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Novel Advancements for Improving Sprout Safety

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NOVEL ADVANCEMENTS FOR IMPROVING SPROUT SAFETY

A Dissertation Presented

By

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NOVEL ADVANCEMENTS FOR IMPROVING SPROUT SAFETY

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Kyle S Landry

ABSTRACT

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All varieties of bean sprouts (mung bean, alfalfa, broccoli, and radish) are classified as a "super-food" and are common staples for health conscious consumers. Along with the proposed health benefits, there is also an inherent risk of foodborne illness. When sprouts are cooked, there is little risk of illness. The purpose of this dissertation was to explore novel techniques to minimize or prevent the incidence of foodborne illness associated with the consumption of sprouts. Three areas were investigated: 1) the use of a biocontrol organism, 2) the use of a novel spontaneous carvacrol nanoemulsion, and 3) the influence of the sprouting environment, antimicrobial treatments, and the presence of pathogens on the microbiota of sprouts. Using a novel strain of *Serratia plymuthica*, the growth of *Salmonella* spp. or *E. coli* O157:H7 were either suppressed or inhibited on sprouts that were co-inoculated with *S. plymuthica* and either pathogen. A novel carvacrol nanoemulsion was developed and tested for its efficacy against contaminated sprouting seeds and storage stability. The initial formulation was able to inactive low levels (≤ 3 log CFU/g) of *S.* Enteritidis or *E. coli* O157:H7 on mung beans, alfalfa, and radish sprouting seeds, but not broccoli. It was

found that pH and high levels (≥10% v/v) of organic load significantly influenced the antimicrobial properties of the emulsion. With the addition of 50 mM acetic or levulinic acid, the treatment was able to inactivate 4 log CFU/g and 2 log CFU/g of pathogens on mung beans or broccoli seeds, respectively. The emulsion was found to be stable and still effective up to 30 days of storage at room temperature. Microbial population studies, utilizing a terminal restricted fragment length polymorphism analysis, showed that the microbiota differed between sprouting seed varieties. During the course of aseptic germination, there was a population shift which resulted in a less diverse population, mainly compose of Pseudomonadaceae. Sprouts that were commercially germinated had a more diverse population than aseptically germinated sprouts when seeds from the same distributer were used, suggesting that the sprouting environment can influence the final microbiota. The presence of pathogens resulted in a microbiota predominantly composed of Pseudomonadaceae and Enterobacteriaceae. Sprouting seeds that were treated with the carvacrol nanoemulsion resulted in a population comprised of mostly Pseudomonadaceae. Seeds that were initially contaminated with *S.* Enteritidis and treated with the carvacrol nanoemulsion had no detectable *Salmonella* restricted fragments or viable cells, suggesting complete inactivation of the pathogen. Sprouts will continue to a food for health conscious consumers. They will also be scrutinized for their chronic correlation with foodborne pathogens such as *Salmonella* spp. and *E. coli* O157:H7. It is vital that research continues in the areas of prevention, disinfection, and detection of pathogens on produce.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF IMAGES

Image **Page**

LIST OF DIAGRAMS

Diagrams **Page**

CHAPTER 1

INTRODUCTION

All varieties of bean sprouts (mung bean, alfalfa, broccoli, and radish) are classified as a “super-food” and are common staples for health conscious consumers. Along with the proposed health benefits, there is also an inherent risk of foodborne illness. When sprouts are cooked, there is little risk of illness. However, when consumed raw, the probability of foodborne illness is exacerbated. The sprouting process is ideal for microbial growth, lending to final aerobic counts of ≥ 9 log CFU/g of sprouts. Sprouting seeds containing any pathogenic bacteria, even at concentrations ≤ 0.1 CFU/g, may reach ≥ 8 log CFU/g of sprouts following germination.

As a result of frequent sprout related outbreaks, the Food and Drug Administration (FDA) released guidelines in 1999 which recommended treating sprouting seeds with a 20,000 ppm calcium hypochlorite soak prior to germination. Even after the implementation of these guidelines, sprout related foodborne illness continues to be a pertinent problem within the produce industry. As a result, research into acceptable and effective sprout disinfecting techniques continues to be a major focal point within the scientific community. The development of novel chemical, physical, and biological treatment techniques continues to advance produce safety.

Understanding the influence of current treatment techniques, environmental microbiota found during production, and pathogens in the final microbiome of sprouts is also an important aspect of sprout safety. Within the literature, papers regarding the bacterial population of commercial and lab grown sprouts can be found (65, 144, 151).

However, studies comparing the microbiota of sprouts from various distributers, growing environments, and/or antimicrobial treatment types are limited. It is unknown if the final sprout microbiota is a direct result of the bacterial communities present on the seeds, sprouting environment or both.

Each of these factors may promote or discourage the presence of pathogens and/or spoilage organisms. For example, seeds from a distributer from the west coast may have a different microbial community than seeds from the east coast. The same can be said for the environmental microbiota in processing plants and water sources between growers. The use of aquafer water, compared to the use of treated water may significantly influence the final microbial population. Understanding the effects of various pre-germination seed treatments on the final microbiome of the sprout is also limited. Calcium hypochlorite treatments may be more effective against one portion of the microbiota but not another, resulting in a change in the final microbiota of the sprout when compared to an untreated batch. These changes may be better, or they may provide the necessary foothold for more undesirable bacteria like pathogens and/or spoilage organisms. As previously mentioned, seeds contaminated with extremely low pathogen levels may have a final pathogen count greater than 8 log CFU/g of sprout (54, 86). It would be greatly beneficial to the sprout industry and food safety experts to understand how the initial low levels of pathogens affect the final phyllosphere.

1.1 Objectives

The purpose of this research is to explore and develop novel technologies that can be used to reduce the incidence of foodborne illness associated with the consumption of raw sprouts. With this said the objectives are as follows:

1) Determine the effectiveness of the antagonistic organisms *Serratia plymuthica* EJ against foodborne pathogens on contaminated sprouting seeds
2) Develop and determine the effectiveness of a novel carvacrol nanoemulsion against foodborne pathogens on contaminated sprouting seeds
3) Study the differences between lab grown and commercially grown sprout microbiota using the Terminal Restricted Fragment Length Polymorphism (T-RFLP) analysis

CHAPTER 2

LITERATURE REVIEW

2.1 The Lure of Sprouts: A Simple Functional Food

The number of consumers who are actively aware of their health and well-being has dramatically increased over the past few decades. As more foods are dubbed "super-foods", the idea that commonly available foods can treat or prevent certain medical conditions will become more predominate within the food industry (220, 226). With a continued awareness that eating healthy may help manage or prevent certain illness, consumers will not only look for foods to satisfy hunger but also to improve their overall health (162).

One of the most readily available groupings of functional foods are fruits and vegetables (225). All fruits and vegetables contain numerous functional compounds, many of which have been linked to improved health (105). With an increase in nutritional self-awareness, the evolution of the diet-health model has moved towards more minimally processed, additive-free, natural foods. Sprouts, such as mung bean, radish, broccoli, and alfalfa, have been of great interest for their proposed health benefits, resulting in a resurgence of consumer interest throughout Europe and the United States of America (76, 93, 108).

Sprouts are naturally high in secondary metabolites such as flavonoids, glucosinolates, S-methylcystine sulfoxide, and anthocyanins (124, 148). There is evidence that sprouts contain active compounds which may slow aging (15, 176) and help prevent or limit Alzheimer's disease (122), angiogenesis (253), asthma (198), and

various forms of cancer (69, 160, 202, 204). Out of all the phytochemicals found in sprouts, sulforaphanes have been the most recent and exciting addition to sprouts list of health claims. Sulforaphanes have been shown to increase the expression of cytoprotective genes that regulate numerous endogenous cellular defense mechanisms (127, 174, 233). It is believed that sulforaphanes are one of the main bioactive compounds responsible for the alleged health benefits of sprouts (105). As a result of increased research, awareness, and marketing of sulforaphanes functional properties, sprout manufactures are allowed to market certain varieties of sprouts as a cancer preventive dietary supplement (105).

The proposed health benefits unfortunately do not mitigate the fact that spouts are considered a "high-risk" food product by multiple agencies. The ever pressing demand for minimally processed "super foods" has elevated sprouts to a trending health product. As sprouts are incorporated into more diets of individuals throughout the world, the need for an effective but consumer and environmental friendly antimicrobial treatment is imperative.

2.2 Microbial Risks Associated with Sprouts

With an increased consumption of sprouts, there is a proportion increase in the risk of foodborne illness. Since the first report of foodborne illness linked to sprout based products in 1973, there have been numerous outbreaks worldwide (236, 241, 262). The consumption of raw sprouts was responsible for over 10% of produce related foodborne illness during the 12 year span between 1990 and 2002 (120). The majority were caused by *Salmonella* spp. and *Escherichia coli* contamination although other

pathogens such as *Listeria monocytogenes*, *Bacillus cereus,* and *Staphylococcus aureus* have also been linked to outbreaks (54, 62, 84, 228).

All varieties of sprouts are considered a raw agricultural product and subsequently, have the apparent microbiological risks associated with such commodities. It has been widely established and recognized that contaminated sprouting seeds are the major source of foodborne pathogens (62, 84, 98). The initial source or point of contamination throughout the sprouting process can be hard to establish. Firstly, sprouting seeds are harvested from fields and either directly transported to sprouting facilities or sent to a distribution facility. It is possible for seeds to be contaminated during this time with various pathogenic microorganisms such as *E. coli* O157:H7, *Bacillus cereus,* and/or *Salmonella* spp. (147, 152, 191). It has been demonstrated that sprouting seeds, such as alfalfa, mung bean, and radish, can harbor bacterial populations of 6 log CFU/gram of seed (98, 189, 194). These populations are often comprised of ubiquitous, non-pathogenic organisms, however there have been cases where non-O157 *E. coli* have been isolated from sprouting seeds suggesting some form of fecal contamination (5, 194, 217).

Unfortunately, the sprouting process is also ideal for microbial proliferation. Seeds are often soaked for 2 – 4 hrs in lukewarm water and germinated for up to 5 days at room temperature (58, 207). During this time, even extremely low pathogen concentrations will reach ≥ 5 log CFU/g in 24 hrs (84). The testing of seeds prior to germination for the presence of pathogens is a common practice for many sprout producers (58, 207). However, the sample of seeds taken from each batch is not exactly

representative of all seeds, since seeds can often have extremely low levels of pathogens (≤ 0.1 CFU/g) (84). Pathogens can also become internalized within the sprout during germination, further complicating the process (91, 255). Once internalized, the use of a topical disinfectant is ineffective. To fully understand the potential public health risk, one must focus on the two most common foodborne pathogens associated with sprout products, E. *coli* O157:H7 and *Salmonella enterica*.

2.2.1 *Escherichia Coli* O157:H7

Escherichia coli is a ubiquitous, generally harmless organism present in the environment and the GI tract of animals. However there are several variants of *E. coli* that commonly cause severe cases of foodborne illness resulting in gastroenteritis, urinary tract infections, and kidney failure. The major variants are: 1) enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and enterohemorrhagic *E. coli* (EHEC), with the latter being the most severe.

Within the EHEC family, *E. coli* O157:H7 is most often linked to foodborne outbreaks and recalls. *Escherichia coli* O157:H7 is a Gram negative, facultative anaerobic, rod shaped bacterium first isolated in 1982 from individuals who had consumed partially cooked hamburgers (158). *E. coli* O157:H7 is an uncommon, yet serious foodborne pathogen that is not bound to any specific food commodity (158, 188). *E. coli* O157:H7 virulence can be attributed to its ability to produce at least one of two possible Shiga toxins (Stx1 and/or Stx2). The severity of an infection, often mediated by the age and overall health of an individual, can range from asymptomatic

to lethal and anywhere in-between. Asymptomatic infections have been documented in varies outbreaks across the world. During an outbreak in England, over 15% of the tested population were found to be asymptomatic carriers of *E. coli* O157:H7 (101). A similar percentage (12% of the tested population) of asymptomatic carriers were also noted after an outbreak in Scotland that ultimately infected over 250 individuals and resulted in 17 deaths (48).

Even with reported asymptomatic infections, the majority of the infected population are symptomatic. Generally, healthy individuals would experience abdominal pain and non-bloody diarrhea for 1-4 days eventually progressing to bloody diarrhea (158, 237). In roughly 15% of those infected, the infection leads to hemolytic urea syndrome (HUS) 5-14 days following initial infection(237). Hemolytic urea syndrome is the diminishing of renal function leading to hypouresis or anuria (237). Renal impairment to this degree leads to increased urea and creatinine concentrations in the blood serum which results in the aggregation of platelets and fibrin in the microtubule vessels found within the kidneys (150). As red blood cells pass through the restricted blood vessels, they are fragmented and rendered inactive resulting in a condition known as microangiopathic hemolytic anemia (150, 188, 237).

In regards to foodborne HUS related instances, the most commonly affected age group is children under the age of five; the elderly and immunocompromised are also at an elevated risk (188). The occurrence of HUS in this age bracket may be high but is not definite. During an Irish outbreak, 60% of the asymptomatic infected population were children under the age of five (149). The Russian roulette infection style and potential

severity of *E. coli* O157:H7 infections makes it a high-profile pathogen whose potential presence in the food supply is alarming. The introduction of *E. coli* O157:H7 within the sprout industry is convoluted and unclear since the overall process from field to sprout is a multifaceted production with various opportunities for contamination.

The initial source of *E. coli* O157:H7 is classically linked to ruminant animals, such as cattle and sheep. It is within these where *E. coli* O157:H7 proliferates, with no harm to the ruminant host, and is eventually shed into the environment through fecal excretion. The number of infected animals is inconsistent as indicated by the vast differences in the reported numbers of infected animals around the world. For example, the percentage of infected cattle per herd have ranged from 0.2% to over 45% (114, 115). Wild birds, such as European Starlings (*Sturnus vulgaris*), are known carriers of *E. coli* O157:H7 and annually cause over $800 million dollars of agricultural damage in the United States (36, 50). The correlation between flocks of birds and contaminated produce, soil, irrigation water and animal feed has been studied for decades, yet a practical solution for this problem has yet to be discovered (36, 252).

Pre-harvest contamination of the mother plant, from which sprouting seeds are obtained, can come from a variety of sources, but the two must common culprits are manure and contaminated irrigation water(24). The use of improperly composted manure has been shown to dramatically increase the risk of contamination and is largely due to the fact that enteric pathogens can survive for extended periods of time in animal feces (109, 116, 168). Irrigation water may become contaminated when fecal excrement enters the supply during seasonal flooding or heavy rain storms. The transfer

of contaminated water sediments from one water supply to another has also been linked to crop contamination(95).

2.2.2 *Salmonella enterica*

Salmonella enterica is a Gram negative, facultative anaerobe with over 2500 serovars in 6 subgroupings (47). *S. enterica* is the leading cause of foodborne illness and is suspected of causing roughly 1.3 billion cases of foodborne illness annually, with symptoms ranging from mild intestinal discomfort to bacteremia and death (47). Among the serovars, typhoidal/enteric fever variants are the most concerning. The bacterium *S. enterica* serovar Typhi is responsible for human typhoid. Fever, malaise, intestinal distress, and nausea are the initial symptoms of typhoid which are often experienced a week or two after consuming *S.* Typhi contaminated food (47). As the disease progresses, the fever worsens along with an increased risk of hepatosplenomegaly and abdominal tenderness (49, 111). In some cases, salmonellosis may result in a reactive form of arthritis known as Reiter's syndrome 3-4 weeks after the initial infection. In healthy individuals the infection can be controlled by the body's defenses, however fluoroquinolones are often prescribed to reduce the duration of illness (111).

Non-typhoidal *Salmonella* serovars are responsible for an estimated 3 million deaths worldwide each year (179). Non-typhoidal strains, like typhoidal/enteric fever serovars, cause nausea, cramps, and diarrhea during the onset of infection, however symptoms occur between 6 – 72hrs following consumption and the infection does not trigger a fever (47). Non- typhoidal *Salmonella* infections can occur throughout the

whole intestinal track and in some cases result in enterocolitis and Reiter's syndrome (23, 155). If not systemic, infections last about one week in healthy individuals (23). If the infection is not limited to the gut, fluid and electrolyte replenishment plus antibiotics are often used (47).

The risk of *Salmonella* spp. infections will continue to be a major concern for the food industry since *Salmonella* spp. are ubiquitous throughout the farm-scape and are transported through various animal vectors (169). In regards to sprouting seeds, contamination is believed to occur prior to seed harvest or from human handling. Contaminated irrigation water or simply a flock of birds passing over a field can increase the risk of *Salmonella* spp. It was previously thought that *Salmonella* spp. survived poorly on plant surfaces during pre-harvest conditions, where they would be continuously exposed to UV light, desiccation, and temperature fluctuations; however this is not the case (106). Various studies have demonstrated *Salmonella* spp.'s ability to survive and proliferate under these conditions (106). Besides unwelcoming conditions, plant surfaces also have pre-existing aggregates or biofilms from resident organisms that compete with pathogens for essential nutrients (17, 254). It has been shown that *Salmonella* spp. can easily integrate and thrive in pre-existing resident multicellular aggregates on various plant phyllospheres and, as a result, be protected from desiccation stresses such as drying (25, 211).

Salmonella enterica serovars most commonly associated with sprout based outbreaks are of the non-Typhoid type, however there was a case in Colorado which linked *S.* Typhi to contaminated alfalfa sprouts (140). There have been a variety of non-

Typhoid serovars associated with sprouts, yet there seems to be no logical pattern between serovar, sprout variety, and/or location. A few reoccurring serovars include *S.* Newport, Saint Paul, Havana, Montevideo and Enteritidis (5, 31, 236).

2.2.3 Sprout Based Outbreaks

The apparent risk of foodborne illness from the consumption of sprouts is illuminated by the number of outbreaks over the past few decades. In July of 1996, school lunches prepared with white radish sprouts were responsible for 9,451 reported cases of *E. coli* O157:H7 infections throughout Japan (163). During this incident, 12 school children died and hundreds were hospitalized with complications (163). A year earlier, a major outbreak of *S.* Stanley in Finland and the Unites States was linked to contaminated alfalfa seeds obtained through the same distributor (147). Alfalfa sprouts were again responsible for an outbreak of hemorrhagic *E. coli* O157 in Minnesota and Colorado in 2003 (19, 63). Improper seed handling and disinfecting were to blame (63). Improper alfalfa seed disinfecting was also to blame for an *E. coli* O157:H7 outbreak in Virginia and Michigan between June and July of 1997 (26, 195). During the two month time frame, 82 people were infected, 36 of which required hospitalized and 4 experienced HUS (26).

One of the largest reported outbreaks linked to sprouting seeds occurred between May and June of 2011 in Germany and France and involved pathogenic *E. coli* O104:H4 (230). There were over 4,300 confirmed cases with 852 resulting in HUS and a total of 50 deaths (203). The source of *E. coli* O104:H4 was linked to a lot of fenugreek

seeds that had been imported from Egypt, and as a precautionary measure the German authorities banned the import of sprouting seeds from Egypt for 10 months (230).

The emergence of *E. coli* O104:H4 as a potential pathogen linked to sprouts is an ever greater indication that food safety practices are necessary to minimize the public health risks of sprouts. Unlike *E. coli* O157:H7, the *E. coli* O104:H4 strain from the German outbreak of 2011 was a hybrid organisms that had both characteristics of enterohemorrhagic and enteroaggregative *E. coli* (22). Termed an entero-aggregative-hemorrhagic *E. coli* (EAHEC), *E. coli* O104:H4 has the ability to produce Shiga-toxins (stx) and fimbriae used to adhere to the intestinal walls (22, 80). This hemorrhagic ability combined with a highly effective attachment/effacement mechanism makes *E. coli* O104:H4 a severe threat to food safety (45, 230).

Salmonella spp. is responsible for more produce based outbreaks than any other bacterium, making up over 50% of produce related outbreaks in both Europe and the United States (31, 222). *Salmonella* spp. was responsible for 70% and 65% of the sprout related outbreaks in the United States and Europe during 2004–2012 (31). During 2001, a multi-state outbreak of *S.* Enteritidis in Florida and Minnesota was connected to contaminated mung bean sprouts purchased at Asian markets and restaurants (243). It was determined that the mung beans, originally imported from China, distributed to Florida growers from a Kentucky based broker was the source of *S.* Enteritidis (243). The seed source was also the reason behind the *Salmonella enterica* serotype Bovismorbificans outbreak in Finland during 2009 (199). Alfalfa seeds purchased from an Italian grower/distributer had *S.* Bovismordificans concentrations of 4.3 MPN/g of seeds

(199). Unknown to the Finnish growers, the Italian grower/distributer did not test seed lots for *Salmonella* spp. prior to sale. In hindsight, this may have prevented the 42 confirmed cases of salmonellosis (199).

Salmonella enterica serovar Mbandaka was responsible for 82 confirmed cases of salmonellosis from January to April of 1999 in Oregon, Washington, Idaho, and California (98). Normally, the incidence of *S.* Mbandaka in Oregon was low, with an average of 1.5 cases per year attributed to that specific serovar (98). The rapid influx of *S.* Mbandaka cases in Oregon and surrounding states triggered an in-depth investigation that ultimately lead to the uncovering of a multistate outbreak (98). The contaminated seeds came from one California based farmer that was found to have multiple "probable" points for seed contamination which included river water and wild and/or domestic animal contact points (98).

The sheer number of sprout related outbreaks is quite alarming. The realization that sprouting seeds are the common vehicle for sprout contamination has redirected the focus of sprout safety on pathogen detection and the disinfection of sprouting seeds. Although it may seem simple in concept, the practical application, use and overall acceptance of disinfection techniques for sprouting seeds is quite challenging.

2.3 Current and Potential Disinfection Techniques for Sprouting Seeds

2.3.1 Challenges Associated with Sprouting Seeds

The majority of foodborne outbreaks associated with sprouts is linked to the sprouting seeds. The seeds of each sprout variety have different sizes, shapes, and

surface characteristics which may explain why there are more frequent outbreaks with certain varieties. For example, alfalfa have a rough or wrinkled topography which may enhance bacterial adhesion and protect pathogens from sanitizers (43, 74). Mung beans on the other hand, have a hard generally smooth seed coat that may prove easier to disinfect than rough seed varieties (43). Also, within in each seed lot there are variations between seeds; some seeds may have experienced excessive drying or physical damage resulting in surface cracks, allowing for the internalization of pathogens (43, 74, 255). Even an undamaged seed can allow for internalization. All seeds have a micropyle who's function is to allow water to enter the seed and trigger germination (14). The micropyle may also serve as an entering point for pathogens present in sprouting water. Seeds also have a large scar tissue area called the hilum (14). The size of the hilum is dependent on seed variety, however with all seeds, the hilum is a rough, porous area that can serve as an attachment site for microorganisms (**Image 1.1**).

The actual method of bacterial effacement to produce is complicated and still under debate. Pathogens must be able to quickly attach and successfully proliferate or survive under less than ideal conditions. The general seed surface, known as the testa, is a hard structure designed to limit dehydration and infiltration by phytopathogens and insects (14, 197). Pathogens, such as *Salmonella* spp., can attach to these surfaces and other produce surfaces in just a few hours (10, 118, 184, 246).

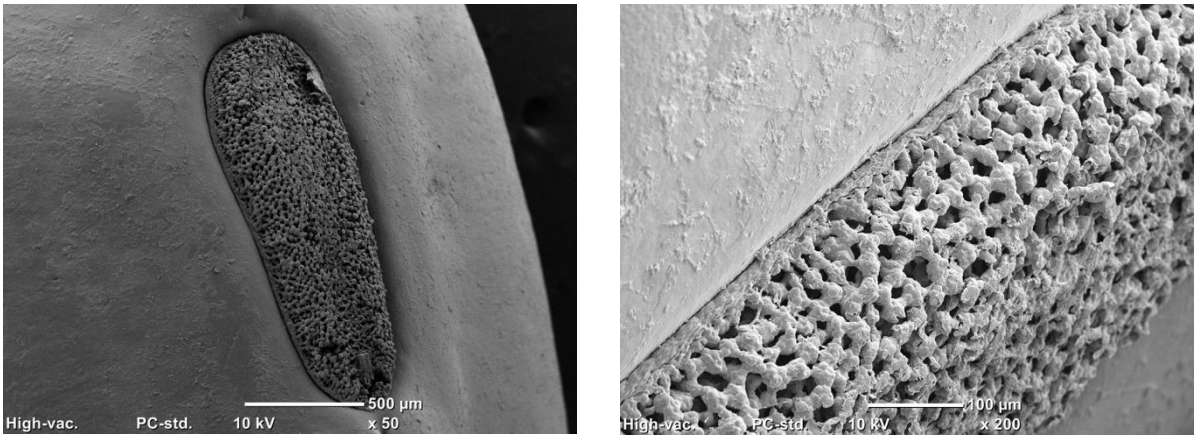

Image 1.1. Scanning microscopy of the hilum on a mung bean. The porous nature of the hilum may act as a point of entry and/or attachment for various pathogens.

When the testa is disrupted, a second seed coat, the tegmen, is exposed. Unlike the testa, the tegmen membrane is thin and papery in nature and possibly susceptible to bacterial penetration (213).

The farm to grower pipeline may be just as significant of a contributor to sprout based outbreaks as seed characteristics. Sprouting seeds can be obtained two ways; 1) they could be grown domestically or 2) they could be imported from various nations worldwide (21, 98, 199, 207). Domestically produced sprouting seeds could be contaminated by various means such as animal and/or human contact, inadequate farming practices, or contaminated run-off water. Even with these present risks, farms within the United States are still regulated by various agencies that instill and enforce farming and safety polices designed to limit risks (74, 76, 93). Though present, the safety polices of foreign countries from which sprouting seeds are often imported may not be as stringent as our domestic regulations, however the same can be possible for our own exported seeds. Imported seeds from China, Egypt, and Italy have been the cause of

multiple outbreaks around the world and were the result of seed lots that were not screened for the presence of pathogens (199, 222, 230).

Testing seed lots for the presence of pathogens is unreliable, as indicated by the number of outbreaks. First, the sample size often taken for microbiological testing is extremely small (5 – 10 grams) compared to the size of the seed lot (54, 98). Pathogens can also be underneath the seed coat, internalized, or severely stressed limiting their ability to be detected during pre-enrichment (199). Coupled with the fact that pathogens are heterogeneously distributed throughout seed lots, there is a high chance of a false negative (43, 236).

2.3.2 Current and Proposed Disinfecting Techniques

Due to the correlation between outbreaks and sprouts, the Food and Drug Administration (FDA) released a set of guidelines stressing the importance of applying a disinfectant to sprouting seeds prior to germination, citing and recommending a 20,000 ppm calcium hypochlorite soak (74, 78, 170). After implementation, sprout based outbreaks are still prevalent and continue to drive the search for a suitable, effective treatment.

Sprouting seed treatments fall into three main categories: chemical, physical, and biological, all of which have their own benefits and limitations.

2.3.3 Chemical Treatment Techniques for Sprouting Seeds

Antimicrobial chemical treatments are a widely used and studied method to combat microbial loads on all types of surfaces. Due to its proven effectiveness, it is only natural that chemical treatments would be suggested and strongly studied as a sprouting seed disinfectant. One major advantage of any chemical treatment is the convenience and affordability. Unfortunately, chemically based treatments are dependent on contact time, product – chemical ratio, stirring, and the presence of inhibiting or sequestering compounds (20, 54).

The treatment of sprouting seeds with a 20,000 ppm calcium hypochlorite soak prior to germination is the current recommended treatment as presented in the FDA's guide on reducing microbial food safety hazards for sprouting seeds (74). Variable *in vitro* data and the continued incidence of outbreaks suggests that the calcium hypochlorite is not a cure-all treatment for sprouting seeds. A quick literature search will reveal the inconsistencies with the calcium hypochlorite treatment. In one case, a 10 min 20,000 ppm calcium hypochlorite treatment was able to generate ~5 log reduction in *Salmonella* Stanley on alfalfa seeds (92). A later study, published in 2009, was only able to produce a 2.8 log reduction of a *Salmonella* spp. cocktail on alfalfa seeds following a 45 min soak in 20,000 ppm calcium hypochlorite (137). Over the past few decades, the reported pathogen reduction on sprouting seeds using the recommended hypochlorite treatment ranged between 0.5 – 6.90 log CFU/g (54).

