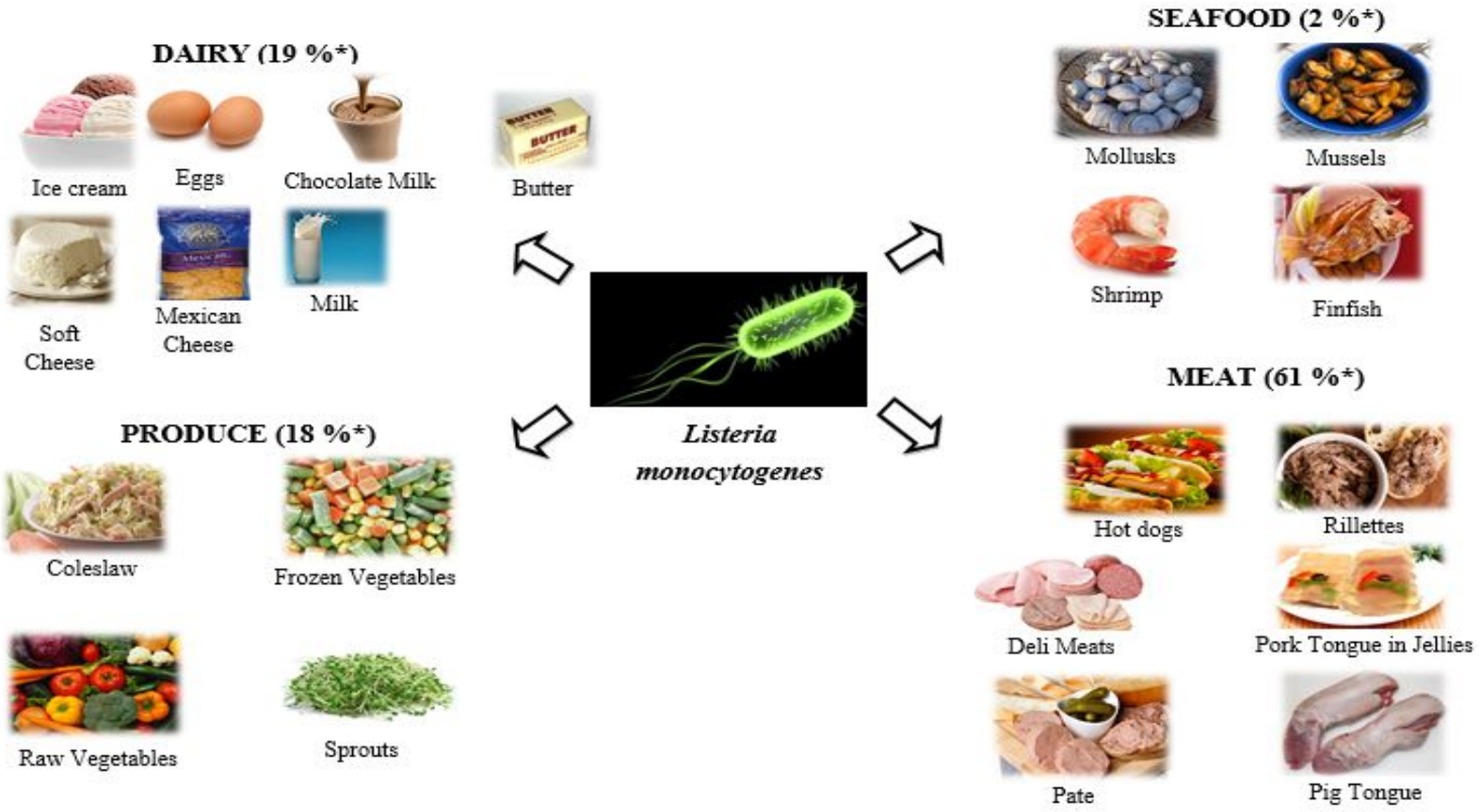


Figure 3. Some of the common foods associated with *Listeria* outbreaks since 1981

* Percentage of listeriosis cases associated with the food category since 1990 (De Valk and others 2005; Cartwright and others 2013; Silk and others 2013)

Table 3. Key findings from some *Listeria monocytogenes* studies

Food matrix	Key findings	Reference
Raw milk	Prevalence of *Listeria* was highest in raw milk (22.5%; most common species observed was *Listeria innocua* (57.8%)	Jamali and others (2013)
RTE and refrigerated vegetables	Prevalence of *Listeria* (20 %); *Listeria* detected in one cut red cabbage sample among 19 cabbage samples.	Kovacevic and others (2013)
Ready-to-eat poultry products	high phage concentration effectively inactivates target pathogen on food surfaces	Bigot and others (2011)
Raw chicken and RTE chicken products in Jordan	Prevalence rate of *Listeria* was 50 % among 280 tested samples and highest contamination of *Listeria monocytogenes* (18.2 %)	Osaili and others 2011 (2011)
White cheese	Overall incidence rate - 33.1 % of 142 tested samples	Arslan and Özdemir (2008)
Retail foods (RTE meats, raw chicken, fresh produce)	*L. monocytogenes* serotype 1/2b - 41 %; serotype 4b - 32 % and serotype 1/2a 26 %. Serotype 4b was found in all food categories.	Zhang and others (2007)
Alfalfa sprouts	The *L. monocytogenes* levels reached 10^6 CFU/g during germination within 48 h and remained constant at 4 °C for 7 days.	Palmai and Buchanan (2002)
Dairy products	Milk sampling surveys indicated 3-4 % *Listeria* contamination	Kozak and others (1996)
Fresh vegetables	Populations of *L. monocytogenes* increased during storage at 4 and 15 °C but Controlled Atmosphere Storage (CAS) did not influence the rate of growth	Berrang and others (1989)

2.1.10 Listeria monocytogenes Zero-Tolerance Policy

The FDA has a zero-tolerance policy against *L. monocytogenes* in ready-to-eat foods (USFDA 2003). This policy states that a food product is considered adulterated if any *L. monocytogenes* is detected. However, it is difficult to completely eliminate

Listeria contamination in a food processing environment due to the ubiquitous nature of the pathogen. The USDA Food Safety and Inspection Service (FSIS) supports the fact that achieving zero levels of *Listeria* is very difficult for post-processing environments even under strict HACCP plans. Hence, the FDA is reviewing the petition put forward by trade associations to set a limit for *L. monocytogenes* of 100 CFU/g for foods which do not support the pathogen growth (Gandhi and Chikindas 2007). Chen and others (2003) reported a 1.82 % prevalence rate for over 31,000 ready-to-eat retail foods surveyed among eight food categories. Thus, regulating the number of *L. monocytogenes* can increase the food safety standards.

2.1.11 Factors affecting the growth of Listeria

The growth of *Listeria* is highly dependent on external environmental factors. The range of temperature for *Listeria* growth extends from 2-45 °C with optimum temperature range of 30 – 37 °C (Gray and Killinge 1966). The survival of the organism in refrigerated conditions is a great concern to the food industry (Smith and others 2013; Gandhi and Chikindas 2007). *Listeria* growth as a function of pH studied by Boziaris and Nychas (2006) suggested that the minimum pH required for pathogen growth was 4.81 at a temperature range of 25-35 °C and a high water activity (a_w) of 0.992. Although pathogens such as *L. monocytogenes, Salmonella typhimurium, E. coli* O157:H7, and *Shigella flexneri* grow best under neutral pH conditions, facultative intracellular pathogens such as *Salmonella* and *Listeria* can tolerate low pH conditions (Abee and Wouters 1999). Gray and Killinge (1966) report that *Listeria* grows optimally in neutral to alkaline medium within a pH range of 5.6 - 9.6.

The growth of *Listeria* is also dependent on carbohydrates as the primary energy source and the function of carbohydrates at low pH is of particular concern. Vital processes such as DNA transcription, protein synthesis and enzyme activities are mainly controlled by the intracellular pH (pHi) value of the organism (Shabala and others 2002). According to Ita and Hutkins (1991), *L. monocytogenes* maintained pH_i of 0.5 to 0.7 over extracellular pH (pH_0) of 5.0 – 6.0. This higher growth potential of *Listeria* towards large ΔpH makes it more tolerant to acidic environments (Shabala and others 2002). The inoculum size is also important for initiation of bacterial growth in a food matrix (Koutsoumanis and Sofos 2005). Augustin and others (2000) studied the significance of inoculum size on the lag phase of *L. monocytogenes* growing in relatively poor growth medium and reported and extended lag time under small inoculation size. Robinson and others (2001) reported an increase in cell requirement to initiate *L. monocytogenes* growth from one cell for medium in optimum conditions to 10^5 cells for medium supplemented with 1.8 M NaCl. The addition of chemical antimicrobials such as sodium propionate, potassium sorbate, and sodium benzoate extended the *L. monocytogenes* lag phase for 18, 27 and 21 d, respectively, at 4 °C (Razavilar and Genigeorgis 1998).

2.2 ALFALFA SPROUTS

The demand for fresh produce has been increasing and gaining popularity as an important source of nutrients, vitamins and fiber in humans (Brackett 1999; Olaimat and Holley 2012). According to the 2010 Dietary Guidelines for Americans (McGuire 2011), 4-5 servings of fruits and vegetables are recommended daily based on a 2000 kcal diet. Fruits, vegetables, and legumes provide bioactive phytochemicals that also help in reducing the risk of developing chronic diseases (Kuo and others 2004). Besides the

health benefits of fruit and vegetable consumption, the availability of domestic and imported produce throughout the year (Olaimat and Holley 2012) and increasing trend of food preparation through ready-to-eat products (Tournas 2005) have further enhanced the popularity and demand for fresh fruits and vegetables.

The alfalfa plant (*Medicago sativa*) is a perennial legume (NRCS 2002). However, when alfalfa seeds are germinated under optimum temperatures of 18-25 °C, the seeds undergo metamorphosis and loose the toxins associated with the legume. The seed germination allows the alfalfa seed to mature to a green stage where it is no longer a legume and now considered as a green plant (Meyerowitz 1999). The USDA National Nutrient Database (NDB 2012) classifies raw alfalfa sprouts under the food group "Vegetable and Vegetable products".

Legumes are a good source of dietary proteins, carbohydrates, vitamins and minerals but also contain certain anti-nutritional factors such as trypsin inhibitors, which reduce trypsin activity and thereby reduce amino acid availability, tannins which reduce the activity of lysine and methionine (essential amino acids) by forming cross-links with proteins, and phytate, which reduces bioavailability of minerals (Vidalvalverde and others 1994). To remove some of these anti-nutritional factors, soaking, cooking and germination have been studied (Vidalvalverde and others 1994). The germination process increases the digestibility, reduces antinutritional factors and increases antioxidant compounds (Penas and others 2009). At the same time, germination provides suitable environmental conditions (temperature, humidity, and nutrients released from seeds) for growth and proliferation of bacteria (Piernas and Guiraud 1998). Sprout seeds such as alfalfa, mung bean, and radish generally show a high microbial load (aerobic plate count)

ranging from 10^3 to 10^6 CFU/g (Penas and others 2009). These levels tend to increase under ideal conditions during sprouting, reaching up to $10^8 – 10^{11}$ CFU/g (Taormina and others 1999). This results in a reduced shelf-life of the product and increased susceptibility of consumers to bacterial infections (Penas and others 2009).

Sprouts provide a nutritional, inexpensive, natural, convenient and rich source of proteins, minerals, carbohydrates and vitamins (Penas and others 2009; Yang and others 2013). Minimal processing involves preserving foods by non-thermal techniques to extend shelf life without compromising on nutritional and sensory quality of the product (Ramos and others 2013). Sprouts require minimal processing and are often consumed raw; however, as a result the risk of pathogen contamination increases (Olaimat and Holley 2012). Minimal processing ideally involves simple operations such as cleaning, washing, trimming, peeling, cutting, sanitizing and packaging which creates the need for freezing or refrigeration as an alternative and easy preservation technique especially in case of sprouts (Ramos and others 2013; Tournas 2005). Storage of sprouts under temperature-abused conditions (>10 °C) facilitates bacterial growth further. Microbial contamination and growth have the potential to occur in sprouts throughout the food supply chain, depending on safety measures adopted at each stage (Olaimat and Holley 2012). For example, contamination and growth may occur during production, harvest, processing, storage, transportation, handling, and/or consumption at home/restaurants.

2.2.1 Glass jar method is the most preferred sprouting technique used for alfalfa sprouts germination

Sprouts are popular in the American kitchen not only because of their nutritional benefits but also for their ease of production in the kitchen (Mansour 1993). Sprouts are produced by soaking seeds in warm water for 3-7 d under controlled temperature for germination and growth, with exact conditions depending on the type of sprout used (USFDA 2006b). Among four sprouting techniques studied for yield, labor requirement and microbial quality of sprouts, Sawyer and others (1985) reported the largest yield, smallest bacterial growth, and smallest total direct labor time using the glass container method of sprouting in contrast to the tray method using either cheese cloth cover or paper towel cover (on top or bottom of the tray). Sawyer and others (1985) suggested that the glass container method was best sprouting technique to adopt for food service applications. Mansour (1993) studied three methods (Glass jar method, paper towel method, sprinkle method) for growing vegetable sprouts and recommended the glass jar method, with a temperature range of 21 – 29 °C, rinsing 3-4 times per day, a sprouting time of 3-5 days and a harvesting length of 1-2 inches for alfalfa sprouts.

2.2.2 Techniques used for microbial decontamination in sprouts

2.2.2.1 *Chemical methods*

Chemical intervention methods involve the use of chemical sanitizers on the surface of produce for decontamination followed by rinsing with potable water (Ramos and others 2013). The prominent category of sanitizers used in processing plants are chlorine-based sanitizers particularly chlorine dioxide (Sikin and others 2013). According

to USDA recommendations, seed treatment with 20,000 ppm active chlorine, $Ca(OCl_2)$ for 15 min can control the risk of sprout-associated food borne illnesses (USFDA 1999). However, chlorine treatment has been reported to reduce germination of seeds and sprout quality (Fransisca and others 2012) and when in contact with organic substance in water, chlorine has been reported to form carcinogenic byproducts such as trihalomethanes (THM) (Li and others 1996). Organic acids, electrolyzed oxidizing water, and ozone are some of the other commonly used chemical interventions for microbial safety (Sikin and others 2013). An overview of chemical intervention strategies are given in Table 4.

Table 4. Efficacy of chemical methods in reducing microbial contamination in seeds and sprouts.

Chemical used	**Seed / Sprout type**	**Treatment conditions**	**Target pathogen**	**Microbial reduction (log CFU/g)**	**Reference**
Acetic Acid (AA)	Alfalfa seeds	5% AA, 10 min, 42 °C	*E. coli* O157 : H7	6.3	Lang and others (2000)
	Radish seeds	8.7% AA (gaseous), 3 h, 55 °C	*Salmonella*	5	Nei and others (2011)
	Mung bean sprouts	2% AA, 48 h, 24 °C	*Salmonella*	7 to 8	Pao and others (2008)
Acidic electrolyzed water (AEW)	Alfalfa sprouts	84 ppm active chlorine, pH 2.4, 24 °C; ORP: + 1081 mV, 10 min + sonication + seed coats removed	*Salmonella*	3.7	Kim and others (2003)

Chlorine dioxide	Alfalfa sprouts	0.5% fumaric acid+ 50 ppm of ClO_2 (10 min)	Total aerobic bacteria	3.18	Kim and others (2009)
			E. coli O157 : H7	4.06	
			Salmonella typhimurium	3.57	
			L. monocytogenes	3.69	
	Mung bean sprouts	100 ppm of ClO_2, 5 min	*S. typhimurium*	3	Jin and Lee (2007)
			L. monocytogenes	1.5	
Fatty Acids	Alfalfa seeds	15X fatty acid-based sanitizer (250 ppm peroxyacid, 1000 ppm caprylic and capric acids, 1000 ppm lactic acid, 500 ppm glycerol monolaurate) + 3 min exposure	*L. monocytogenes*	>6.92	Pierre and Ryser (2006)
			E. coli O157 : H7	>5.45	
			S. typhimurium, DT104	>5.62	
Lactic Acid (LA)	Alfalfa seeds	5% LA, 10 min, 42 °C	*E. coli* O157 : H7	6.6	Lang and others (2000)

Malic Acid (MA), Thiamine Dilauryl Sulfate (TDS)	Alfalfa seeds	10% MA, 1 % TDS, 20 min	*E. coli* O157 : H7	4.41	Fransisca and others (2012)
Ozone	Alfalfa Sprouts	20 ppm ozone + sparging, 4 °C, 20 min	*L. monocytogenes*	0.94	Wade and others (2003)
Phytic Acid (PA) and Oxalic Acid (OA)	Mung bean, broccoli, alfalfa seeds	Dry heat (50 °C, 17 h) + soaking in 0.03% PA	*E. coli* O157 : H7	5	Bari and others (2009a)

Source: Sikin and others (2013)

2.2.2.2 Physical methods

One of the major drawbacks of the chemical methods of preservation is that they act on the seed surface which limits the efficiency of the treatment against bacteria that is not exposed to the chemical during the treatment. Besides, consumers have negative perceptions of the use of synthetic chemicals when associated with foods, especially fresh produce (Sikin and others 2013). As a result, there is an ever-growing consumer preference for reduction in the use of chemicals or synthetic additives and increased use of natural antimicrobials/preservatives in foods (Oms-Oliu and others 2010). Physical interventions such as irradiation, heat, and pressure treatments help decontaminate sprouts against pathogens protected by the food matrix itself, or internalized bacteria

(Sikin and others 2013). Physical treatments such as dry heat/hot water treatment, high hydrostatic pressure, gamma irradiation and supercritical carbon dioxide have been reported to be effective at decontamination of seed sprouts in various studies (Yang and others 2013; Sikin and others 2013). An overview of the major studies reporting the physical intervention strategies are given in Table 5.

Table 5. Efficacy of physical methods in reducing microbial contamination in seeds and sprouts.