With hypochlorites and any other reactive compound, the effectiveness is based on the amount of active compound available to interact with bacterial cells. The

antimicrobial activity of hypochlorite is based on two main reactions: 1)saponification and 2) chloramination (59). With saponification, hypochlorite breaks down triglycerides into fatty acid salts and glycerol (59). Hypochlorites solvent like properties can destroy key components needed for membrane function. Hypochlorites also neutralize amino acids which results in the release of hydroxyl ions forming hypochlorous acid (HOCl-) and hypochlorite ions (OCl-) (59). When hypochlorous acid comes in contact with organic tissue, chlorine is released and combines with amino groups forming chloramines (59). Chloramines interfere with cellular metabolism by oxidizing various cellular components, such as enzymes, lipid membranes, and nucleic acids.

These highly reactive, oxidative species are free to interact with various compounds/molecules in the surrounding environment with no degree of specificity (79, 83). Generated free radicals stay at the formation site and rarely diffuse throughout the system, however more oxidative species and radicals can be generated through chain-reaction based mechanisms and/or various secondary reactions (79, 83, 219). If a substantial number of free radicals are generated on a bacterium, cell death would result due to cytoplasmic membrane oxidation, protein denaturation, and oxidative destruction of DNA (60, 79, 87). The high pH (~12) of hypochlorites, from the rapid release of hydroxyl ions also promotes oxidation of bacterial cells (59).

The presence of an organic load, such as extraneous carbohydrates, lipids, proteins, or pre-existing bacteria, could sequester the majority of generated hypochlorous acid, hypochlorite and hydroxyl ions and prevent interaction with pathogenic cells (99, 215). Depending on the practices of sprout growers, the

continued presence of organic materials during seed decontamination may be limiting the overall effectiveness. Produce particulates, biofilms, and recycled wash water have all been successful at reducing the efficacy of hypochlorites and can be present during sprouting (5, 19, 96, 143, 175). There have been studies looking at increasing the concentration of hypochlorite and/or treatment time, however this approach is not ideal. High concentrations of hypochlorites and/or increased contact time has negative effects on sprout yield. The use of higher concentrations of hypochlorites can also have unfavorable environmental impacts and is not morally accepted by the majority of organic sprout producers (54, 257).

The demand for an alternative chemical treatment has resulted in rapid progress in this area of research. The diversity of proposed chemical treatments is vast. Seed treatment with organic acids (131, 264), fatty acids (38, 190), ammonia (110), alcohols (20), and electrolyzed water (113, 117) have all been studied as a potential alternative to hypochlorite. Reductions of some proposed treatments were as high as 7 log CFU/g, however the cost, reliability, and practicality of the alternative treatments were not fully realized (38, 170). Germination rates, sprout yield, quality, and flavor profiles are all important factors that may be affected and must be studied in order to judge the practicality of any proposed treatment.

The majority of proposed treatments focus on germination rates as a means of determining the effect of treatments on sprout quality. Germination rates are important for identifying the destructive nature of a treatment on the viability of post-treated seeds, but it is not indicative of overall sprout quality. Sprout yield, size, and

palatability are not represented by studying germination rates. Like pathogens, chemical compounds can bind to or become internalized within produce, which may alter the final flavor profile of the product (139, 193). Proposed sprouting seed treatments that utilize free fatty acids or essential oils fall victim to possible undesirable flavor changes (28, 29, 38, 190).

Free fatty acids are often synonymous with rancid aromas and taste sensations such as sour, bitter, pungent, and astringent; all flavor descriptors that are not necessarily ideal for consumers (37). Similarly, essential oils are pungent in aroma and flavor (28). Post-treatment effects on produce flavor and overall quality is limited in the literature, however the use of essential oils as an antimicrobial treatment is a very active research area (28).

Essential oils are natural compounds isolated from various plant sources that have gained wide acceptance as flavorings and antioxidant compounds (9, 28, 164). They also are natural, and in some cases, food-grade generally recognized as safe (GRAS) compounds which demonstrate a wide range of antimicrobial activity (28). Due to their standing, essential oils are of great interest to the food industry as a potential natural alternative to caustic disinfect treatments that have negative stereotypes with consumers (28, 54). There have been numerous studies addressing the antimicrobial properties of essential oils (28, 103). However, as with all oils, essential oils suffer from limited solubility in aqueous environments and it is not desirable to use pure compounds as an antimicrobial treatment for food products.

2.3.4 Physical Treatment Techniques for Sprouting Seeds

The use of physical methods to inactivate pathogens in food systems are well established and widely accepted throughout the food industry. During the past few decades heat and/or high-pressure have been studied as potential treatments for pathogen inactivation on sprouting seeds. The use of a heat treatment for mung beans is very common in many Asian countries (11, 12, 112). Mung beans are soaked in 85 ^{o}C water for 10 sec to reduce both pathogenic levels and spoilage organisms (57). Due to their thick seed coats, mung beans can withstand short durations of treatment with ≤85 ^{o}C water, making them ideal candidates for hot water treatments (234). Treatments ≥ 90 ^{o}C have been shown to have greater disinfection rates but results in a significant decrease in sprout yield (12). Other seed varieties, such as alfalfa and radish seeds do not have a thick outer coating and are more sensitive to hot water treatments (234). Hot water treatments of these varieties can result in decreased germination and sprout yields (119, 214).

High pressure treatments are another well studied methodology for disinfecting sprouting seeds. Bacterial reductions of ~3.5 log CFU/g of seed have been observed when alfalfa seeds were treated with 600 MPa of pressure for 2 min (172). When treated with less pressure (100 – 250 MPa) a 2 log CFU/g reduction on both mung beans and alfalfa seeds was observed (186). Higher pressure treatments (≥ 600 MPa) provide the greatest microbial reduction values (~5 log) but significantly decreases the germination rates of all seed varieties by up to 60% (7). The use of a pressure based treatment was found to be most effective when used in combination with another

treatment, such as a heat or chemical based treatment (171, 187). These hurdle based approaches resulted in ≥ 6 log and ≥ 5 log reduction in pathogenic bacterial respectively (171, 187).

The use of a physically based treatment protocol is more consistent, and in some cases more effective than commonly used chemical methods (54). However, unlike chemical treatments, physically based treatments require large, expensive, specialized equipment. Seed pasteurizers can cost in excess of $150,000, not include additional infrastructure or training costs.

2.3.5 Biological Treatment Techniques for Sprouting Seeds

The concept of using a bio-control agent to control fungal phytopathogens in agricultural settings is a well-researched area within the agricultural community. The perceived natural alternative to harsh pesticides is very attractive in concept and practicality, and may be another tool future farmers can use to help reduce pesticide usage (82, 141, 210). The use of bio-control organisms to prevent foodborne illness on fresh produce is an interesting area. There have been two proposed mechanisms for which biological control agents could be selected and applied: 1) isolate, identify, and characterize an organism(s) that is naturally antagonistic towards you target pathogen(s) or 2) use large inoculums or mixtures of a culture(s) that would thrive and out-compete any present or introduced pathogen(s). Both methods have been used successfully. For example, *Pseudomonad* spp. have been used as an antagonistic bio-control against various phytopathogens (258) and a mixture of competitive exclusion

organisms was used to prevent *Salmonella* spp. colonization in young poultry and swine (3, 94, 142).

The incidence of foodborne illness associated with the consumption of fresh produce has continued to increase, this is especially true for sprouts. Sprouts are produced in a warm, moist, hydroponic process which is also optimal for bacterial growth. Following the sprouting process, it is common for native microbial loads to reach as high as 6 – 9 log CFU/g (54, 68, 85). The same can be said for pathogens. It has been shown that seeds inoculated with less than 1 CFU/g yielded sprouts with pathogen counts over 6 log CFU/g (151, 262). The addition of bio-control organisms before or during the sprouting process may reduce or eliminate any present or accidently introduced pathogens.

Studies exploring the use of competitive exclusion and/or antagonistic organisms during the sprouting process seem to be the most successful at reducing/eliminating pathogenic bacteria. For example, a study present by Matos and Garland explored microbial communities of mature sprouts as a potential source for competitive exclusion organisms(151). They found that germinating sprouting seeds in water inoculated with an established mature sprout microbiota significantly reduced *Salmonella* spp. numbers in the final product(151). The group also demonstrated that the microbial communities found on mature sprouts had greater fitness in the sprouting environment than *Salmonella* spp.. They hypothesized that the native microbiota could process and utilize the available nutrients more efficiently than *Salmonella* spp., significantly reducing the amount of available nutrients (151). Various

strains of *Pseudomonades* spp. have demonstrated potential for being a suitable biocontrol agent for sprouts, generating a ~5 log reduction in *Salmonella* spp.. (64, 151).

Lactic Acid Bacteria (LAB) are a well-studied group of organisms that have potential as a bio-control organism. LAB are considered food grade and GRAS by the FDA and have been used to preserve various dairy and meat products(232). Their ability to inhibit the growth of pathogenic bacteria is due to the production of various antagonistic compounds such as organic acids, bacteriocins, hydrogen peroxide, and diacetyl (16, 46, 232). The use of LAB on produce as antagonistic organisms against foodborne pathogens is well established in the literature. Apples, leafy greens, melons, and other various fresh produce have been used as model systems to demonstrate the potential uses for LAB (138, 177, 244). With sprouts, the use of LAB as a means of reducing the incidence of foodborne illness from sprouts is limited. Numerous studies have demonstrated that LAB only reduce final numbers of *Salmonella* spp. or *E. coli* by ~2 log CFU/g (18, 260). Though a significant reduction in pathogenic cell numbers, there is still an inherent risk of foodborne illness.

More recently, it was demonstrated that *Salmonella* spp. levels could be controlled on mung bean and alfalfa sprouts using a combination of the antagonistic bacterium *Enterobacter asburiae* and lytic bacteriophages (262). When contaminated (initial inoculum of 6 log CFU/g) sprout seeds were germinated in the presence of both the antagonistic bacteria and lytic phages, no *Salmonella* spp. was detected via enrichment. Treatment with just the *E. asburiae* did not completely eradicate

Salmonella, but did result in a 7 log reduction when compared to the control. A similar reduction was observed when contaminated mung bean seeds where sprouted in the presence of *E. asburiae*.

CHAPTER 3

EFFECT OF *SERRATIA PLYMUTHICA* ON CONTAMINATED SPROUTING SEEDS

3.1 Abstract

There has been a multitude of *Salmonella* spp. and *E. coli* O157:H7 outbreaks associated with the consumption of bean sprouts throughout the world. The use of antagonistic, non-pathogenic organisms may help aid in the reduction of pathogens on bean sprouts, and ultimately limit the incidence of foodborne disease. In this study, the effectiveness of a novel stain (EJ) of *Serratia plymuthica* was evaluated against *S.* Enteritidis and *E. coli* O157:H7 contaminated sprouting seeds. Seeds were co-inoculated with various concentrations of pathogens and *S. plymuthica* and germinated for 5 days. Following germination, sprouts were tested for the presence of pathogens. Final *S.* Enteritidis and *E. coli* O157:H7 levels of < 3 CFU/g were found on mung bean and radish sprouts that had initial seed inoculums of 2 log CFU/g *S.* Enteritidis and 8 log CFU/g *S. plymuthica* EJ. The co-inoculated of mung bean or radish seeds with *S. plymuthica* EJ (8 log CFU/g) and 5 log CFU/g of *S.* Enteritidis reduced or maintained the final pathogen levels on sprouts at 4 log and 5 CFU/g respectively. The inoculation of broccoli seeds with *S. plymuthica* EJ resulted in significant reductions (> 3 log CFU) in final pathogen levels on broccoli sprouts. The novel strain of *S. plymuthica* was also found to produce extracellular siderophores and have the gene necessary for the production of the antagonistic compound pyrrolnitrin.

3.2 Introduction

As the demand for fresh produce increases, so does the inherent risk of foodborne illness. As outbreaks continue to be a common occurrence within our food

supply, the development of alternative treatment methodologies will remain a pertinent area of research. Classically, chemical and/or physical treatments were the main area of focus for new antimicrobial technologies. However, the practical application of biocontrol organisms has shown to be a potential alternative to the traditional areas of study.

Microbial based preservation methods have be used since the dawn of civilization; one such example is food fermentation. In today's health conscious society, the use of natural, old-world preservation techniques are more appealing than ever. The overall mindset surrounding bio-preservation is that the impact on food safety, food quality, and the environment are less than chemical and/or physio-chemical preservation methods (88). As for technical advantages, the use of bio-preservation techniques require little in advanced equipment and are, in theory, more cost effective (88). Secondly, the use of biological organisms may help reduce the propagation of antibiotic resistance among pathogenic species (81).

The mechanism by which antagonistic organisms function is broad. The lactic acid bacteria (LAB) are the most widely studied biocontrol organisms and have been shown to produce organic acids, anti-fungal compounds and various forms of bacteriocins (173). Though their main antagonistic mechanism is the production of organic acids, other inhibitory compounds such as diacetyl, propionate, bacteriocins and bacteriocins-like compounds are quite effective (173). Nisin, pediocin AcH/PA-1, lacticin 3147, and cyclic peptide enterocin AS-48 are currently available or have promising potential for commercial applications (4, 90).

There have been numerous studies assessing the effectiveness of antagonistic organisms against various foodborne pathogens on produce such as leafy greens, peppers, carrots, potatoes, and sprouts (135, 136, 242). The effectiveness of biocontrol organisms against foodborne pathogens is influenced by numerous factors including initial microbial populations, food matrices, and storage conditions (88, 221). These variables are believed to be the reasoning behind the wide range in effectiveness between tested produce varieties and between *in vitro* and *in vivo* models. Within a model system, *Pseudomonas* fluorescens, when co-inoculated with *L. monocytogenes*, was able to generate a > 3 log reduction in pathogenic cell numbers (27). Similar log reductions have also been noted for *Salmonella* spp., *E. coli* O157:H7, and phytopathogenic bacteria when tested using *in vitro* model systems (208, 245).

As previously stated, when a biocontrol organism's effectiveness is evaluated on actual food products the perceived results are often less encouraging than *in vitro* results (221). For example, the effectiveness of *Lactococcus lactis* against *L. monocytogenes* on alfalfa sprouting seeds was found to be significantly less than previous reported *in vitro* results(180). The group found that *L. monocytogenes* levels reached >6 log CFU/g of sprouts after 48 hrs when initially inoculated with 2 log CFU/g of seed(180). When co-inoculated (2 log CFU/g of seed) with *L. lactis*, final numbers of *L. monocytogenes* were only reduced by 1 log CFU/g (180). Similar results were also reported by Cai *et al.*(30). In both cases the effectiveness of *L. lactis* against contaminated alfalfa sprouting seeds was significantly less than previous reports in model systems (180, 221). The successful use of LAB to eliminate pathogenic organisms

on fresh produce has yet to be demonstrated. However, significant reductions and suppression of pathogenic growth has been widely demonstrated throughout the literature (88, 177, 245). Though impressive, the presence of any pathogenic organisms on produce would still present an inherent risk of illness.

The use of novel biocontrol organisms and/or other bio-preservation methods (i.e. bacteriophage and antagonistic peptides) have been demonstrated as potential alternatives for the treatment of produce. A cocktail of lytic phage specific for various *Salmonella* serotypes was evaluated as a treatment for contaminated sprouting seeds (183). Even though reductions in pathogen numbers were seen after 4 hrs post treatment, pathogen levels reached > 5 log CFU/g of seed after 24 hrs (183). However, the combined use of *Enterobacter asburiae* and lytic bacteriophage were able to reduce *Salmonella* spp. below detectable limits on both mung bean and alfalfa sprouting seeds (262). Similar results were reported when *Enterobacter asburiae* and lytic bacteriophage were used to treat *S.* Javiana contaminated tomatoes (263).

The use of bio-preservation techniques for bean sprouts is very attractive from both the industrial and consumer prospective. Currently, bean sprout growers are encouraged to soak sprouting seeds in a 2% calcium hypochlorite solution prior to germination (54). The implementation of this treatment has yet to curb the reoccurring incidence of sprout related foodborne illness. Novel physical and chemical treatment methodologies are promising areas of produce safety research, yet their practicality is often limited due to expensive, specialized equipment and/or potentially hazardous active ingredients. Bio-preservation techniques circumvent these problems, making it a

highly desirable and easily applicable commercial treatment. Sprout growers would be able to inoculate their seeds with biocontrol cultures and potentially prevent the growth of pathogens without the use of expensive equipment or caustic chemical treatments or additives. Also, any natural, environmentally friendly treatment would be rated highly by earth-friendly and health conscious consumers.

Recently, a strain of *Serratia plymuthica* was isolated from lettuce harvested from a local farm (Amherst, MA) which demonstrated antagonistic properties against *S.* Enteritidis and *E. coli* O157:H7 (56). The antagonistic properties of *S. plymuthica* EJ were found to inducible and heat resistant (56). Based on the strong antagonistic properties, *S. plymuthica* EJ was studied as a possible biocontrol organisms for sprouting seeds.

3.3 Materials and Methods

3.3.1 Bacterial strains and culture conditions

The bacterial strains used in the presented experiments were *Salmonella* enterica subspecies enterica serovar Enteritidis (ATCC BAA-1045), an enhanced green fluorescent protein (EGFP) expressing *Escherichia coli* O157:H7 (ATCC 42895), and the previously isolated *Serratia plymuthica* EF (56, 192). Stock cultures of each organism were stored at -80 ^{o}C in tryptic soy broth (TSB; BD Diagnostic Systems, Cat# DF0064-07-6) containing 25% (v/v) glycerol. Monthly, frozen stock cultures were transferred to working cultures by plating on tryptic soy agar (TSA; BD Diagnostic Systems, Cat# DF0370-075) slants/plates and incubating at 37 ^{o}C for 24 hrs. Following incubation, single colonies of E. coli O157:H7 were picked and transferred to Luria broth (Lennox,

LB) (Fisher BioReagents Cat# BP9724-500) plates containing 500 µg/mL ampicillin (Fisher Scientific Cat# BP1760-5). The absorbance at 600 nm was used to determine cells numbers, with an absorbance of 0.5 equal to 1.0 x 10^8 CFU as determined by plate counts.

Periodically, working cultures were streaked on differential media to ensure purity. For *S*. Enteritidis, cultures were spread on xylose, lysine, deoxycholate (XLD) agar (Remel Cat# R459902). For *E. coli* O157:H7, cultures were spread on LB (Fisher BioReagents Cat# BP9724-500) plates containing 500 µg/mL ampicillin (Fisher Scientific Cat# BP1760-5) and 20 µg/mL IPTG (Thermo Scientific Cat# FERR0392) and observed under UV light. Cultures were incubated overnight in TSB at 37 ^{o}C on a rotary shaker set at 150 RPM. All cultures were diluted with TSB to the desired cell numbers.

3.3.2 Generation of a 100 ug/mL nalidixic acid resistant S. plymuthica EJ strain

The isolated *S. plymuthica* EJ was inoculated in TSB containing 10 µg/mL nalidixic acid and incubated overnight at 32 ^{o}C. Overnight growth was streaked on TSA plates containing 10 µg/mL nalidixic acid and incubated overnight at 32 ^{o}C. An isolated colony was picked and inoculated in TSB containing 20 µg/ mL nalidixic acid and incubated overnight at 32 ^{o}C. This procedure was continued using 10 µg/mL intervals of nalidixic acid until a 100 µg/mL strain was obtained.

3.3.3 Induction of antagonistic properties of a 100 µg/mL S. plymuthica EJ strain

A single vertical streak of nalidixic acid resistant *S. plymuthica* EJ strain was plated in the middle of a TSA plate and allowed to incubate overnight at 32 ^{o}C. Following

incubation, the agar plate was aseptically flipped and a single left-to-right streak moving from top-to-bottom of either *S.* Enteritidis or *E. coli* O157:H7 was plated and incubated overnight at 32 °C. Antagonistic colonies were picked using a sterile needle and plated on TSA containing 100 µg/mL nalidixic acid.

3.3.4 Influence of S. plymuthica EJ supernatant on growth of foodborne pathogens

A single vertical streak of nalidixic acid *S. plymuthica* EJ strain was plated in the middle of a TSA plate and allowed to incubate overnight at 32 °C. Following incubation the agar plate was aseptically flipped and a single left-to-right streak moving top-to-bottom of either *S.* Enteritidis or *E. coli* O157:H7 was plated and again incubated overnight at 32 °C. Plates demonstrating antagonistic properties were aseptically transferred to sterile 250 mL Whirl-Pack bag containing 50 mL of TSB and stomached for 60 sec. The blended solution was then transferred to sterile 50 mL centrifuge tubes and centrifuged for 25 min at 13,400 RPM. The remaining supernatant was filter sterilized through a 0.45 µm syringe filter and transferred to a sterile 50 mL conical tube which was stored at 2 °C. Prior to use, the supernatant was tested for the presence of antagonistic properties by dropping 20 µL of supernatant on TSA seeded with 4 log CFU/mL of either *S.* Enteritidis or *E. coli* O157:H7 and incubated overnight at 32 °C. Antimicrobial activity was visualized by a zone of clearing in and around the drop area.

Overnight growth of either *S.* Enteritidis or *E. coli* O157:H7 was added to a test tube containing TSB and various concentrations of antagonistic extract (0%, 25%, 50%, or 75% (%v: %v); 9 mL total volume) for a final inoculum of 7 log CFU/mL. The cell

mixture (150 µL) was transferred to a 96-well plate (previously sterilized with 70% ethanol), covered, and incubated for various time intervals (6, 12, or 18 hrs) at 37 ^{o}C. At each time point the absorbance was read at 570 nm using a Bio-Tek ELX 800 plate reader (Bio-Tek; Winooski, VT).

3.3.5 Storage viability of S. plymuthica EJ on mung beans

Batches of beans (25 g) were soaked in a 50 mL suspension of 100 ug/ml nalidixic acid resistant *S. plymuthica* EJ (9 log CFU/mL) for 20 mins for a final inoculum level of 8 log CFU/g. The inoculated beans were then transferred to a sterile glass petri dish containing sterile filter paper within a biological safety cabinet, and allowed to dry overnight at ambient temperature. Once dried the inoculated beans were stored for various time intervals (2, 4, 6, 12, 20, 25, or 30 days) at either 20 ^{o}C or 4 ^{o}C.

To determine surviving cell numbers, each batche of beans were transferred to sterile 250 mL beakers containing 50 mL of 0.1% peptone water and placed on a rotary shaker set to 125 RPM for 15 mins. A dilution series was created and plated on the appropriate media. Spread plates were incubated at 32 ^{o}C for 24 hrs.

3.3.6 Suppression of *Salmonella* Enteritidis *or Escherichia coli* 0157:H7 on Sprouting Seeds

All beans/seeds were generously provided by Jonathan's Organics (Rochester, MA) and each treatment condition was tested and sprouted in triplicate. Beans/seeds (25g) were soaked in 50 mL (6 log, or 3 log CFU/mL) of *S.* Enteritidis or CyGe *E. coli* O157:H7 for 20 mins. Inoculated beans/seeds were transferred to 50 mL suspension of

100 ug/ml nalidixic acid resistant *S. plymuthica* EJ (9 log CFU/mL) and soaked for an additional 20 mins. The inoculated beans/seeds were then transferred to a sterile glass petri dish containing sterile filter paper within a biological safety cabinet, and allowed to dry overnight at ambient temperature.

For mung beans, inoculated beans were placed in a 500 mL container and soaked in 150 mL of distilled water at 20^{o}C for 24 hrs. The water was removed, and sprouting continued for 4 more days, with daily water by a 5-min soak in 150 ml of distilled water.

Alfalfa, broccoli, and radish seeds (20 g) were poured in sterile 250 mL beakers and soaked in 150 mL of distilled water at 20 ^{o}C for 24 hrs. The water was drained, and seeds were transferred to sterile plastic trays lined with paper towels and germinated for 5 days at 20 ^{o}C. Seeds were watered four times a day with sterile deionized water.

Sprouts were weighed to determine their yield and two 25 g batches were taken for microbiological testing. The samples were suspended in 225 mL of 0.1% peptone water and stomached for 60 s. A dilution series was created and plated on the appropriate media. For *S.* Enteritidis, dilutions were spread on XLD. For *E.* coli O157:H7, dilutions were spread on LB plates containing 500 µg/mL ampicillin and 20 µg/mL IPTG and observed under UV light. For *S. plymuthica* EJ, dilutions were spread on TSA containing 100 ug/mL nalidixic acid. Samples were incubated at either 32 or 37 ^{o}C for 24 hrs. For samples with low *S.* Enteritidis *or E. coli* 0157:H7 inoculation levels a most probable number (MPN) assay was used. Samples were appropriately diluted in LB *Salmonella* enrichment or LB broth containing 500 µg/mL ampicillin and incubated

overnight. Any positive tubes were streaked for confirmation. Total aerobic counts were determined by plating dilutions on TSA.

3.3.7 Detection of the antagonistic prnC pyrrolnitrin gene

The PCR was used to determine the possible production of the antagonistic compound pyrrolnitrin. Primers sets were designed by aligning the prnC gene of *S. plymuthica* (GenBank: JF274257.1 and NC_021659.1) and *P. fluorescens* (GeneBank: DQ058621.1 and DQ058619.1) using Clustal in SeaView (100). The prnC gene was amplified using 20 µL Fast-Mix French PCR pre-mix tubes (Bulldog Bio Cat# 25185) with primers prnCF (5'-CAG GAG CAC GAC CCG AAG GAG TT-3') and prnCR (5'-GGT AGG ACG GGT GCA TCC AGT GC-3'), using the manufactures recommended procedure .

Amplification was performed with a C1000 Thermal Cycler (Bio Rad, Hercules, CA) using a cycle program consisting of a 3 min initial denaturation step (95 ^{o}C); 30 cycles of 95 ^{o}C for 60 sec, 75 ^{o}C for 15 sec, and 72 ^{o}C for 30 sec; and an 8 min final extension step at 70 ^{o}C. Amplification products were separated on a 1% agarose gel and visualized under UV light using Midori Green nucleic acid stain (Bulldog Bio, Cat #MG06). *P. fluorescens* was used as a positive control.

3.3.8 Detection of extracellular siderophores

S. Plymuthica EJ was plated on CAS media which was prepared according to Schwyn and Neilands (209). Inoculated plates were incubated at 32 ^{o}C for 24 hrs. The presence of a pink halo surrounding the colony was indicative of siderophore activity.

3.4 Results

3.4.1 Effect of S. plymuthica EJ extract on the growth of S. Enteritidis and E. coli O157:H7 in vitro

The antagonistic properties of *S. plymuthica* EJ were only observed when grown in the presence of either pathogen. Therefore, *S. plymuthica* EJ was grown with either pathogen and a cell fee extract was prepared as explained in section 3.3.4. Prior to each test, the antimicrobial effect of the extract was tested by dropping 20 µL of extract on a TSA seeded with 4 log CFU/mL either *S.* Enteritidis or *E. coli* O157:H7. Antimicrobial activity was visualized by a zone of clearing in and around the drop, as seen in **Figure 3.1**.

Figure 3.2 shows the effect of the antagonistic supernatant on the growth of either *S.* Enteritidis or *E. coli* O157:H7. The addition of antagonistic supernatant had a significant effect on the growth of both pathogens. Following an 18 hr incubation, there was no significant difference in growth of *S*. Enteritidis in TSB containing a final volume consisting of 75% antagonistic extract when compared to Time 0. The growth of *E. coli* O157:H7 in the presence of 75% antagonistic extract was reduced by ~20% when compared to the control.

3.4.2 Suppression of S. Enteritidis or E.coli O157:H7 on sprouting seeds

The effectiveness of *S. plymuthica* EJ on contaminated mung bean, alfalfa, broccoli, and radish seeds are summarized in **Tables 3.1 – 3.8**. Based on the results of an MPN assay, final *S*. Enteritidis and *E. coli* O157:H7 levels of < 3 CFU/g were found on

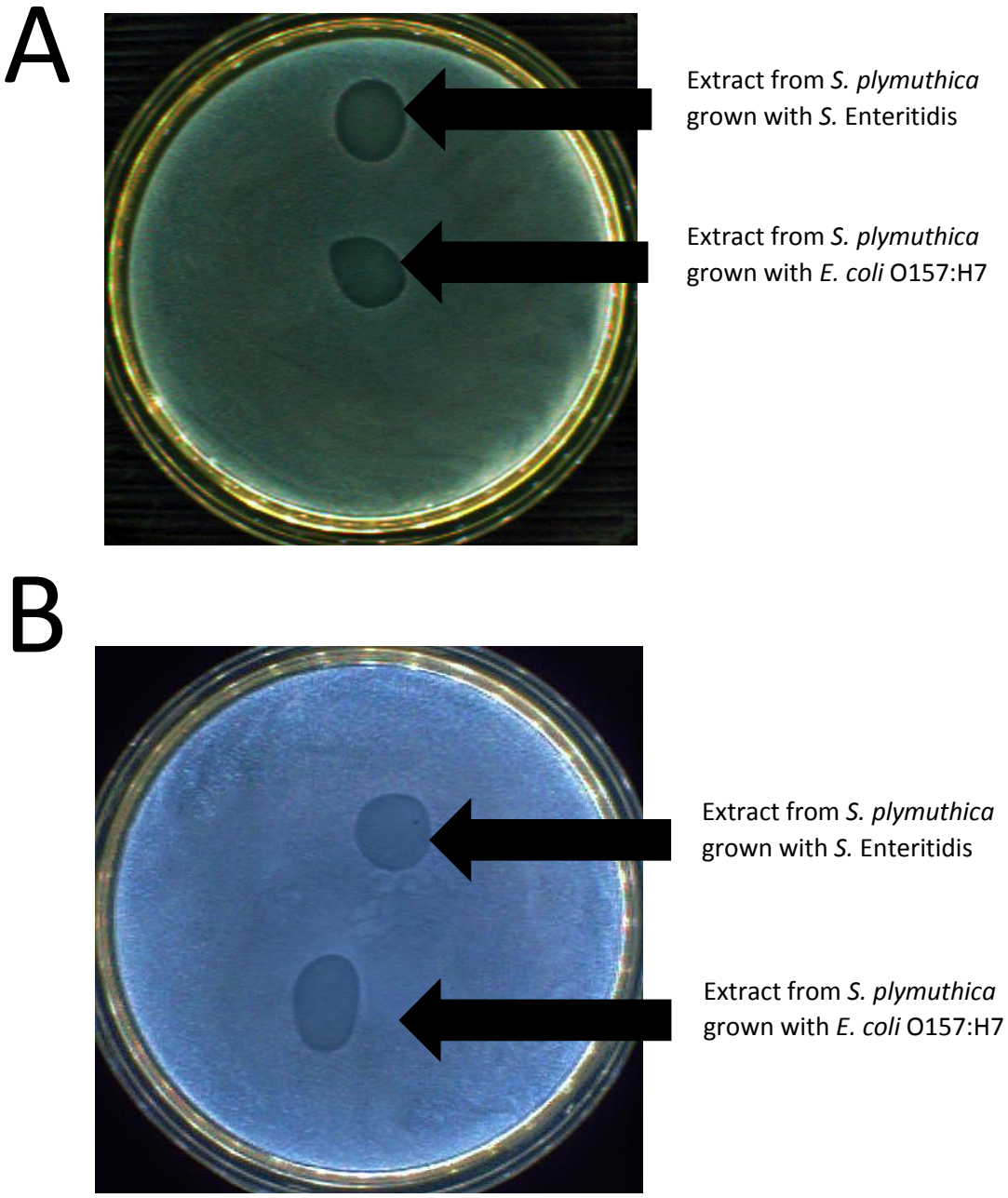

Figure 3.1. The effect of a *S. plymuthica* extract on either *S.* Enteritidis (A) or *E. coli* O157:H7 (B) seeded (4 log CFU/mL) TSA plates. A 20 µL drop of *S. plymuthica* extract was placed on a seeded TSA plate and incubated overnight at 32 ^{o}C.

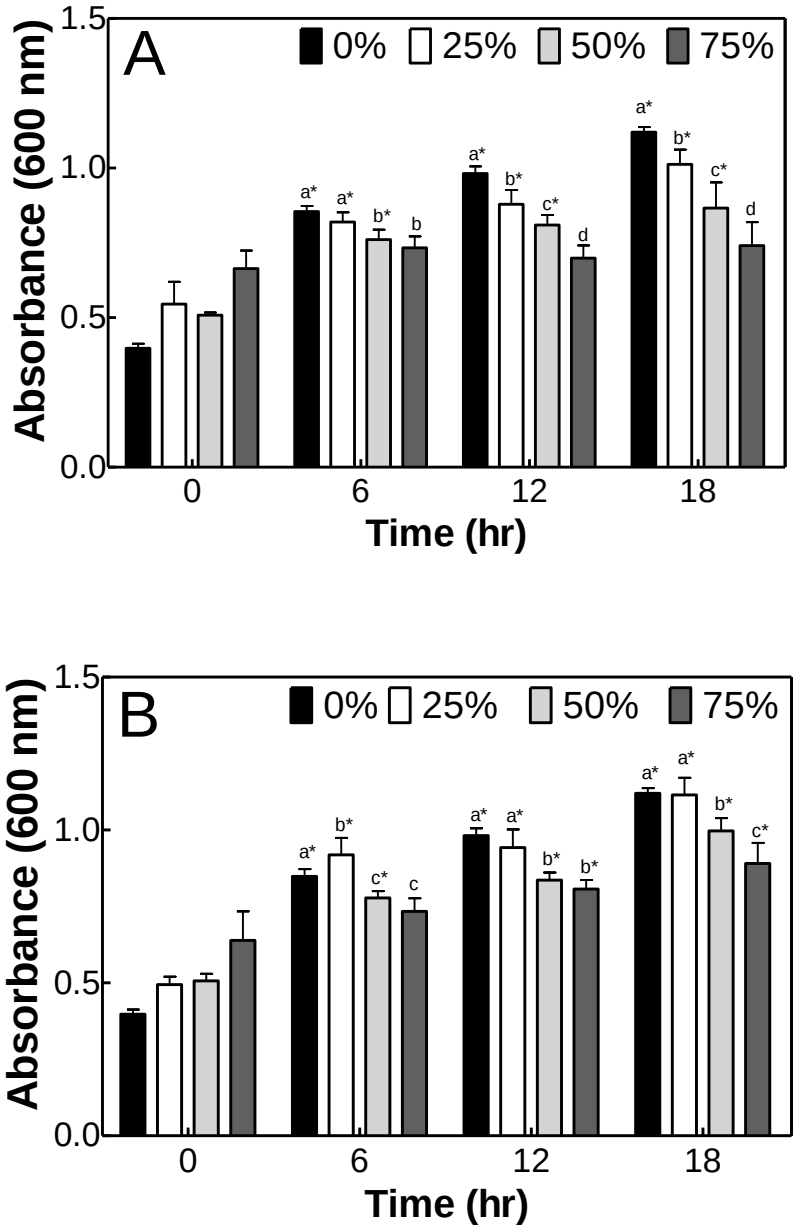

Figure 3.2. The effect of a *S. plymuthica* extract on either *S.* Enteritidis (A) or *E. coli* O157:H7 (B) growth in relation to absorbance. A cell suspension (100 µL) with various concentrations of extract was transferred to a sterile 96-well plate and incubated at 37 ^{o}C for various time intervals. The absorbance at each time point was read at 570 nm using a Bio-Tek ELX 800 plate reader.

mung bean and radish sprouts that had initial seed inoculums of 2 log CFU/g *S.* Enteritidis and 8 log CFU/g *S. plymuthica* EJ (**Tables 3.1 – 3.4**). When *S. plymuthica* EJ (8 log CFU/g) was co-inoculated on mung bean or radish seeds containing 5 log CFU/g of *S.* Enteritidis, final pathogen levels on sprouts were reduced to or maintained at 4 log and 5 CFU/g respectively. The inoculation of broccoli seeds with *S. plymuthica* EJ resulted in significant reductions (> 3 log CFU) in final pathogen levels on broccoli sprouts when co-inoculated with 2 log CFU/g of either *E. coli* O157:H7 or *S.* Enteritidis (**Tables 3.7 and 3.8**) . However, when 8 log CFU/g of *S. plymuthica* EJ was co-inoculated with 5 log CFU/g of either pathogen, final pathogen levels grew > 8 log CFU/g of sprouts. Co-inoculation of *S. plymuthica* EJ with *E. coli* O157:H7 or *S.* Enteritidis on alfalfa seeds did not result in any reduction in final pathogen levels, regardless of the initial pathogen inoculation concentration (**Tables 3.5 and 3.6**).

3.4.3 Detection of antagonistic compounds present in S. plymuthica EJ

The potential presence of two antagonistic compounds, pyrrolnitrin and extracellular siderophores were investigated. The presence of the gene *prnC*, which is necessary for the production of pyrrolnitrin was determined using the PCR with *P. fluorescence* as a positive control. As seen in **Figure 3.3**, the PCR resulted in bands within the size for the targeted gene for both *S. plymuthica* EJ and *P. fluorescence.* Even though the gene *prnC* is present within the genome of *S. plymuthica* EJ, further studies are needed validate the expression and production of pyrrolnitirin. *S. plymuthica* EJ tested positive for the production of extracellular siderophores as indicated by the pink halo present on CAS media (**Figure 3.4**).

Table 3.1. Effect of *S. plymuthica* EJ against *S.* Enteritidis contaminated mung beans[1]

Sample	Bean Yield (g)	*S.* Enteritidis (log CFU/g)	*S. plymuthica* EJ (log CFU/g)	Total Aerobic Count (log CFU/g)
Control	78.4 ± 1.7	ND	ND	9.21 ±0.17
S. Enteritidis (2 log CFU/g)	75.2 ± 7.2	9.03 ± 0.65	ND	9.33 ± 0.35
S. Enteritidis (5 log CFU/g)	77.6 ± 3.45	8.22 ± 0.30	ND	9.42 ± 0.27
S. plymuthica (8 log CFU/g)	77.3 ± 2.2	ND	9.15 ± 0.17	9.10 ± 0.48
S. Enteritidis (5 log CFU/g) *S. plymuthica* (8 log CFU/g)	79.6 ± 2.5	4.31 ± 0.05	9.18 ± 0.15	9.17 ± 0.75
S. Enteritidis (2 log CFU/g) *S. plymuthica* (8 log CFU/g)	76.5 ± 2.15	ND	9.05 ± 0.25	9.19 ± 0.40

[1]All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g.

Table 3.2. Effect of *S. plymuthica* EJ against *E. coli* O157:H7 contaminated mung beans[1]

Sample	Bean Yield (g)	*E. coli* O157:H7 (log CFU/g)	*S. plymuthica* EJ (log CFU/g)	Total Aerobic Count (log CFU/g)
Control	75.5 ± 1.4	ND	ND	9.32 ±0.09
E. coli O157:H7 (2 log CFU/g)	71.9 ± 3.5	8.97 ± 0.32	ND	9.58 ± 0.12
E. coli O157:H7 (5 log CFU/g)	77.4 ± 1.8	8.37 ± 0.11	ND	9.12 ± 0.19
S. plymuthica (8 log CFU/g)	76.3 ± 1.3	ND	9.25 ± 0.17	9.19 ± 0.53
E. coli O157:H7 (5 log CFU/g) *S. plymuthica* (8 log CFU/g)	75.5 ± 2.9	4.16 ± 0.14	9.18 ± 0.15	9.09 ± 0.10
E. coli O157:H7 (2 log CFU/g) *S. plymuthica* (8 log CFU/g)	76.8 ± 1.14	ND	9.17 ± 0.10	9.29 ± 0.63

[1]All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g.

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Table 3.3. Effect of *S. plymuthica* EJ against *S.* Enteritidis contaminated radish seeds[1]

Sample	Bean Yield (g)	*S.* Enteritidis (log CFU/g)	*S. plymuthica* EJ (log CFU/g)	Total Aerobic Count (log CFU/g)
Control	80.2 ± 8.5	ND	ND	9.17 ±0.27
S. Enteritidis (2 log CFU/g)	84.6 ± 6.5	8.91 ± 0.49	ND	9.51 ± 0.43
S. Enteritidis (5 log CFU/g)	76.3 ± 6.4	9.61 ± 0.95	ND	9.01 ± 0.23
S. plymuthica (8 log CFU/g)	84.3 ± 4.7	ND	9.26 ± 0.25	9.75 ± 0.58
S. Enteritidis (5 log CFU/g) *S. plymuthica* (8 log CFU/g)	85.2 ± 4.3	5.88 ± 0.61	9.27 ± 0.79	9.30 ± 0.20
S. Enteritidis (2 log CFU/g) *S. plymuthica* (8 log CFU/g)	83.5 ± 13.5	ND	9.40 ± 0.70	9.79 ± 0.45

[1]All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g.

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Table 3.4. Effect of *S. plymuthica* EJ against *E. coli* O157:H7 contaminated radish seeds[1]

Sample	Bean Yield (g)	*E. coli* O157:H7 (log CFU/g)	*S. plymuthica* EJ (log CFU/g)	Total Aerobic Count (log CFU/g)
Control	80.2 ± 3.04	ND	ND	9.41 ±0.27
E. coli O157:H7 (2 log CFU/g)	86.3 ± 7.1	9.40 ± 0.30	ND	9.36 ± 0.27
E. coli O157:H7 (5 log CFU/g)	78.5 ± 6.7	9.31 ± 0.43	ND	9.61 ± 0.35
S. plymuthica (8 log CFU/g)	82.1 ± 3.6	ND	8.89 ± 0.28	9.77 ± 0.05
E. coli O157:H7 (5 log CFU/g) *S. plymuthica* (8 log CFU/g)	81.1 ± 2.06	5.15 ± 0.44	9.82 ± 0.60	9.51 ± 0.48
E. coli O157:H7 (2 log CFU/g) *S. plymuthica* (8 log CFU/g)	83.2 ± 4.5	ND	9.42 ± 0.17	9.33 ± 0.51

[1]All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g.

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Table 3.5. Effect of *S. plymuthica* EJ against *S.* Enteritidis contaminated alfalfa seeds[1]

Sample	Bean Yield (g)	*S.* Enteritidis (log CFU/g)	*S. plymuthica* EJ (log CFU/g)	Total Aerobic Count (log CFU/g)
Control	107.8 ± 11.7	ND	ND	9.63 ±0.14
S. Enteritidis (2 log CFU/g)	112.0 ± 6.5	8.95 ± 0.25	ND	9.13 ± 0.12
S. Enteritidis (5 log CFU/g)	101.8 ± 3.3	9.57 ± 0.33	ND	9.63 ± 0.49
S. plymuthica (8 log CFU/g)	115.8 ± 13.2	ND	9.64 ± 0.68	9.70 ± 0.12
S. Enteritidis (5 log CFU/g) *S. plymuthica* (8 log CFU/g)	108.0 ± 9.23	9.26 ± 0.16	8.99 ± 0.12	9.44 ± 0.68
S. Enteritidis (2 log CFU/g) *S. plymuthica* (8 log CFU/g)	97.8 ± 6.74	8.91 ± 0.13	9.39 ± 0.26	9.57 ± 0.44

[1]All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g.

Table 3.6. Effect of *S. plymuthica* EJ against *E.coli* O157:H7 contaminated alfalfa seeds[1]

Sample	Bean Yield (g)	*E. coli* O157:H7 (log CFU/g)	*S. plymuthica* EJ (log CFU/g)	Total Aerobic Count (log CFU/g)
Control	99.8 ± 1.5	ND	ND	9.48 ±0.32
E. coli O157:H7 (2 log CFU/g)	104.3 ± 9.1	9.19 ± 0.27	ND	9.34 ± 0.15
E. coli O157:H7 (5 log CFU/g)	102.9 ± 5.8	9.12 ± 0.63	ND	9.70 ± 0.65
S. plymuthica (8 log CFU/g)	109.5 ± 20.1	ND	9.23 ± 0.25	9.30 ± 0.37
E. coli O157:H7 (5 log CFU/g) *S. plymuthica* (8 log CFU/g)	99.9 ± 12.3	9.07 ± 0.31	9.19 ± 0.21	9.49 ± 0.19
E. coli O157:H7 (2 log CFU/g) *S. plymuthica* (8 log CFU/g)	110.4 ± 12.5	8.97 ±0.39	9.11 ± 0.42	9.17 ± 0.16

[1]All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g.

Table 3.7. Effect of *S. plymuthica* EJ against *S.* Enteritidis contaminated broccoli seeds[1]

Sample	Bean Yield (g)	*S.* Enteritidis (log CFU/g)	*S. plymuthica* EJ (log CFU/g)	Total Aerobic Count (log CFU/g)
Control	93.3 ± 9.7	ND	ND	9.63 ±0.52
S. Enteritidis (2 log CFU/g)	92.9 ± 3.5	9.01 ± 0.50	ND	9.06 ± 0.36
S. Enteritidis (5 log CFU/g)	107.2 ± 8.5	9.26 ± 0.24	ND	9.20 ± 0.28
S. plymuthica (8 log CFU/g)	95.7 ± 8.1	ND	9.65 ± 0.39	9.28 ± 0.15
S. Enteritidis (5 log CFU/g) *S. plymuthica* (8 log CFU/g)	104.8 ± 14.5	8.94 ± 0.43	9.32 ± 0.13	9.39 ± 0.39
S. Enteritidis (2 log CFU/g) *S. plymuthica* (8 log CFU/g)	101.7 ± 12.1	1.42 ± 0.14	9.45 ± 0.15	9.69 ± 0.36

[1]All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g.

Table 3.8. Effect of *S. plymuthica* EJ against *E.coli* O157:H7 contaminated broccoli seeds[1]

Sample	Bean Yield (g)	*E. coli* O157:H7 (log CFU/g)	*S. plymuthica* EJ (log CFU/g)	Total Aerobic Count (log CFU/g)
Control	102.9 ± 4.1	ND	ND	9.35 ±0.10
E. coli O157:H7 (2 log CFU/g)	103.9 ± 6.7	9.14 ± 0.17	ND	9.41 ± 0.07
E. coli O157:H7 (5 log CFU/g)	98.2 ± 2.7	9.58 ± 0.19	ND	9.36 ± 0.37
S. plymuthica (8 log CFU/g)	100.6 ± 12.8	ND	9.03 ± 0.11	9.88 ± 0.80
E. coli O157:H7 (5 log CFU/g) *S. plymuthica* (8 log CFU/g)	97.7 ± 4.4	8.96 ± 0.40	9.63 ± 0.44	9.97 ± 0.20
E. coli O157:H7 (2 log CFU/g) *S. plymuthica* (8 log CFU/g)	97.2 ± 5.7	1.41 ±0.50	9.44 ± 0.32	9.84 ± 0.34

[1]All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g.

3.4.4 Storage viability of S. plymuthica EJ on mung beans

If *S. plymuthica* is to be used as a biocontrol agent, it is important to understand the survival of this organism on the surface of sprouting seeds. The storage viability of *S. plymuthica* EJ dried on mung beans and stored at 4 ^{o}C and 20 ^{o}C can be seen in **Figure 3.5**. Mung beans stored for 30 days at 4 ^{o}C had a 2.1 log CFU/g reduction in *S. plymuthica* EJ whereas beans stored at 20 ^{o}C had a 3.6 log CFU/g reduction. Storage at 20^{o}C resulted in significantly ($p < 0.05$) fewer viable cells than refrigerated storage.

3.5 Discussion

With an increased annual consumption and constant risk of foodborne illness, the ability to prevent the growth of and/or inactivate foodborne pathogens on sprouts is of great importance. The potential use of biocontrol organisms as a means of reducing the incidence of foodborne illness is a promising technique. Various manuscripts have demonstrated the effectiveness and practicality of biocontrol organism as a preventative measure against foodborne pathogens (18, 27, 102, 133). The organisms *S. plymuthica* EJ has demonstrated strong antagonistic activity against *S.* Enteritidis and *E. coli* O157:H7 in both *in vitro* and *in vivo* settings. **Figure 3.6** shows the antagonistic nature of *S. plymuthica* EJ against *S.* Enteritidis when utilizing the flip-plate method previously described in the materials and methods section. Germinated mung bean sprouts, whose seeds had been co-inoculated with pathogens and *S. plymuthica* EJ, continued to demonstrate antagonistic properties when placed on an agarose plate seeded with 4 log CFU/mL of *E. coli* O157:H7 or *S.* Enteritidis (**Figure 3.7**).

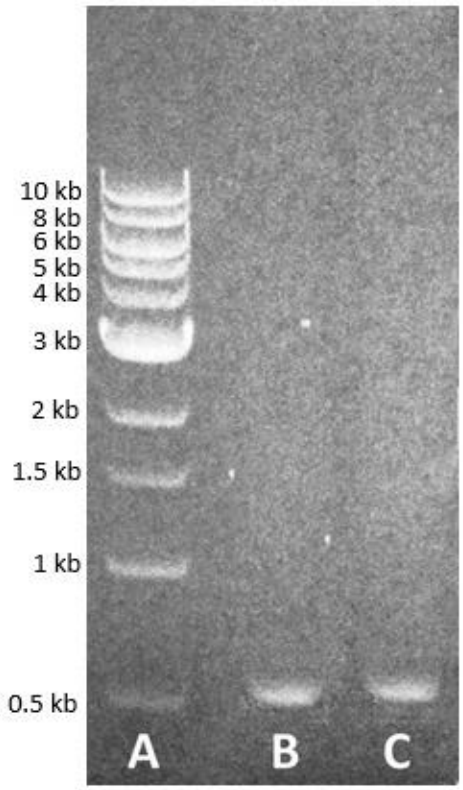


Figure 3.3. Gel electrophoresis of the amplified *prnC* gene of *S. plymuthica* EJ (B: 525 bp) and *P. fluorescence* (C: 525 bp). DNA ladder (A): 10, 8, 6, 5, 4, 3, 2, 1.5, 1, and 0.5 kb.

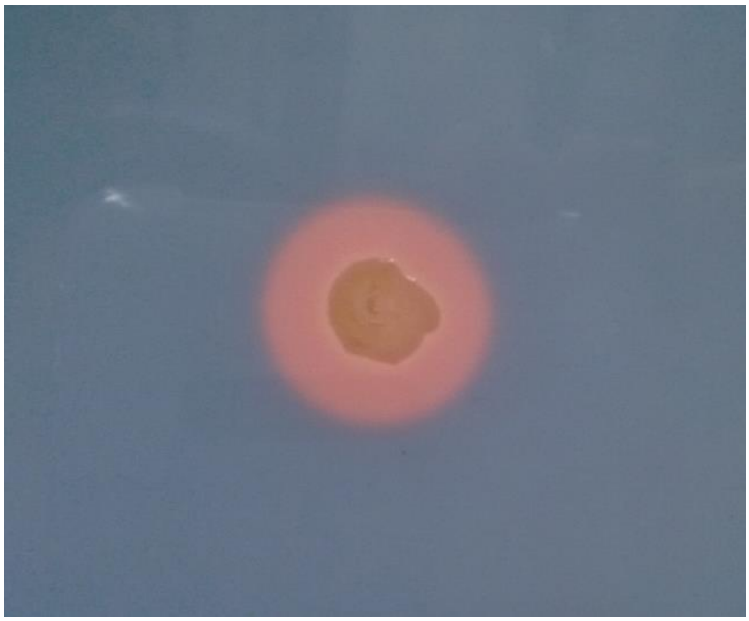


Figure 3.4. Siderophore activity of *S. plymuthica* EJ demonstrated on CAS media.

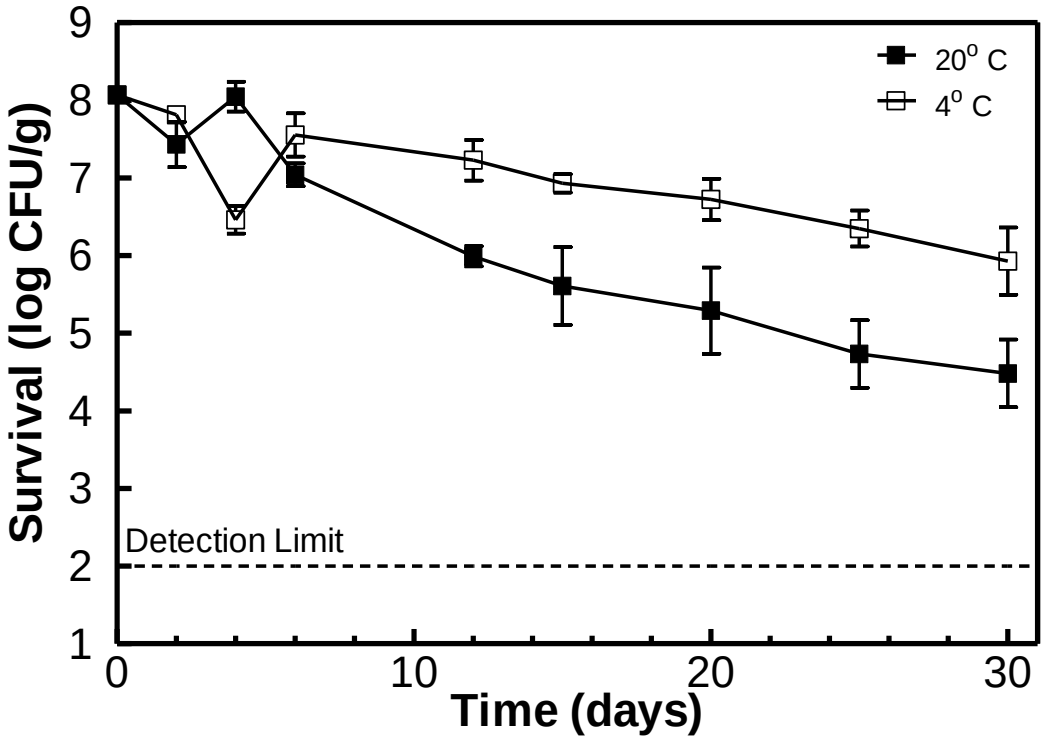


Figure 3.5. Viability of *S. plymuthica* EJ on mung bean seeds when stored at either or 20 °C for 30 days. Beans were inoculated with 100 ug/ml nalidixic acid resistant *S. plymuthica* EF for a final concentration of 8 log CFU/g of seed and stored at 4 °C or 20 °C for up to 30 days. Following storage, seeds were transferred to sterile 250 mL beakers containing 50 mL of 0.1% peptone water and placed on a rotary shaker set to 125 RPM for 15 mins. A dilution series was created and plated on the appropriate media.