Physical method	**Seed / Sprout type**	**Treatment conditions**	**Target pathogen**	**Microbial reduction (log CFU/g)**	**Reference**
Hot water	Radish, Alfalfa seeds	58 °C, 6 min	*E. coli* O157 : H7, *Salmonella*	5	Weiss and Hammes (2005)
	Mung beans	85 °C, 40 s	*E. coli* O157 : H7	3.69	Bari and others (2009b)
			Salmonella	3.84	
	Alfalfa sprouts	70 °C, 10 s	*Salmonella*	7.6	Pao and others (2008)
		80 °C, 5 s			
		90 °C, 3 s			
		100 °C, 3 s			
Dry heat	Mung bean seeds	50 °C,1 h	*E. coli* O157 : H7	1	Bari and others (2003)
	Alfalfa seeds	55 °C, 6 days	*E. coli* O157 : H7	5	Feng and others (2007)
			Salmonella	5	

	Alfalfa seeds, Broccoli seeds	50 °C, 17 h	*E. coli* O157 : H7	5	Bari and others (2009a)
Irradiation	Alfalfa Seeds	8 kGy gamma radiation	*E. coli* O157 : H7	5.03	Kim and others (2006)
	Mung bean sprouts	1.5 kGy gamma radiation	*E. coli* O157 : H7	5.97	Bari and others (2004)
	Broccoli sprouts	1 kGy gamma radiation	*L. monocytogenes*	4.88	Bari and others (2005)
	Alfalfa sprouts	3.3 kGy gamma radiation	*L. monocytogenes*	6	Schoeller and others (2002)
Ultraviolet light	Clover sprouts	1 kJ/ m^2 UV-C (254 nm), 3.3 min	Total aerobic bacteria	1	Kim and others (2009)
			E. coli O157 : H7	1.02	
			Salmonella	1.06	
High Pressure treatment	Alfalfa seeds	475 MPa, 40 °C, 8 min	*E. coli* O157 : H7	2	Ariefdjohan and others (2004)
	Alfalfa seeds	500 MPa, 45 °C, 2 min	*E. coli* O157 : H7	5.2	Neetoo and Chen (2010)
			Salmonella	5.8	
Supercritical Carbon dioxide ($SC\text{-}CO_2$)	Alfalfa Seeds	15 MPa, 45 °C, 10 min	*L. monocytogenes*	7	Jung and others (2009)

Source: (Sikin and others 2013)

2.2.2.3 Biological methods

The development of antibiotic resistant strains of bacteria since 1970's has triggered the need to find alternative approaches for controlling pathogenic bacteria in

foods. Biological interventions such as the use of bacteriophages and protective bacterial cultures such as *Lactobacillus spp.* along with antimicrobials produced by microorganisms such as bacteriocins and organic acids have provided an alternative to other physical and chemical methods of preservation in foods (Sikin and others 2013). The biological preservation methods not only control growth of pathogenic bacteria but also reduce the use of synthetic additives as preservatives in food (Sikin and others 2013). Biological methods of improving microbial safety involve the use of various bio control agents to extend shelf life using microorganisms and/or their metabolites (Ramos and others 2013). Razavi Rohani and others (2011) have reported the use of antibacterial compounds such as nisin against bacterial contamination. The emergence of hurdle technology has enhanced the use of biological preservation techniques in combination with other preservation methods to reduce bacterial loads in a variety of foods (Leistner 2000). Some common bio control agents used against target pathogens in seeds and sprouts are given in Table 6.

Table 6. Efficacy of biological methods in reducing microbial contamination in seeds and sprouts.

Bio control method	**Seed / Sprout type**	**Treatment conditions**	**Microbial reduction (log CFU/g)**	**Reference**
		Salmonella		
Bacteriophage	Alfalfa Seeds	Phage SSP6, 70 MOI, 12 h	1.0	Kocharunchitt and others (2009)

	Mustard seeds	Phage A , 10^8 PFU/mL, 25 °C, 24 h	1.37	Pao and others (2004)
	Mung bean seeds	Bacteriophage mixture, 10^6 PFU/mL, 4 days	3.41	Ye and others (2010)
Protective bacterial culture	Alfalfa seeds	*E. asburiae* JXI (10^6 CFU/mL) + bacteriophage mixture (10^6 PFU/mL), 4 days	7.62	Ye and others (2010)
		Listeria monocytogenes		
Bacteriocin	Alfalfa, soybean sprouts	225 ppm of AS-48 + chemical preservatives (soak), room temperature, 5 min	2.70	Molinos and others (2005)
	Mung bean sprouts	50 ppm nisin, 48 ppm pediocin + 0.02% phytic acid, pH 2.3, 1 min	2.31	Bari and others (2005)

Source: (Sikin and others 2013)

2.3 BACTERIOPHAGE

Bacteriophages are naturally occurring viruses that specifically attack bacterial targets (Hagens and Loessner 2007). Bacteriophages are extremely host specific and it is this specificity that enables elimination of the target organism without affecting the viability of other organisms in a food matrix (Guenther and others 2009). The antimicrobial activity of bacteriophages was first discovered by the efforts of Ernest Hankin (1896) and Frederick Twort (1915). In recent years it has been found that bacteriophages have several applications in the food industry including use as antimicrobials and tool for detecting pathogenic bacteria. The advantages of

bacteriophages include the following: i) they kill the bacterial target ii) they do not cross genus boundaries and thus are specific in their action, and iii) they breakdown into non-toxic proteins and nucleic acids as their final product (Rossi and others 2011). Bacteriophage use in the food industry is advantageous for a number of reasons. They are ubiquitously present in nature, and can replicate easily. Bacteriophages can be used to detect pathogens and act against biofilm production. In addition they can act as an excellent source of potential antimicrobials. Due to these bio sanitizing and bio preservative properties, they have versatile use in the food supply chain (Arachchi and others 2014; Garcia and others 2008). Bacteriophages can also be considered an important tool in hurdle technology when used with a combination of different methods of preservation. Leverentz and others (2003) found that a combination of nisin (a bacteriocin) and phage mixture (LM-103 and LMP-102) resulted in reduction of *L. monocytogenes* by up to 2.3 log units in apple slices. Similarly, Soni and others (2012) reported a reduction in *L. monocytogenes* by 2-4 log CFU/cm^2 in Queso fresco cheese when treated with a combination of bacteriophage LISTEX™ P100 and antimicrobials, lauric alginate and potassium lactate-sodium diacetate. Phages are present in food and water and are regularly consumed unknowingly without any undesirable effects. The abundance of phage on earth (estimated to be 10^{31} phage particles), coupled with their relatively safe use against harmful bacteria have made phages an increasing area of research in the field of food safety (Wommack and Colwell 2000).

The phage discovery begin when d'Herelle (1917) found microbes which caused bacterial lysis in liquid culture and showed distinct patches when grown on agar containing bacteria. He called these killing zones 'plaques' and considered the microbes

as ‘ultraviruses’ which were antagonistic to bacteria and multiplied after bacteria lysis. He termed these ultraviruses “Bacteriophages” (d’Herelle 1917). In 1931, he also studied bacterial mutations on *Salmonella typhimurium* and found that the association of bacteriophage with the bacteria caused the variations and thus stated that bacteriophage are the infectious agents which act as obligate intracellular parasites to bacteria (d’Herelle 1917). Following the studies of d’Herelle, many companies began commercial production of phages against bacterial pathogens. However, the emergence of antibiotics and its immediate impact in the modern world prompted a decline in use of phage therapy against bacterial pathogens (Garcia and others 2008). The concern of antibiotic resistance of bacterial pathogens has led to renewed interest in use of bacteriophages as biocontrol agents of foodborne pathogens (Hagens and Loessner 2007).

In 2007, the U.S. Food and Drug Administration (FDA) approved and granted generally recognized as safe (GRAS) status to the use of the bacteriophage LISTEX™ P100 in raw and ready-to-eat foods as an antimicrobial agent against *L. monocytogenes* (USFDA 2007). In a study conducted on Brazilian fresh sausage, Rossi and others (2011) reported that application of bacteriophage LISTEX™ P100 (3×10^7 PFU/g) on *L. monocytogenes* 1/2a inoculated Brazilian sausage (2.1×10^4 CFU/g) at 4 °C for 10 days reduced *L. monocytogenes* counts by 2.5 log. For contamination-sensitive ready-to-eat foods, Guenther (2009) reported that the *Listeria* counts dropped below count from 10^3 CFU/g for liquid foods such as chocolate milk and mozzarella cheese brine when treated with phage A511 (3×10^6 to 3×10^8 PFU/g) at 6 °C for 6 days whereas on solid foods (hot dogs, sliced turkey meat, smoked salmon, sliced cabbage, and lettuce) a 5-log bacterial reduction was observed. In another study of using bacteriophage as a bio control

in combination with other food preservation methods, Leverentz and others (2003) observed that a phage-nisin mixture reduced the *L. monocytogenes* population by 5.7 log on honeydew melon slices and 2.3 log on apple slices while nisin (bacteriocin) alone reduced the *Listeria* population by 3.2 log on honeydew melon slices and 2.0 log on apple slices. Thus, bacteriophages can be used either alone or in combination, without compromising the compatibility of other control measures, to reduce bacterial contamination. Some of the other studies using bacteriophages as a control for food borne pathogens are given in Table 7.

Table 7. Key findings from previous bacteriophage studies

Food matrix	**Bacteriophage**	**Key results**	**Author**
		L. monocytogenes	
Cooked Turkey and roast beef	LISTEX™ P100	Cooked turkey - 7.5 $\log_{10}$ CFU/cm^2 reduction	Chibeu and others (2013)
	LISTEX™ P100	Roast beef - 7.2 $\log_{10}$ CFU/cm^2 reduction	
Queso fresco cheese	LISTEX™ P100	Combination treatment with other antimicrobials resulted in 2–4 $\log_{10}$ CFU/cm^2 reduction	Soni and others (2012)
Ready-to-eat poultry products	Phage isolated from sheep feces	2.5 $\log_{10}$ CFU/cm^2 reduction of pathogen	Bigot and others (2011)
Brazilian fresh sausage	LISTEX™ P100	2.5 $\log_{10}$ CFU/cm^2 reduction of pathogen	Rossi and others (2011)
Raw Salmon Fillet	LISTEX™ P100	1 $\log_{10}$ CFU/g reduction	Soni and Nannapaneni (2010)

Ready-to-eat products	Phage A511, Phage P100	Hot dogs, sliced turkey meat, smoked salmon, seafood, sliced cabbage, lettuce leaves – up to 5 log_{10} CFU/cm^2 reduction	Guenther and others (2009)
Soft Cheeses	Phage P100	3.5 log_{10} CFU/cm^2 reduction of *L. monocytogenes* *Salmonella*	Carlton and others (2005)
Alfalfa sprouts	SSP5 and SSP6	1 log_{10} CFU/g reduction	Kocharunchitt and others (2009)
Mung bean and alfalfa sprouts	Phages isolated from pig or cattle farm	Combination of antagonistic bacteria and bacteriophage resulted in 5.7 to 6.4 log_{10} CFU/g reduction	Ye and others (2010)

Biology of Phages

Phages are viruses that infect bacteria for their survival and multiplication. Bacteriophages are very host specific owing to specific cell wall receptors (Strauch and others 2007). However, in nature some bacteriophages exhibit diverse host range whereas some are highly specific to a single bacterial strain. This diversity emphasizes the importance of understanding the morphology and host-phage interaction. A typical bacteriophage structure is divided into two main regions - head or capsid, and a tail (Figure 4). Most bacteriophages have DNA as nucleic acid, but some phages have been known to have RNA instead. In most phages double stranded DNA is observed but some phage may possess single-stranded DNA or RNA (Strauch and others 2007). The head region (usually icosahedron shape) encapsulates the DNA or RNA and acts as a protective covering. The tail is connected to the head via the collar. The tail in most bacteriophages is a contractile sheath which is often connected to six tail fibers

containing specific receptors at the tip which help in attaching to the receptor sites on the host bacterial surface (Hanlon 2007).

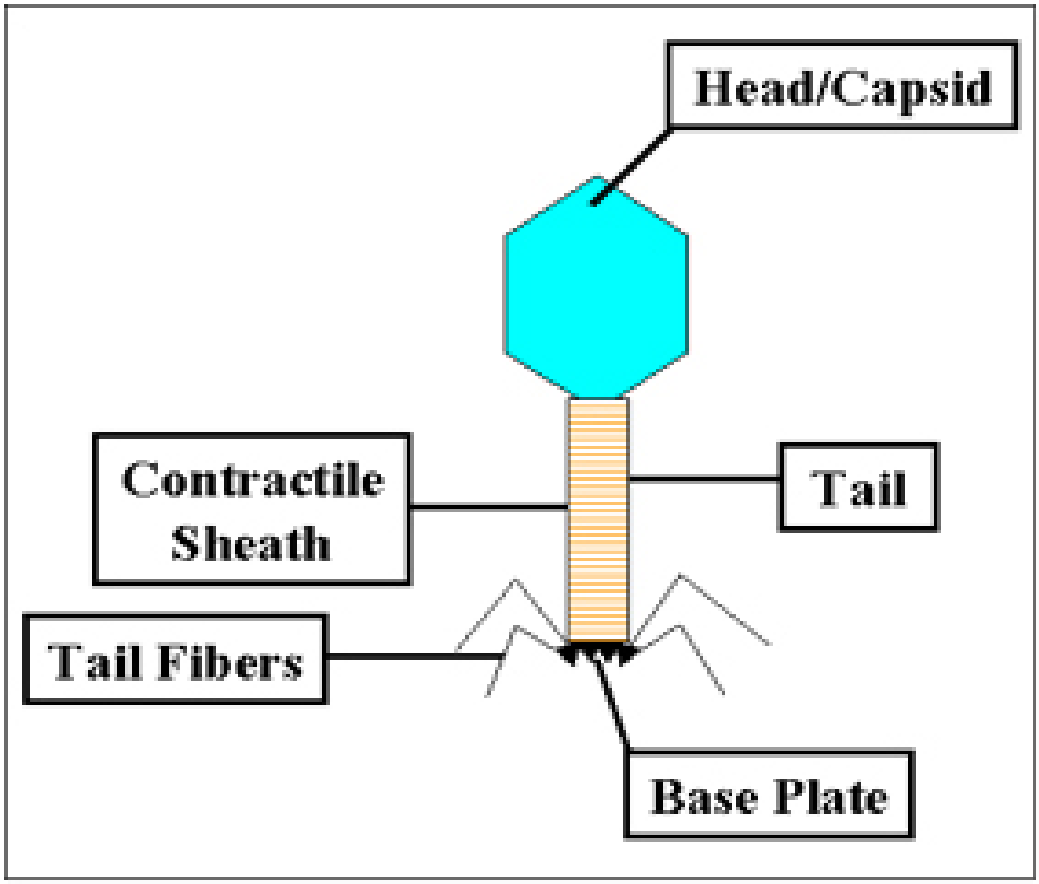


Figure 4. Typical structure of a bacteriophage Source: Stewart (2003)

2.3.1 Life Cycle of a Phage

A typical life cycle of phage starts with an irreversible adsorption of phage to the bacterial cell wall where the receptors of the tail fiber are attached to the receptor site on the host cell. These receptor sites may include cell surface components such as protein, peptidoglycan, oligosaccharide, lipopolysaccharide, capsules, flagella or teichoic acid (Hanlon 2007). For phages lacking the tail fibers, the adsorption step is executed by some

form of analogous structure present in the phage. After the initial attachment, the phage injects the DNA or RNA into the bacterial cell wall with the help of contractile motion and formation of a hole within the bacterial cell wall by enzymatic action of nuclease enzymes present in the phage. The nucleic acid enters the bacterial cell whereas the body of the phage remains outside the cell. The phage genome is then replicated, synthesized and assembled within the bacterial cell to ultimately result in formation of new capsids. The extensive formation of these capsids within the cell wall results in cell lysis and release of new phage particles which are ready for infection (Strauch and others 2007). The release of the newly formed phage particles is controlled by the enzymes which form holes in the host cell wall through peptidoglycan digestion. Due to the breakdown of its cytoplasmic membrane, the host cell cannot withstand high internal pressure and this results in hypotonic lysis of the cell (Fischetti 2005). Each bacterial cell can release 100-1000 new phage particles, each capable of infecting another bacterial cell. The number of new phage particles released per bacterial cell is referred as ‘burst size’ (Hanlon 2007).

Depending upon the morphology and life cycle, phages can either undergo a lytic (virulent) or lysogenic (temperate) life cycle. Phages undergoing lytic cycle (virulent phages) kill the bacterial host at the end of cycle whereas the temperate phages infect the cells without killing the host. The virulent phage injects the nucleic acid into the host cell after the initial adsorption stage. The phage nucleic acid is expressed within the host and eventually takes control over the biosynthetic machinery of the host cell to produce more phage particles. The structural proteins are synthesized and nucleic acid is embedded within the newly formed head. The remaining phage components such as the tail, collar, etc., are assembled to form a new mature phage particle ready for infection. This process

of assembly is termed as maturation. The phage enzymes cause the breakdown of host cell wall and facilitate the release of the new infective phage particles. Temperate phages on the other hand, form an integral part of the host DNA, replicate along with the host DNA and also remain part of the successive daughter cells. Under favorable environmental conditions, the phage DNA can be reactivated and re-enter the lytic cycle to ultimately cause formation of new virus particles and lysis of host bacterium (Hanlon 2007; Strauch and others 2007; Skurnik and Strauch 2006).

2.3.2 Listeria bacteriophages

*2.3.2.1 **LISTEX™ P100***

Bacteriophage LISTEX™ P100 is a broad range, lytic bacteriophage capable of infecting 95% of common *L. monocytogenes* strains of serovars ½ and 4 (Klumpp and others 2008). It belongs to the morphotype A1 of the *Myoviridae* family comprising an isometric capsid and a long, contractile chain (Calendar 2006). This phage was first isolated from sewage of a dairy plant in southern Germany and found to exhibit lytic action on *L. monocytogenes* strains (Carlton and others 2005). According to MICREOS, the manufacturer of phage P100, marketed under trade name LISTEX™ P100, has been concentrated and selected from a collection of food grade phages taking into the consideration the broadest sensitivity to pathogenic strains of *L. monocytogenes*. LISTEX™ P100 is active for a temperature range of 1 – 35 °C with optimum activity at 30 °C. It can act on a wide pH range of 5.5 to 9.5 with optimum pH of 7.7 for efficient control. The phage can survive high salt levels and prefers a minimum water activity (a_w) of 0.92. It can survive best at 4 °C for long periods of time and is stable over an extended

storage period (Soni and Nannapaneni 2010; Kocharunchitt and others 2009). LISTEX™ P100 is a strictly virulent phage which causes complete lysis of the bacterial host and is unable to transduce bacterial DNA making it safe to use in the food system (Carlton and others 2005). In 2006, the FDA approved GRAS status for application of LISTEX™ P100 to foods (Guenther and others 2009). LISTEX™ P100 can also be regarded as a processing aid thereby exempting it from food labeling (MICREOS 2011).

2.3.2.2 GRAS status of bacteriophage LISTEX™ P100

A food substance, under sections 201(s) and 409 of the Federal Food, Drug and Cosmetic Act (FD&C Act) and regulations in 21 CFR 170.3 and 21 CFR 170.30, can be termed GRAS through scientific procedures thoroughly reviewed by a qualified, scientifically trained, and experienced panel (USFDA 2011). According to FD&C Act of 1958, a food additive can be defined as any substance whose intended use may result in the substance being part of the food or affecting other components of food directly or indirectly (Gaynor 2005). The GRAS substances are safe to use under the conditions of their intended use and the safety is not compromised by their excessive levels in the food. The GRAS substances are therefore not considered as food additives as per the FD&C Act, 1958 and thereby are exempt from premarket approval by the FDA (Gaynor 2005). To determine whether an ingredient is GRAS, it has to achieve a common consensus among qualified experts regarding its use and safety under the conditions of its intended use (Gaynor 2005).Thus, the FDA determines the ingredient safety for food additives whereas, GRAS determination is done by experts outside the government (Gaynor 2005).

On December 21, 2006, EBI Food Safety, Wageningen, Nederlands submitted a notice to the FDA for bacteriophage LISTEX™ P100 preparation from *L. innocua* to be considered GRAS under the regulation 21 CFR 170.36 (USFDA 2007). In its letter to the FDA, EBI Food Safety stated the intended use of bacteriophage LISTEX™ P100 as an antimicrobial in controlling *L. monocytogenes* population in food in general, and up to levels of 10^9 plaque forming units per gram (PFU/g) of food (USFDA 2007).

In its letter to the FDA, EBI described the commercial production process of LISTEX™ P100. The following bacteriophage LISTEX™ P100 production process was adapted from EBI Food Safety, Wageningen, Nederlands (USFDA 2006a). The bacteriophage preparation involved use of isolates from wastewater sources such as sewage water. A non-pathogenic strain of the genus *Listeria, L. innocua* was used for cultivation of bacteriophage LISTEX™ P100. The bacteriophage LISTEX™ P100 has been found to infect and kill 95 percent of strains of serovars 1/2a, 4b (*L. monocytogenes*) and 5 (*L. ivanovii*) (Guenther and others 2009). The manufacture of bacteriophage LISTEX™ P100 begun as a batch fermentation process involving culturing of *L. innocua* to a known density followed by phage application which amplified upon further incubation. The bacterial cultures were grown in standard media and enumerated by standard procedures. The cell debris and host cells were filtered after a series of filtrations and removed to obtain a high titer bacteriophage LISTEX™ P100 solution. A detailed characterization and bioinformatics analysis of the LISTEX™ P100 genome revealed that the genes or proteins of *Listeria* known to be toxins, pathogenic, antibiotic resistant, or allergens had no correlation with proteins of bacteriophage LISTEX™ P100 (Carlton and others 2005). The study also observed no potential harmful effects of

repeated dose oral toxicity (such as abnormal biological symptoms or mortality) in rats thereby supporting the safe use of LISTEX™ P100 in foods (Carlton and others 2005).

2.3.2.3 Phage and host identity of LISTEX™ P100

The bacterial host used in LISTEX™ P100 preparation is *L. innocua*. According to the German classification system, *L. innocua* is associated with Risk Group 1 (Anonymous 1983). The type strain and registry numbers include SLCC 3379, NCTC 11288, ATCC 33090 and DSM 20649 (Anonymous 1983). The phage belongs to the Order Caudovirales, Family Myoviridae, and Species P100 (USFDA 2006a). The bacteriophage P100 is host specific to *L. monocytogenes*, *L. innocua*, and other *Listeria* spp. (USFDA 2006a).

The bacteriophage P100 is marketed under the trade name LISTEX™ P100 by MICREOS Food Safety, WA, Nederlands. It is most stable (>2 year stability) under refrigerated conditions at a temperature range of 2-8 °C. The product specifications of commercially available LISTEX™ P100 are summarized in Table 8.

Table 8. Physical, chemical, and microbiological properties of LISTEX™ P100 obtained from MICREOS Food Safety, WA, Nederlands.

LISTEX™ P100 – Product Specification	
Physical Properties	
Product description	Suspension formulated in propylene glycol

Source	Batch fermentation process
Initial concentration	2 x 10^{11} phage/mL
Chemical Properties	
Heavy metals	<10 ppm
Lead	<1 ppm
Arsenic	<1 ppm
Mercury	<0.5 ppm
Microbiological properties	
Yeasts and molds	Less than 10/mL
Enterobacteriaceae	Negative in 1 mL
Salmonella	Negative in 25 mL
Listeria spp.	Negative in 25 mL
Staphylococcus aureus	Negative in 1 mL
E. coli	Negative in 1 mL

Source: (USFDA 2006a)

2.3.2.4 Taxonomy of LISTEX™ P100

The shape, size and nucleic acid are the major basis for phage taxonomy (Skurnik and Strauch 2006). LISTEX™ P100 is classified under the group I (dsDNA) and belongs to family Myoviridae, subfamily Spounavirinae, genus Twort like virus, species P100 (ICTV 2012).

2.3.3 Listeria biocontrol by phage

The recent approval of bacteriophage LISTEX™ P100 in 2007 by FDA has triggered interest within the scientific community in use of phages as a bio control agent in the control of *Listeria monocytogenes*. One of the first bacteriophages (LMP-102) approved as food additive by FDA contained a cocktail of six *Listeria*-specific phages capable of killing 170 different *Listeria* strains. A recommended phage preparation of 1 mL per 500 m^2 surface of RTE and poultry products before packaging could kill *Listeria* colonies in the product (Bren 2006).

The efficacy of broad host range phages A511 and P100 were studied against *Listeria monocytogenes*-infected RTE food samples (Guenther and others 2009). In solid foods such as hot dogs, turkey, smoked salmon, seafood, sliced cabbage, and lettuce leaves up to 5-log reduction in *L. monocytogenes* counts was observed when these foods were stored at 6 °C for six days with either phages (Guenther and others 2009). When tested against liquid foods such as chocolate and Mozzarella cheese brine, the *L. monocytogenes* counts reached below detection level suggesting that the food matrix plays an essential role in phage application. The phage concentration also affected the efficacy as high dose concentration of 10^8 PFU/g resulted in optimum efficiency (Coffey and others 2010). Phage resistance studies performed for LISTEX™ P100 demonstrated a 3.5-log reduction in *L. monocytogenes* count without any bacterial resistance (Guenther and others 2009). Soni and Nannapaneni (2010) demonstrated antilisterial activity of LISTEX™ P100 on raw salmon fillet tissue and reported a 1 log CFU/g reduction at a phage concentration of 10^8 PFU/g when samples were stored at 4 °C over a 10-day storage period. Similarly, phage application of phages SSP5 and SSP6 over contaminated

alfalfa seeds resulted in 1-log reduction of *Salmonella* after 3 h of phage application (Kocharunchitt and others 2009). Thus, phages have tremendous potential as bio control agents against *Listeria* contamination in a variety of food matrices.

2.3.4 Factors affecting efficacy of phage

The development of antibiotic-resistant bacteria strains has prompted a renewed interest in commercial production of bacteriophages to be used as antimicrobial agents in phage therapy. However, the application of phage has not always been fruitful. The efficacy of phage depends on several factors such as bacteriophage: bacteria host ratio, phage resistance and the physico-chemical properties (pH, aw, temperature) of the food matrix. Phage characteristics such as initial dose, phage adsorption rate, burst size, latent period, and eclipse period also determine the magnitude of bacterial lysis. Phage specificity plays a major role in the ability of phage to infect the host cell. Some studies have found that a cocktail of broad-spectrum phages increased the phage activity (Pao and others 2004). The activity of phages was higher in liquid foods such as chocolate milk and mozzarella cheese brine when compared with solid foods such as hot dogs, turkey meat, smoked salmon, sliced cabbage and lettuce leaves (Guenther and others 2009). Due to the complexity within the food matrix with respect to its physical structure, the accessibility of phage to the target bacteria is affected. Phage efficiency is also affected by enzymes present in the food system; for example, O'Flaherty and others 2005found that the whey proteins in raw milk prevented the adsorption of phage on bacterial cells. Furthermore, antibodies present in a food can neutralize the phages and render them ineffective (Ly-Chatain 2014). The external physical and chemical factors such as temperature, acidity, ions, and a_w also affect the host-phage interaction. Most

phages are stable to external environmental conditions and exhibit maximum efficiency around neutral pH (6-8) and a temperature range of 5-35 °C. Some phages are also capable of surviving the pasteurization process and high temperatures (40-90 °C) but cannot survive low pH conditions found in the guts of mice (Watanabe and others 2007). The temperature plays an important role in attachment, penetration, multiplication and the length of the latent period (Jonczyk and others 2011). Salt concentrations, on the other hand did not significantly impact the phage titer when monovalent salt (LiCl, NaCl and KCl) concentrations were increased to 1.0 M (Mylon and others 2010). Thus, the external and internal factors combined with the biochemical composition of the food matrix studied largely determined the ability of phage to infect the bacteria (Ly-Chatain 2014).

2.3.5 Phage resistance

The bacteria cell wall has specific receptor sites which help in the initial attachment of phage to its host. The loss or inactivity of these receptor sites due to gene mutation can lead to development of phage resistance in bacteria. However, according to Skurnik and Strauch (2006), this loss of receptor site would be beneficial to decrease the virulence of the bacteria as the receptors act as virulence determinants. The other possible form of phage resistance is through restriction-modification immunity where the restriction endonucleases in bacteria degrade and stop the activity of the injected phage nucleic acid, thereby stopping the phage lytic cycle (Skurnik and Strauch 2006; Levin and Bull 2004). Some bacteria develop partial phage resistance by formation of mucoid colonies which protect the bacterial host against phage adsorption. However, phage resistance can be overcome by using a cocktail of different phages, as different phages

have different receptors and would thus require individual mutation to each phage. Also, phages evolve continuously and therefore are capable of overcoming the bacterial resistance over time. Further studies are required to confirm the phage ecology and host-phage interaction of resistant bacteria.

2.3.5.1 *Listeria* lysins

Lysins are the enzymes that are encoded by phages and digest the bacterial cell wall to release of the newly formed phages. Lysins are effective against killing of Gram-positive bacteria due to their ability to target the peptidoglycan bonds in the bacterial cell wall. Lysins do not induce any bacterial resistance and thus offer an advantage over other methods of bio control. However, lysins in their use as bio preservative have certain challenges and difficulties such as proteolysis in some foods, unproven safety issues and costly technology to adopt (Coffey and others 2010).

2.3.6 Recent advances - Hurdle Technology

The deliberate and intelligent combination of hurdles in food preservation technology to improve the microbial stability, sensory quality as well as nutritional and economic properties of food is one of the latest trends in food preservation techniques and is known as hurdle technology (Leistner 2000). A combined use of several physical and chemical methods for safer foods is a common practice (Lee 2004). The recent trends for “healthier” and convenient foods which require little or minimal processing and are “natural” without the addition of chemical preservatives has given rise to adoption of hurdle technology in food processing plants (Lee 2004). The important hurdles in food preservation that influence the quality and safety of foods include temperature, water

activity (a_w), pH, redox potential, preservatives and competitive microflora (e.g. Lactic acid bacteria) (Leistner 2000). The multiplication of microorganisms is adversely affected when the hurdle factors disturb the homeostatic mechanism. When multiple stress conditions are created, the microorganisms are unable to cope and remain inactive or even die. Thus, determining the major factors that contribute to microbial safety and quality is necessary. At the same time, bacteriophages can be used as an additional hurdles to enhance microbial safety and quality of foods (Chibeu and others 2013).

3 RATIONALE AND SIGNIFICANCE

Microbial food safety has been a rising concern in the food industry for the past few years as reported in the outbreaks associated with various pathogens such as *Listeria monocytogenes*, *Salmonella*, *E. coli,* and *Vibrio* by the Center for Disease Control and Prevention (CDC 2013a). In 2014, a multistate outbreak caused by the consumption of *L. monocytogenes* contaminated bean sprouts resulted in 2 deaths and 5 hospitalizations (CDC 2014b). Alfalfa sprouts are one of the most consumed sprouts and the probability of an outbreak is higher than other sprouts (Yang and others 2013). Also, the seed germination process provides conducive environmental conditions for the pathogen to grow and proliferate (Palmai and Buchanan 2002). Several physical, chemical, and biological techniques have been implemented on sprouts in order to control pathogenic growth. However, factors such as antibiotic resistance, development of undesirable organoleptic features, and consumer preference of “natural” preservation techniques have shifted the focus on use of natural antimicrobial agents such as bacteriophages in foods. The use of bacteriophages in foods has increased after the FDA GRAS status approval of a *Listeria*-specific bacteriophage LISTEX™ P100 to be used in all food products

(USFDA 2006a). Soni and Nannapaneni (2010) reported a 3.5 log CFU/g reduction of *L. monocytogenes* in raw salmon fillet tissue by LISTEX™ P100 stored at 4 or 22 °C for a 10-day storage period. Kocharunchitt and others (2009) observed a 1 log CFU/g reduction of *Salmonella oranienburg* in alfalfa sprouts 3 h after phage (SSP6) application during sprouting. Several studies have reported the use of LISTEX™ P100 to control *L. monocytogenes* in various food matrices such as cheese (Carlton and others 2005), catfish fillet (Soni and others 2010), cooked beef and turkey (Chibeu and others 2013), sausages (Rossi and others 2011), and variety of ready-to-eat (RTE) foods (Guenther and others 2009). However, no study has demonstrated the antimicrobial susceptibility of *L. monocytogenes* to bacteriophage LISTEX™ P100 in alfalfa sprouts.

The increase in outbreaks associated with the sprout industry has led to extensive research on methods for microbial decontamination (Yang and others 2013). Bacteriophages provide a safe, natural, and environmentally friendly alternative against bacteria in foods (Guenther and others 2009). The increasing applications of hurdle technology have further enhanced the role of bacteriophages as they can provide an additional hurdle to hinder the growth of pathogenic bacteria in foods (Leistner 2000). Understanding the efficacy of bacteriophage against *L. monocytogenes* cells in a complex food matrix such as alfalfa seed sprouts would substantiate the potential use of phages in bacterial decontamination of foods. The *overall goal of this study* was to evaluate the antimicrobial susceptibility of *L. monocytogenes* to bacteriophage on alfalfa sprouts during seed germination and subsequent refrigerated storage at 4 °C. The specific aims established to achieve this overall goal were:

I. Determine the efficacy of phage against *L. monocytogenes* in nutrient broth. Guenther and others (2009) reported rapid reduction (up to 5 log units) of *L. monocytogenes* in liquid foods such as chocolate milk and mozzarella cheese brine when treated with phage A511 and samples were stored for 6 days at 6 °C. However, solid foods such as turkey breast and hot dogs showed a significantly lower reduction (up to 2.3 log units) than the liquid foods. In this study, LISTEX™ P100 was hypothesized to work against L. monocytogenes in alfalfa sprouts due to lytic activity of the bacteriophage, as reported by Guenther and others (2009).

II. Determine the effect of LISTEX™ P100 on *L. monocytogenes* in sprouts. Kocharunchitt and others (2009) reported a 1 log CFU/g reduction of *Salmonella* in alfalfa sprouts 3 h after phage SSP6 application during sprouting. The working hypothesis for this aim was that when bacteriophages come in contact with the target bacteria (*L. monocytogenes*), they will penetrate the bacterial cells, replicate the phage DNA within the bacterial cells, and eventually cause cell lysis (Fischetti 2005).