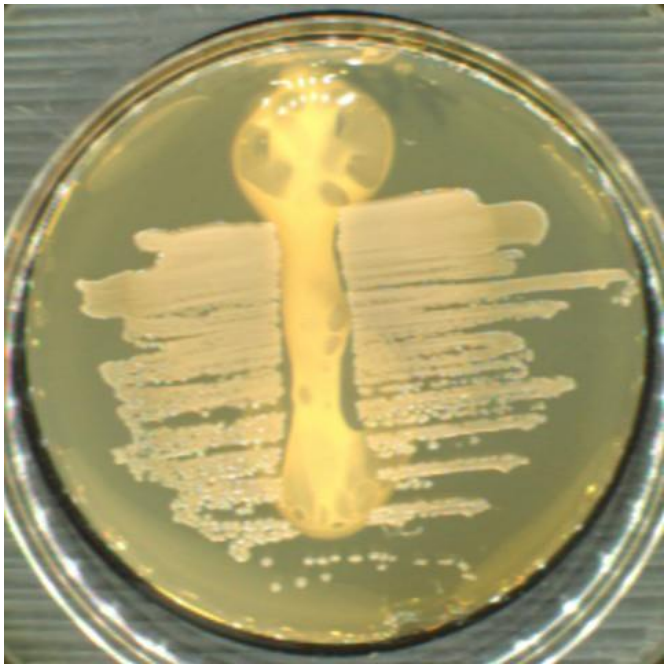


Figure 3.6. Flip-plate method: A typical representation of the antagonistic activity of *S. plymuthica* against *S.* Enteritidis.

Figure 3.7. A typical representation of the antagonistic properties of a mung bean containing ≥ 8 log CFU/g of *S. plymuthica* EJ when placed and incubated on a seeded (4 log CFU/mL *S.* Enteritidis) tryptic soy agarose plate at 32 ^{o}C.

The gene *prnC*, which is responsible for the antibiotic compound pyrrolnitrin, was found to be present in *S. plymuthica* EJ. Though primarily an anti-fungal compound, pyrrolnitirin has also been shown to effective against various Gram positive and negative opportunistic human pathogens and may play a role in the antagonistic nature of *S. plymuthica* EJ (44). The production of extracellular siderophores are a method by which a bacterium can sequester metals from the surrounding environment. Siderophores have been studied as a potential antimicrobial compound due to the fact that they can sequester and render iron unavailable for other microorganisms (107, 178, 227). The production of extracellular siderophores and the potential expression of pyrrolnitrin may aid in the antagonistic nature of the isolated strain but are probably not the main mechanisms. Various bacteriocins, organic acids, and bacteriocin-like compounds are more likely the key compounds responsible for *S. plymuthica* EJ's anti-*Salmonella* and *E. coli* O157:H7 properties. Identification, purification, and characterization of any such compounds must be performed to fully understand the antagonistic mechanism of *S. plymuthica* EJ.

As for the practical application of biocontrol organisms, there has been a push to further develop and investigate the use of such techniques on food based systems such as produce and ready-to-eat meats (88, 221, 227). The findings from this study illuminate the potential of such technology. This specific strain was able to suppress the growth of both *E. coli* O157:H7 and *S.* Enteritidis on mung bean, radish, and broccoli sprouts. Sprouts grown from mung beans or radish seeds had a final product that tested negative for the presence of either pathogen when initial inoculated with 2 log CFU/g

pathogen and 8 log CFU/g of *S. plymuthica* EJ. The robust storage viability of *S. plymuthica* EJ on sprouting seeds is reasonably impressive, with only a 3 log CFU/g and 1.5 log CFU/g reduction when stored for 30 days at either 20 ^{o}C or 4 ^{o}C respectively. Due to the estimated low levels of pathogens present on sprouting seeds (≤ 1 log CFU/g), seeds with ≥ 5 log CFU/g *S. plymuthica* EJ may still be able to limit or prevent the proliferation of any present pathogen; further studies are needed to validate this claim.

The utilization of a biocontrol organism(s) to help minimize the risk associated with minimally processed produce is just one of the many areas currently studied addressing this problem. With the increase of self-educated, health conscious consumers, “natural” solutions may be at the top of their list compared to traditional chemical and/or physical treatments. However, the influence of any artificially introduced biocontrol organisms on the soil and human microflora along with the production of any potentially hazardous compounds must be taken into consideration.

CHAPTER 4
EFFECTIVENESS OF A NOVEL SPONTANEOUS CARVACROL NANOEMULSION AGAINST *SALMONELLA* ENTERICA ENTERITIDIS AND *ESCHERICHIA COLI* O157:H7 ON CONTAMINATED MUNG BEAN AND ALFALFA SEEDS

4.1 Abstract

Outbreaks of foodborne illness from consumption of sprouts have been linked to contaminated seeds prior to germination. Due to the long sprouting period at ambient temperatures and high humidity, germinating seeds contaminated with low pathogen levels (0.1 log CFU/g) can result in sprouts with high numbers (≥10^8 CFU/g) of pathogens. Currently, the recommended treatment method involves soaking seeds in 20,000 ppm (2 %) calcium hypochlorite prior to germination. In this study, an alternative treatment involving soaking seeds in a carvacrol nanoemulsion was tested for its efficacy against *S.* Enteritidis (ATCC BAA-1045) or EGFP expressing *E. coli* O157:H7 (ATCC 42895) contaminated mung bean and alfalfa seeds. The antimicrobial treatment was performed by soaking inoculated seed batches in the spontaneous nanoemulsion (4,000 or 8,000 ppm) for 30 or 60 minutes. The spontaneous nanoemulsion was formed by titrating the oil phase (carvacrol and medium chain triglycerides) and water-soluble surfactant (Tween 80®) into sodium citrate buffer. Following treatment the numbers of surviving cells were determined by suspending the seeds in TSB and performing plate counts and/or Most Probable Number (MPN) enumeration. Treated seeds were sprouted and tested for the presence of the appropriate pathogen. This treatment successfully inactivated low levels (2 and 3 log CFU/g) of *S.* Enteritidis and *E. coli* on

either seed types when soaked for either 30 or 60 min at nanoemulsion concentrations corresponding to 4,000 (0.4 %) or 8,000 (0.8 %) ppm carvacrol. Inoculated alfalfa seeds treated with 4,000 ppm nanoemulsion, required a 60 minute treatment time to show a similar 2-3 log reduction. Complete inactivation was confirmed by germinating treated seeds and performing microbiological testing. Total sprout yield was not compromised by any of the tested treatments. These results show that carvacrol nanoemulsions may be an alternative antimicrobial treatment method for mung bean and alfalfa seeds.

4.2 Introduction

Bean sprouts, specifically mung and alfalfa sprouts, have been associated with outbreaks of foodborne illness throughout the world (34, 35). The most frequent pathogen involved is *Salmonella* spp.. One of the most wide-spread, sprout based outbreak of salmonellosis occurred throughout Ontario in 2005 and resulted in over 600 reported cases (35). The primary source of contamination is often linked to the seeds prior to sprouting (86, 98). This may be due to the fact that seeds from several producers are often consolidated into a single lot and sold to multiple growers. It has been shown that even extremely low levels of initial contamination (0.1 log CFU/g) will grow to substantial numbers during the sprouting process (86). In addition, pathogens can be internalized during sprouting, protecting them for sanitation processes following germination (67, 255). Therefore, it is necessary to find a safe and effective method to either decontaminate seeds prior to germination or a way to inactivate and retard the growth of pathogens during the sprouting process.

Currently, a 20,000 ppm hypochlorite soak is used as a pre-treatment prior to germination to lower the risk of foodborne disease (241). However, chlorine is easily de-activated in conditions with high organic loads and therefore requires constant monitoring to ensure that the proper concentration is maintained. Also, the actual topography and condition of the seeds along with the presence of protective sites protect bacteria from disinfectants (241). Soaking seeds in 20,000 ppm calcium hypochlorite for 15 min has an average reduction of 3.08 log CFU/g, but its efficacy reported in recent literature reports reductions ranging from 0.51 – 6.90 log CFU/g (11, 13, 54). Treatment with such high levels of calcium hypochlorite is also considered unacceptable for the production of certified organic sprouts and is banned in some European countries (54).

One alternative method to using calcium hypochlorite is the use of antimicrobial essential oils. Essential oils are naturally occurring substances produced by various aromatic plants that have been shown to have antioxidant, antiradical, and antimicrobial properties (28). These substances are considered “natural”, their use for commercial applications is attractive since many consumers are now concerned about the addition of synthetic compounds to foods (28, 42). The essential oil carvacrol has been shown to reduce cell numbers and inactive *S*. Enteritidis and *E. coli* O157:H7 in broth and on food samples (145, 185, 196, 261). In an emulsified form, essential oils may be applied as an aqueous-based treatment. In fact, fine droplets may improve the delivery of antimicrobial compounds to seeds because they may be able to penetrate into the cracks and crevices on the seeds surfaces.

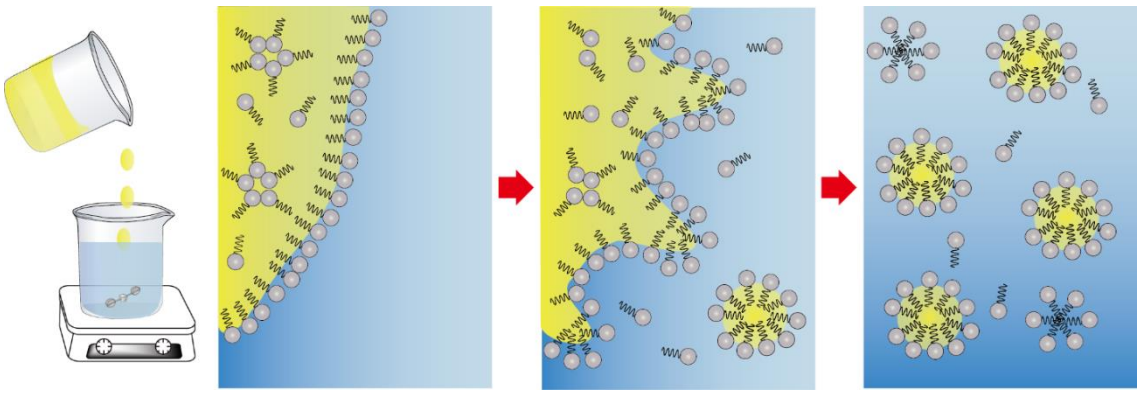


Diagram 4.1. Schematic representation of the spontaneous emulsification process used to form antimicrobial nanoemulsions.

Emulsions containing very fine droplets (radius ≈ 100 nm) are referred to as nanoemulsions. Nanoemulsions can be formed from both high-energy and low-energy methods (154). High energy methods require specialized mechanical devices that are capable of generating intense mechanical forces that can intermingle and disrupt the oil and water phases, such as sonicators, high pressure valve homogenizers, or microfluidizers. Low-energy methods rely on the spontaneous formation of fine oil droplets due to physicochemical processes that occur when certain combinations of surfactant, oil, and water are combined under appropriate conditions The use of low-energy methods is highly attractive for preparing nanoemulsions for many applications because of its low cost and simplicity (41). A number of low-energy methods are available for producing nanoemulsions, e.g., spontaneous emulsification, emulsion inversion point, phase inversion temperature, and phase inversion composition methods (6, 51). The spontaneous emulsification method is one of the more suitable for commercial implementation since it simply involves titrating a mixture of oil and water-soluble surfactant into water (**Diagram 4.1**) (51, 154). This method has recently been reported for fabricating effective antimicrobial nanoemulsions from essential oils (41). Based on these findings and the fact that carvacrol is a potent antimicrobial agent, the spontaneous emulsification method was used to produce carvacrol nanoemulsions. The emulsions storage stability and efficacy against artificially contaminated mung bean and alfalfa seeds was determined.

4.3 Materials and Methods

4.3.1 Bacterial strains and culture conditions

The bacterial strains used in the presented experiments were *Salmonella* enterica subspecies enterica serovar Enteritidis (ATCC BAA-1045) and an enhanced green fluorescent protein (EGFP) expressing *Escherichia coli* O157:H7 (ATCC 42895) (192). Stock cultures of each organism were stored at -80 ^{o}C in tryptic soy broth (TSB; BD Diagnostic Systems, Cat# DF0064-07-6) containing 25% (v/v) glycerol. Monthly, frozen stock cultures were transferred to working cultures by plating on tryptic soy agar (TSA; BD Diagnostic Systems, Cat# DF0370-075) slants/plates and incubating at 37 ^{o}C for 24 hrs. Following incubation, single colonies of E. coli O157:H7 were picked and transferred to Luria broth (Lennox, LB) (Fisher BioReagents Cat# BP9724-500) plates containing 500 µg/mL ampicillin (Fisher Scientific Cat# BP1760-5). The absorbance at 600 nm was used to determine cells numbers, with an absorbance of 0.5 equal to 1.0 x 10^{8} CFU for both *E. coli* O157:H7 and *S.* Enteritidis as determined by plate counts.

Periodically, working cultures were streaked on differential media to ensure purity. For *S*. Enteritidis, cultures were spread on xylose, lysine, deoxycholate (XLD) agar (Remel Cat# R459902). For *E. coli* O157:H7, cultures were spread on LB (Fisher BioReagents Cat# BP9724-500) plates containing 500 µg/mL ampicillin (Fisher Scientific Cat# BP1760-5) and 20 µg/mL IPTG (Thermo Scientific Cat# FERR0392) and observed under UV light. Cultures were incubated overnight in TSB at 37 ^{o}C on a rotary shaker set at 150 RPM. All cultures were diluted with TSB to the desired cell numbers.

4.3.2 Formation of antimicrobial nanoemulsions

The preparation of the antimicrobial nanoemulsion was based on the optimized system presented by Chang, McLandsborough, and McClements (41). Preparation was as follows. Carvacrol (4 g) (Sigma-Aldrich, Cat# W224502-100G-K) was added to 6 g medium chain triglyceride (MCT) oil (Miglyol 812, Witten, Germany) and thoroughly mixed for 5 min. Once mixed, Tween 80® (10 g) (Sigma-Aldrich, Cat# P1754-500ml) was added to the oil mixture and mixed for another 5 min. The oil/Tween 80 mixture (20 g) was titrated, at a rate of 2 mL/min, into 80 g of 5.0 mM sodium citrate buffer (pH 3.5) containing a magnetic stirring bar set to 600 RPM and allowed to mix for an additional 15 min. The emulsion was filter sterilized through a sterile 0.45 µm syringe filter (Fisher Scientific Cat# 09-719-005) and stored in sterile 50 mL tubes at 2 - 5 ^{o}C for up to 3 weeks. Droplet size was measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK).

4.3.3 Storage stability of nanoemulsions

The nanoemulsion (20 mL) was placed in pre-sterilized test tubes and incubated at 20 ^{o}C. For thirty consecutive days, size measurements were recorded using a dynamic light scattering instrument (ZetasizerNano ZS, Malvern Instruments, UK). This instrument determines the particle size from intensity-time fluctuations of a laser beam (633 nm) scattered from a sample at an angle of 173°. Each individual measurement was an average of 13 runs. To determine if diluting the emulsion would have an impact on stability, stock nanoemulsion was diluted 5-fold in 5.0 mM sodium citrate buffer (pH

3.5) and size measurements were recorded for thirty consecutive days as described above.

4.3.4 Sensitivity of S. Enteritidis and E.coli O157:H7 to the carvacrol nanoemulsion in vitro.

Overnight growth (9 log CFU/mL) of either *S.* Enteritidis or *E.coli* O157:H7 was added to test tubes containing TSB (9 mL) and various concentrations of the carvacrol nanoemulsion (8000, 4000, 2000, 1000, 500, 250, and 125 PPM final carvacrol concentration), to give an initial level of approximately 6 log CFU/mL. The tubes were incubated at 37 ^{o}C for 24 hrs. A dilution series was created and plated on the appropriate media. For *S*. Enteritidis, dilutions were spread on XLD (Remel Cat# R459902). For *E. coli* O157:H7, dilutions were spread on LB (Fisher BioReagents Cat# BP9724500) plates containing 500 µg/mL ampicillin (Fisher Scientific Cat# BP1760-5) and 20 µg/mL IPTG (Thermo Scientific Cat# FERR0392) and observed under UV light. It was from this procedure that the MIC for both *E. coli* O157:H7 and *S.* Enteritidis were determined.

4.3.5 Effectiveness of carvacrol nanoemulsion on S. Enteritidis or E. coli O157:H7 contaminated mung beans and alfalfa seeds

All beans/seeds used in this study were generously provided by Jonathan's Organics (Rochester, MA). Beans/seeds were inoculated and sprouted using a modified version of the method presented by Ye *et al*. (262). Batches (20 g) of beans /seeds were soaked in 50 mL of diluted *S*. Enteritidis or *E. coli* O157:H7 for 20 min resulting in final inoculums of 8, 5, 3, or 2 log CFU/g. The inoculated beans/seeds were then transferred

to a sterile glass petri dish containing sterile filter paper within a biological safety cabinet, and allowed to dry overnight at ambient temperature. The inoculated bean/seed batches were placed in 250-mL beakers and treated by soaking in 50 mL of nanoemulsion (4,000 or 8,000 ppm) for 30 or 60 mins. For the control, contaminated batches were soaked in 5.0 mM, pH 3.5 sodium citrate buffer. After treatment, the batches were rinsed once with 50 mL of sterile deionized water and transferred to a Whirl-Pack bag containing 50 mL of TSB. The Whirl-Pack bag was placed on a rotary shaker set to 50 RPM for 15 min. After agitation, a dilution series was created and plated on the appropriate media and incubated at 37 ^{o}C for 24 hrs. For samples with low *S*. Enteritidis or *E. coli* O157:H7 inoculation levels (2 and 3 log CFU/g), a three tube Most Probable Number (MPN) assay was used in conjunction with spread plating according to the FDA's *Bacteriological Analytical Manual* (BAM) (250). Samples were appropriately diluted in Lactose Broth (BD BBL Cat# DF0004-17-7) *Salmonella* enrichment or Luria Broth (Lennox) broth (Fisher Scientific Cat# BP9722-500) containing ampicillin (Fisher Scientific Cat# BP1760-5) (500 µg/mL) and incubated overnight. Dilution sets were checked for turbidity. Any positive tubes were streaked on selective/differential media for confirmation. Both treated inoculated and uninoculated samples were then sprouted.

Treated inoculated and uninoculated mung beans (20 g) and remaining broth were transferred to a sterile 1000 mL bottle and soaked in 150 mL of distilled water at 20 ^{o}C for 24 hrs. The water was removed, and sprouting continued for 4 days at 20 ^{o}C, with daily water by a 5-min soak in 150 mL of distilled water. After four days, two 25 g

batches of sprouts were taken for microbiological testing. The samples were suspended in 225 mL of 0.1% peptone water and stomached for 1 min. A dilution series was created and plated on the appropriate media as previously described.

Treated inoculated and uninoculated alfalfa seeds (20 g) and remaining broth were poured in a sterile 250 mL beaker and soaked in 150 mL of distilled water at 20 ^{o}C for 24 hrs. The water was drained, and seeds were transferred to sterile plastic trays lined with paper towels and germinated for 5 days at 20 ^{o}C. Seeds were watered four times a day with sterile deionized water. After sprouting, two 25 g batches of sprouts were suspended in 225 mL of 0.1% peptone water and stomached for 1 min. A dilution series was created and plated on the appropriate media as previously described.

4.3.6 Statistical Analysis

One-way ANOVA followed by Tukey's multiple comparisons test (95% confidence interval) for total log reduction and total sprout yield was performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, La Jolla California USA.

4.4 Results

4.4.1 Formation and stability of carvacrol nanoemulsions

Carvacrol nanoemulsions were prepared using the spontaneous emulsification method described in Section 4.2.2. The freshly prepared nanoemulsions had mean droplet diameters (Z-average) around 100 nm as determined by dynamic light scattering, which increased to around 200 nm after 30 days storage at 20 ^{o}C (**Figure 4.1**). These results indicated that nanoemulsions containing small droplets could initially be

formed, but are unstable resulting in droplet growth during long-term storage. The growth in droplet size during storage could be inhibited by diluting the nanoemulsion 5-fold with sterile sodium citrate buffer prior to storage (**Figure 4.2**). A 5-fold dilution of the nanoemulsion corresponds to a carvacrol level of 8,000 PPM, which is the amount required for observed antimicrobial efficacy in this system. In addition, the *in vitro* antimicrobial efficacy of the stored nanoemulsions was not significantly different from that of the fresh nanoemulsions (data not shown). These dilution and antimicrobial studies suggest that a nanoemulsion could be prepared and used for some time before it would need to be replaced, but further studies are needed to confirm this.

4.4.2 Effectiveness of carvacrol nanoemulsions against S. Enteritidis or E. coli 0157:H7 contaminated mung bean and alfalfa seeds

Preliminary experiments were carried out using bacteria dispersed within broth (rather than inoculated on seeds) to establish appropriate usage levels. The minimum inhibitory concentration (MIC) of the antimicrobial nanoemulsions in broth was found to be ≈ 500 PPM carvacrol, with inactivation below the detectable level occurring at concentrations ≥ 4000 PPM carvacrol (**Figure 4.3**). Based upon these results, levels of 4000 and 8000 PPM carvacrol were selected to test as a disinfection agent for sprouting seeds. The effectiveness of the carvacrol nanoemulsion treatments on mung beans and alfalfa seeds is summarized in **Tables 4.1** and **4.2**. Using an MPN assay, final *S.* Enteritidis and *E. coli* O157:H7 levels of ≤ 3 CFU/g were found on mung bean seeds that had an initial inoculum of 3 log CFU/g or less when 4000 or 8000 PPM of carvacrol were used (**Table 4.1** and **4.2**). Treatment of contaminated alfalfa seeds with 8000 PPM for either

30 or 60 min yielded similar results for *S.* Enteritidis and *E. coli* O157:H7 with an initial inoculum of 3 log CFU/g or less. However, only the 60 min 4000 PPM carvacrol treatment was successful against both pathogens on alfalfa seeds (**Table 4.3** and **4.4**).

All treatments that resulted in MPN numbers of ≤ 3 CFU/g were germinated and tested again for the presence of the pathogens using both plate counts and MPN. Following germination and microbiological testing, neither *Salmonella* nor *E. coli* O157:H7 were detected. These findings indicate that complete inactivation of both pathogens was achieved when treated with 8000 PPM. Total sprout yield was not compromised by any of the emulsion treatments. As seen in Table 5, there was no significant difference between treated and untreated sprout germination yields. It has been shown that some treatment methods such as high chlorine concentrations and pasteurization decontamination methods impact the germination rate of treated seeds and results in sub-par germination yields. The spontaneous carvacrol nanoemulsion was able to inactivate pathogens without affecting the sprout yield.

4.5 Discussion

The use of carvacrol as a potential food antimicrobial has been studied in various food systems including but not limited to fish, milk, and produce (123, 126, 201, 249). For example, treatment of kiwifruit with 15 mM of carvacrol resulted in a 3 log reduction in spoilage organisms and significantly increased the fruits shelf life (201). The ability of carvacrol to inactive *Salmonella* spp. on celery was studied by Ravishankar *et al.* (196). The group found that treatment with 10,000 ppm (1%) carvacrol resulted in a 5 log reduction of the pathogen when compared to the negative controls (196).

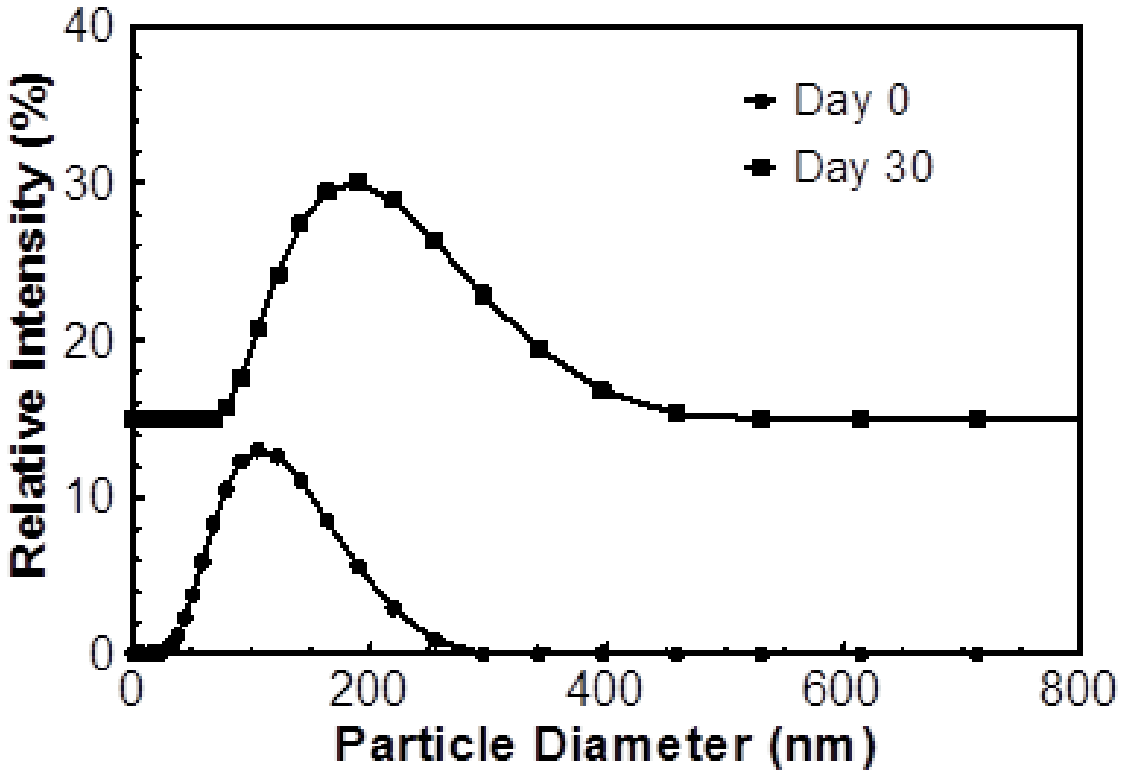


Figure 4.1. Comparison of the mean diameter (Z-average) of freshly prepared and stored (30 days) carvacrol nanoemulsions. To distinguish between the samples 15 units were added to the relative intensity of the 30 day sample.

The potential use of carvacrol as an antimicrobial, in combination with existing treatment methods has been shown to be promising for the sprout industry. When a carvacrol treatment was combined with high temperature and pressure treatment protocols reductions of more than 5 log CFU/g where recorded (187). This treatment is highly effective, yet results in a significant decrease in sprout germination rates (187).

The use of carvacrol alone as an alternative treatment method for mung bean or alfalfa seeds has not been studied. Carvacrol itself is not water soluble and treatment with pure carvacrol is not economically ideal. The utilization of spontaneous nanoemulsion technology solubilizes carvacrol, demonstrating significant reductions in cell numbers with lower concentrations of the essential oil.

For the commercial application of this technology, we envision that a nanoemulsion treatment would be applied by simply pouring emulsified carvacrol into an aqueous solution containing one or more batches of seeds in a well-ventilated area. Initially, we therefore tested the formation and stability of the antimicrobial nanoemulsion. The efficacy of a spontaneous antimicrobial nanoemulsion is directly influenced by the amount of lipid phase and surfactant in the system (41). If not properly optimized, the emulsion system may be subject to coalescence, Ostwald ripening, or flocculation, all of which can greatly reduce storage stability and antimicrobial activity (40). The emulsion stability data presented in this manuscript is very similar to the previously reported data for this optimized system (41).