III. Determine the stability of phage over the sprouting and storage period of alfalfa seeds and sprouts. The working hypothesis is that phages would remain stable during the entire sprouting and storage period since each phage particle will liberate progeny phage particles via the lytic cycle (Fischetti 2005). Similar results were reported by Kocharunchitt and others (2009), where the phage (SSP6) remained stable throughout the sprouting period of alfalfa. Soni and Nannapaneni (2010) obtained similar results for LISTEX™ P100, which showed

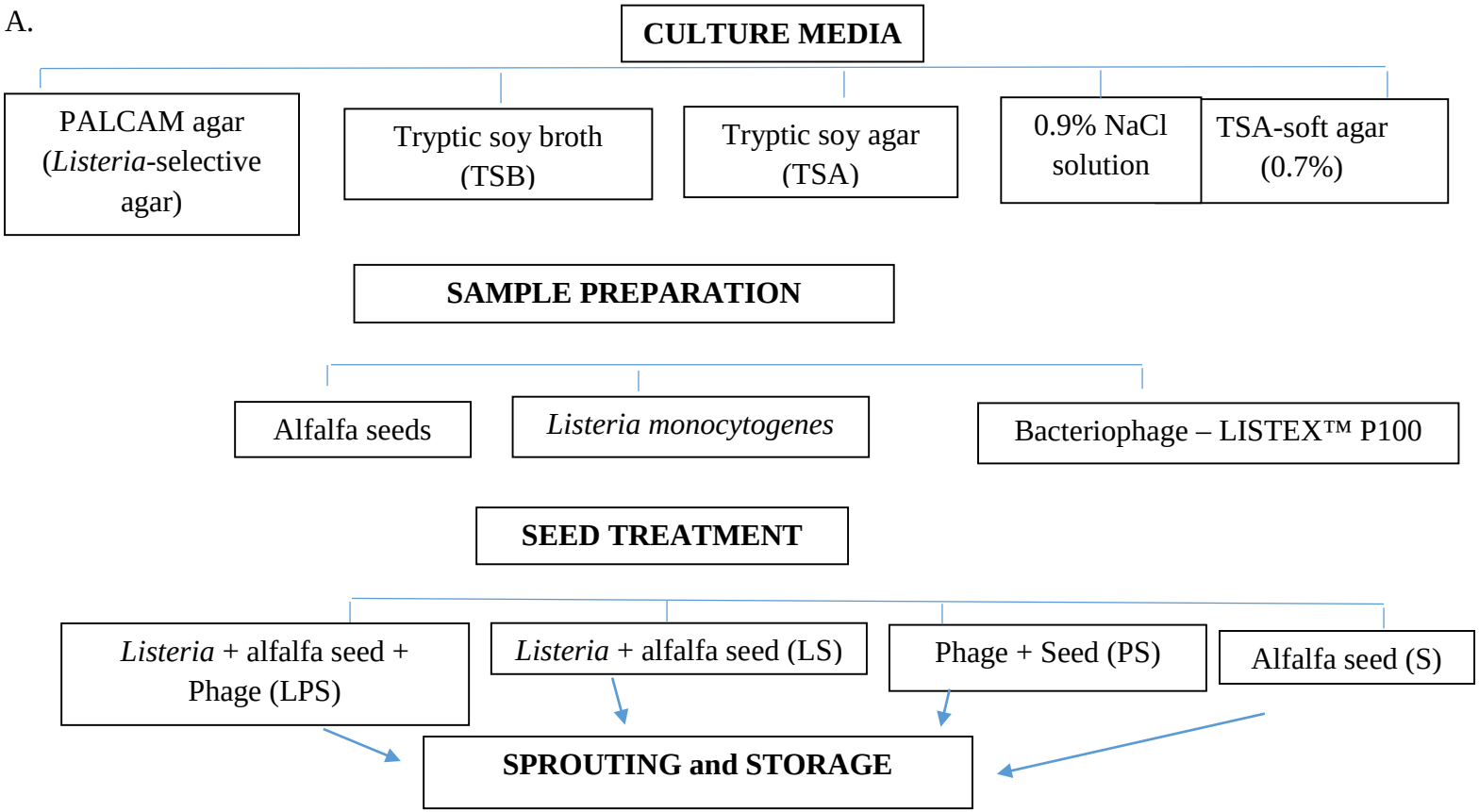
only a marginal decrease of 0.6 log PFU/g from the initial inoculation of 8 log PFU/g in raw salmon fillet tissue stored at 4 °C for 10 days.

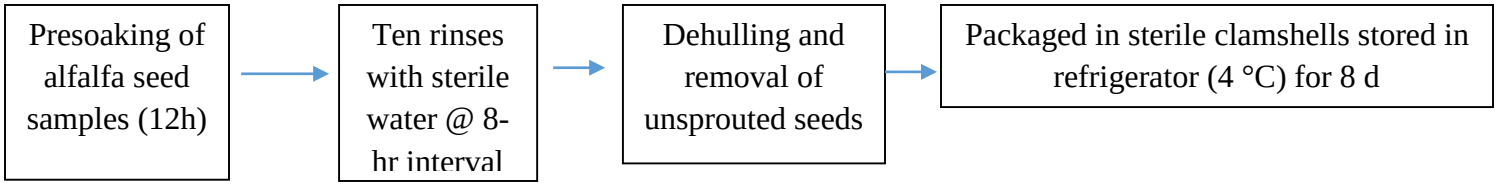
4 MATERIALS AND METHODS

4.1 *Experimental Analysis*

Raw alfalfa seeds were dipped in *Listeria monocytogenes* inoculum before dipping in LISTEX™ P100 phage solution. The treated and untreated seeds were further dried and used for seed sprouting using the glass jar method.

A.





B.

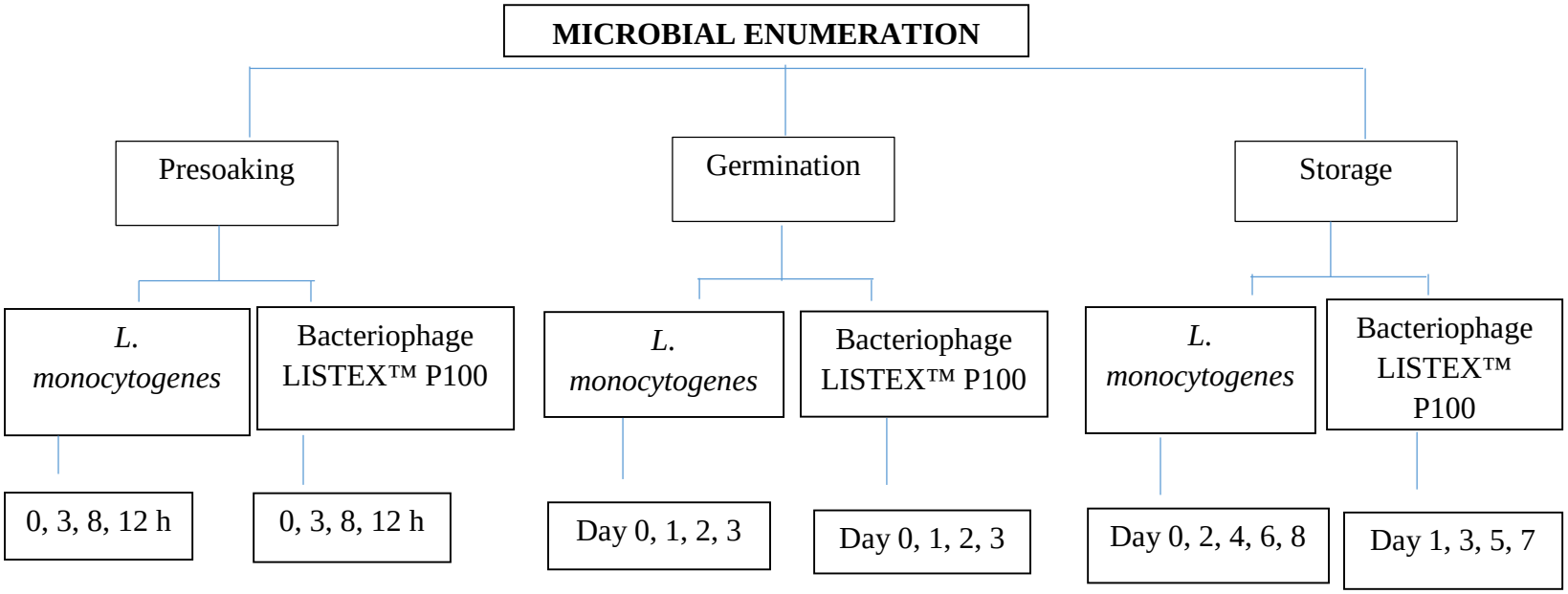


Figure 5. Experimental design of (A) *Listeria* inoculated-alfalfa seed with or without phage, (B) Microbial and phage enumeration in seed and sprout samples.

4.2 *Preparation of media and cultures*

Two of the three *L. monocytogenes* serogroup commonly associated with food borne illness (Type 1 and Type 4) were used for this study. The cultures of *L. monocytogenes* were obtained from the Food and Drug Administration (FDA) Pacific Regional Laboratory Southwest and were stored at -80 °C. Each culture was transferred to 25 mL tryptic soy broth (TSB) and incubated at 37 °C for 24 h before cultivating by streaking on the *Listeria*-specific polymyxin acriflavine lithium chloride ceftazidimeaesculin mannitol (PALCAM) agar plates at 37 °C for 48 h. A single isolated *L. monocytogenes* colony of each serogroup was transferred to TSB before centrifuging at 4000 rpm for 15 min to obtain cell pellets. The cell pellets were resuspended in 0.9 % NaCl solution to obtain bacterial stocks with concentrations of 5.8 $\log_{10}$ CFU/mL and 5.6 $\log_{10}$ CFU/mL for T1 and T4, respectively as determined by the standard plate count using PALCAM agar combined with the standard curve from the turbidimetric method (described below). An equal volume of each bacterial solution was mixed to obtain a final bacterial inoculum at concentration of 5.7 $\log_{10}$ CFU/mL for use in seed inoculation.

4.3 *Determination of cell concentration by the turbidimetric method*

The final concentration of bacterial inoculum for each *L. monocytogenes* serogroup (T1 and T4) was determined by the turbidimetric method using a BioPhotometer plus (Eppendorf, Hauppauge, NY, USA) and the standard plate count as described by Brown and Benson (2007). The stock solution of *L. monocytogenes* serogroup T1 and T4 was serially diluted in tryptic soy broth (TSB) medium and 1 mL aliquot from each dilution was transferred individually into specialized cuvettes called

'Uvettes' (Eppendorf, Hauppauge, NY, USA) and optical density (O.D) was read at 595 nm. For the standard plate count, a 1 mL aliquot from each dilution was pour-plated using sterile PALCAM agar and incubated for 48 h at 37 °C. Using a standard plate counter (American Optical Corporation, Southbridge, MA, USA), the number of colonies in each plate were counted and used for determining cell concentration. The *Listeria* concentration values were expressed in CFU/mL. The values from the spectrophotometer and the standard plate count were correlated to prepare a standard curve of absorbance vs. inoculum concentration. This standard curve (Figure 6) was used for determination of bacterial concentrations in future inoculums by recording the absorbance values at 595 nm.

4.4 *Bacteriophage preparation*

The bacteriophage LISTEX™ P100 was obtained from MICREOS Safety (NieuweKanaal, Wageningen, Nederlands) and stored under refrigerated (4 °C) conditions. The phage stock solution had an initial concentration of 11.3 $\log_{10}$ PFU/mL (2 x 10^{11} PFU/mL) stored under saline buffer according to the manufacturer. The phage solution was serially diluted using sterile SM buffer [100 mM NaCl, 8 mM MgSO4 and 50 mM Tris-HCl (pH 7.5)]. The final phage concentration was determined by standard phage titer assay – soft agar overlay method (Soni and Nannapaneni 2010). A cocktail mixture of two *L. monocytogenes* serogroup T1 and T4 was prepared by adding 1 mL of each serogroup at concentrations of 7.84 $\log_{10}$ CFU/mL ($O.D_{595}$ = 0.704) and 7.79 $\log_{10}$ CFU/mL ($O.D_{595}$ = 0.620), respectively. A mixture of 4 mL soft agar (0.7% agar), 100 µL of each phage dilution (10^{-5}, 10^{-6}, 10^{-7}), and 100 µL *Listeria* cocktail was poured over pre-warmed (37 °C) Tryptic Soy Agar (TSA) plates and incubated at 30 °C for 24 h.

After the incubation period, the number of visible plaques in each plate were counted and the dilution factor was multiplied to determine the PFU/ml.

4.5 *LISTEX™ P100 treatment in nutrient broth*

LISTEX™ P100 was inoculated in nutrient broth to study its inhibitory effect on *L. monocytogenes*. The two serogroups of *L. monocytogenes* were tested separately. A mixture of 8 mL TSB, 1 mL *L. monocytogenes* serogroup T1 (5.8 $\log_{10}$ CFU/mL) or T4 (5.6 $\log_{10}$ CFU/mL) cultured in sterile tryptic soy broth (TSB) (described above), and 1 mL LISTEX™ P100 in sterile SM buffer at 8 $\log_{10}$ PFU/mL was prepared. The untreated control broth received *L. monocytogenes* serogroup T1 or T4, and 1 mL of sterile SM buffer instead of phage. The phage control broth was prepared by adding 1 mL sterile TSB instead of the *Listeria* inoculum. The negative control broth was comprised of 9 mL sterile TSB and 1 mL sterile SM buffer (with no *Listeria* or phage). All broth samples were incubated at 37 °C for 24 h. Bacterial counts of *L. monocytogenes* were then determined by pour plating samples on PALCAM agar plates in triplicate and incubating plates at 37 °C for 48 h .

4.6 *Alfalfa seed treatment with LISTEX™ P100*

4.6.1 Seed inoculation with L. monocytogenes

A total of four sets of alfalfa seeds were prepared for use in this study: *Listeria*-inoculated seeds treated with phage (LPS), *Listeria*-inoculated seeds with no phage (LS), uninoculated seeds treated with phage (PS), and uninoculated seeds with no phage (S). To prepare the *Listeria*-inoculated seeds (LPS and LS), alfalfa seeds (Fisher Scientific,

Nazareth, PA) were aseptically weighed into a sterile beaker to obtain 40 g and then mixed with 200 mL of the *Listeria* inoculum described above (5.7 $\log_{10}$ CFU/mL) and kept as such for 1 h. The seeds were then removed from the inoculum and allowed to dry at ambient temperature for 16-18 h under a Biosafety hood (SterilGard II, Class II Type A/B3, The Baker Co., Sanford, ME) on aluminum foil. The process was repeated for the two sets of uninoculated seeds (PS and S) using sterile 0.9% NaCl solution instead of bacterial inoculum.

4.6.2 Bacteriophage treatment with LISTEX™ P100

The bacteriophage treatment on raw alfalfa seeds was adopted from Kocharunchitt and others (2009) with some modifications. The sets of alfalfa seeds to be treated with phage (LPS and PS) were each soaked in 240 mL sterile water containing bacteriophage at a concentration of 7.72 $\log_{10}$ PFU/mL for 12 h. The sets of alfalfa seeds that were not treated with phage (LS and S) were dipped in 240 mL sterile water without bacteriophage. After 12 h of immersion, the seeds were allowed to dry on sterile trays under the Bio-safety hood for 30 min before the first rinse for the seed sprouting process.

4.6.3 Seed germination of alfalfa seeds

The method used for germination (sprouting) of alfalfa seeds was adopted from Sawyer (1985), with some modifications. After the 12 h immersion step described above, the seeds were removed from solution and rinsed with sterile water by the twirling method in glass jars covered with cheesecloth. The jars were then placed in an inverted position at a 45° angle at room temperature (24.5 ± 0.6 °C) in the dark in a flat, deep, autoclaved glass tray. At every 8 h interval, water was rinsed nine times to obtain a

cumulative time of 72 h of sprout growth. After the ninth rinse, the jars were exposed to visible light for 8 h for a total of 80 h of growing time. Next, the water was drained-off and sprouts were placed in a bowl of sterile deionized water, stirred vigorously to loosen the hulls and allowed to stand for 10 min to separate the hulls, which float on the surface. The treated alfalfa seeds were then removed from the solution and dried for 30 min at room temperature on aluminum foil under a Biosafety hood. After drying, the sprouts were packaged in plastic clam shells (Sprout House, New York, USA) and stored at 4 °C. The clamshells were sterilized by ultraviolet (UV) light for 30 min prior to use. All samples were held for a storage period of 8 d in the refrigerator (Fisher Scientific, Pittsburg, PA, USA) at 4 °C.

4.7 *Microbial enumeration*

The microbial enumeration method was adopted from Kocharunchitt and others (2009). Samples of alfalfa seeds/sprouts were collected immediately after 12 h presoaking but before the first rinse for day 0. Subsequently, seed samples were collected at 24 h (day 1), 48 h (day 2), and sprout sample at 72 h (day 3) after the first rinse. After seed sprouting, the sprouts samples were collected immediately after the last rinse (day 0) for enumerating *L. monocytogenes* during refrigerated storage. Following the first sprout sample collection, further samples of sprouts were collected at day 2, day 4, day 6, and day 8 of the refrigerated storage. For bacterial enumeration of seed samples, a 1.0 g seed sample was placed in a stomacher bag containing 9 mL of sterile buffered peptone water (BPW) (Thermo Scientific, Remel products, Lenexa, KS, USA) and mixed for 30 s at 230 RPM in a 400C Seward Stomacher (Seward Stomacher, England). Similarly, for bacterial enumeration of sprouts, 10 g of sprout sample was placed in a stomacher bag containing

90 mL of sterile BPW and mixed for 30 s in a 400C Seward Stomacher. The obtained seed or sprout homogenates were then serially diluted in sterile BPW and pour-plated on PALCAM plates, followed by incubation at 37 °C for 48 h and enumeration of *L. monocytogenes* colonies.

4.8 *LISTEX™ P100 Stability*

The phage stability determination method was adopted from Kocharunchitt and others (2009). Phage stability was determined throughout the germination and storage periods. Water samples (2 mL) for each treatment solution (LPS and PS) were obtained at 0, 3, 8 and 12 h during the presoaking period after phage application. The seed samples were taken at Day 1, 2, and 3 during germination and sprout samples were taken at Day 1, 3, 5 and 7 during refrigerated storage. Each set of seeds was added to a separate sterile stomacher bag containing 9 mL of BPW. For sprouted samples, 10 g sprouted seed sample and 90 mL of BPW were added to a sterile stomacher bag. The mixtures were stomached for 30 s at 230 RPM in a 400C Seward Stomacher. Each homogenate was filter-sterilized with a sterile syringe and 0.22 µM filters to obtain filtrate in a sterile test tube for further testing. This filtrate was serially diluted in sterile BPW and plated on TSA using the soft agar overlay technique described above. After incubation, the visible phage plaques of LISTEX™ P100 were enumerated to obtain PFU/g.

4.9 *Statistical Analysis*

L. monocytogenes and LISTEX™ P100 phage counts were converted into $\log_{10}$ CFU/g and $\log_{10}$ PFU/g, respectively, to normalize the data. Data were analyzed using one-way analysis of variance (ANOVA), Tukey's test to compare growth of *L.*

monocytogenes in the treatment and control groups across the 3-day germination period. These tests were carried out with IBM SPSS Statistics 21 (IBM SPSS Inc., Armonk, NY, USA) and the significance was based on a confidence interval of 95% ($p < 0.05$). The statistical analysis comparing sample sets LPS and PS to determine phage stability in alfalfa seeds and sprouts was performed using a two-tailed Student's t-test, at a confidence interval of 95% ($p < 0.05$) carried out in MS-Excel 2013.

5 RESULTS AND DISCUSSION

5.1 *L. monocytogenes* inhibition by LISTEX™ P100 in nutrient broth

The inoculated broth sample treated with bacteriophage showed no growth of *L. monocytogenes* after incubation at 37 °C for 48 h, indicating that the phage was able to lyse the bacteria. The untreated control broth (*Listeria* without phage application) showed *L. monocytogenes* counts of 7.68 $\log_{10}$ CFU/mL for *L. monocytogenes* T1 and 7.90 $\log_{10}$ CFU/mL for *L. monocytogenes* T4 on PALCAM plates as compared to the initial *L. monocytogenes* inoculum of 8.2 $\log_{10}$ CFU/mL for both T1 and T4. The phage control (phage without bacteria) and negative control (no bacteria, no phage) of broth samples did not show *L. monocytogenes* growth as confirmed by pour plating on PALCAM plates.

5.2 *Antimicrobial activity of LISTEX™ P100 on L. monocytogenes during germination and storage of alfalfa seeds sprouts*

As shown in Figure 7 and Figure 8, the cocktail mixture of *L. monocytogenes* serogroups T1 and T4 survived the pre-soaking, germination and storage periods of

alfalfa seeds and sprouts in LPS and PS samples. The phage significantly reduced the *L. monocytogenes* population by 1.6 $\log_{10}$ CFU/g in the LPS sample when compared to the PS sample after 24 h of seed germination at room temperature (24.5 ± 0.6 °C). Thereafter, the phage failed to cause significant reductions in *L. monocytogenes* numbers as shown in Figure 7, with no significant difference between *Listeria* counts in LPS and LS samples for day 2 after the first rinse during seed germination. On the last germination day (day 3), the *L. monocytogenes* counts in the LPS sample were found to be significantly ($p < 0.05$) higher than those for the LS sample. The PS and S samples, did not show growth of *L. monocytogenes* throughout the seed germination period (80 h), except at day 3, where the *L. monocytogenes* population in the PS sample was significantly greater than the S sample indicating a potential contamination due to error in handling of samples. The LS samples showed a spike (8.0 $\log_{10}$ CFU/g) in *L. monocytogenes* growth on day 2 of germination following which no further increases in the *Listeria* population were observed until the end of seed germination period.

As seen in Figure 8, the LPS and LS samples showed no significant difference in *L. monocytogenes* levels at day 0 of alfalfa sprout storage at 4 °C. A similar trend followed for the subsequent time points at days 2 and 4 of the refrigerated storage as no significant reduction in *L. monocytogenes* counts was observed between LPS and LS samples. However, LISTEX™ P100 was able to cause a statistically significant reduction of 0.5 $\log_{10}$ CFU/g and 0.1 log10 CFU/g of *L. monocytogenes* in LPS samples when compared to the LS samples on day 6 and day 8 of storage, respectively (Figure 8). During the entire refrigerated storage of 8 days for alfalfa sprouts, the highest *L. monocytogenes* counts in LPS and LS were enumerated at 5.26 $\log_{10}$ CFU/g and 5.75

$\log_{10}$ CFU/g respectively, at day 0. However, there was only a marginal decrease in *L. monocytogenes* counts at the end of the 8-day storage period for the sprouts treated with phage, indicating an inefficacy of LISTEX™ P100 to lyse *L. monocytogenes* cells in a complex food matrix such as alfalfa sprouts.

5.3 *Stability of LISTEX™ P100 on alfalfa seeds and sprouts*

The phage counts on *Listeria*-inoculated (LPS) alfalfa seeds and sprouts at the end of the sprouting period were significantly higher than the phage counts of phage-treated uninoculated alfalfa seeds and sprouts (PS). Figure 9 shows the stability of LISTEX™ P100 during the pre-soaking, germination and storage of alfalfa sprouts in both LPS and PS samples. For LPS samples, the initial phage titer at the presoaking stage was 7.4 $\log_{10}$ PFU/g and at the end of the sprouting period it was 7.3 $\log_{10}$ PFU/g. For PS samples, the initial phage titer was enumerated at 7.3 $\log_{10}$ PFU/g, with a decrease in phage numbers to 5.7 $\log_{10}$ PFU/g at the end of sprouting period. The phage numbers in both LPS and PS samples further decreased to 5.4 $\log_{10}$ PFU/g and 4.4 $\log_{10}$ PFU/g at the end of day 7 of refrigerated storage for LPS and PS samples, respectively. The means of LPS and PS samples at all time-points showed significant difference except at 12 h (presoaking) and day 5 (storage). These results indicate that the phage remained stable throughout the seed germination process for LPS samples, however, in case of PS samples, the phage numbers began to decline after day 2.

Discussion

A variety of foodborne pathogens cause contamination in sprouts (Yang and others 2013). Schoeller and others (2002) confirmed the growth of *L. monocytogenes*

during sprouting and subsequent survival during refrigerated storage. The alfalfa seeds used for sprouting are considered a raw agricultural commodity (Taormina and others 1999). These seeds further go through the usual distribution chain from farm to the sprouting facilities, as with other grains, fruits and vegetables (Taormina and others 1999). This transport system can serve as a source of contamination for raw seeds with pathogenic bacteria such as *L. monocytogenes* (Montville and Schaffner 2005). Moreover, owing to the environmental conditions during the sprouting of alfalfa seeds, the risk of pathogen contamination during this phase is enhanced (Kocharunchitt and others 2009). During sprouting, raw seeds are rinsed with water at regular intervals and are held at specific temperatures to improve sprouting efficiency. Such conditions are supportive for growth, proliferation and spread of pathogens such as *L. monocytogenes* within the food matrix (Montville and Schaffner 2005). For example, Palmai and Buchanan (2002) demonstrated the proliferation of *L. monocytogenes* in germinating alfalfa seeds to the level of 10^6 CFU/g from an initial load of 10^2 CFU/g within 24 h of germination. Similar growth levels were seen in the current study, where the *L. monocytogenes* population increased from 10^5 CFU/g to 10^8 CFU/g after 24 h of seed germination. The slightly greater growth rate observed by Palmai and Buchanan (2002) may be attributed to the type of method used for germination, as they used a mini-sprouter for germinating alfalfa seeds which may have resulted in ideal moisture levels for bacteria to grow.

Several physical and chemical intervention strategies have been proposed to minimize bacterial populations in sprouts at both pre-harvest and postharvest stages (Yang and others 2013). Chemical interventions such as chlorous acid (268 ppm) have

been reported to reduce *L. monocytogenes* populations by 5-log in mung bean sprouts after 9-day refrigerated storage (Lee and others 2002). A combined treatment of aqueous chlorine dioxide and modified atmosphere packaging in vacuum, N_2, or CO_2 resulted in a 1.5 $\log_{10}$ CFU/g reduction of *L. monocytogenes* in mung bean sprouts during refrigerated storage at 5 ± 2 °C (Jin and Lee 2007). Supercritical carbon dioxide (SC-CO_2) treatment has also been used to inhibit pathogen growth without affecting the seed germination potential (Jung and others 2009). An SC-CO_2 treatment at 20 MPa and 45 °C for 15 min on raw alfalfa seeds resulted in >7 log CFU/g reduction of *L. monocytogenes* without adversely affecting the germination rate (Jung and others 2009). Although these interventions have shown promise in reducing the bacterial load in seed sprouts, there is a demand among consumers for the use of natural antimicrobials in food safety.

Bacteriophages are host specific and naturally lyse the target bacterial cell thereby providing a promising alternative for biocontrol of sprouts (Strauch and others 2007; Sikin and others 2013). LISTEX™ P100, a listericidal phage, has been concentrated and selected from a collection of food grade phages taking into the consideration the broadest sensitivity to pathogenic strains of *L. monocytogenes* (USFDA 2007). LISTEX™ P100 is active for a temperature range of 1 – 35 °C with optimum activity at 30 °C (USFDA 2007). The phage can survive best at 4 °C for long periods of time and is stable over an extended storage period (Soni and Nannapaneni 2010). In this study, the ability of phage LISTEX™ P100 to lyse *L. monocytogenes* during germination of alfalfa seeds was determined. The efficacy of LISTEX™ P100 in reducing *L. monocytogenes* levels has been studied with several other foods, including turkey, beef, salmon fillets, catfish, cheeses, ready-to-eat foods etc. (Chibeu and others 2013; Guenther and others 2009;

Rossi and others 2011; Soni and Nannapaneni 2010). As discussed previously, the type of food matrix plays an important role in determining the efficacy of phage biocontrol (Guenther and others 2009). In the present study, the effectiveness of phage against *L. monocytogenes* was higher in broth (7 $\log_{10}$ CFU/mL reduction after 48 h) as compared to a complex food matrix such as alfalfa sprouts (1.6 $\log_{10}$ CFU/mL reduction after 24 h during seed germination). Greater effectiveness in the broth sample was expected due to the increased diffusivity of phage in a liquid medium resulting in effective attachment of phage to the host cell (Guenther and others 2009). Similar results were obtained by Guenther and others (2009), when liquid foods such as chocolate milk and mozzarella cheese brine treated with wide-host-range phages A511 and P100 resulted in 7.6 log CFU/mL and 5.1 log CFU/mL reduction of *L. monocytogenes* respectively, as compared to reduction levels of up to 5 $\log_{10}$ CFU/g in solid foods such as hot dogs, sliced turkey meat, smoked salmon, seafood, lettuce leaves, and sliced cabbage. Reduced diffusivity in a food matrix can result in immobilization and subsequent inactivation of phages (Guenther and others 2009). Also, the broth samples were incubated at a higher temperature of 37 °C than the germination temperature (24.5 ± 0.6 °C) or commercial refrigeration storage temperature (4 °C). The optimum activity of LISTEX™ P100 is reported to be at 30-35 °C according MICREOS, the manufacturer of the phage. Although LISTEX™ P100 is able to lyse bacteria at lower temperatures, the higher temperatures are more conducive to the growth and efficacy of lytic bacteriophages such as P100. Soni and others (2012) observed a higher efficacy of phage P100 in reducing *L. monocytogenes* counts by 5.6 log10 CFU/mL at 30 °C against a *L. monocytogenes* reduction of 3.1 $\log_{10}$ CFU/mL at 4 °C in TSB broth after 24 h compared to an untreated

control. An additional explanation for the reduced effectiveness of phage in the alfalfa seeds during germination is the presence of favorable environmental conditions conducive to *L. monocytogenes* proliferation. In the current study, the average temperature was 24.5 ± 0.6 °C throughout the entire sprouting period. *L. monocytogenes* has shown growth at a temperature range of 0-45 °C (USFDA 2003). The moist environment conditions during the sprouting of seeds contribute greatly to the rapid growth and proliferation of pathogenic bacteria such as *L. monocytogenes*. In the current study, although the sample treated with LISTEX™ P100 showed a significant reduction in the *L. monocytogenes* population by 1.4 log10 CFU/g when compared to the untreated bacteria control after 24 h of sprouting, the effectiveness of the phage did not last throughout the germination period. In a similar observation, Kocharunchitt and others (2009) reported rapid growth (1 $\log_{10}$ CFU/g) of *Salmonella* on experimentally contaminated alfalfa seeds after the first 12 h of phage application.

The initial phage concentration level used in this study was based on a previous study by Leverentz and others (2003), which used a host culture of *L. monocytogenes* (5 x 10^7 CFU/ml) against a phage concentration of 1 x 10^7 PFU/ml to give a Multiplicity of Infection (MOI) of 0.2 PFU/CFU. In the current study, a *Listeria* concentration of 5.7 $\log_{10}$ CFU/g was used against a phage concentration of 7.7 $\log_{10}$ PFU/g, resulting in an MOI of 100 PFU/CFU. The use of higher MOIs has been found to result in greater bacterial reduction due to the presence of more phage particles (Garcia and others 2008; Guenther and others 2009; Soni and Nannapaneni 2010). For example, Bigwood and others (2008) reported a significant reduction of >5.9 log CFU/g at 24 °C in *Salmonella*-inoculated raw and cooked beef when treated with phage at an MOI of 10^4 after 24 h.

Similarly, the same study found that a high MOI of 10^5 resulted in reduction of *Camplyobacter jejuni* to below detectable levels in artificially contaminated chicken skin. In the current study LISTEX™ P100 was able to cause a significant reduction in *L. monocytogenes* population for the LPS sample only for the initial 24 h of sprouting compared to the LS sample. The LS sample showed a spike on day 2 of sprouting after which the *L. monocytogenes* counts remained stable throughout the sprouting period. Perhaps, increasing the MOI would help to improve the efficacy of the phage against *L. monocytogenes* throughout the sprouting period. For example, a LISTEX™ P100 dose of 10^8 PFU/g on salmon fillets inoculated with 10^4 CFU/g *L. monocytogenes* resulted in a ~3.5 log CFU/g reduction in the bacterial population, with an MOI of 10^4 (Soni and Nannapaneni 2010). However, lowering the phage concentration to 10^7 PFU/g while keeping the same *L. monocytogenes* concentration (10^4 CFU/g) resulted in a bacterial reduction of only 2 log CFU/g (Soni and Nannapaneni 2010). Kim and others (2007) observed regrowth of *Enterobacter sakazakii* in infant formula to 3.29 ± 1.48 and 4.94 ± 0.64 log CFU/ml from an initial inoculation of 2 log CFU/mL when treated with phage levels of 10^8 and 10^7 PFU/mL, respectively, whereas a higher phage concentration of 10^9 PFU/ml reduced the counts to non-detectable levels.

As reported by Chibeu and others (2013), subsequent progeny phages developed after bacterial lysis demonstrated lower phage efficiency than the initial infecting phage. This may explain the observed reduction in phage effectiveness after the initial 24 h in the current study. Furthermore, the surface complexity of alfalfa seeds could act as a hindrance for newly formed phages to access the target bacterial cells (Ly-Chatain 2014). The other factors contributing to the inefficacy of LISTEX™ P100 in alfalfa sprouts

could be the neutralization of phages by host antibodies (Sulakvelidze and others 2001) and temporary phage resistance (Kocharunchitt and others 2009). However, it is unclear at this point if phage antibodies (if present) can cause a barrier for bacterial lysis by slowing the doubling time of bacteria (Sulakvelidze and others 2001).

In order for a technology to be applicable as a method of bio preservation, the stability of reacting particles over the food surface is of utmost importance. Soni and Nannapaneni (2010) found that the stability of LISTEX™ P100 did not differ significantly from an initial concentration of 8 log PFU/g to a final 7.4 log PFU/g in salmon fillets over 10-day storage at 4 °C. Similarly, Soni and others (2010) observed only a reduction in bacteriophage numbers from 7.3 $\log_{10}$ PFU/g to 5.5 $\log_{10}$ PFU/g and 5.2 $\log_{10}$ PFU/g in catfish fillets stored at 4 °C and 10 °C, respectively, for 10 days. Kocharunchitt and others (2009) observed that although phage SSP6 survived the presoaking and germination period of alfalfa seed sprouts, the phage numbers decreased by ~3 $\log_{10}$ PFU/g. In this study, similar results for the phage stability were obtained for seed sprouts throughout the sprouting and storage period. The phage numbers decreased from an initial concentration of 7.4 $\log_{10}$ PFU/g to 5.4 $\log_{10}$ PFU/g by day 7 of storage in LPS sample whereas the phage numbers decreased from 7.4 $\log_{10}$ PFU/g to 4.4 $\log_{10}$ PFU/g in PS sample. The higher decrease in phage numbers in PS sample could be attributed to unavailability of host bacteria for the phage to lyse. Also, the uneven surface areas of alfalfa seeds coupled with periodical rinsing during the sprouting process might have caused phage particles to detach from the seed surface and wash-off with the rinsing water. The average phage numbers throughout the presoaking, sprouting and storage period for LPS sample and PS samples were 6.64 ± 0.98 $\log_{10}$ PFU/g and 5.85 ± 1.18

$\log_{10}$ PFU/g respectively (Figure 9). The results indicate that the phage survived and remained stable for the entire presoaking, sprouting and storage period of alfalfa seed sprouts.

6 CONCLUSION

Although bacteriophages have shown great potential as an alternative strategy in controlling foodborne diseases, the efficacy of phage-based biocontrol highly depends upon the complexity of the food matrix. This study has shown that LISTEX™ P100 may serve as a potential hurdle in controlling the proliferation of *Listeria monocytogenes* in alfalfa sprouts during the initial stages of germination. However, further understanding of phage delivery and phage optimization specifically for seed sprouts needs to be considered for effective biocontrol of foodborne pathogens. The use of lytic bacteriophages can act as an additional hurdle in decontamination and help in enhancing the safety of seeds and sprouts.

7 POTENTIAL PROBLEMS, ALTERNATIVE STRATEGIES, AND FUTURE INVESTIGATIONS

The potential problems linked to this study can be associated with prior microbial contamination arising due to presence of aerobic bacteria on the working platform. In spite of the utmost care during operation of the aseptic techniques for microbial enumeration, the possibility of environmental contamination cannot be denied due to several factors. The use of non-specific agar such as TSA for the soft-agar overlay technique can help in the growth of external, undesired microorganisms that can cause potential errors in phage enumeration. Seed sprouts are reported to carry high background

microbiota, with an aerobic plate count (APC) of 10^8 - 10^9 CFU/g in alfalfa and mung bean sprouts available at retail stores, as reported by Patterson and Woodburn (1980). The microflora associated with these seeds can cause competitive interaction between *L. monocytogenes* as well as a hindrance to attachment of phage particles to the target bacteria thereby reducing the phage efficacy. Another potential problem during sprouting of alfalfa seeds is the dehulling step during the process. Commercial sprouts are dehulled before packaging to enhance the marketability of the product by separating the unsprouted seeds from the sprouted. In a lab setting, effective dehulling can be very difficult to achieve while simultaneously working in an aseptic environment. The glass jar method of producing alfalfa sprout may increase the potential of external contamination due to partially uncovered mouth of the jar (kept for aeration).

The external microbial contamination during the experiment can be prevented using appropriate aseptic techniques during the entire operation. The use of a Biosafety hood for the purpose of bacteria and phage inoculation as well as for rinsing water during seed sprouting can reduce aerobic contamination in the samples. The use of a home-sprouting apparatus for germinating alfalfa seeds can be an alternate approach instead of glass jar method. A mechanical sprouter would have better advantage over control of other sprouting factors such as irrigation water, moisture control, etc. (Palmai and Buchanan 2002). In the current study, microbial enumeration was carried out using a standard pour-plate technique. However, the use of an automated spiral plater for microbial enumeration in both treated and control sample would have given a more accurate data on microbial numbers in each sample.