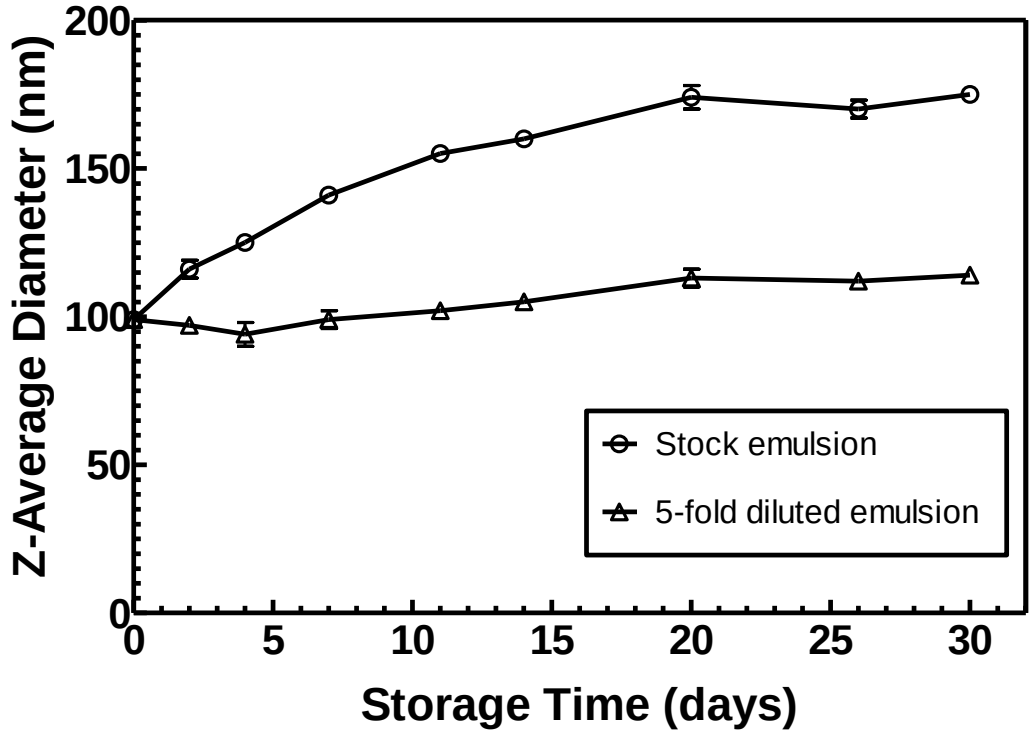


Figure 4.2. Comparison of the increase in mean droplet diameter (Z-average) of undiluted and 5-fold diluted spontaneous nanoemulsions when stored for 30 days. All plotted means and standard deviations are from triplicate studies.

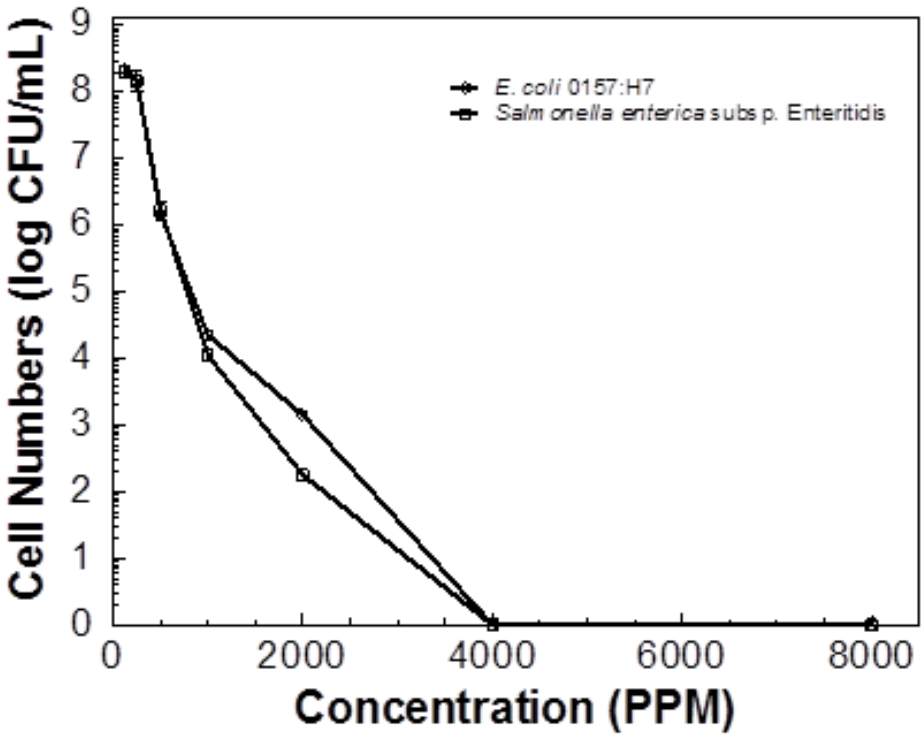


Figure 4.3. Sensitivity of *Salmonella* Enteritidis and *Escherichia coli* O157:H7 to the carvacrol nanoemulsion in TSB. Overnight growth (6 log CFU/mL) of either *S.* Enteritidis or *E. coli* O157:H7 was added to test tubes containing TSB and various concentrations of the carvacrol nanoemulsion. The tubes were incubated at 37 ^{o}C for 24 hrs and a dilution series was created and plated on the appropriate media. All plotted means and standard deviations are from triplicate studies.

Table 4.1. Effect of carvacrol emulsion on *E. coli* O157:H7 contaminated mung bean seeds[1,2]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	8.2 ± 0.14	7.3 ± 0.15	0.86 ± 0.24^{A}	+
		5.5 ± 0.32	4.3 ± 0.22	1.3 ± 0.32^{A}	+
		3.2 ± 0.12	2.5 ± 0.27	0.89 ± 0.20^{A}	+
		2.6 ± 0.29	1.4 ± 0.20	1.4 ± 0.26^{A}	+
	60	8.2 ± 0.15	7.4 ± 0.14	0.83 ± 0.11^{A}	+
		5.6 ± 0.22	4.5 ± 0.36	1.1 ± 0.34^{A}	+
		3.4 ± 0.21	2.4 ± 0.35	0.94 ± 0.22^{A}	+
		2.4 ± 0.24	1.1 ± 0.10	1.3 ± 0.28^{A}	+
8000 ppm	30	8.2 ± 0.13	5.2 ± 0.15	3.0 ± 0.18^{A}	+
		5.3 ± 0.25	3.1 ± 0.35	2.2 ± 0.22^{B}	+
		3.2 ± 0.14	ND	3.2 ± 0.14^{A}	-
		2.4 ± 0.21	ND	2.4 ± 0.21^{B}	-
	60	8.2 ± 0.03	4.6 ± 0.29	3.6 ± 0.29^{A}	+
		5.1 ± 0.06	2.5 ± 0.31	2.6 ± 0.35^{B}	+
		3.1 ± 0.12	ND	3.1 ± 0.12^{A}	-
		2.3 ± 0.29	ND	2.3 ± 0.29^{B}	-
4000 ppm	30	8.3 ± 0.17	5.7 ± 0.47	2.6 ± 0.57^{A}	+
		5.3 ± 0.22	3.5 ± 0.43	1.8 ± 0.57^{B}	+
		3.1 ± 0.03	ND	3.1 ± 0.03^{A}	-
		2.4 ± 0.34	ND	2.4 ± 0.34^{A}	-
	60	8.5 ± 0.32	5.5 ± 0.24	3.0 ± 0.55^{A}	+
		5.4 ± 0.24	2.3 ± 0.21	3.1 ± 0.45^{A}	+
		3.4 ± 0.33	ND	3.4 ± 0.33^{A}	-
		2.2 ± 0.05	ND	2.2 ± 0.05^{B}	-

[1] All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g. A "+" indicates that pathogens were detected after germination. A "-"indicates that no pathogens were detected after germination.

[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval.

Table 4.2. Effect of carvacrol emulsion on *S.* Enteritidis contaminated mung bean seeds[1,2]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	8.1 ± 0.06	7.3 ± 0.30	0.84 ± 0.32^{A}	+
		5.4 ± 0.37	4.4 ± 0.23	1.0 ± 0.59^{A}	+
		3.3 ± 0.40	2.4 ± 0.33	0.90 ± 0.73^{A}	+
		2.2 ± 0.07	1.4 ± 0.22	0.77 ± 0.27^{A}	+
	60	8.3 ± 0.22	7.5 ± 0.43	0.79 ± 0.34^{A}	+
		5.4 ± 0.31	4.3 ± 0.35	1.2 ± 0.62^{A}	+
		3.4 ± 0.38	2.4 ± 0.34	1.0 ± 0.62^{A}	+
		2.5 ± 0.29	1.3 ± 0.14	1.2 ± 0.33^{A}	+
8000 ppm	30	8.3 ± 0.39	5.2 ± 0.37	3.1 ± 0.11^{A}	+
		5.7 ± 0.26	3.1 ± 0.20	2.6 ± 0.36^{A}	+
		3.2 ± 0.19	ND	3.2 ± 0.19^{A}	-
		2.1 ± 0.09	ND	2.1 ± 0.09^{B}	-
	60	8.5 ± 0.31	4.01 ± 0.20	4.5 ± 0.11^{A}	+
		5.6 ± 0.36	2.22 ± 0.27	3.4 ± 0.40^{B}	+
		3.4 ± 0.28	ND	3.4 ± 0.28^{B}	-
		2.8 ± 0.29	ND	2.8 ± 0.29^{B}	-
4000 ppm	30	8.2 ± 0.16	5.8 ± 0.23	2.4 ± 0.12^{A}	+
		5.4 ± 0.31	3.3 ± 0.30	2.1 ± 0.45^{A}	+
		3.7 ± 0.28	ND	3.7 ± 0.28^{B}	-
		2.2 ± 0.17	ND	2.2 ± 0.17^{A}	-
	60	8.7 ± 0.24	5.2 ± 0.32	3.5 ± 0.40^{A}	+
		5.2 ± 0.12	2.5 ± 0.41	2.8 ± 0.52^{A}	+
		3.6 ± 0.41	ND	3.6 ± 0.41^{A}	-
		2.2 ± 0.29	ND	2.2 ± 0.29^{B}	-

[1] All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g. A "+" indicates that pathogens were detected after germination. A "-"indicates that no pathogens were detected after germination.
[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval.

Table 4.3. Effect of carvacrol emulsion on *E. coli* 0157:H7 contaminated alfalfa seeds[1,2]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	8.2 ± 0.17	7.9 ± 0.73	0.34 ± 0.89 [A]	+
		5.7 ± 0.29	4.8 ± 0.77	0.90 ± 0.59[A]	+
		3.8 ± 0.24	3.2 ± 0.11	0.57 ± 0.26[A]	+
		2.1 ± 0.18	1.3 ± 0.22	0.79 ± 0.40[A]	+
	60	8.3 ± 0.30	8.2 ± 0.09	0.11 ± 0.38[A]	+
		5.6 ± 0.54	5.0 ± 0.18	0.66 ± 0.62[A]	+
		3.8 ± 0.28	3.0 ± 0.15	0.80 ± 0.42[A]	+
		2.3 ± 0.24	1.3 ± 0.16	0.99 ± 0.40[A]	+
8000 ppm	30	8.7 ± 0.31	5.7 ± 1.17	2.9 ± 0.86[A]	+
		5.7 ± 0.26	3.4 ± 0.33	2.2 ± 0.10[A]	+
		3.5 ± 0.43	ND	3.5 ± 0.43[A]	-
		2.2 ± 0.17	ND	2.2 ± 0.17[A]	-
	60	8.4 ± 0.10	5.3 ± 1.22	3.2 ± 1.13[A]	+
		5.8 ± 0.55	3.2 ± 0.32	2.8 ± 0.38[A]	+
		3.3 ± 0.23	ND	3.3 ± 0.23[A]	-
		2.4 ± 0.46	ND	2.4 ± 0.46[A]	-
4000 ppm	30	9.0 ± 0.13	5.6 ± 0.53	3.4 ± 0.65[A]	+
		5.5 ± 0.24	4.1 ± 0.58	1.5 ± 0.47[B]	+
		3.5 ± 0.45	2.4 ± 0.69	1.1 ± 0.58[B]	+
		2.4 ± 0.46	1.6 ± 0.41	0.84 ± 0.49[B]	+
	60	8.8 ± 0.15	5.0 ± 0.23	3.8 ± 0.37[A]	+
		5.7 ± 0.10	3.6 ± 0.51	1.8 ± 0.73[B]	+
		3.3 ± 0.15	ND	3.3 ± 0.15[A]	-
		2.2 ± 0.14	ND	2.2 ± 0.14[B]	-

[1] All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g. A "+" indicates that pathogens were detected after germination. A "-"indicates that no pathogens were detected after germination.

[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval.

Table 4.4. Effect of carvacrol emulsion on *S.* Enteritidis contaminated alfalfa seeds[1,2]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	8.4 ± 0.19	8.2 ± 0.54	0.16 ± 0.38^{A}	+
		5.8 ± 0.72	5.0 ± 0.18	0.76 ± 0.55^{A}	+
		3.2 ± 0.17	2.9 ± 0.03	0.31 ± 0.14^{A}	+
		2.3 ± 0.32	1.3 ± 0.13	1.0 ± 0.24^{A}	+
	60	8.4 ± 0.42	8.2 ± 0.54	0.20 ± 0.50^{A}	+
		5.7 ± 0.29	4.8 ± 0.55	1.04 ± 0.84^{A}	+
		3.45 ± 0.15	2.9 ± 0.58	0.58 ± 0.46^{A}	+
		2.6 ± 0.27	1.3 ± 0.14	1.3 ± 0.23^{A}	+
8000 ppm	30	8.4 ± 0.35	5.8 ± 1.17	3.3 ± 0.72^{A}	+
		5.9 ± 0.27	3.4 ± 0.33	2.2 ± 0.31^{B}	+
		3.5 ± 0.28	ND	3.5 ± 0.28^{A}	-
		2.1 ± 0.17	ND	2.1 ± 0.17^{B}	-
	60	8.9 ± 0.21	4.7 ± 0.80	4.2 ± 0.92^{A}	+
		5.3 ± 0.45	3.1 ± 0.36	2.2 ± 0.09^{B}	+
		3.7 ± 0.58	ND	3.7 ± 0.58^{A}	-
		2.4 ± 0.43	ND	2.4 ± 0.43^{B}	-
4000 ppm	30	8.5 ± 0.33	5.4 ± 0.35	3.1 ± 0.34^{A}	+
		5.8 ± 0.18	4.0 ± 0.51	1.8 ± 0.34^{B}	+
		3.6 ± 0.27	2.3 ± 0.19	1.3 ± 0.15^{B}	+
		2.6 ± 0.51	1.4 ± 0.23	1.2 ± 0.63^{B}	+
	60	8.6 ± 0.13	4.7 ± 0.73	3.9 ± 0.63^{A}	+
		5.7 ± 0.22	3.3 ± 0.59	2.4 ± 0.75^{B}	+
		3.2 ± 0.17	ND	3.2 ± 0.17^{A}	-
		2.2 ± 0.16	ND	2.2 ±0.16^{B}	-

[1] All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a limit of detection of ≤ 3 MPN/g. A "+" indicates that pathogens were detected after germination. A "-"indicates that no pathogens were detected after germination.

[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval.

The recommended FDA treatment method currently implemented by the industry is soaking seeds in 20,000 PPM calcium hypochlorite prior to germination. In theory, these high concentrations should inactive any pathogens present. However, this may not always be the case since the number of outbreaks has actually increased after the release of this recommendation. The antimicrobial nanoemulsions developed in this research have similar log reductions (2 -3 CFU/g) as reported for alternative chemical and physical method (54). It should be appreciated that this reduction was achieved without costly equipment while still being label friendly.

A potential advantage of the nanoemulsions is their effectiveness against bacteria on both alfalfa and mung beans despite their large differences in physical characteristics. Mung bean seeds have a smooth surface, whereas alfalfa seeds have a rough surface which can act as a site for bacterial attachment and protection. In addition, when alfalfa seeds are added to water the seeds tend to clump together, which may provide additional protection for any bacteria present. Interestingly, clumping was not observed when alfalfa seeds were added to the carvacrol nanoemulsion, which may be due to surfactant inhibiting the hydrophobic attraction that normally occurs between bare seed surfaces. As a result, any bacteria on the surfaces of the seeds may be more exposed to antimicrobial agents present in the surrounding aqueous phase. In addition, the small size of these droplets (d ~ 100 nm) may allow them to enter any cracks or crevices on the seed thereby enabling them to inactivate bacteria trapped within the seeds. Nevertheless, further research is needed

Table 4.5. Effect of carvacrol emulsion on sprout yield[1,2]

Treatment	Treatment Time (min)	Mung Bean Yield (g)	Alfalfa Yield (g)
Control	30	71.16 ± 7.00[A]	100.16 ± 12.66[A]
	60	74.13 ± 5.14[A]	97.63 ± 4.42[A]
8000 PPM	30	75.23 ± 5.60[A]	104.00 ± 12.85[A]
	60	73.38 ± 9.02[A]	102.95 ± 9.64[A]
4000 PPM	30	73.96 ± 4.41[A]	97.96 ± 16.97[A]
	60	77.22 ± 8.29[A]	99.72 ± 14.88[A]

[1] Bean yield averages were compared using Tukey's Test with a 95% confidence interval.
[2] All means and standard deviations are from triplicate studies.

to establish the precise physicochemical basis of interaction between nanoemulsion droplets and seed coats.

It has been shown that both intrinsic and extrinsic properties of a food matrix can influence the effectiveness of essential oils, such as carvacrol (28, 229, 238). For example, carvacrol is insoluble in water and like many other essential oils, can easily be dispersed or attracted to lipid phases present in a food system (28, 159). The production of a carvacrol nanoemulsion may help limit this problem by keeping carvacrol in the aqueous phase, where it can act on present pathogens (28, 41, 154). The susceptibility of bacteria to essential oils has been shown to be dependent on the pH of the treatment (224). A lower pH not only adds stress to bacterial cells but also increases the transfer of essential oils to bacterial membranes, increasing its observed effectiveness (121, 224). The carvacrol nanoemulsion treatment put forth in this study has a pH of 3.5 which utilizes the synergistic effects of both pH stress and an increased affinity of carvacrol to the bacterial membrane. The development of a novel antimicrobial treatment composed of all food grade and GRAS components may have important commercial implications. The use of a treatment method that is label friendly and as effective as the currently used recommended treatment may prove to be valuable for both the traditional and organic sprout industry.

In summary, a food-grade, GRAS carvacrol nanoemulsion was tested for its efficacy against *S.* Enteritidis and *E. coli* O157:H7 contaminated mung bean and alfalfa seeds. The antimicrobial nanoemulsions successfully inactivated the bacteria on contaminated seeds that were soaked for at least 30 min in systems containing 8000

PPM carvacrol. As described earlier, there have been several types of sprout seeds associated with outbreaks of either *S.* Enteritidis and/or *E. coli* O157:H7. Due to various factors such as seed topography, the nature of germination, and lack of reliable decontamination processes, the need to find a successful way to reduce foodborne illness is essential. It would therefore be useful in future studies to test the efficacy of the antimicrobial nanoemulsions developed in this work against other foodborne pathogens such as *Listeria monocytogenes* and on other seed types to establish its range of efficacy.

CHAPTER 5

EFFECTIVENESS OF A SPONTANEOUS CARVACROL NANOEMULSION AGAINST *SALMONELLA* ENTERICA ENTERITIDIS AND *ESCHERICHIA COLI* O157:H7 ON CONTAMINATED BROCCOLI AND RADISH SEEDS

5.1 Abstract

The incidence of foodborne illness associated with the consumption of fresh produce has continued to increase over the past decade. Sprouts, such as mung bean, alfalfa, radish, and broccoli, are minimally processed and have been sources for foodborne illness. Currently, a 20,000 ppm calcium hypochlorite soak is recommended for the treatment of sprouting seeds. In this study, the efficacy of an antimicrobial carvacrol nanoemulsion was tested against *S.* Enteritidis (ATCC BAA-1045) or EGFP expressing *E. coli* O157:H7 (ATCC 42895) contaminated sprouting seeds. Antimicrobial treatments were performed by soaking inoculated seeds in nanoemulsions (4,000 or 8,000 ppm) for 30 or 60 minutes. Following treatment, surviving cells were determined by performing plate counts and/or Most Probable Number (MPN) enumeration. Treated seeds were sprouted and tested for the presence of pathogens. Treatment successfully inactivated low levels (2 and 3 log CFU/g) of *S.* Enteritidis and *E. coli* on radish seeds when soaked for 60 min at concentrations ≥ 4,000 (0.4 %) ppm carvacrol. This treatment method was not affective on contaminated broccoli seeds. Total sprout yield was not influenced by any treatments. These results show that carvacrol

nanoemulsions may be an alternative treatment method for contaminated radish seeds.

5.2 Introduction

The incidence of foodborne illness associated with the consumption of fresh produce has continued to increase over the past decade. Between 1990 and 2005, there have been over 700 outbreaks, resulting in roughly 34,000 cases of foodborne illness (206). One potential reason for the increase in outbreaks may be due to the change in social eating habits and the accessibility of fresh produce. For example, the per capita demand and consumption of fresh produce has dramatically increased compared to past decades (93). Also, the produce industry has experienced a rapid globalization in its supply chain, making the implementation of universal protocols challenging (93). Yet, these sociological and supply changes do not fully explain the increased outbreaks seen in fresh produce. The incidence of outbreaks in particular produce systems, such as leafy greens and sprouts, have increased 4- fold compared to the increase in consumption (35, 108).

The demand for minimally processed, natural produce has continued to increase despite the inherent risk of foodborne illness. At the forefront of this resurgence are sprouts. Sprouts, such as mung bean, alfalfa, radish, and broccoli, are minimally processed and can be vectors for both *Salmonella* spp. and *E. coli* O157:H7 (165). Prior to sprouting, seeds are generally soaked in lukewarm water (32 – 35 ^{o}C) for 2 – 4 hrs or allowed to soak at room temperature for 24 hrs (207).

Unfortunately, this necessary step not only triggers seed germination, but can also act as an enrichment step for any present human pathogen. To help minimize the potential of foodborne illness, a 2% calcium hypochlorite soak is recommended prior to sprouting (241). However, rapid sequestering of free chlorine by organic load, inadequate pH adjustments, and seed topography limit its effectiveness (76, 207, 259).

A potential alternative to current recommended methods, is the use of emulsified essential oils. Essential oils are natural compounds that are isolated from various plant sources such as thyme, oregano, and basil, that demonstrate antimicrobial activity (28). One essential oil that has been shown to have promising antimicrobial properties against a variety of foodborne pathogens is carvacrol (28, 130, 145, 185). Essential oils, alone, have minimal solubility in water. The spontaneous emulsification of carvacrol, originally put forth by Chang *et al.*(41), is simple to produce and requires minimal equipment and training (51, 154). The nanoemulsion has also been shown to be effective against foodborne pathogens in both *in vitro* and *in vivo* sprout settings. In a broth based system, the minimal inhibitory concentration for the spontaneous carvacrol nanoemulsion was found to be 500 ppm, with complete inactivation at concentrations greater than or equal to 4000 ppm. When applied to a sprout based system, a 60 min treatment in 4,000 or 8,000 ppm carvacrol nanoemulsion resulted in complete inactivation of both *S.* Enteritidis and *E. coli* O157:H7 on both mung bean and alfalfa seeds. Based on these findings, the efficacy of the spontaneous carvacrol nanoemulsion at similar concentrations was tested against

S. Enteritidis and *E. coli* O157:H7 contaminated radish and broccoli seeds. The influence of organic loads on the emulsions antimicrobial efficiency *in vitro* was also studied.

5.3 Materials and Methods

5.3.1 Bacterial strains and culture conditions

The bacterial strains used in the presented experiments were *S.* Enteritidis (ATCC BAA-1045) and an enhanced green fluorescent protein (EGFP) expressing *E. coli* O157:H7 (ATCC 42895) (192). Stock cultures of each organism were stored at -80 ^{o}C in tryptic soy broth (TSB; BD Diagnostic Systems, Cat# DF0064-07-6) containing 25% (v/v) glycerol. Monthly, frozen stock cultures were transferred to working cultures by plating on tryptic soy agar (TSA; BD Diagnostic Systems, Cat# DF0370-075) slants/plates and incubating at 37 ^{o}C for 24 hrs. Following incubation, single colonies of *E. coli* O157:H7 were picked and transferred to Luria broth (Lennox, LB) (Fisher BioReagents Cat# BP9724-500) plates containing 500 µg/mL ampicillin (Fisher Scientific Cat# BP1760-5). The absorbance at 600 nm was used to determine cells numbers, with an absorbance of 0.5 equal to 1.0 x 10^{8} CFU for both *E. coli* O157:H7 and *S.* Enteritidis as determined by plate counts.

Periodically, working cultures were streaked on differential media to ensure purity. For *S.* Enteritidis, cultures were spread on xylose, lysine, deoxycholate (XLD) agar (Remel Cat# R459902). For *E. coli* O157:H7, cultures were spread on LB (Fisher BioReagents Cat# BP9724-500) plates containing 500 µg/mL ampicillin (Fisher Scientific Cat# BP1760-5) and 20 µg/mL IPTG (Thermo Scientific Cat# FERR0392) and observed

under UV light. Cultures were incubated overnight in TSB at 37 ^{o}C on a rotary shaker set at 150 RPM. All cultures were diluted with TSB to the desired cell numbers.

5.*3.2 Formation of antimicrobial nanoemulsions*

Carvacrol (4 g) (Sigma-Aldrich, Cat# W224502-100G-K) was added to 6 g of medium chain triglyceride (MCT) oil (Miglyol 812, Witten, Germany) and thoroughly mixed for 5 min at 125 RPM. Once mixed, Tween 80® (10 g) (Sigma-Aldrich, Cat# P1754-500ml) was added to the oil mixture and mixed for another 5 min at 125 RPM. The oil/Tween 80 mixture (20 g) was titrated, at a rate of 2 mL/min, into 80 g of 5.0 mM sodium citrate buffer (pH 3.5) containing a magnetic stirring bar set to 600 RPM and allowed to mix for an additional 15 min. The emulsion was filter sterilized through a sterile 0.45 µm syringe filter (Fisher Scientific Cat# 09-719-005) and stored in sterile 50 mL tubes at 2 - 5 ^{o}C for up to 3 weeks. Droplet size was measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK) to ensure that the mean droplet diameter was ≈ 100 nm.

5.3.3. Effect of organic load on the effectiveness of the carvacrol nanoemulsion against S. Enteritidis and E. coli 0157:H7 in vitro.

Overnight growth (9 log CFU/mL) of either *S.* Enteritidis or *E. coli* O157:H7 was added to test tubes containing TSB (9 mL), carvacrol nanoemulsion (8000, 4000, 2000, 1000, and 500 ppm final carvacrol concentration), and various concentrations of organic load (2%, 10%, or 20% v/v) to give an initial level of approximately 6 log CFU/mL. The tubes were incubated at 37 ^{o}C for 24 hrs. A dilution series was created and plated on the appropriate media. For *S*. Enteritidis, dilutions were spread on XLD (Remel Cat#

R459902). For *E. coli* O157:H7, dilutions were spread on LB (Fisher BioReagents Cat# BP9724500) plates containing 500 µg/mL ampicillin (Fisher Scientific Cat# BP1760-5) and 20 µg/mL IPTG (Thermo Scientific Cat# FERR0392) and observed under UV light.

Organic loads were simulated with either horse serum (Thermo Scientific Cat# R55075) or homogenized mung bean sprouts purchased from a local supermarket. To produce the homogenized mung bean extract, 50 g of mung beans and 50 mL of sterile water were homogenized in an Oster Osterizer Classic ® blender (Oster: Boca Raton, FL, USA) for 45 sec.

5.3.4 Effectiveness of a carvacrol nanoemulsion on contaminated seeds

All seeds used in this study were generously provided by Jonathan's Organics (Rochester, MA) and each treatment condition was tested and sprouted in triplicate. Seeds were inoculated and sprouted using a modified version of the method presented by Ye et al.(262). Batches (20 g) of seeds were soaked in 50 mL of diluted *S.* Enteritidis or *E. coli* O157:H7 for 20 min resulting in final inoculums of 8, 5, 3, or 2 log CFU/g. The inoculated seeds were then transferred to a sterile glass petri dish containing sterile filter paper within a biological safety cabinet, and allowed to dry overnight at ambient temperature. The inoculated bean/seed batches were placed in 250-mL beakers and treated by soaking in 50 mL of nanoemulsion (4,000 or 8,000 ppm) with agitation (125 RPM) for 30 or 60 mins. For the control, contaminated batches were soaked in 5.0 mM, pH 3.5 sodium citrate buffer. After treatment, the batches were rinsed once with 50 mL of sterile deionized water and transferred to a Whirl-Pack bag containing 50 mL of TSB.