According to MICREOS, the manufacturer of LISTEX™ P100, the optimum pH range for anti-listerial activity of LISTEX™ P100 is 5.6 – 9.6. Although this study did not consider the pH values on the alfalfa seed surface, the future investigations might consider pH as one of the variable in method design. Combination of various physical, chemical, and biological treatments to obtain a synergistic effect in reducing microbial populations has been studied in the past (Penas and others 2009). The use of bacteriophage as part of a combination treatment may prove effective for reducing bacterial growth in sprouts. Future studies could focus on investigating the effective combinations of several physical, chemical, and biological interventions in addition to application of bacteriophages for decontamination of sprouts. The use of a multi-phage cocktail against single bacterial pathogens could also reduce the potential of developing bacteriophage insensitive mutants (BIM) and thereby prevent an acquired phage resistance (Sharma 2013). Further investigation on phage cocktails against specific bacteria could help optimize phage therapy especially in produce. Lastly, the use of LISTEX™ P100 as a processing aid against decontaminating food processing equipment is still largely unreported. Exploring the potential of suppressing the growth of *L. monocytogenes* on food processing equipment surfaces could help advance microbiological food safety.

FIGURES

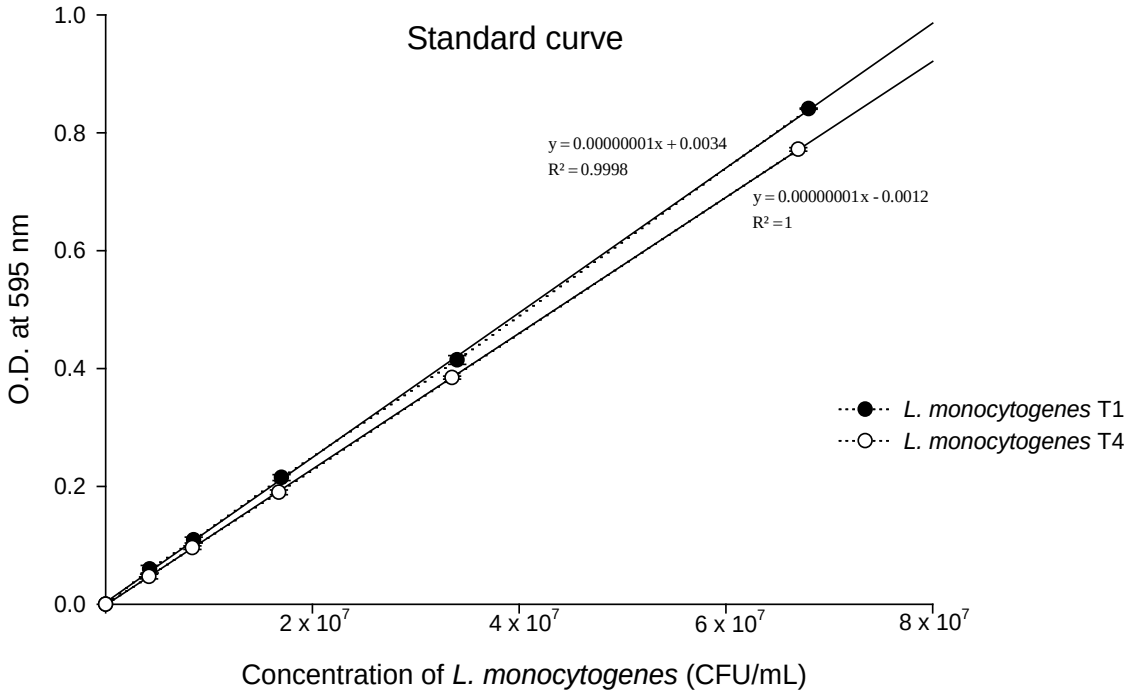


Figure 6. Standard curve showing O.D at 595 nm against *L. monocytogenes* concentration

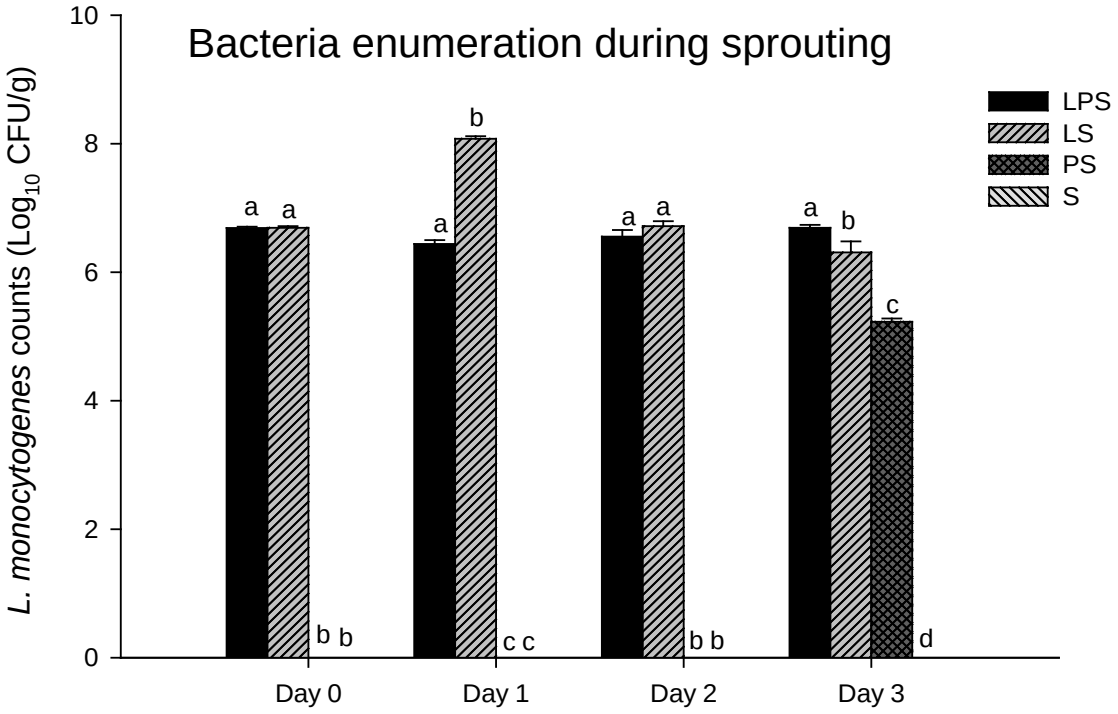


Figure 7. *L. monocytogenes* growth during alfalfa seed germination (24.5 ± 0.6 °C) compared between LPS (*Listeria monocytogenes* + P100 + alfalfa seeds), LS *(Listeria monocytogenes* + Alfalfa seeds), PS (P100 + alfalfa seeds) and S (Alfalfa seeds only) samples. Means with the same letter are not statistically different ($p < 0.05$) from other samples groups tested at each time point.

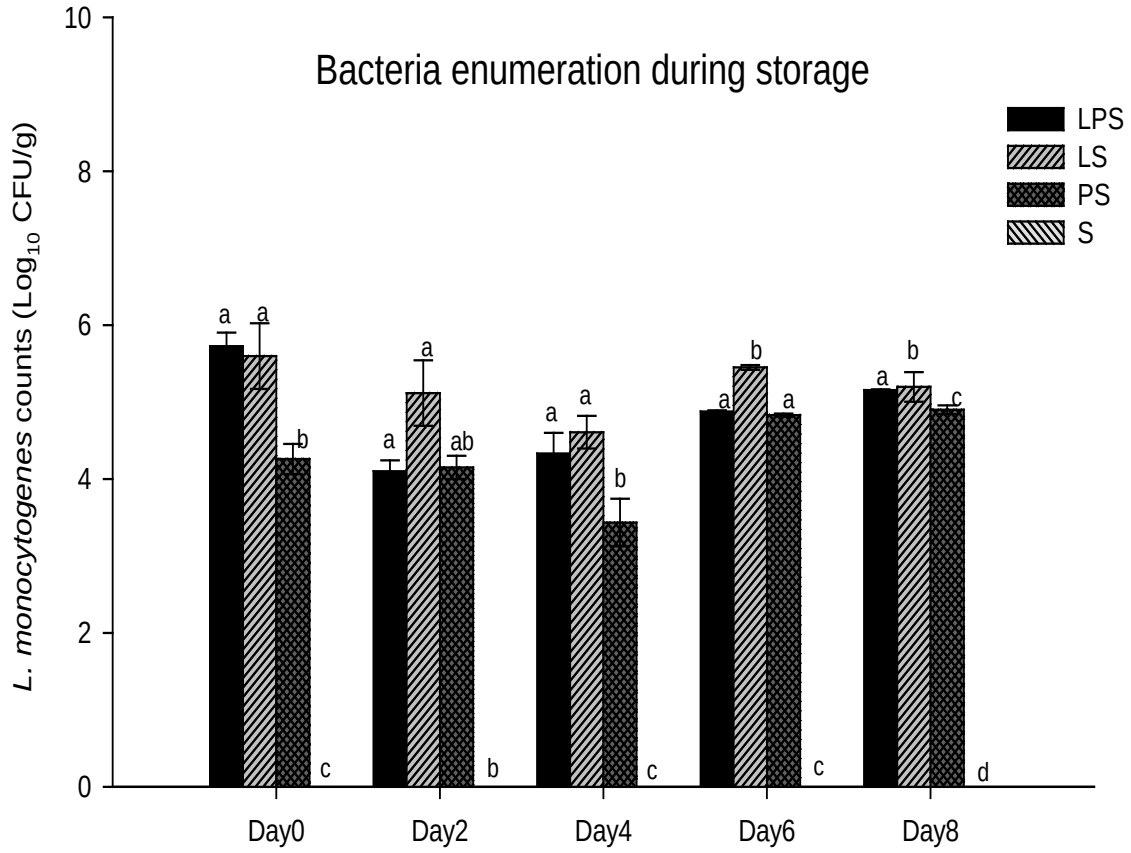


Figure 8. *L. monocytogenes* growth during alfalfa sprout storage (4 °C) compared between LPS (*Listeria monocytogenes* + P100 + alfalfa seeds), LS (*Listeria monocytogenes* + Alfalfa seeds), PS (P100 + alfalfa seeds) and S (Alfalfa seeds only) samples. Means with the same letter are not statistically different ($p < 0.05$) from other samples groups tested at each time point.

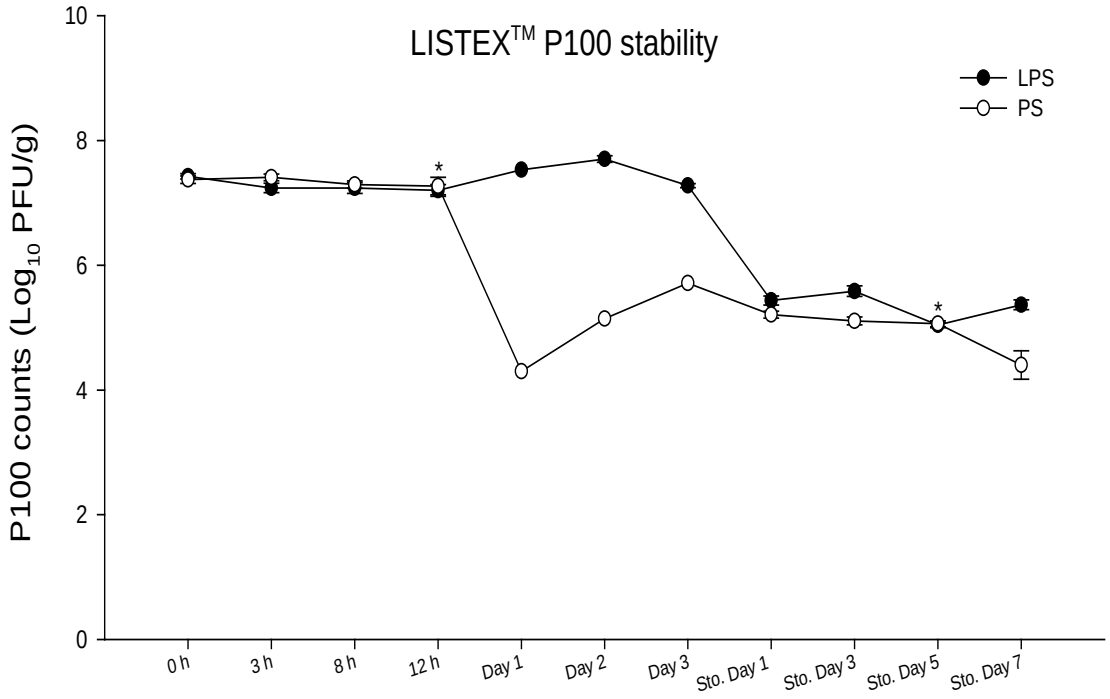


Figure 9. LISTEX™ P100 stability during seed germination and storage of alfalfa seed sprouts between LPS (*Listeria* monocytogenes + P100 + alfalfa seeds) and PS (P100 + alfalfa seeds). * indicates no significant difference ($p < 0.05$) between LPS and PS samples at the given time point.

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9 MANUSCRIPT DRAFT

Antimicrobial Susceptibility of *Listeria monocytogenes* to Bacteriophage P100 during Germination and Storage of Alfalfa Sprouts (*Medicago sativa*)

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Abstract

The seed germination process during sprout production provides suitable environmental conditions for the growth of pathogenic bacteria, such as *Listeria monocytogenes.* A potential way to control this bacterial growth is through the use of bacteriophages, which are naturally occurring viruses that specifically attack bacterial targets and have been shown to be effective antimicrobials in some foods. Therefore, the objective of this study was to evaluate the antimicrobial susceptibility of *L. monocytogenes* to bacteriophage on alfalfa sprouts during seed germination and subsequent refrigerated storage at 4 °C. Alfalfa sprout seeds were dip-inoculated with 5.5 x 10^5 CFU/ml *L. monocytogenes* serogroups 1 and 4. This was followed by treatment with the commercial bacteriophage LISTEX™ P100 at a concentration of 5.3 x 10^7 PFU/ml. The seeds were then soaked and germinated for 80 h using the glass jar method. The concentration of *L. monocytogenes* was determined every 24 h using PALCAM agar plated in triplicate. When compared to the spiked, untreated control, treatment of sprout seeds with LISTEX™ P100 resulted in a statistically significant ($p < 0.05$) reduction of 1.6 $\log_{10}$ CFU/g *L. monocytogenes* after the initial 24 h of germination. However, the bacteriophage did not show a lasting inhibitory effect, with no statistically significant reductions in *L. monocytogenes* growth as compared to the control at subsequent time points. The bacteriophage remained stable over the entire germination and storage period. Although biocontrol of *Listeria* with bacteriophages has high potential to serve as an alternative strategy to control foodborne illnesses, factors such as phage delivery and dose optimization in sprouts need to be further investigated.

Keywords

Listeria monocytogenes, alfalfa sprouts, bacteriophage, LISTEX P100, germination

Introduction

Listeria monocytogenes is a Gram-positive, ubiquitous, facultative anaerobic bacterium that can grow at refrigerated temperatures and survive in a wide range of growth conditions such as low pH and high salt levels (USFDA 2012). *L. monocytogenes* causes a foodborne illness called listeriosis, which involves diarrhea, fever, headaches, stiffness, and nausea. In some cases, it can lead to more serious diseases like pneumonia and meningitis, as well as spontaneous abortions and still births among pregnant women (USFDA 2012). Human listeriosis is responsible for 28% of all deaths due to foodborne illness and it has the highest hospitalization rate (92 %) among food borne pathogens in United States (Scallan and others 2011). The susceptible populations for listeriosis are pregnant women, elderly and immunocompromised populations.

Since food borne listeriosis was first reported in 1981, numerous outbreaks caused by *L. monocytogenes* have been reported in the United States (Silk and others 2013). The majority of these outbreaks have been caused by the food groups such as meat, dairy, poultry and produce (Conter and others 2009). In 2013, alfalfa sprouts from Sprouters Northwest of Kent were found to be contaminated with *L. monocytogenes,* resulting in an immediate recall of over 1,953 lbs of alfalfa and other sprouts (USFDA 2013). A potential means for controlling *L. monocytogenes* in sprouts is through the use of bacteriophages, which are naturally occurring viruses that specifically attack bacterial

targets. In the past decade, it has been found that bacteriophages have several applications in the food industry as biocontrol, biopreservative, and biosanitation agents as well as for detecting pathogenic bacteria in foods (Garcia and others 2008). The advantages of bacteriophages include the following: i) they kill the bacterial target, ii) they do not cross genus boundaries and thus are specific in their action, and iii) they breakdown into non-toxic proteins and nucleic acids as their final product (Rossi and others 2011). The regulatory agencies in several countries have begun to approve the use of bacteriophages to prevent bacterial contamination in fresh produce (Sharma 2013). In 2006, the U.S. Food and Drug Administration (FDA) approved and granted generally recognized as safe (GRAS) status to the use of the LISTEX™ P100 in raw and ready-to-eat foods as an antimicrobial agent against *L. monocytogenes* (USFDA 2007). Bacteriophages have been proven to be effective at controlling pathogens such as *Salmonella*, *Escherichia coli*, and *L. monocytogenes* in a variety of food products (Hagens and Loessner 2007). The efficacy of LISTEX™ P100 was evaluated in broth and on raw salmon fillet tissues for control of *L. monocytogenes* as a function of storage temperature (Soni and Nannapaneni 2010). A 2.3 log CFU/g reduction in *L. monocytogenes* was observed with application of phage LISTEX™ P100 on raw salmon fillet tissue over a 10 d storage period at 4 °C. The potential of bacteriophages has also been studied with alfalfa sprouts where a 1-log CFU/g reduction in *Salmonella oranienburg* was observed after a 3 h phage application during sprouting (Kocharunchitt and others 2009). However, no studies have evaluated the effectiveness of LISTEX™ P100 as an antimicrobial agent against *L. monocytogenes* in alfalfa sprouts. The goal of this study was to evaluate the antimicrobial susceptibility of *Listeria monocytogenes* to

the bacteriophage LISTEX™ P100 on alfalfa sprouts (*Medicago sativa*) at refrigerated temperature (4 °C).

Materials and Methods

Preparation of media and cultures

Two of the three *L. monocytogenes* serogroup commonly associated with food borne illness (Type 1 and Type 4) were used for this study. The cultures of *L. monocytogenes* were obtained from the Food and Drug Administration (FDA) Pacific Regional Laboratory Southwest and were stored at -80 °C. Each culture was transferred to 25 mL tryptic soy broth (TSB) and incubated at 37 °C for 24 h before cultivating by streaking on the *Listeria*-specific polymyxin acriflavine lithium chloride ceftazidimeaesculin mannitol (PALCAM) agar plates at 37 °C for 48 h. A single isolated *L. monocytogenes* colony of each serogroup was transferred to TSB before centrifuging at 4000 rpm for 15 min to obtain cell pellets. The cell pellets were resuspended in 0.9 % NaCl solution to obtain bacterial stocks with concentrations of 5.8 $\log_{10}$ CFU/mL and 5.6 $\log_{10}$ CFU/mL for T1 and T4, respectively as determined by the standard plate count using PALCAM agar combined with the standard curve from the turbidimetric method (described below). An equal volume of each bacterial solution was mixed to obtain a final bacterial inoculum at concentration of 5.7 $\log_{10}$ CFU/mL for use in seed inoculation.

Determination of cell concentration by the turbidimetric method

The final concentration of bacterial inoculum for each *L. monocytogenes* serogroup (T1 and T4) was determined by the turbidimetric method using a BioPhotometer plus (Eppendorf, Hauppauge, NY, USA) and the standard plate count as

described by Brown and Benson (2007). The stock solution of *L. monocytogenes* serogroup T1 and T4 was serially diluted in tryptic soy broth (TSB) medium and 1 mL aliquot from each dilution was transferred individually into specialized cuvettes called 'Uvettes' (Eppendorf, Hauppauge, NY, USA) and optical density (O.D) was read at 595 nm. For the standard plate count, a 1 mL aliquot from each dilution was pour-plated using sterile PALCAM agar and incubated for 48 h at 37 °C. Using a standard plate counter (American Optical Corporation, Southbridge, MA, USA), the number of colonies in each plate were counted and used for determining cell concentration. The *Listeria* concentration values were expressed in CFU/mL. The values from the spectrophotometer and the standard plate count were correlated to prepare a standard curve of absorbance vs. inoculum concentration. A standard curve was used for determination of bacterial concentrations in future inoculums by recording the absorbance values at 595 nm.

Bacteriophage preparation

The bacteriophage LISTEX™ P100 was obtained from MICREOS Safety (NieuweKanaal, Wageningen, Nederlands) and stored under refrigerated (4 °C) conditions. The phage stock solution had an initial concentration of 11.3 $\log_{10}$ PFU/mL (2 x 10^{11} PFU/mL) stored under saline buffer according to the manufacturer. The phage solution was serially diluted using sterile SM buffer [100 mM NaCl, 8 mM $MgSO_4$ and 50 mM Tris-HCl (pH 7.5)]. The final phage concentration was determined by standard phage titer assay – soft agar overlay method (Soni and Nannapaneni 2010). A cocktail mixture of two *L. monocytogenes* serogroup T1 and T4 was prepared by adding 1 mL of each serogroup at concentrations of 7.84 $\log_{10}$ CFU/mL ($O.D_{595}$ = 0.704) and 7.79 $\log_{10}$ CFU/mL ($O.D_{595}$ = 0.620), respectively. A mixture of 4 mL soft agar (0.7% agar), 100

125 µL of each phage dilution (10^{-5}, 10^{-6}, 10^{-7}), and 100 µL *Listeria* cocktail was poured over
126 pre-warmed (37 °C) Tryptic Soy Agar (TSA) plates and incubated at 30 °C for 24 h.
127 After the incubation period, the number of visible plaques in each plate were counted and
128 the dilution factor was multiplied to determine the PFU/ml.

129 ***LISTEX™ P100 treatment in nutrient broth***

130 LISTEX™ P100 was inoculated in nutrient broth to study its inhibitory effect on
131 *L. monocytogenes*. The two serogroups of *L. monocytogenes* were tested separately. A
132 mixture of 8 mL TSB, 1 mL *L. monocytogenes* serogroup T1 (5.8 $\log_{10}$ CFU/mL) or T4
133 (5.6 $\log_{10}$ CFU/mL) cultured in sterile tryptic soy broth (TSB) (described above), and 1
134 mL LISTEX™ P100 in sterile SM buffer at 8 $\log_{10}$ PFU/mL was prepared. The untreated
135 control broth received *L. monocytogenes* serogroup T1 or T4, and 1 mL of sterile SM
136 buffer instead of phage. The phage control broth was prepared by adding 1 mL sterile
137 TSB instead of the *Listeria* inoculum. The negative control broth was comprised of 9 mL
138 sterile TSB and 1 mL sterile SM buffer (with no *Listeria* or phage). All broth samples
139 were incubated at 37 °C for 24 h. Bacterial counts of *L. monocytogenes* were then
140 determined by pour plating samples on PALCAM agar plates in triplicate and incubating
141 plates at 37 °C for 48 h .

142 ***Alfalfa seed treatment with LISTEX™ P100***

143 *Seed inoculation*

144 A total of four sets of alfalfa seeds were prepared for use in this study: *Listeria*-
145 inoculated seeds treated with phage (LPS), *Listeria*-inoculated seeds with no phage (LS),
146 uninoculated seeds treated with phage (PS), and uninoculated seeds with no phage (S).

To prepare the *Listeria*-inoculated seeds (LPS and LS), alfalfa seeds (Fisher Scientific, Nazareth, PA) were aseptically weighed into a sterile beaker to obtain 40 g and then mixed with 200 mL of the *Listeria* inoculum described above (5.7 $\log_{10}$ CFU/mL) and kept as such for 1 h. The seeds were then removed from the inoculum and allowed to dry at ambient temperature for 16-18 h under a Biosafety hood (SterilGard II, Class II Type A/B3, The Baker Co., Sanford, ME) on aluminum foil. The process was repeated for the two sets of uninoculated seeds (PS and S) using sterile 0.9% NaCl solution instead of bacterial inoculum.

Bacteriophage treatment

The bacteriophage treatment on raw alfalfa seeds was adopted from Kocharunchitt and others (2009) with some modifications. The sets of alfalfa seeds to be treated with phage (LPS and PS) were each soaked in 240 mL sterile water containing bacteriophage at a concentration of 7.72 $\log_{10}$ PFU/mL for 12 h. The sets of alfalfa seeds that were not treated with phage (LS and S) were dipped in 240 mL sterile water without bacteriophage. After 12 h of immersion, the seeds were allowed to dry on sterile trays under the Bio-safety hood for 30 min before the first rinse for the seed sprouting process.

Seed Germination

The method used for germination (sprouting) of alfalfa seeds was adopted from Sawyer (1985), with some modifications. After the 12 h immersion step described above, the seeds were removed from solution and rinsed with sterile water by the twirling method in glass jars covered with cheesecloth. The jars were then placed in an inverted position at a 45° angle at room temperature (24.5 ± 0.6 °C) in the dark in a flat, deep,

autoclaved glass tray. At every 8 h interval, water was rinsed nine times to obtain a cumulative time of 72 h of sprout growth. After the ninth rinse, the jars were exposed to visible light for 8 h for a total of 80 h of growing time. Next, the water was drained-off and sprouts were placed in a bowl of sterile deionized water, stirred vigorously to loosen the hulls and allowed to stand for 10 min to separate the hulls, which float on the surface. The treated alfalfa seeds were then removed from the solution and dried for 30 min at room temperature on aluminum foil under a Biosafety hood. After drying, the sprouts were packaged in plastic clam shells (Sprout House, New York, USA) and stored at 4 °C. The clamshells were sterilized by Ultra-violet (UV) light for 30 min prior to use. All samples were held for a storage period of 8 d in the refrigerator (Fisher Scientific, Pittsburg, PA, USA) at 4 °C.

Microbial enumeration

The microbial enumeration method was adopted from Kocharunchitt and others (2009). Samples of alfalfa seeds/sprouts were collected immediately after 12 h presoaking but before the first rinse for day 0. Subsequently, seed samples were collected at 24 h (day 1), 48 h (day 2), and sprout sample at 72 h (day 3) after the first rinse. After seed sprouting, the sprouts samples were collected immediately after the last rinse (day 0) for enumerating *L. monocytogenes* during refrigerated storage. Following the first sprout sample collection, further samples of sprouts were collected at day 2, day 4, day 6, and day 8 of the refrigerated storage. For bacterial enumeration of seed samples, a 1.0 g seed sample was placed in a stomacher bag containing 9 mL of sterile buffered peptone water (BPW) (Thermo Scientific, Remel products, Lenexa, KS, USA) and mixed for 30 s at 230 RPM in a 400C Seward Stomacher (Seward Stomacher, England). Similarly, for bacterial

enumeration of sprouts, 10 g of sprout sample was placed in a stomacher bag containing 90 mL of sterile BPW and mixed for 30 s in a 400C Seward Stomacher. The obtained seed or sprout homogenates were then serially diluted in sterile BPW and pour-plated on PALCAM plates, followed by incubation at 37 °C for 48 h and enumeration of *L. monocytogenes* colonies.

LISTEX™ P100 Stability

The phage stability determination method was adopted from Kocharunchitt and others (2009). Phage stability was determined throughout the germination and storage periods. Water samples (2 mL) for each treatment solution (LPS and PS) were obtained at 0, 3, 8 and 12 h during the presoaking period after phage application. The seed samples were taken at Day 1, 2, and 3 during germination and sprout samples were taken at Day 1, 3, 5 and 7 during refrigerated storage. Each set of seeds was added to a separate sterile stomacher bag containing 9 mL of BPW. For sprouted samples, 10 g sprouted seed sample and 90 mL of BPW were added to a sterile stomacher bag. The mixtures were stomached for 30 s at 230 RPM in a 400C Seward Stomacher. Each homogenate was filter-sterilized with a sterile syringe and 0.22 µM filters to obtain filtrate in a sterile test tube for further testing. This filtrate was serially diluted in sterile BPW and plated on TSA using the soft agar overlay technique described above. After incubation, the visible phage plaques of LISTEX™ P100 were enumerated to obtain PFU/g.

Statistical Analysis

L. monocytogenes and LISTEX™ P100 phage counts were converted into $\log_{10}$ CFU/g and $\log_{10}$ PFU/g, respectively, to normalize the data. Data were analyzed using

one-way analysis of variance (ANOVA), Tukey's test to compare growth of *L. monocytogenes* in the treatment and control groups across the 3-day germination period. These tests were carried out with IBM SPSS Statistics 21 (IBM SPSS Inc., Armonk, NY, USA) and the significance was based on a confidence interval of 95% ($p < 0.05$). The statistical analysis comparing sample sets LPS and PS to determine phage stability in alfalfa seeds and sprouts was performed using a two-tailed Student's t-test, at a confidence interval of 95% ($p < 0.05$) carried out in MS-Excel 2013.

Results

L. monocytogenes inhibition by LISTEX™ P100 in nutrient broth

The inoculated broth sample treated with bacteriophage showed no growth of *L. monocytogenes* after incubation at 37 °C for 48 h, indicating that the phage was able to lyse the bacteria. The untreated control broth (*Listeria* without phage application) showed *L. monocytogenes* counts of 7.68 $\log_{10}$ CFU/mL for *L. monocytogenes* T1 and 7.90 $\log_{10}$ CFU/mL for *L. monocytogenes* T4 on PALCAM plates as compared to the initial *L. monocytogenes* inoculum of 8.2 $\log_{10}$ CFU/mL for both T1 and T4. The phage control (phage without bacteria) and negative control (no bacteria, no phage) of broth samples did not show *L. monocytogenes* growth as confirmed by pour plating on PALCAM plates.

Antimicrobial activity of LISTEX™ P100 on L. monocytogenes during germination and storage of alfalfa seeds sprouts

As shown in Figure 1 and Figure 2, the cocktail mixture of *L. monocytogenes* serogroups T1 and T4 survived the pre-soaking, germination and storage periods of

alfalfa seeds and sprouts in LPS and PS samples. The phage showed effectiveness in significantly reducing the *L. monocytogenes* population by 1.6 $\log_{10}$ CFU/g in the LPS sample when compared to the PS sample after 24 h of seed germination at room temperature (24.5 ± 0.6 °C). Thereafter, the phage failed to cause significant reductions in *L. monocytogenes* numbers as shown in Figure 1, with no significant difference between *Listeria* counts in LPS and LS samples for day 2 after the first rinse during seed germination. On the last germination day (day 3), the *L. monocytogenes* counts in the LPS sample were found to be significantly ($p < 0.05$) higher than those for the LS sample. The PS and S samples, did not show growth of *L. monocytogenes* throughout the seed germination period (80 h), except at day 3, where the *L. monocytogenes* population in the PS sample was significantly greater than the S sample indicating a potential contamination due to error in handling of samples. The LS samples showed a spike (8.0 $\log_{10}$ CFU/g) in *L. monocytogenes* growth on day 2 of germination following which no further increases in the *Listeria* population were observed until the end of seed germination period.

As seen in Figure 2, the LPS and LS samples showed no significant difference in *L. monocytogenes* levels at day 0 of alfalfa sprout storage at 4 °C. A similar trend followed for the subsequent time points at days 2 and 4 of the refrigerated storage as no significant reduction in *L. monocytogenes* counts was observed between LPS and LS samples. However, LISTEX™ P100 was able to cause a statistically significant reduction of 0.5 $\log_{10}$ CFU/g and 0.1 log10 CFU/g of *L. monocytogenes* in LPS samples when compared to the LS samples on day 6 and day 8 of storage, respectively (Figure 2). During the entire refrigerated storage of 8 days for alfalfa sprouts, the highest *L.*

monocytogenes counts in LPS and LS were enumerated at 5.26 $\log_{10}$ CFU/g and 5.75 $\log_{10}$ CFU/g respectively, at day 0. However, there was only a marginal decrease in *L. monocytogenes* counts at the end of the 8-day storage period for the sprouts treated with phage, indicating an inefficacy of LISTEX™ P100 to lyse *L. monocytogenes* cells in a complex food matrix such as alfalfa sprouts.

Stability of LISTEX™ P100 on alfalfa seeds and sprouts

The phage counts on *Listeria*-inoculated (LPS) alfalfa seeds and sprouts at the end of the sprouting period were significantly different than the phage counts of phage-treated uninoculated alfalfa seeds and sprouts (PS). Figure 3 shows the stability of LISTEX™ P100 during the pre-soaking, germination and storage of alfalfa sprouts in both LPS and PS samples. For LPS samples, the initial phage titer at the presoaking stage was 7.4 $\log_{10}$ PFU/g and at the end of the sprouting period it was 7.3 $\log_{10}$ PFU/g. For PS samples, the initial phage titer was enumerated at 7.3 $\log_{10}$ PFU/g, with a decrease in phage numbers to 5.7 $\log_{10}$ PFU/g at the end of sprouting period. The phage numbers in both LPS and PS samples further decreased to 5.4 $\log_{10}$ PFU/g and 4.4 $\log_{10}$ PFU/g at the end of day 7 of refrigerated storage for LPS and PS samples, respectively. The student t-test at confidence interval of 95% ($p < 0.05$) showed a statistical difference between the means of LPS and PS samples at all time-points except at 12 h (presoaking) and day 5 (storage). These results indicate that the phage remained stable throughout the seed germination process for LPS samples, however, in case of PS samples, the phage numbers began to decline after day 2.

Discussion

A variety of foodborne pathogens have been known to cause contamination in fresh produce and sprouts in particular (Yang and others 2013). Schoeller and others (2002) confirmed the growth of *L. monocytogenes* during sprouting and subsequent survival during refrigerated storage. The alfalfa seeds used for sprouting are considered a raw agricultural commodity (Taormina and others 1999). These seeds further go through the usual distribution chain from farm to the sprouting facilities, as with other grains, fruits and vegetables (Taormina and others 1999). This transport system can serve as a source of contamination for raw seeds with pathogenic bacteria such as *L. monocytogenes* (Montville and Schaffner 2005). Moreover, owing to the environmental conditions during the sprouting of alfalfa seeds, the risk of pathogen contamination during this phase is enhanced (Kocharunchitt and others 2009). During sprouting, raw seeds are supplemented with water at regular intervals and are held at specific temperatures to improve sprouting efficiency. Such conditions are supportive for growth, proliferation and spread of pathogens such as *L. monocytogenes* within the food matrix (Montville and Schaffner 2005). For example, Palmai and Buchanan (2002) demonstrated the proliferation of *L. monocytogenes* in germinating alfalfa seeds to the level of 10^6 CFU/g from an initial load of 10^2 CFU/g within 24 h of germination. Similar growth levels were seen in the current study, where the *L. monocytogenes* population increased from 10^5 CFU/g to 10^8 CFU/g after 24 h of seed germination. The slightly greater growth rate observed by Palmai and Buchanan (2002) may be attributed to the type of method used for germination. The authors used a mini-sprouter for germinating alfalfa seeds which may have resulted in ideal moisture levels for bacteria to grow.

303 Several physical and chemical intervention strategies have been proposed to
304 minimize bacterial populations in sprouts at both pre-harvest and postharvest stages
305 (Yang and others 2013). Chemical interventions such as chlorous acid (268 ppm) have
306 been reported to reduce *L. monocytogenes* populations by 5-log in mung bean sprouts
307 after 9-day refrigerated storage (Lee and others 2002). A combined treatment of aqueous
308 chlorine dioxide and modified atmosphere packaging in vacuum, N_2, or CO_2 resulted in a
309 1.5 $\log_{10}$ CFU/g reduction of *L. monocytogenes* in mung bean sprouts during refrigerated
310 storage at 5 ± 2 °C (Jin and Lee 2007). Supercritical carbon dioxide (SC-CO_2) treatment
311 has also been used to inhibit pathogen growth without affecting the seed germination
312 potential (Jung and others 2009). An SC-CO_2 treatment at 20 MPa and 45 °C for 15 min
313 on raw alfalfa seeds resulted in >7 log CFU/g reduction of *L. monocytogenes* without
314 adversely affecting the germination rate (Jung and others 2009). Although these
315 interventions have shown promise in reducing the bacterial load in seed sprouts, there is a
316 demand among consumers for the use of natural antimicrobials in food safety.