The Whirl-Pack bag was placed on a rotary shaker set to 50 RPM for 15 min. After agitation, a dilution series was created and plated on the appropriate media and incubated at 37 ^{o}C for 24 hrs. For samples with low *S.* Enteritidis or *E. coli* O157:H7 inoculation levels (2 and 3 log CFU/g), a three tube most probable number (MPN) assay was used in conjunction with spread plating according to the FDA's *Bacteriological Analytical Manual* (BAM) (250). Samples were appropriately diluted in Lactose Broth (BD BBL Cat# DF0004-17-7) *Salmonella* enrichment or Luria Broth (Lennox) broth (Fisher Scientific Cat# BP9722-500) containing ampicillin (Fisher Scientific Cat# BP1760-5) (500 µg/mL) and incubated overnight. Dilution sets were checked for turbidity. Any positive tubes were streaked on selective/differential media for confirmation. Both treated inoculated and uninoculated samples were then sprouted.

Both seed types were sprouted in a similar fashion as described by Fransisca *et al.*(81). Batches (10 g) of inoculated seeds were transferred to a sterile 250 mL beaker and soaked in 150 mL of distilled water at 20 ^{o}C for 24 hours. The water was removed, and the seeds transferred to 3 pieces of sterile filter paper (Fisher Scientific Cat# 09-803-6D) on top of a sterile plastic test tube rack in a sterile stainless steel container with a lid. The seeds were sprouted in the dark at 20 ^{o}C for 72 hrs. The seeds were watered with 15 mL of distilled water every 8 hrs with a plastic spray bottle (Fisher Scientific Cat# 03-438-12A). After sprouting, two 25 g batches of sprouts were suspended in 225 mL of 0.1% peptone water and stomached for 1 min. A dilution series was created and plated on the appropriate media as previously described.

5.3.5 Statistical Analysis

One-way ANOVA followed by Tukey's multiple comparison test (95% confidence interval) for total log reduction and total sprout yield. Differences in reduction between emulsion with organic load and without were compared using an unpaired t-test with a 95% confidence interval. Statistical analysis was performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, La Jolla California USA.

5.4 Results

5.4.1 Effect of organic load on the efficacy of the carvacrol nanoemulsions

The minimal inhibitory concentration (MIC) of the carvacrol nanoemulsion was found to be 500 ppm in a broth system. The influence of organic load on the effectiveness of carvacrol nanoemulsions against *S.* Enteritidis and *E. coli* O157:H7 was studied because antimicrobials are often utilized in complex environments in practical applications. Initially, we used purified horse serum as a model fluid that contained appreciable quantities of organic matter. The effect of horse serum on the efficacy of the nanoemulsions can be seen in **Figures 5.1A** and **5.1B**.

The addition of 2% (v/v) horse serum had no significant effect on the efficacy of the antimicrobial nanoemulsions against *S.* Enteritidis or *E. coli* O157:H7, with the results being similar to the control (**Figures 5.1A** and **5.1B**). Comparable growth inhibition to that of the control, for both pathogens, occurred with carvacrol concentrations between 500 and 1000 ppm. The addition of 10 and 20% (v/v) horse serum actually led to a significant decrease in the effectiveness of the emulsion at the lowest carvacrol

concentration tested (500 ppm) when compared to the control. The addition of 20% (v/v) horse serum also greatly decreased the efficacy of the antimicrobial nanoemulsion through the tested concentrations. Only with 8000 ppm carvacrol were both pathogens reduced to undetectable levels.

At 500 PPM carvacrol, significant growth occurred for both *S.* Enteritidis ($p=0.005$) and *E. coli* O157:H7 ($p = 0.04$) in samples containing ≥ 10% (v/v) horse serum. The minimum inhibitory concentration (MIC) for samples containing 10% (v/v) horse serum was found to be 900 ppm carvacrol for both pathogens (**Figures 5.1A** and **5.1B**). Reduction to undetectable levels was still observed at 4000 and 8000 ppm. Samples with 20% (v/v) horse serum demonstrated the most substantial decrease in effectiveness. The MIC for samples with 20% (v/v) horse serum was 2000 ppm carvacrol nanoemulsion and the reduction of pathogens to undetectable levels was only achieved with 8000 ppm carvacrol nanoemulsion (**Figures 5.1A** and **5.1B**).

The effect of homogenized mung bean sprouts on the efficacy of the spontaneous carvacrol nanoemulsion can be seen in **Figures 5. 1C** and **5.1D**. As with horse serum, sprout homogenate concentrations ≤ 2% (v/v) did not significantly decrease the emulsions effectiveness against both pathogens. With the addition of ≥ 10% (v/v) sprout homogenate, the previously determined MIC of 500 ppm was not valid, and a significant decrease in effectiveness was observed when compared to the control.

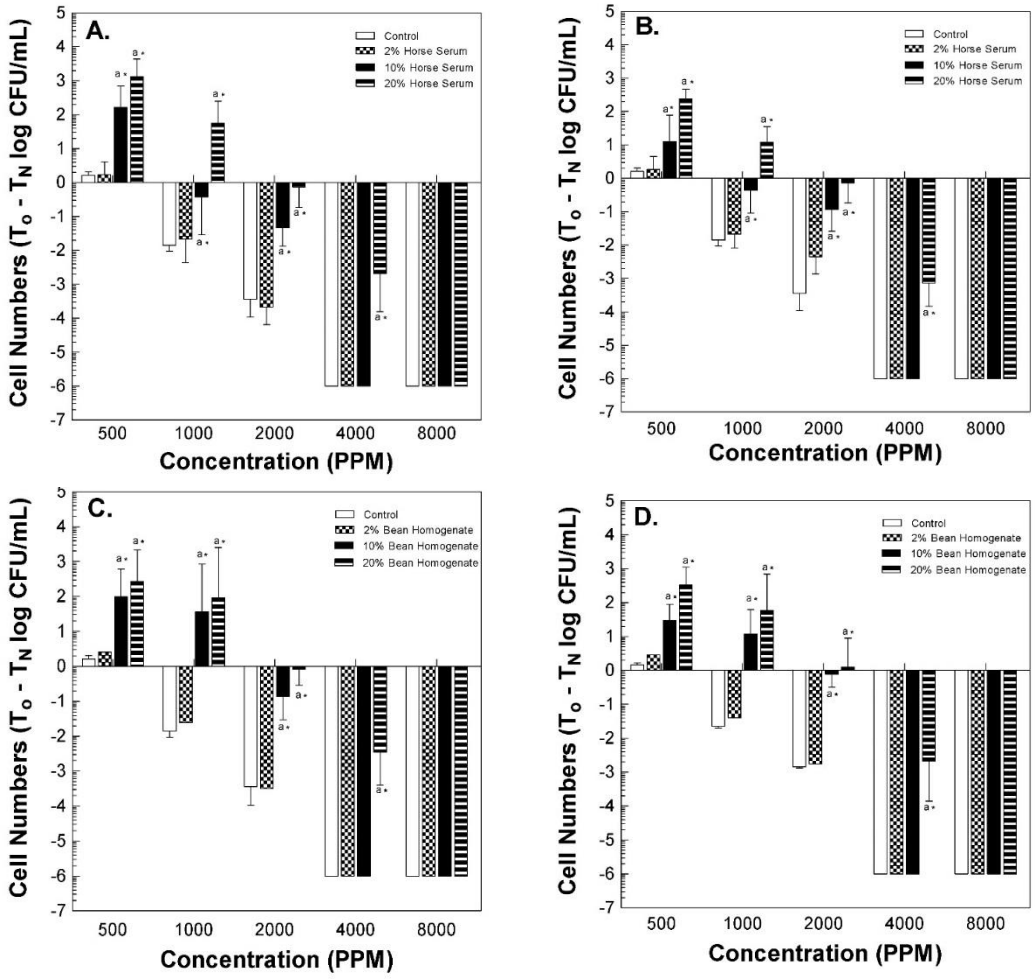


Figure 5.1. The effect of horse serum or bean homogenate on the effectiveness of the spontaneously emulsified carvacrol nanoemulsion against *S.* Enteritidis (A, C) and *E. coli* O157:H7 (B, D). All tests were performed in triplicate. A "*" designates that there was a statistically significant difference between the sample and control when compared using a t-test with a 95% confidence interval. An "a" indicates that the sample was subject to creaming following incubation. The designation log T_n CFU/mL indicates the initial cells numbers (T_n = 6 log CFU/mL) and log T_o CFU/mL is the observed numbers following a 24 hour incubation period at 37 ^oC.

At 500 and 1000 ppm, significant growth was observed for *S.* Enteritidis and *E. coli* O157:H7 in samples containing ≥ 10% (v/v) sprout homogenate when compared to the control. The MIC for *S.* Enteritidis and *E. coli* O157:H7 in samples containing 10% (v/v) sprout homogenate were found to be 1500 and 2000 ppm respectively. In samples containing 10% (v/v) sprout homogenate, pathogens were reduced to undetectable levels following treatment with 4000 or 8000 ppm carvacrol nanoemulsion (**Figures 5.1C** and **5.1D**). However, the reduction of *S.* Enteritidis or *E. coli* O157:H7 to undetectable levels in samples containing 20% (v/v) sprout homogenate was only observed following treatment with 8000 ppm carvacrol nanoemulsion (**Figures 5.1C** and **5.1D**).

The addition of an organic load not only effected the emulsions efficacy, but also its physical stability. As seen in **Figures 5.1** and **5.2**, both horse serum and sprout homogenate concentrations of 20% (v/v) resulted in destabilization of the emulsion at ≤ 4000 ppm carvacrol. A pictorial representation of the observed creaming in the presence of 20% (v/v) horse serum can be seen in **Figure 5. 2**. Samples with a 10% (v/v) organic load (horse serum or sprout homogenate) only exhibited creaming and decreased effectiveness at concentrations > 4000 ppm carvacrol. These results indicated that when the emulsion stability is reduced, the antimicrobial efficacy is also reduced.

5.4.2 Effectiveness of carvacrol nanoemulsion against S. Enteritidis or E. coli 0157:H7 contaminated broccoli and radish seeds.

The effectiveness of the carvacrol nanoemulsion treatments on broccoli and radish seeds is summarized in **Tables 5.1-5.4**. Based on the results of an MPN assay, final *S.* Enteritidis and *E. coli* O157:H7 levels of ≤ 3 CFU/g were found on radish seeds that had an initial inoculum of 3 log CFU/g or less when treated with either 4000 or 8000 ppm of carvacrol nanoemulsion for 60 min (**Tables 5.1 and 5.2**).

All treatments that resulted in MPN numbers ≤ 3CFU/g were germinated and tested again for the presence of the pathogens using both plate counts and MPN. Following germination and enumeration neither pathogen were detected using standard plate counts (detection limit of 2 log CFU/mL) when radish seeds with low levels of contamination were treated with 4000 or 8000 ppm carvacrol nanoemulsion for 60 min. It should be noted that seeds containing < 2 log CFU/g of pathogen following treatment, always resulted in > 8 log CFU/g of pathogen following germination. As indicated by the results of Tukey's test, the treatment of contaminated radish seeds with 8000 ppm carvacrol nanoemulsion for 60 or 30 min consistently resulted in a 2.9 ± 0.36 log reduction for both pathogens. However, treatment with 4000 ppm carvacrol nanoemulsion for 30 min only resulted in a 2.07 ± 0.38 log reduction for both pathogens.

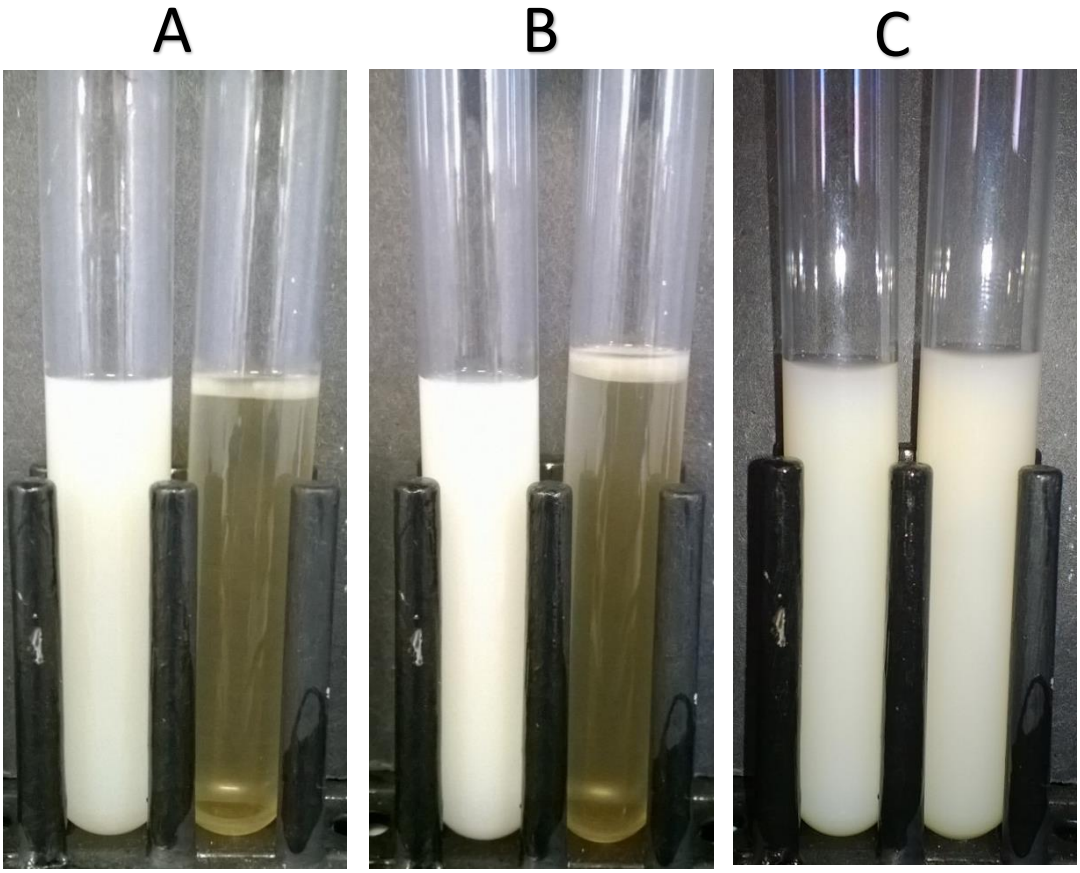


Figure 5.2. The effect of 20% (v/v) horse serum on the stability of the spontaneous carvacrol nanoemulsion at various concentrations following a 24 hr incubation at 37 ^{o}C: (A) comparison between a 2000 ppm carvacrol nanoemulsion with (right) and without (left) 20% (v/v) horse serum, (B) comparison between a 4000 ppm carvacrol nanoemulsion with (right) and without (left) 20% (v/v) horse serum, (C) comparison between a 8000 ppm carvacrol nanoemulsion with (right) and without (left) 20% (v/v) horse serum.

The treatment of broccoli seeds contaminated with high initial numbers of pathogens (8 or 5 log CFU/g) with either 4000 or 8000 ppm carvacrol nanoemulsion resulted in a significant reduction (≥ 2 log) of both pathogens when compared to the control (**Table 5.3 and 5.4**). However, at low cell concentrations (3 and 2 log CFU/g) the effectiveness of the nanoemulsion was comparable to that of the control. As seen in **Table 5.5**, there was no significant difference between treated and untreated sprout germination yields.

5.5 Discussion

The use of essential oils, such as carvacrol, as antimicrobials is appealing because these compounds are a "natural" alternative to traditional treatment methods (28). The effectiveness of carvacrol against various foodborne pathogens has been reported in numerous studies (28, 41, 129, 145, 185). The antimicrobial efficacy of carvacrol has been attributed to its ability to permeabilize and depolarize the cytoplasmic membrane (247, 248, 261). This phenomenon is a result of the hydrophilic hydroxyl group on the phenolic ring, which allows carvacrol to dissolve into and disrupt cytoplasmic membrane function (218, 247). However, even with this hydrophilic moiety, carvacrol is still predominantly hydrophobic and therefore has low water-solubility. The spontaneous emulsification process utilized in this and previous studies, allows for the dispersion of essential oils into aqueous phases in the form of small oil droplets (41, 130). In a water-dispersible form, carvacrol is able to act on any pathogens also present in the surrounding aqueous phase or at surfaces (28, 41, 154).

Currently, the Food and Drug Administration (FDA) recommends a 20,000 ppm (2%) calcium hypochlorite treatment step prior to germination (241). Even after the implementation of this recommended treatment step, the number of sprout based foodborne outbreaks has increased (54, 241). One of the problems with the currently recommend treatment is the fact that calcium hypochlorite is easily deactivated by soluble organics. As a result of various treatment environments and conditions (206), the reported reductions of a 20,000 ppm calcium hypochlorite treatment on sprouting seeds ranges from 0.51 – 6.90 log CFU/g (28, 54, 206).

To determine if the carvacrol nanoemulsions suffered from a similar reduction in efficacy in the presence of organic matter, the effect of organic load was tested using horse serum, the FDA standard for simulating organic load (250). In addition, homogenized mung bean sprouts were also studied as another potential source of organic load. It was found that high organic loads (10 and 20% v/v) physically destabilized the emulsion, with rapid phase separation being observed visibly (**Figure 5.2**). This phenomenon is usually the result of droplet aggregation (such as coalescence and flocculation), and then rapid creaming due to the increase in net particle size. Droplet aggregation may be induced by a number of different mechanisms in emulsions, such as electrostatic screening, bridging, depletion, or binding effects.

Table 5.1. - Effect of carvacrol emulsion on *E. coli* O157:H7 contaminated broccoli seeds[12]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	5.6 ± 0.25	4.2 ± 0.85	1.4 ± 0.55^{A}	+
		3.5 ± 0.23	2.5 ± 0.91	1.0 ± 0.73^{A}	+
		2.7 ± 0.21	1.9 ± 0.27	0.86 ± 0.47^{A}	+
	60	5.4 ± 0.40	4.7 ± 0.28	0.81 ± 0.12^{A}	+
		3.6 ± 0.10	2.7 ± 0.20	0.84 ± 0.26^{A}	+
		2.6 ± 0.12	2.0 ± 0.12	0.66 ± 0.12^{A}	+
8000 ppm	30	5.4 ± 0.48	3.6 ± 0.44	1.8 ± 0.82^{AB}	+
		3.4 ± 0.46	2.4 ± 0.27	1.0 ± 0.54^{B}	+
		2.6 ± 0.07	1.6 ± 0.17	1.0 ± 0.14^{B}	+
	60	5.3 ± 0.40	3.1 ± 0.52	2.2 ± 0.42^{A}	+
		3.5 ± 0.63	2.3 ± 0.28	1.2 ± 0.78^{AB}	+
		2.7 ± 0.14	1.6 ± 0.06	1.0 ± 0.12^{B}	+
4000 ppm	30	5.1 ± 0.56	4.1 ± 0.30	1.0 ± 0.34^{A}	+
		3.5 ± 0.44	2.7 ± 0.18	0.81 ± 0.40^{A}	+
		2.6 ± 0.12	1.7 ± 0.22	0.88 ± 0.26^{A}	+
	60	5.2 ± 0.41	3.8 ± 0.28	1.4 ± 0.68^{A}	+
		3.9 ± 0.42	3.5 ± 0.15	0.87 ± 0.51^{A}	+
		2.7 ± 0.15	2.5 ± 0.01	0.85 ± 0.06^{A}	+

[1] All means and standard deviations are from triplicate studies. Following treatment, seeds were germinated and tested for the presence of pathogens. A "ND" indicates that no pathogens were detected using a three-tube MPN assay. A "+" indicates that pathogens were detected after germination. A "-"indicates that no pathogens were detected after germination.

[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval. Groupings of statistical significance are indicated by superscripted letters.

Table 5.2. - Effect of carvacrol emulsion on *S.* Enteritidis contaminated broccoli seeds [12]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	5.8 ± 0.10	4.6 ± 0.13	1.14 ± 0.23^{A}	+
		3.8 ± 0.51	2.9 ± 0.24	0.89 ± 0.26^{A}	+
		2.6 ± 0.10	2.1 ± 0.45	0.51 ± 0.54^{B}	+
	60	5.7 ± 0.24	4.5 ± 0.57	1.2 ± 0.60^{A}	+
		3.4 ± 0.17	2.4 ± 0.23	0.95 ± 0.38^{A}	+
		2.7 ± 0.17	2.1 ± 0.57	0.52 ± 0.69^{B}	+
8000 ppm	30	5.6 ± 0.20	3.3 ± 0.78	2.3 ± 0.97^{A}	+
		3.4 ± 0.17	2.6 ± 0.07	0.85 ± 0.19^{B}	+
		2.5 ± 0.13	1.9 ± 0.23	0.34 ± 0.11^{B}	+
	60	5.7 ± 0.65	3.8 ± 0.08	1.9 ± 0.63^{A}	+
		3.8 ± 0.27	2.4 ± 0.08	1.4 ± 0.19^{A}	+
		2.6 ± 0.39	1.4 ± 0.06	1.2 ± 0.43^{A}	+
4000 ppm	30	5.4 ± 0.39	4.5 ± 0.74	0.95 ± 0.37^{A}	+
		3.6 ± 0.40	2.8 ± 0.16	0.81 ± 0.32^{A}	+
		2.8 ± 0.20	2.0 ± 0.16	0.86 ± 0.17^{A}	+
	60	5.5 ± 0.55	4.4 ± 0.70	1.0 ± 0.89^{A}	+
		3.5 ± 0.31	2.3 ± 0.31	1.2 ± 0.58^{A}	+
		2.6 ± 0.39	1.4± 0.05	1.1 ± 0.34^{A}	+

[1] All means and standard deviations are from triplicate studies. Following treatment, seeds were germinated and tested for the presence of pathogens. A "ND" indicates that no pathogens were detected using a three-tube MPN assay. A "+" indicates that pathogens were detected after germination. A "-"indicates that no pathogens were detected after germination.
[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval. Groupings of statistical significance are indicated by superscripted letters.

Table 5.3. - Effect of carvacrol emulsion on *E. coli* O157:H7 contaminated radish seeds[1]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	5.8 ± 0.32	4.8 ± 0.21	1.0 ± 0.26[A]	+
		3.3 ± 0.54	2.5 ± 0.14	0.87 ± 0.54[A]	+
		2.8 ± 0.37	2.0 ± 0.06	0.72 ± 0.43[A]	+
	60	5.4 ± 0.38	4.6 ± 0.52	0.80 ± 0.80[A]	+
		3.4 ± 0.72	2.8 ± 0.15	0.70 ± 0.57[A]	+
		2.8 ± 0.27	1.9 ± 0.38	0.86 ± 0.43[A]	+
8000 ppm	30	5.3 ± 0.53	2.6 ± 0.11	2.6 ± 0.58[A]	+
		3.1 ± 0.28	ND	3.1 ± 0.28[A]	-
		2.7 ± 0.28	ND	2.7 ± 0.28[A]	-
	60	5.6 ± 0.27	2.8 ± 0.27	2.8 ± 0.36[A]	+
		3.4 ± 0.52	ND	3.4 ± 0.52[A]	-
		2.6 ± 0.28	ND	2.6 ± 0.28[A]	-
4000 ppm	30	5.5 ± 0.61	3.8 ± 0.95	1.8 ± 0.49[A]	+
		3.6 ± 0.84	1.7 ± 0.23	1.9 ± 0.65[A]	+
		2.1 ± 0.50	ND	2.1 ± 0.50[A]	-
	60	5.3 ± 0.71	2.6 ± 0.20	2.7 ± 0.76[A]	+
		3.0 ± 0.22	ND	3.0 ± 0.22[A]	-
		2.6 ± 0.19	ND	2.6 ± 0.19[A]	-

[1] All means and standard deviations are from triplicate studies. Following treatment, seeds were germinated and tested for the presence of pathogens. A "ND" indicates that no pathogens were detected using a three-tube MPN assay. A "+" indicates that pathogens were detected after germination. A "-"indicates that no pathogens were detected after germination

[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval. Groupings of statistical significance are indicated by superscripted letters.

Table 5.4. - Effect of carvacrol emulsion on *S.* Enteritidis contaminated radish seeds[12]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	5.6 ± 0.45	4.9 ± 0.31	0.66± 0.72[A]	+
		3.6 ± 0.40	3.1 ± 0.16	0.45 ± 0.11[A]	+
		2.6 ± 0.10	2.5± 0.05	0.17 ± 0.05[A]	+
	60	5.2 ± 0.35	4.3 ± 0.47	0.85± 0.83[A]	+
		3.5 ± 0.82	2.7 ± 0.25	0.88 ± 1.03[A]	+
		2.8 ± 0.35	2.2± 0.24	0.70 ± 0.53[A]	+
8000 ppm	30	5.5 ± 0.70	2.9 ± 0.17	2.6 ± 0.72[A]	+
		3.2 ± 0.27	ND	3.2 ± 0.27[A]	-
		2.2 ± 0.47	ND	2.2 ± 0.47[A]	-
	60	5.4 ± 0.54	3.1 ± 0.33	2.3 ± 0.41[A]	+
		3.2 ± 0.75	ND	3.2 ± 0.75[A]	-
		2.7 ± 0.07	ND	2.7 ± 0.07[A]	-
4000 ppm	30	5.0 ± 0.54	2.9 ± 0.19	2.1 ± 0.69[A]	+
		3.4 ± 0.66	2.0 ± 0.47	1.4 ± 0.59[A]	+
		2.3 ± 0.54	ND	2.3 ± 0.54[A]	-
	60	5.4 ± 0.77	3.1 ± 0.43	2.3 ± 0.47[A]	+
		3.2 ± 0.54	ND	3.2 ± 0.54[A]	-
		2.5 ± 0.10	ND	2.5 ± 0.10[A]	-

[1] All means and standard deviations are from triplicate studies. Following treatment, seeds were germinated and tested for the presence of pathogens. A "ND" indicates that no pathogens were detected using a three-tube MPN assay. A "+" indicates that pathogens were detected after germination. A "-"indicates that no pathogens were detected after germination.

[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval. Groupings of statistical significance are indicated by superscripted letters.

Table 5.5. Effect of carvacrol emulsion on sprout yield[12]

Treatment	Treatment Time (min)	Radish Yield (g)	Broccoli Yield (g)*
Control	30	83.77 ± 7.49[A]	102.94 ± 9.22[A]
	60	82.19 ± 11.6[A]	114.19 ± 14.79[A]
8000 PPM	30	80.35 ± 11.7[A]	92.99 ± 8.57[A]
	60	76.6 ± 6.96[A]	90.88 ± 11.17[A]
4000 PPM	30	78.80 ± 10.5[A]	96.77 ± 7.07[A]
	60	81.36 ± 7.17[A]	88.39 ± 15.20[A]

[1] Bean yield averages were compared using Tukey's Test with a 95% confidence interval.
[2] All means and standard deviations are from triplicate studies.
*Yields from sprouting 25 g of broccoli seeds

Electrostatic screening is important in electrostatically-stabilized emulsions, and occurs when the ionic strength is increased due to binding of counter-ions with the charged surfaces. Bridging occurs when a charged polymer binds to oppositely charged emulsion droplets. Depletion occurs when there is a sufficiently high concentration of a non-adsorbed polymer in the aqueous phase due to an osmotic exclusion effect that induces an osmotic attraction between the droplets. Binding effects may be the result of the surfactant molecules binding to other substances in the system, and thereby losing their ability to stability the emulsion droplets.