317 Bacteriophages are host specific and naturally lyse the target bacterial cell thereby
318 providing a promising alternative for biocontrol of sprouts (Strauch and others 2007;
319 Sikin and others 2013). LISTEX™ P100, a listericidal phage, has been concentrated and
320 selected from a collection of food grade phages taking into the consideration the broadest
321 sensitivity to pathogenic strains of *L. monocytogenes* (USFDA 2007). LISTEX™ P100 is
322 active for a temperature range of 1 – 35 °C with optimum activity at 30 °C (USFDA
323 2007). It can survive best at 4 °C for long periods of time and is stable over an extended
324 storage period (Soni and Nannapaneni 2010). In this study, the ability of phage
325 LISTEX™ P100 to lyse *L. monocytogenes* during germination of alfalfa seeds was

326 determined. The efficacy of LISTEX™ P100 in reducing *L. monocytogenes* levels has
327 been studied with several other foods, including turkey, beef, salmon fillets, catfish,
328 cheeses, ready-to-eat foods etc. (Chibeu and others 2013; Guenther and others 2009;
329 Rossi and others 2011; Soni and Nannapaneni 2010). As discussed previously, the type of
330 food matrix plays an important role in determining the efficacy of phage biocontrol
331 (Guenther and others 2009). In the present study, the effectiveness of phage against *L.*
332 *monocytogenes* was higher in broth (7 $\log_{10}$ CFU/mL reduction after 48 h) as compared
333 to a complex food matrix such as alfalfa sprouts (1.6 $\log_{10}$ CFU/mL reduction after 24 h
334 during seed germination). Greater effectiveness in the broth sample was expected due to
335 the increased diffusivity of phage in a liquid medium resulting in effective attachment of
336 phage to the host cell (Guenther and others 2009). Similar results were obtained by
337 Guenther and others (2009), when liquid foods such as chocolate milk and mozzarella
338 cheese brine treated with wide-host-range phages A511 and P100 resulted in 7.6 log
339 CFU/mL and 5.1 log CFU/mL reduction of *L. monocytogenes* respectively, as compared
340 to reduction levels of up to 5 $\log_{10}$ CFU/g in solid foods such as hot dogs, sliced turkey
341 meat, smoked salmon, seafood, lettuce leaves, and sliced cabbage. Reduced diffusivity in
342 a food matrix can result in immobilization and subsequent inactivation of phages
343 (Guenther and others 2009). Also, the broth samples were incubated at a higher
344 temperature of 37 °C than the germination temperature (24.5 ± 0.6 °C) or commercial
345 refrigeration storage temperature (4 °C). The optimum activity of LISTEX™ P100 is
346 reported to be at 30-35 °C according MICREOS, the manufacturer of the phage.
347 Although LISTEX™ P100 is able to lyse bacteria at lower temperatures, the higher
348 temperatures are more conducive to the growth and efficacy of lytic bacteriophages such

as P100. Soni and others (2012) observed a higher efficacy of phage P100 in reducing *L. monocytogenes* counts by 5.6 log10 CFU/mL at 30 °C against a *L. monocytogenes* reduction of 3.1 $\log_{10}$ CFU/mL at 4 °C in TSB broth after 24 h compared to an untreated control. An additional explanation for the reduced effectiveness of phage in the alfalfa seeds during germination is the presence of favorable environmental conditions conducive to *L. monocytogenes* proliferation. In the current study, the average temperature was 24.5 ± 0.6 °C throughout the entire sprouting period. *L. monocytogenes* has shown growth at a temperature range of 0-45 °C (USFDA 2003). The moist environment conditions during the sprouting of seeds contribute greatly to the rapid growth and proliferation of pathogenic bacteria such as *L. monocytogenes*. In the current study, although the sample treated with LISTEX™ P100 showed a significant reduction in the *L. monocytogenes* population by 1.4 log10 CFU/g when compared to the untreated bacteria control after 24 h of sprouting, the effectiveness of the phage did not last throughout the germination period. In a similar observation, Kocharunchitt and others (2009) reported rapid growth (1 $\log_{10}$ CFU/g) of *Salmonella* on experimentally contaminated alfalfa seeds after the first 12 h of phage application.

The initial phage concentration level used in this study was based on a previous study by Leverentz and others (2003), which used a host culture of *L. monocytogenes* (5 x 10^7 CFU/ml) against a phage concentration of 1 x 10^7 PFU/ml to give a Multiplicity of Infection (MOI) of 0.2 PFU/CFU. In the current study, a *Listeria* concentration of 5.7 $\log_{10}$ CFU/g was used against a phage concentration of 7.7 $\log_{10}$ PFU/g, resulting in an MOI of 100 PFU/CFU. The use of higher MOIs has been found to result in greater bacterial reduction due to the presence of more phage particles (Garcia and others 2008;

Guenther and others 2009; Soni and Nannapaneni 2010). For example, Bigwood and others (2008) reported a significant reduction of >5.9 log CFU/g at 24 °C in *Salmonella*-inoculated raw and cooked beef when treated with phage at an MOI of 10^4 after 24 h. Similarly, the same study found that a high MOI of 10^5 resulted in reduction of *Camplyobacter jejuni* to below detectable levels in artificially contaminated chicken skin. In the current study LISTEX™ P100 was able to cause a significant reduction in *L. monocytogenes* population for the LPS sample only for the initial 24 h of sprouting compared to the LS sample. The LS sample showed a spike on day 2 of sprouting after which the *L. monocytogenes* counts remained stable throughout the sprouting period. Perhaps, increasing the MOI would help to improve the efficacy of the phage against *L. monocytogenes* throughout the sprouting period. For example, a LISTEX™ P100 dose of 10^8 PFU/g on salmon fillets inoculated with 10^4 CFU/g *L. monocytogenes* resulted in a ~3.5 log CFU/g reduction in the bacterial population, with an MOI of 10^4 (Soni and Nannapaneni 2010). However, lowering the phage concentration to 10^7 PFU/g while keeping the same *L. monocytogenes* concentration (10^4 CFU/g) resulted in a bacterial reduction of only 2 log CFU/g (Soni and Nannapaneni 2010). Kim and others (2007) observed regrowth of *Enterobacter sakazakii* in infant formula to 3.29 ± 1.48 and 4.94 ± 0.64 log CFU/ml from an initial inoculation of 2 log CFU/mL when treated with phage levels of 10^8 and 10^7 PFU/mL, respectively, whereas a higher phage concentration of 10^9 PFU/ml reduced the counts to non-detectable levels.

As reported by Chibeu and others (2013), subsequent progeny phages developed after bacterial lysis demonstrated lower phage efficiency than the initial infecting phage. This may help to explain the observed reduction in phage effectiveness after the initial 24

h in the current study. Furthermore, the surface complexity of alfalfa seeds could act as a hindrance for newly formed phages to access the target bacterial cells (Ly-Chatain 2014). The other factors contributing to the inefficacy of LISTEX™ P100 in alfalfa sprouts could be the neutralization of phages by host antibodies (Sulakvelidze and others 2001) and temporary phage resistance (Kocharunchitt and others 2009). However, it is unclear at this point if phage antibodies (if present) can cause a barrier for bacterial lysis by slowing the doubling time of bacteria (Sulakvelidze and others 2001).

In order for a technology to be applicable as a method of bio preservation, the stability of reacting particles over the food surface is of utmost importance. Soni and Nannapaneni (2010) found that the stability of LISTEX™ P100 did not differ significantly from an initial concentration of 8 log PFU/g to a final 7.4 log PFU/g in salmon fillets over 10-day storage at 4 °C. Similarly, Soni and others (2010) observed only a reduction in bacteriophage numbers from 7.3 $\log_{10}$ PFU/g to 5.5 $\log_{10}$ PFU/g and 5.2 $\log_{10}$ PFU/g in catfish fillets stored at 4 °C and 10 °C, respectively, for 10 days. Kocharunchitt and others (2009) observed that although phage SSP6 survived the presoaking and germination period of alfalfa seed sprouts, the phage numbers decreased by ~3 $\log_{10}$ PFU/g. In this study, similar results for the phage stability were obtained for seed sprouts throughout the sprouting and storage period. The phage numbers decreased from an initial concentration of 7.4 $\log_{10}$ PFU/g to 5.4 $\log_{10}$ PFU/g by day 7 of storage in LPS sample whereas the phage numbers decreased from 7.4 $\log_{10}$ PFU/g to 4.4 $\log_{10}$ PFU/g in PS sample. The higher decrease in phage numbers in PS sample could be attributed to unavailability of host bacteria for the phage to lyse. Also, the uneven surface areas of alfalfa seeds coupled with periodical rinsing during the sprouting process might have

caused phage particles to detach from the seed surface and wash-off with the rinsing water. The average phage numbers throughout the presoaking, sprouting and storage period for LPS sample and PS samples were 6.64 ± 0.98 $\log_{10}$ PFU/g and 5.85 ± 1.18 $\log_{10}$ PFU/g respectively (Figure 3). The results indicate that the phage survived the presoaking, sprouting and storage period of alfalfa seed sprouts.

Conclusions

Although bacteriophages have shown great potential as an alternative strategy in controlling foodborne diseases, the efficacy of phage-based biocontrol highly depends upon the complexity of the food matrix. This study has shown that LISTEX™ P100 may serve as a potential hurdle in controlling the proliferation of *Listeria monocytogenes* in alfalfa sprouts during the initial stages of germination. However, further understanding of phage delivery and phage optimization specifically for seed sprouts needs to be considered for effective biocontrol of foodborne pathogens. The use of lytic bacteriophages can act as an additional hurdle in decontamination and help in enhancing the safety of seeds and sprouts.

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Figures

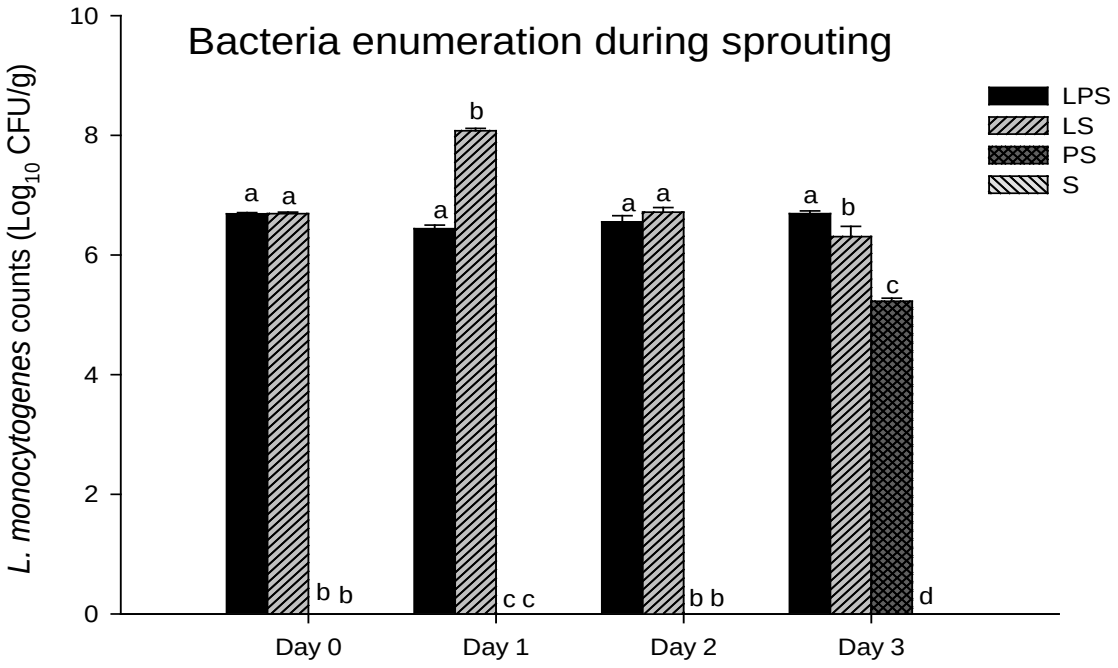

Figure 10. *L. monocytogenes* growth during alfalfa seed germination (24.5 ± 0.6 °C) compared between LPS (*Listeria monocytogenes* + P100 + alfalfa seeds), LS (*Listeria monocytogenes* + Alfalfa seeds), PS (P100 + alfalfa seeds) and S (Alfalfa seeds only) samples. Means with the same letter are not statistically different ($p < 0.05$) from other samples groups tested at each time

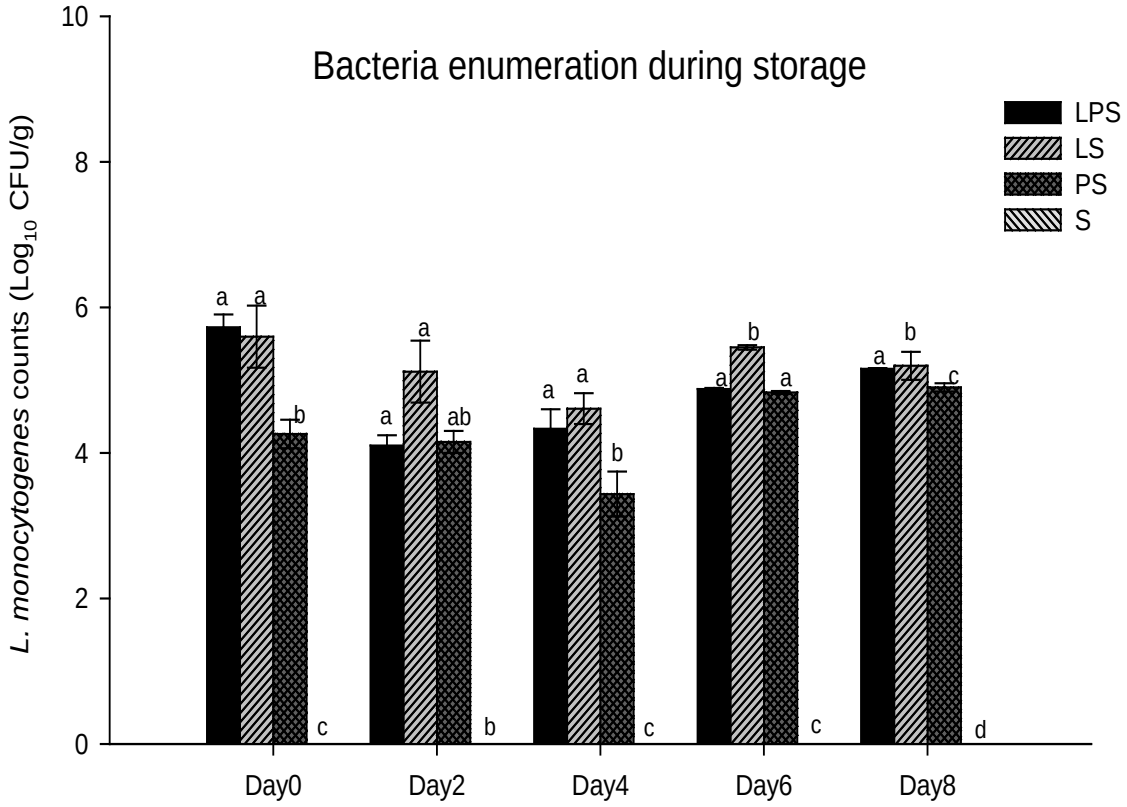

Figure 11. *L. monocytogenes* growth during alfalfa sprout storage (4 °C) compared between LPS (*Listeria monocytogenes* + P100 + alfalfa seeds), LS (*Listeria monocytogenes* + Alfalfa seeds), PS (P100 + alfalfa seeds) and S (Alfalfa seeds only) samples. Means with the same letter are not statistically different ($p < 0.05$) from other samples groups tested at each time point.

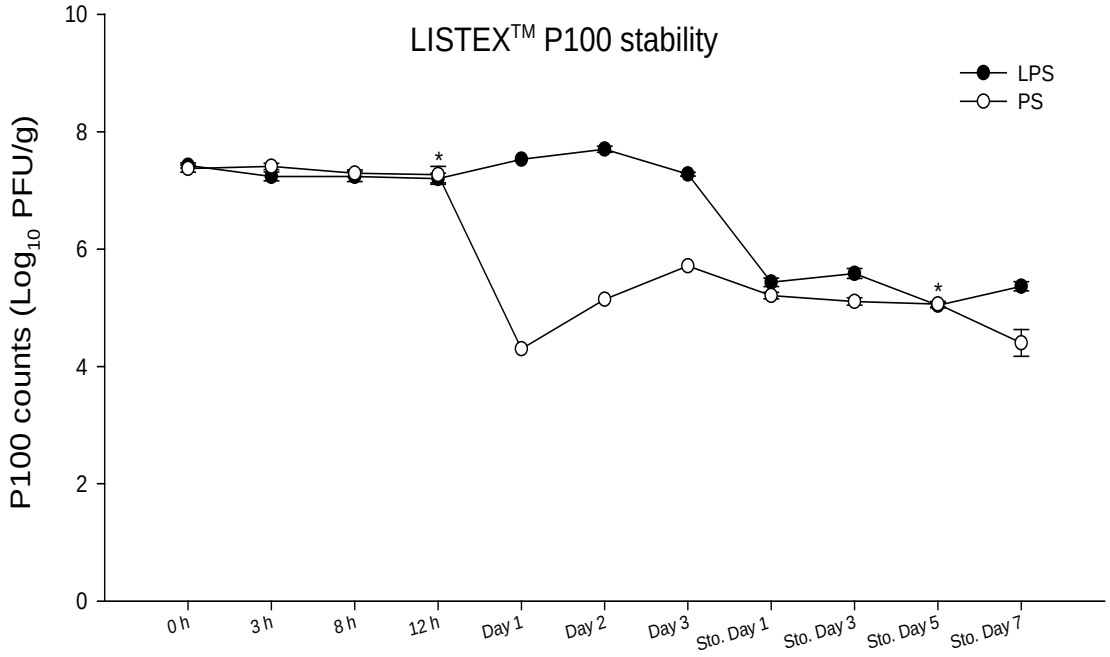

Figure 12. LISTEX™ P100 stability during seed germination and storage of alfalfa seed sprouts between LPS (*Listeria monocytogenes* + P100 + alfalfa seeds) and PS (P100 + alfalfa seeds). * indicates no significant difference ($p < 0.05$) between LPS and PS samples at the given time point.