The aqueous solutions containing organic matter used in this study (*i.e.,* horse serum and homogenized mung bean) are likely to have contained a variety of constituents that could promote emulsion instability through these mechanisms. The presence of mineral ions could promote instability through electrostatic screening effects, whereas the presence of proteins or polysaccharides could induce bridging or depletion flocculation. Any substances with hydrophobic groups could bind surfactants, and thereby strip them from the oil droplet surfaces, resulting in droplet coalescence. We hypothesize that once the carvacrol droplets had creamed to the top of the systems (**Figure 5.2**) their ability to interact with the bacterial cells in the aqueous phase was greatly reduced. This would explain why there was an observed reduction in efficacy with creamed samples containing higher concentrations of organic load.

As previously reported, a treatment using carvacrol nanoemulsions was successful at inactivating both *S.* Enteritidis and *E. coli* O157:H7 on contaminated mung bean and alfalfa seeds. To further establish the range of effectiveness, radish and

broccoli seeds contaminated with *S.* Enteritidis or *E.coli* O157:H7 were tested under similar treatment conditions. It was found that low levels (2 and 3 log CFU/g) of pathogens were inactivated when radish seeds were treated with 4,000 ppm and 8,000 ppm carvacrol for 60 min. More interesting, is the fact that similar results could not be obtained on broccoli seeds. Treatment of broccoli seeds inoculated with high levels (8 and 5 log CFU/g) of pathogens consistently produced a ≈ 2.0 log reduction. However, at lower inoculum levels the emulsions effectiveness was similar to that of the control. This "tailing-off" effect has been reported before with similar treatment methods (54). The lack of antimicrobial action on contaminated broccoli seeds may be contributed to compounds found within the seeds. Broccoli seeds/sprouts have significantly higher concentrations of glucosinolates, isothiocyanates, and sulforaphane than other sprout varieties (61, 134, 212). These charged compounds may be destabilizing the nanoemulsion or interacting with the reactive hydroxyl group of the carvacrol. Charged species can have a dramatic effect on nanoemulsion formation, stability, and functionality (39, 128). It has also been shown that the binding/repulsion of essential oils to various charged species significantly decreases the observed antimicrobial effectiveness (161). Nevertheless, further research is needed to establish the physicochemical interaction and effect of these compounds on the stability and efficacy of antimicrobial nanoemulsions.

The development of a food grade, generally recognized as safe (GRAS) antimicrobial treatment is of great interest to the food industry. The use of an essential oil or combination of essential oils may provide a label friendly alternative to some of

the currently used methods. The use of spontaneously emulsified essential oils as an antimicrobial treatment is relatively new and requires further studies. The interaction between carvacrol nanoemulsions and various food compounds and matrices has to be studied to better understand the physiochemical properties of these systems. With greater understanding of the system as a whole, the use of antimicrobial nanoemulsions may find a broad range of applications within the food industry.

CHAPTER 6

MULTICOMPONENT ANTIMICROBIAL NANOEMULSIONS: INACTIVATION OF *SALMONELLA* SPP. ON SPROUTING SEEDS USING A SPONTANEOUS CARVACROL NANOEMULSION ACIDIFIED WITH ORGANIC ACIDS

6.1 Abstract

Over the past decade, there has been an increased demand for natural, minimally processed produce including sprout-based products. A 20,000 ppm calcium hypochlorite is currently recommend for all sprouting seeds prior to germination to limit sprout-related foodborne outbreaks. A potentially promising alternative is acidified spontaneous essential oil nanoemulsions. In this study, the efficacy of an acidified carvacrol nanoemulsion was tested against mung beans and broccoli seeds artificially contaminated with a *S.* Enteritidis cocktail (ATCC BAA-709, ATCC BAA-711 and ATCC BAA-1045). Treatments were performed by soaking inoculated seeds in acidified (50 mM acetic or levulinic acid) carvacrol nanoemulsions (4,000 or 8,000 ppm) for 30 or 60 min. Following treatment, surviving cells were determined via plate counts and/or Most Probable Number (MPN) enumeration. Treated seeds were sprouted and tested for the presence of pathogens and sprout yield. Treatment successfully reduced 4 log CFU/g of *S.* Enteritidis on mung beans and 2 log CFU/g on broccoli seeds below our detection limit (≤ 3 MPN/g) when soaked for 30 min and produced a final sprout product with no detectable pathogens. Total sprout yield was not influenced by any treatment.

6.2 Introduction

Over the past few decade, there has been an increased demand for natural, minimally processed produce (97). The consumption of sprouted produce, like mung bean, alfalfa, broccoli, and radish sprouts, has dramatically increased, with millions of pounds being consumed in the United States alone (93, 207). Since 1995, there have been at least 40 reported outbreaks of foodborne illness, in the United States, associated with sprout consumption. The majority were caused by *Salmonella* and *E. coli* contamination, although other pathogens such as *Listeria monocytogenes*, *Bacillus cereus,* and *Staphylococcus aureus* have also been linked to outbreaks (54, 170, 228).

With intentions on limiting sprout related outbreaks, government agencies modified existing and created regulatory guidelines targeting both industry and consumer audiences. First, the U.S. Department of Health and Human Services issued several reports on the risks associated with the consumption of raw or lightly cooked sprouts (72). Around the same time, the Food and Drug Administration (FDA) released updated manufacturing guidelines based on independent analyses on the science behind sprout related outbreaks (54, 73, 75). To help minimize the potential of foodborne illness, a 20,000 ppm calcium hypochlorite soak is currently recommended for seeds prior to sprouting (241). However, rapid sequestering of free chlorine by organics, inadequate pH adjustments, and seed topography all limit its effectiveness (76,

207, 259). Even with improved manufacturing guidelines and consumer education campaigns, the threat, incidence, and severity of sprout related illness is prevalent.

The possibility and incidence of sprout related illness is a major concern within the food safety community. The increase in consumer demand for healthy, natural, and sustainable products like sprouts has put pressure on industry and regulatory agencies to find more effective methods for preventing and limiting sprout associated illnesses(77). The primary source of contamination is often linked to the sprouting seeds (86, 98). It has been shown that low levels of initial contamination (0.1 log CFU/g) will grow to substantial numbers during the sprouting process (86). Pathogens can be internalized during sprouting, protecting them from any post-germination treatment steps (67, 255). Soaking seeds in the recommended 20,000 ppm calcium hypochlorite for 15 min has a reported reduction range of 0.51 – 6.90 log CFU/g, but still has not been able to significantly reduce the amount of sprout related outbreaks (11, 13, 55). Also, high levels of calcium hypochlorite is also considered unacceptable for the production of certified organic sprouts and is banned in some European countries (54).

A potential alternative to current recommended methods, is the use of emulsified essential oils. Essential oils are natural compounds that are isolated from various plant, sources that demonstrate antimicrobial activity (28). One essential oil that has been shown to have promising antimicrobial properties against a variety of foodborne pathogens is carvacrol (28, 130, 145, 185). Essential oils, alone, have minimal solubility in water. The spontaneous emulsification of carvacrol, originally put forth by Chang *et al.*(41), is simple to produce and requires minimal equipment and training

(154). The nanoemulsion has also been shown to be effective against foodborne pathogens in both *in vitro* and *in vivo* sprout settings. In a broth-based system, the minimal inhibitory concentration for the spontaneous carvacrol nanoemulsion was found to be 500 PPM, with complete inactivation at concentrations greater than or equal to 4000 PPM. When applied to a sprout based system, a 60 min treatment in 4,000 or 8,000 PPM carvacrol nanoemulsion resulted in complete inactivation of both *Salmonella* Enteritidis and *Escherichia coli* O157:H7 on both mung bean and alfalfa seeds. Similar results have been reported on radish seeds, however the carvacrol nanoemulsion did not produce significant log reduction against pathogens on contaminated broccoli seeds.

Previous studies have demonstrated that the combination of organic acids (50 mM levulinic acid) and anionic surfactants (sodium dodecyl sulfate) can inactivate pathogens on alfalfa seeds (264). For the spontaneous carvacrol nanoemulsion, Tween 80® is the main surfactant used in the system. To determine if organic acids may be beneficial, acetic or levulinic acid was added to the nanoemulsion formulation and its efficacy tested on artificially contaminated mung bean and broccoli sprouting seeds.

6.3 Materials and Methods

6.3.1 Bacterial strains and culture conditions

The bacterial cultures used in the presented experiments were *S.* Enteritidis strains 709 (ATCC BAA- 709), 711 (ATCC BAA-711), and 1045 (ATCC BAA-1045). Stock cultures of each organism were stored at -80 ^{o}C in tryptic soy broth (TSB; BD Diagnostic

Systems, Cat# DF0064-07-6) containing 25% (v/v) glycerol. Monthly, frozen stock cultures were transferred to working cultures by plating on tryptic soy agar (TSA; BD Diagnostic Systems, Cat# DF0370-075) slants/plates and incubating at 37 ^{o}C for 24 hrs.

Periodically, working cultures were streaked on differential media to ensure purity. For *S.* Enteritidis, cultures were spread on xylose, lysine, deoxycholate (XLD) agar (Remel Cat# R459902). Cultures were incubated overnight in TSB at 37 ^{o}C on a rotary shaker set at 150 RPM. All cultures were diluted with TSB to the desired cell numbers.

6.3.2 Effect of soaking seeds on organic acid buffers

Beans/seeds (20 g) were placed in 50 mL of organic acid buffer (5, 25, or 50 mM of acetic or levulinic acid) and placed on a shaker set at 125 RPM. The pH was measured every 10 min using a pH meter (Fisher Scientific Accumet Basic, Model: AB15, USA) for a total of 60 min. The pH meter was standardized using 4, 7, and 10 pH buffer solutions (Fisher Scientific, Cat# SB107-500, SB107-500, and SB115-500).

6.3.3 Scanning Electron Microscopy (SEM)

Prior to microscopy, samples were coated with 20 nm of gold by a Cressington Sputter Coater 108auto (Watford, UK) to prevent charging. Micrographs were captured with a JEOL Neoscope JCM 6000 Benchtop SEM (Nikon Instruments, Inc. Melville, NY) while operating at 10 kV.

6.3.4 Formation of antimicrobial nanoemulsions

The preparation of the antimicrobial nanoemulsion was based on a method previously reported by our group, but with a few modifications. Carvacrol (4 g) (Sigma-Aldrich, Cat# W224502-100G-K) was added to 6 g of medium chain triglyceride (MCT) oil (Miglyol 812, Witten, Germany) and thoroughly mixed with a magnetic stir bar for 5 min at 125 RPM. Once mixed, Tween 80® (10 g) (Sigma-Aldrich, Cat# P1754-500ml) was added to the oil mixture and mixed with a magnetic stir bar for another 5 min at 125 RPM. The oil/Tween 80® mixture (20 g) was titrated, at a rate of 2 mL/min, into 80 mL of either 50 mM levulinic (pH 2.8; Sigma-Aldrich, Cat# W262706) or 50 mM acetic acid buffer (pH 2.5; Macron chemical, Cat# V196-05) containing a magnetic stirring bar set to 600 RPM and allowed to mix for an additional 15 min. The emulsion was filter sterilized through a sterile 0.45 µm syringe filter (Fisher Scientific Cat# 09-719-005) and stored in sterile 50 mL conical tubes at 2 - 5 ^{o}C for up to 3 weeks. Droplet size was measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK).

6.3.5 Effectiveness of carvacrol nanoemulsion on contaminated seeds

All beans/seeds used in this study were generously provided by Jonathan's Organics (Rochester, MA). Each strain of *S.* Enteritidis was inoculated in separate test tubes containing TSB (9 mL) and incubated overnight at 37 ^{o}C. Overnight growth was pooled together for a final concentration of 9 log CFU/mL. Batches (20g) of beans/seeds were soaked in 50 mL of diluted *S.* Enteritidis cocktail at a concentration of 9, 6, 5, 4, or 3 log CFU/mL for 20 min to achieve an inoculation level on beans/seeds of 8, 5, 4, 3, or 2 log CFU/g, respectively.

The inoculated beans/seeds were then transferred to a sterile glass petri dish containing sterile filter paper (Fisher Scientific Cat# 09-803-6D) within a biological safety cabinet, and allowed to dry overnight at ambient temperature. For initial cell numbers, contaminated batches (5 g) were soaked in 50 mL of TSB for 15 min on a rotary shaker set to 150 RPM, plated on XLD, and incubated overnight at 37 ^{o}C. To test the effectiveness of the acidified nanoemulsion, inoculated bean/seed batches (15 g) were placed in 250 mL beakers and treated by soaking in 50 mL of acidified nanoemulsion (4,000 or 8,000 ppm) with agitation (125 RPM) for 30 or 60 mins. For the control, contaminated batches were soaked in either 50 mM levulinic (pH 2.8; Sigma-Aldrich, Cat# W262706) or acetic acid (pH 2.5; Macron chemical, Cat# V196-05) buffer. After treatment, the batches were rinsed once with 50 mL of sterile deionized water and transferred to a sterile 250 mL beaker containing 50 mL of TSB. The beaker was placed on a rotary shaker set to 50 RPM for 15 min. After agitation, a dilution series was created and plated on XLD and incubated at 37 ^{o}C for 24 hrs. For treated seed batches that were expected to result in negative plate counts (based upon preliminary data), or that had low initial inoculation levels (2 and 3 log CFU/g), a three tube most probable number (MPN) assay was used in conjunction with spread plating according to the FDA's Bacteriological Analytical Manual (BAM) for a final detection limit of ≤ 3 MPN/g (71). Samples were appropriately diluted in Lactose Broth (BD BBL Cat# DF0004-17-7) Salmonella enrichment and incubated overnight. Dilution sets were checked for turbidity. Any positive tubes were streaked on XLD media (Remel Cat# R459902) for confirmation. Both treated inoculated and uninoculated samples were then sprouted.

Mung Bean Sprouting: Treated inoculated and uninoculated mung beans (20 g) were transferred to a sterile 1000 mL bottle and soaked in 150 mL of distilled water at 20 ^{o}C for 24 hrs. The water was removed, and sprouting continued for 4 days at 20 ^{o}C, with daily water by a 5-min soak in 150 mL of distilled water. After four days, two 25 g batches of sprouts were taken for microbiological testing. The samples were suspended in 225 mL of 0.1% peptone water and stomached for 1 min. A dilution series was created and plated on XLD as previously described.

Broccoli Seed Sprouting: Batches (10 g) of inoculated and uninoculated broccoli seeds were transferred to a sterile 250 mL beaker and soaked in 150 mL of distilled water at 20 ^{o}C for 24 hours. The water was removed, and the seeds transferred to 3 pieces of sterile filter paper (Fisher Scientific Cat# 09-803-6D) on top of a sterile plastic test tube rack in a sterile stainless steel container with a lid. The seeds were sprouted in the dark at 20 ^{o}C for 3 days. The seeds were watered with 15 mL of distilled water every 8 hrs with a plastic spray bottle (Fisher Scientific Cat# 03-438-12A). After sprouting, two 25 g batches of sprouts were suspended in 225 mL of 0.1% peptone water and stomached for 1 min. A dilution series was created and plated on the XLD as previously described.

6.3.6 Influence of acidified nanoemulsion treat on sprout germination yield.

Batches (20 g) of beans/seeds were placed in 250 mL beakers and treated by soaking in distilled water (control) or 50 mL of acidified nanoemulsion (4,000 or 8,000 ppm) with agitation (125 RPM) for 30 or 60 mins. Following treatment, the beans/seeds were rinsed once with 50 mL of sterile deionized water and sprouting as previously

described in section 2.5. Sprouted broccoli or mung beans were transferred to a sterile 250 mL Whirl-pack bag and weighed on a top-load balance.

6.3.7 Storage stability of nanoemulsions

The nanoemulsion (20 mL) was placed in pre-sterilized test tubes and incubated at 20^{o}C. For thirty consecutive days, size measurements were recorded using a dynamic light scattering instrument (ZetasizerNano ZS, Malvern Instruments, UK). To determine if diluting the emulsion would have an impact on stability, stock nanoemulsion was diluted 5-fold in either 50 mM levulinic (pH 2.8; Sigma-Aldrich, Cat# W262706) or 50 mM acetic acid (pH 2.5; Macron chemical, Cat# V196-05) buffer and size measurements were recorded for thirty consecutive days using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK). This instrument determines the particle size from intensity-time fluctuations of a laser beam (633 nm) scattered from a sample at an angle of 173°. Each individual measurements was an average of 13 runs. Samples were measured diluted (50 µL into 5 mL) to avoid multiple scattering effects. The effectiveness of the stored emulsion was evaluated by testing its antimicrobial properties on beans/seed artificially inoculated with 8 log CFU/g of *Salmonella* cocktail.

6.3.8 The effect of pH on the effectiveness of the carvacrol nanoemulsion in vitro

The effectiveness of the acidified carvacrol nanoemulsion (5 mM or 50 mM acetic acid; 1,000 ppm carvacrol) at various pH units was measured monitoring the change log reductions of *S.* enterica. Briefly, 0.1 mL of *S.* enterica (9 log CFU/mL) was transferred to a sterile 2.0 mL conical centrifuge containing 0.9 mL sterile saline (0.85%

NaCl) and centrifuged for 1 min at 10,000 RPM. The supernatant was decanted, pellet suspended in 0.9 mL sterile saline, and centrifuged again for 1 min at 10,000 RPM. Again the supernatant was decanted and the pellet suspended in 0.9 mL sterile saline for a final concentration of 8 log CFU/mL. Carvacrol nanoemulsion (1 mL) and *S.* enterica (1 mL) were combined in a sterile 2.0 mL conical centrifuge for a total volume of 2.0 mL with concentrations of 1,000 ppm carvacrol and 6 log CFU/mL *S.* enterica, respectively. The mixture was vortexed for 5 sec and allowed to incubate a room temperature for 5 mins. Following incubation, serial dilutions were made in neutralizing broth (Remel Cat# R453042) and plated on TSA plates. Plates were allowed to incubate at 37 ^{o}C for 24 hrs.

6.3.9 Statistical Analysis

Statistical analysis between pH values for all samples was performed using an unpaired two-tail t-test with a 95% confidence interval. A one-way ANOVA followed by Tukey's multiple comparison test (95% confidence interval) was used to statistically analyze both total log reduction and total sprout yield. All statistical analyses were performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, La Jolla California USA.

6.4 Results

6.4.1 Influence of seeds on the pH of the organic acid buffers.

In a previous study our group found that the antimicrobial efficiency of carvacrol emulsions prepared in 5 mM citrate buffer were much less effective on broccoli seeds, than observed with mung, alfalfa or radish. It was also observed that brown matter

(assumed to be seed coat materials) would come off broccoli seeds during soaking, while less was seen with other seed types. Therefore, in preparation for reformulation of antimicrobials with acetic and levulinic acid, we were curious if the pH of the solution was changing during treatment. The pH of acid solutions at different molarities was monitored over time (**Figure 6.1**). Mung beans had a slight yet significant effect on the pH of both the acetic and levulinic acid (**Figures 6.1A and B**) after soaking for 60 min regardless of acid concentration. Broccoli seeds, on the other hand, had a dramatic effect on the pH of both acetic and levulinic acid (**Figures 6.1C and 6.1D**) buffers. The greatest increase in pH for both seeds occurred within in the first 10 min for all concentrations and acid types (**Figure 6.1**). When soaked in acetic acid for 10 min, the pH for broccoli seeds soaked in 5 mM acetic acid increased by 1.48 pH units, 1.09 pH units for 25 mM acetic acid, and 0.6 pH units for 50 mM acetic acid. For levulinic acid, the pH increased by 1.6 pH units for 5 mM levulinic acid, 1.2 pH units for 25 mM levulinic acid, and 1 pH unit for 50 mM levulinic when soaked for 10 min. A significant difference was found when comparing the change in pH between seeds for each acid, as seen in **Figures 6.2A** and **6.2B**. Broccoli seed's effect on the pH of both acetic and levulinic acid was significantly greater than that of mung beans (**Figure 6.2D**). There was no significant difference found between the effects of mung beans on the change in pH for each acid at either 5 mM or 25 mM (**Figure 6.2C**). A significant difference was found between the effects of broccoli seeds on the change in pH for both acids at all concentrations tested. (**Figure 6.2D**).

In order to determine if seed coat materials were visually changed after soaking, seeds were observed using scanning electron microscopy. As seen in **Figure 6.3**, After soaking broccoli seeds, a noticeable change in surface structure was observed, with dimpled structures (**Figure 6.3A**) becoming much more defined after soaking in acid buffer (**Figure 6.3B**). No observable changes were noted for mung beans (**Figure 6.4**).

6.4.2 The effect of pH on the effectiveness of the carvacrol nanoemulsion in vitro

The effect of pH on the antimicrobial activity of the acidified (5 mM or 50 mM acetic acid) carvacrol nanoemulsion can be seen in **Figure 6.5.** Maximum antimicrobial activity was achieved for both concentrations at pH 3.5. As the pH of the antimicrobial system increased through the range of 4 - 7, the effectiveness of a carvacrol nanoemulsion acidified with acetic acid was reduced by over 41% and 68% for 5 mM and 50 mM concentrations, respectively. A similar, yet more dramatic trend was observed for a carvacrol nanoemulsion acidified with levulinic acid. The effectiveness of the nanoemulsion against *Salmonella* spp. at a pH of 7was reduced by over 88% and 61% for 5 mM and 50 mM levulinic acid concentrations.

6.4.3 Effectiveness of an acidified carvacrol nanoemulsion against Salmonella spp. contaminated mung bean and broccoli seeds.

Due to the higher buffering capacity, 50 mM acids were selected for reformulation of nanoemulsions. The effectiveness of either a 50 mM levulinic or 50 mM acetic acid acidified carvacrol nanoemulsion can be seen in **Figures 6.6** and **6.7** along with **Tables 6.1 – 6.4**. On mung beans, with either acid system a 5 log reduction was observed with high inoculation levels (8 log CFU/g), however, during the course of

germination, levels of *Salmonella* increased to similar levels as observed on untreated samples.

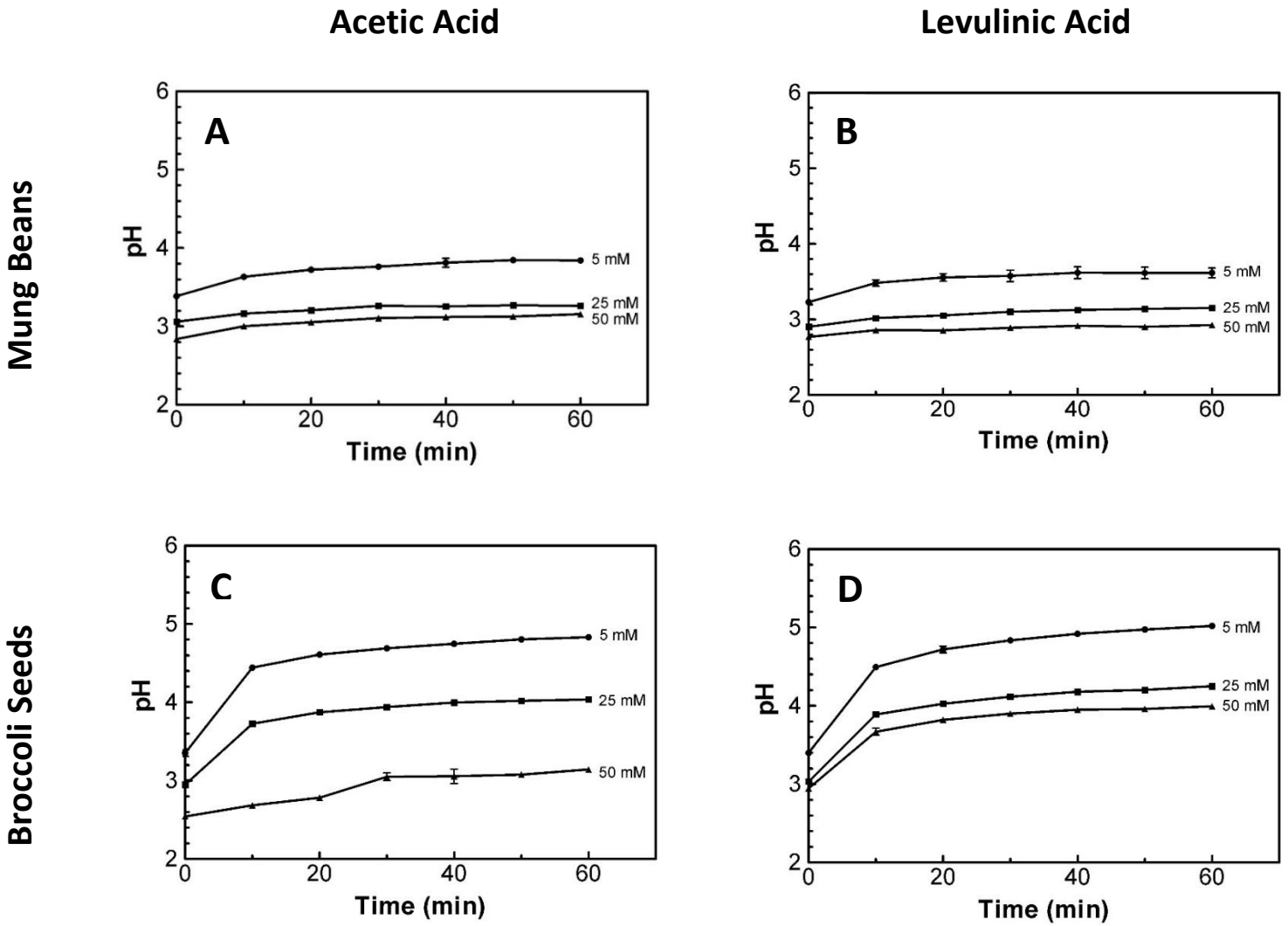


Figure 6.1. The change in pH over time while soaking mung beans or broccoli seeds in either acetic or levulinic acid systems. Beans/seeds were placed in various concentrations of either an acetic or levulinic acid and the pH measured every 10 min. All points are means with standard deviations from triplicate studies.

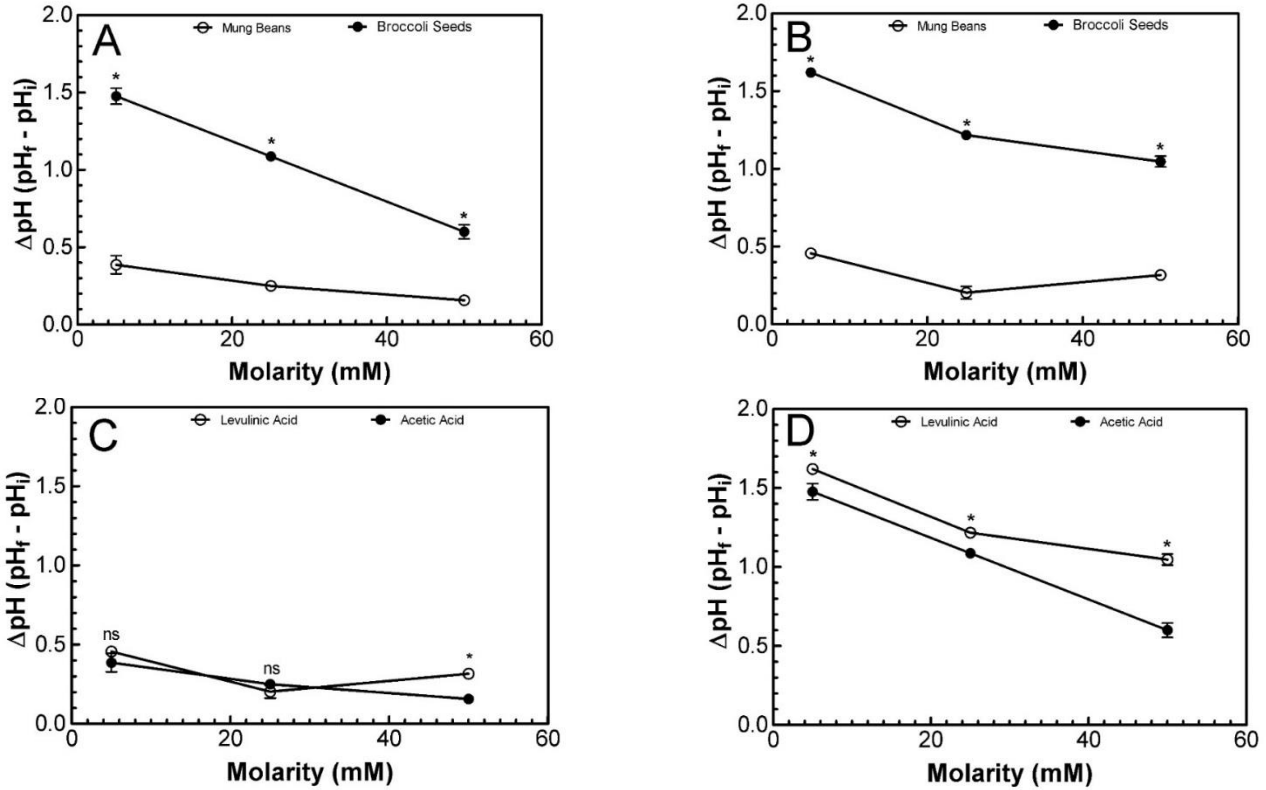


Figure 6.2. Influence of seed/bean and acid type on pH was studied by comparing initial (pH_i) and final pH (pH_f) values. The effect of seed type on the change in pH at various acetic or levulinic acid molarity is represented in A and B respectively. Comparison between initial and final pH of an acetic or levulinic acid solution following a 60 min soak with either mung beans (C) or broccoli seeds (D) was also examined. Statistical analysis between pH values for all samples was performed using an unpaired two-tail t-test with a 95% confidence interval. A "*" indicates that a significant difference was found between samples. An "ns" indicates that there was no significant difference between samples.

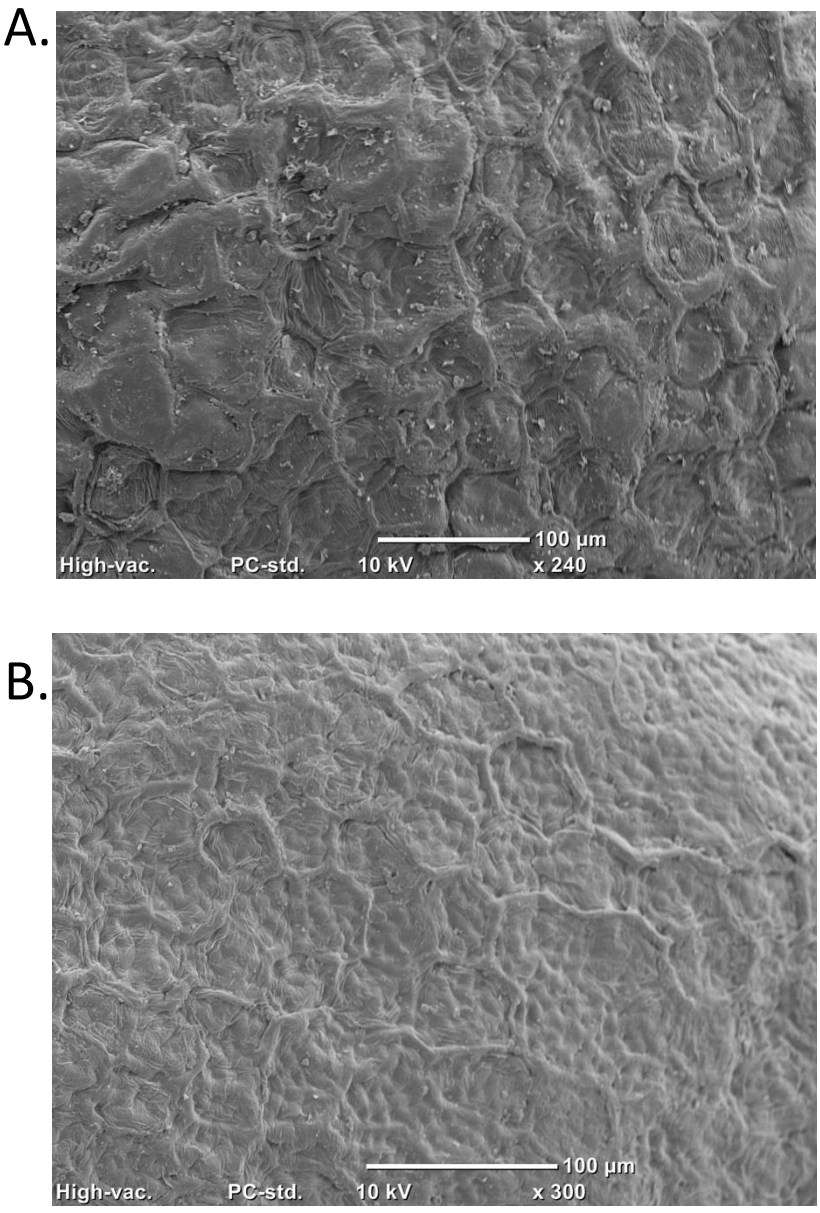
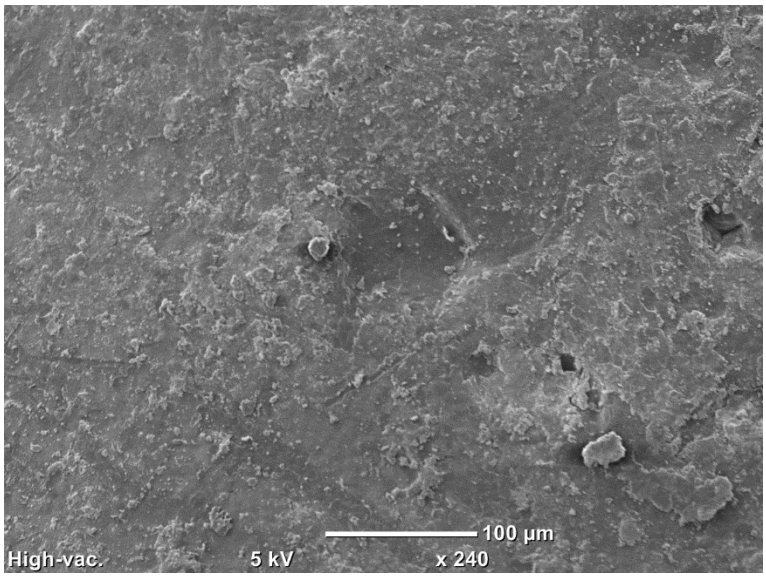


Figure 6.3. Scanning electron microscopy (SEM) images of un-soaked (A) and soaked (B) broccoli seeds. When comparing both figures, an apparent decrease in surface material is observed between samples.

A.



B.

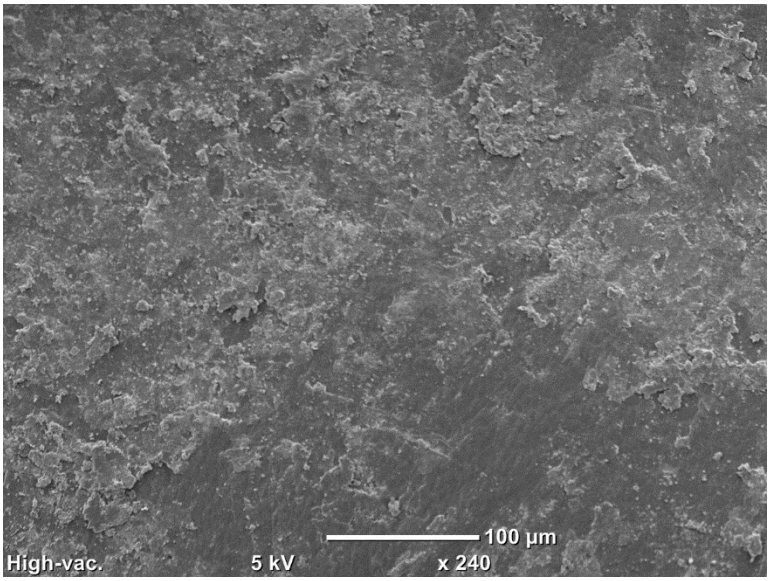


Figure 6.4. Scanning electron microscopy (SEM) images of un-soaked (A) and soaked (B) mung beans. When comparing both figures, no significant difference between surface materials is observed.

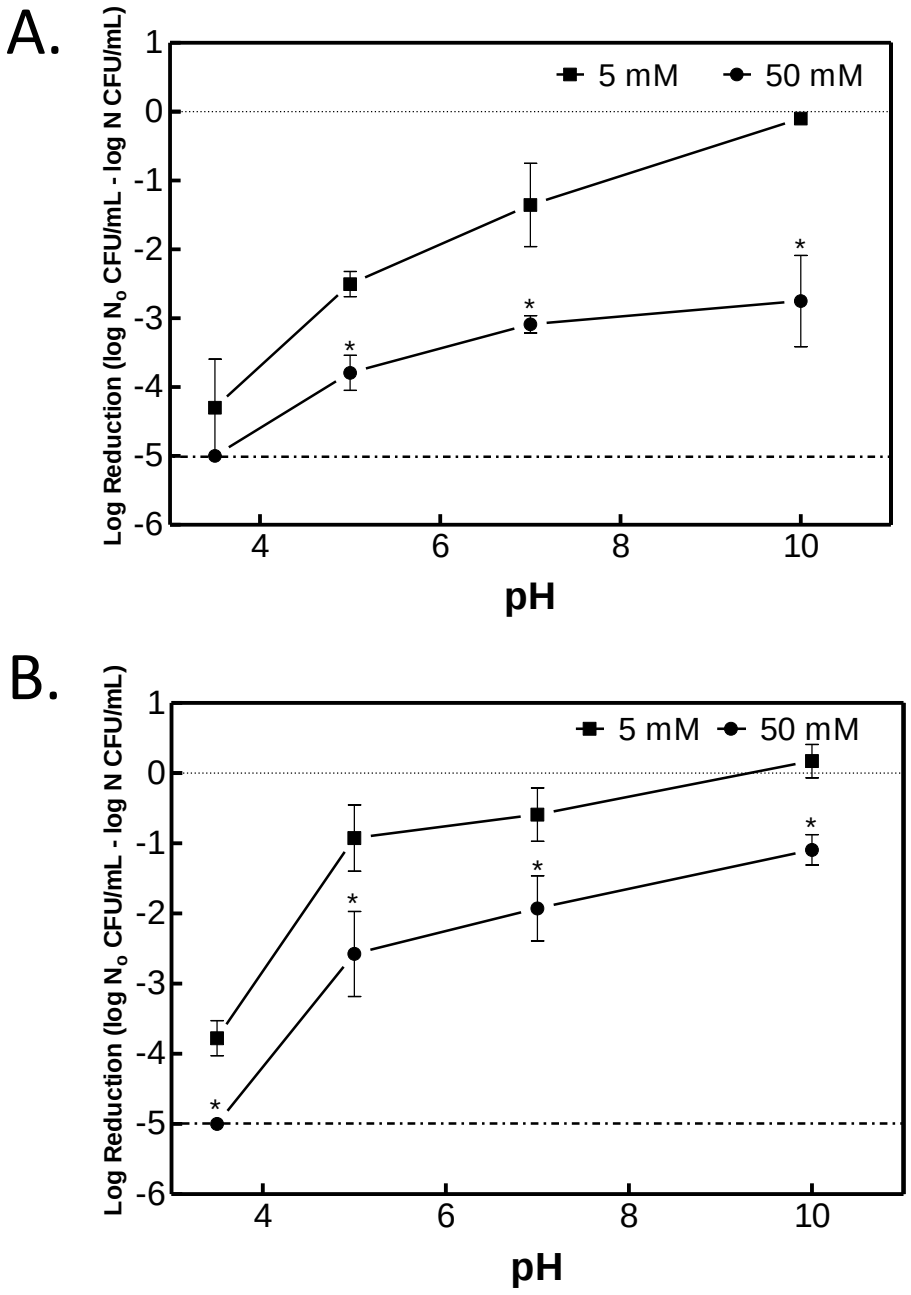


Figure 6.5. The influence of pH on the efficacy of a carvacrol nanoemulsion acidified with either acetic acid (A) or levulininc acid (B) against a *Salmonella* Enteritidis cocktail. The pH was adjusted using 10 M NaOH and each point represents an average value of three replicates. A "*" indicates a significant difference between each sample.

When inoculated with 5 log CFU/g, mung beans treated with all acidified emulsions, reduced levels to 1.5 log CFU/g, however, during the course of germination, levels of *Salmonella* increased to similar levels as observed on untreated samples. With an initial inoculum of 4 log CFU/g, surviving levels of *Salmonella* on inoculated seeds was below detectable levels (less than 3 MPN/g) when treated with 4000 or 8000 ppm of carvacrol acidified with either organic acid (**Figure 6.6A** and **6.6B** and **Tables 6.1** and **6.2**), and remained below detectable limits (1.5 log CFU/g)after germination. Total sprout yield was not compromised by any treatment (**Table 6.5**).

The treatment was less effective on broccoli seeds (**Figures 6.7A** and **6.7B** and **Table 6.3** and **6.4**). On broccoli seeds, with either acid system a 2.6-3.4 log reduction was observed with high inoculation levels (8 log CFU/g) on seeds. While the surviving cells increased during the germination process, the effectiveness of the levulinic acid and acetic acid buffered carvacrol nanoemulsions were more effective than observed when prepared with 5 mM citrate buffer. When an initial inoculum of 2 log CFU/g and treated with 4000 or 8000 ppm of carvacrol acidified with either organic acid, levels of *Salmonella* were reduced to below detectable levels (≤ 3 MPN/g) and remained below detectable limits (1.5 log CFU/g)after germination. Total sprout yield was not compromised by any treatment (**Table 6.6**).

6.4.4 Stability of nanoemulsions

The change in acid concentration can influence the stability of emulsions, and to have practical applications, it is important to assess the emulsion stability. The freshly

prepared stock nanoemulsions with a concentration of 40,000 ppm carvacrol had mean droplet diameters (Z-average) around 100 nm for both 50 mM acetic and levulinic acid compared, to a mean particle diameter of approximately 90 nm when stocks were diluted to a working concentration of 8,000 ppm carvacrol in either acid (**Figures 6.8** and **6.9**). After 30 days, the particle size had increased to approximately 100 nm for diluted emulsions stored at 8,000 ppm and 180 nm for stock emulsions stored concentrated (40,000 ppm). (**Figures 6.8** and **6.9**). Additionally, the polydispersity index decreased over time for all emulsions tested (**Figure 6.10**). These results indicate that while the droplet size is growing, it is also becoming more uniform with time. This is likely due to Ostwald Ripening, as the carvacrol may be moving from the oil droplet into the aqueous phase. However, when efficacy was tested after 30 days of storage the emulsion system was still effective with no significant difference in antimicrobial effectiveness when compared to the freshly prepared emulsion. Previous results showed that growth in droplet size during storage could be inhibited by diluting the nanoemulsion 5-fold with sterile sodium citrate buffer prior to storage. We observed that stability was greatly enhanced with dilution.

6.5 Discussion

The demand for minimally processed, natural produce has continued to increase despite the inherent risk of foodborne illness. At the fore front of this resurgence are sprouts. Sprouts, such as mung bean, alfalfa, radish, and broccoli, are minimally processed and can be vectors for a variety of human pathogens (166). Between 1990 and 2005, there have been over 700 worldwide outbreaks, resulting in roughly 34,000

Table 6.1. Effect of carvacrol emulsion acidified with 50 mM levulinic acid on contaminated mung beans[1,2]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	8.6 ± 0.72	7.7 ± 0.65	0.75 ± 0.43^{A}	+ + +
		5.8 ± 0.68	4.9 ± 0.20	0.68 ± 0.83^{A}	+ + +
		4.3 ± 0.34	3.9 ± 0.53	0.63 ± 0.50^{A}	+ + +
	60	8.8 ± 0.04	7.9 ± 0.50	1.02 ± 0.53^{A}	+ + +
		5.8 ± 0.28	5.3 ± 0.61	0.71 ± 0.62^{A}	+ + +
		4.7 ± 0.28	4.1 ± 0.27	0.64 ± 0.42^{A}	+ + +
8000 ppm	30	8.5 ± 0.53	3.5 ± 0.45	5.01 ± 0.15^{A}	+ + +
		5.3 ± 0.44	1.5 ± 0.10	3.9 ± 0.54^{B}	+ + +
		4.6 ± 0.66	ND	>4.1 ± 0.66^{AB}	- - -
	60	8.4 ± 0.50	3.8 ± 0.35	4.5 ± 0.48^{A}	+ + +
		5.5 ± 0.57	1.3 ± 0.20	4.0 ± 0.60^{A}	+ + +
		4.4 ± 0.60	ND	>3.9 ± 0.60^{A}	- - -
4000 ppm	30	8.4 ± 0.11	3.6 ± 0.76	5.1 ± 0.86^{A}	+ + +
		5.3 ± 0.73	1.4 ± 0.27	3.7 ± 0.47^{B}	+ + +
		4.6 ± 0.73	ND	>4.1 ± 0.73^{AB}	- - -
	60	8.6 ± 0.34	3.6 ± 0.32	5.0 ± 0.22^{A}	+ + +
		5.6 ± 0.84	1.2 ± 0.21	4.0 ± 1.02^{A}	+ + +
		4.5 ± 0.75	ND	>4.0 ± 0.75^{A}	- - -

[1] All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a detection limit of 3 MPN/g. Following treatment, seeds were germinated and tested for the presence of pathogens. A "+" indicates that pathogens were detected in one of triplicate samples and a "-"indicates that no pathogens were detected after germination as determined with a detection limit of 1.5 log CFU/g.

[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval. Groupings of statistical significance are indicated by superscripted letters.

Table 6.2. Effect of carvacrol emulsion acidified with 50 mM acetic acid on contaminated mung beans [1,2]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	8.2 ± 0.08	7.4 ± 0.51	0.87 ± 0.53^{A}	+ + +
		5.2 ± 0.40	4.8 ± 0.88	0.78 ± 0.84^{A}	+ + +
		4.7 ± 0.45	3.8 ± 0.16	0.80 ± 0.64^{A}	+ + +
	60	8.7 ± 0.04	7.9 ± 0.50	1.03 ± 0.53^{A}	+ + +
		5.8 ± 0.68	4.9 ± 0.20	0.69 ± 083^{A}	+ + +
		4.3 ± 0.28	4.0 ± 0.60	0.53 ± 0.82^{A}	+ + +
8000 ppm	30	8.3 ± 0.31	3.6 ± 0.65	4.9 ± 0.52^{A}	+ + +
		5.3 ± 0.46	1.4 ± 0.09	3.7 ± 0.48^{A}	+ + +
		4.5 ± 0.74	ND	>4.0 ± 0.74^{A}	- - -
	60	8.9 ± 0.84	3.9 ± 0.19	4.6 ± 0.91^{A}	+ + +
		5.1 ± 0.58	1.1 ± 0.05	3.8 ± 0.62^{A}	+ + +
		4.0 ± 0.22	ND	>3.5 ± 0.22^{A}	- - -
4000 ppm	30	8.4 ± 0.30	3.3 ± 0.16	5.0 ± 0.30^{A}	+ + +
		5.0 ± 0.47	1.4 ± 0.01	3.5 ± 0.46^{B}	+ + +
		4.2 ± 0.47	ND	>3.7 ± 0.47^{AB}	- - -
	60	8.2 ± 0.40	3.4 ± 0.51	4.9 ± 0.89^{A}	+ + +
		5.1 ± 0.59	1.3 ± 0.20	3.6 ± 0.62^{A}	+ + +
		4.3 ± 0.46	ND	>3.8 ± 0.46^{A}	- - -

[1] All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a detection limit of 3 MPN/g. Following treatment, seeds were germinated and tested for the presence of pathogens. A "+" indicates that pathogens were detected in one of triplicate samples and a "-"indicates that no pathogens were detected after germination as determined with a detection limit of 1.5 log CFU/g.

[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval. Groupings of statistical significance are indicated by superscripted letters.

Table 6.3. Effect of carvacrol emulsion acidified with 50 mM levulinic acid on contaminated broccoli seeds [1]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	8.4 ± 0.43	7.5 ± 0.35	0.83 ± 0.58^{A}	+ + +
		5.7 ± 0.86	4.7 ± 0.38	0.47 ± 0.55^{A}	+ + +
		3.3 ± 0.52	3.0 ± 0.44	0.30 ± 0.91^{A}	+ + +
		2.5 ± 0.13	2.3 ± 0.31	0.29 ± 0.37^{A}	+ + +
	60	8.5 ± 0.42	7.6 ± 0.10	0.74 ± 0.46^{A}	+ + +
		5.6 ± 0.24	5.4 ± 0.67	0.38 ± 0.85^{A}	+ + +
		3.6 ± 0.60	3.3 ± 0.59	0.27 ± 0.22^{A}	+ + +
		2.5 ± 0.12	2.3 ± 0.12	0.20 ± 0.09^{A}	+ + +
8000 ppm	30	8.5 ± 0.23	5.1 ± 0.20	3.4 ± 0.41^{A}	+ + +
		5.6 ± 0.33	3.8 ± 0.38	1.8 ± 0.65^{B}	+ + +
		3.8 ± 0.14	2.8 ± 0.40	1.1 ± 0.52^{B}	+ + +
		2.4 ± 0.09	ND	>1.8 ± 0.09^{B}	- - -
	60	8.3 ± 0.48	5.3 ± 0.11	2.9 ± 0.37^{A}	+ + +
		5.6 ± 0.44	2.8 ± 0.82	2.9 ± 0.88^{A}	+ + +
		3.9 ± 0.54	2.6 ± 0.16	1.2 ± 0.62^{B}	+ + +
		2.1 ± 0.30	ND	>1.6 ± 0.30^{AB}	- - -
4000 ppm	30	8.0 ± 0.12	5.4 ± 0.17	2.6 ± 0.08^{A}	+ + +
		5.9 ± 0.28	3.9 ± 0.43	2.09 ± 0.16^{A}	+ + +
		3.8 ± 0.20	2.8 ± 0.11	0.97 ± 0.31^{B}	+ + +
		2.6 ± 0.09	ND	>2.1 ± 0.09^{A}	- - -
	60	8.1 ± 0.26	5.3 ± 0.23	2.8 ± 0.49^{A}	+ + +
		5.7 ± 0.51	3.9 ± 0.17	1.7 ± 0.46^{B}	+ + +
		3.8 ± 0.38	2.6 ± 0.15	1.1 ± 0.40^{B}	+ + +
		2.6 ± 0.15	ND	>2.1 ± 0.15^{A}	- - -

[1]All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a detection limit of 3 MPN/g. Following treatment, seeds were germinated and tested for the presence of pathogens. A "+" indicates that pathogens were detected in one of triplicate samples and a "-"indicates that no pathogens were detected after germination as determined with a detection limit of 1.5 log CFU/g.

[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval. Groupings of statistical significance are indicated by superscripted letters.

Table 6.4. Effect of carvacrol emulsion acidified with 50 mM acetic acid on contaminated broccoli seeds[1,2]

Treatment	Treatment Time (min)	Initial Inoculum (log CFU/g)	Cell Numbers after Treatment (log CFU/g)	Total Log Reduction	Pathogen Detected after Sprouting
Control	30	8.0 ± 0.38	7.5 ± 0.05	0.45 ± 0.42^{A}	+ + +
		5.0 ± 0.19	4.4 ± 0.31	0.62 ± 0.38^{A}	+ + +
		3.2 ± 0.10	2.9 ± 0.64	0.61 ± 0.60^{A}	+ + +
		2.8 ± 0.53	2.1 ± 0.32	0.69 ± 0.88^{A}	+ + +
	60	8.4 ± 0.43	7.9 ± 0.50	0.48 ± 0.92^{A}	+ + +
		4.9 ± 0.15	4.7 ± 0.69	0.53 ± 0.78^{A}	+ + +
		3.2 ± 0.23	2.8 ± 0.24	0.43 ± 0.42^{A}	+ + +
		2.6 ± 0.08	2.2 ± 0.24	0.45 ± 0.18^{A}	+ + +
8000 ppm	30	8.6 ± 0.30	5.3 ± 0.20	3.2 ± 0.38^{A}	+ + +
		5.7 ± 0.48	3.9 ± 0.73	1.9 ± 0.25^{B}	+ + +
		3.8 ± 0.22	2.7 ± 0.22	1.1 ± 0.08^{B}	+ + +
		2.6 ± 0.15	ND	>2.1 ± 0.15^{B}	- - -
	60	8.7 ± 0.24	5.3 ± 0.36	3.4 ± 0.30^{A}	+ + +
		5.6 ± 0.73	4.0 ± 0.57	1.5 ± 0.82^{B}	+ + +
		3.9 ± 0.22	2.6 ± 0.04	1.3 ± 0.19^{B}	+ + +
		2.6 ± 0.23	ND	>2.1 ± 0.23^{B}	- - -
4000 ppm	30	8.4 ± 0.28	5.6 ± 0.14	2.8 ± 0.27^{A}	+ + +
		5.7 ± 0.41	4.6 ± 0.65	1.2 ± 0.45^{B}	+ + +
		3.8 ± 0.11	2.6 ± 0.30	1.3 ± 0.40^{B}	+ + +
		2.4 ± 0.08	ND	>1.9 ± 0.08^{B}	- - -
	60	8.4 ± 0.66	5.2 ± 0.71	3.1 ± 0.78^{A}	+ + +
		5.7 ± 0.38	4.0 ± 0.36	1.7 ± 0.50^{B}	+ + +
		3.9 ± 0.48	2.5 ± 0.26	1.2 ± 0.33^{B}	+ + +
		2.6 ± 0.10	ND	>2.1 ± 0.10^{AB}	- - -

[1] All means and standard deviations are from triplicate studies. A "ND" indicates that no pathogens were detected using a three-tube MPN assay with a detection limit of 3 MPN/g. Following treatment, seeds were germinated and tested for the presence of pathogens. A "+" indicates that pathogens were detected in one of triplicate samples and a "-"indicates that no pathogens were detected after germination as determined with a detection limit of 1.5 log CFU/g.

[2] Total reduction averages within each treatment grouping were compared using Tukey's Test with a 95% confidence interval. Groupings of statistical significance are indicated by superscripted letters.

Table 6.5. Effect of carvacrol emulsion acidified with 50 mM levulinic acid on sprout yield[1,2]

Treatment	Treatment Time (min)	Mung Bean Yield (g)	Broccoli Yield (g)
Control	30	78.3 ± 8.4^{A}	52.1 ± 5.6^{A}
	60	75.2 ± 9.1^{A}	51.5 ± 11.7^{A}
8000 PPM	30	77.4 ± 5.7^{A}	49.5 ± 3.1^{A}
	60	78.6 ± 8.9^{A}	49.3 ± 1.9^{A}
4000 PPM	30	84.4 ± 12.0^{A}	52.9 ± 11.0^{A}
	60	92.1 ± 18.7^{A}	48.8 ± 3.5^{A}

[1] Bean yield averages were compared using Tukey's Test with a 95% confidence interval.

[2] All means and standard deviations are from triplicate studies.

Table 6.6. Effect of carvacrol emulsion acidified with 50 mM acetic acid on sprout yield[1,2]

Treatment	Treatment Time (min)	Mung Bean Yield (g)	Broccoli Yield (g)
Control	30	78.3 ± 8.4^{A}	52.1 ± 5.6^{A}
	60	75.2 ± 9.1^{A}	51.5 ± 11.7^{A}
8000 PPM	30	81.6 ± 8.7^{A}	51.3 ± 2.5^{A}
	60	79.1 ± 13.4^{A}	54.9 ± 10.2^{A}
4000 PPM	30	84.4 ± 6.8^{A}	54.5 ± 11.7^{A}
	60	83.8 ± 4.2^{A}	54.02 ± 5.5^{A}

[1] Bean yield averages were compared using Tukey's Test with a 95% confidence interval.

[2] All means and standard deviations are from triplicate studies.

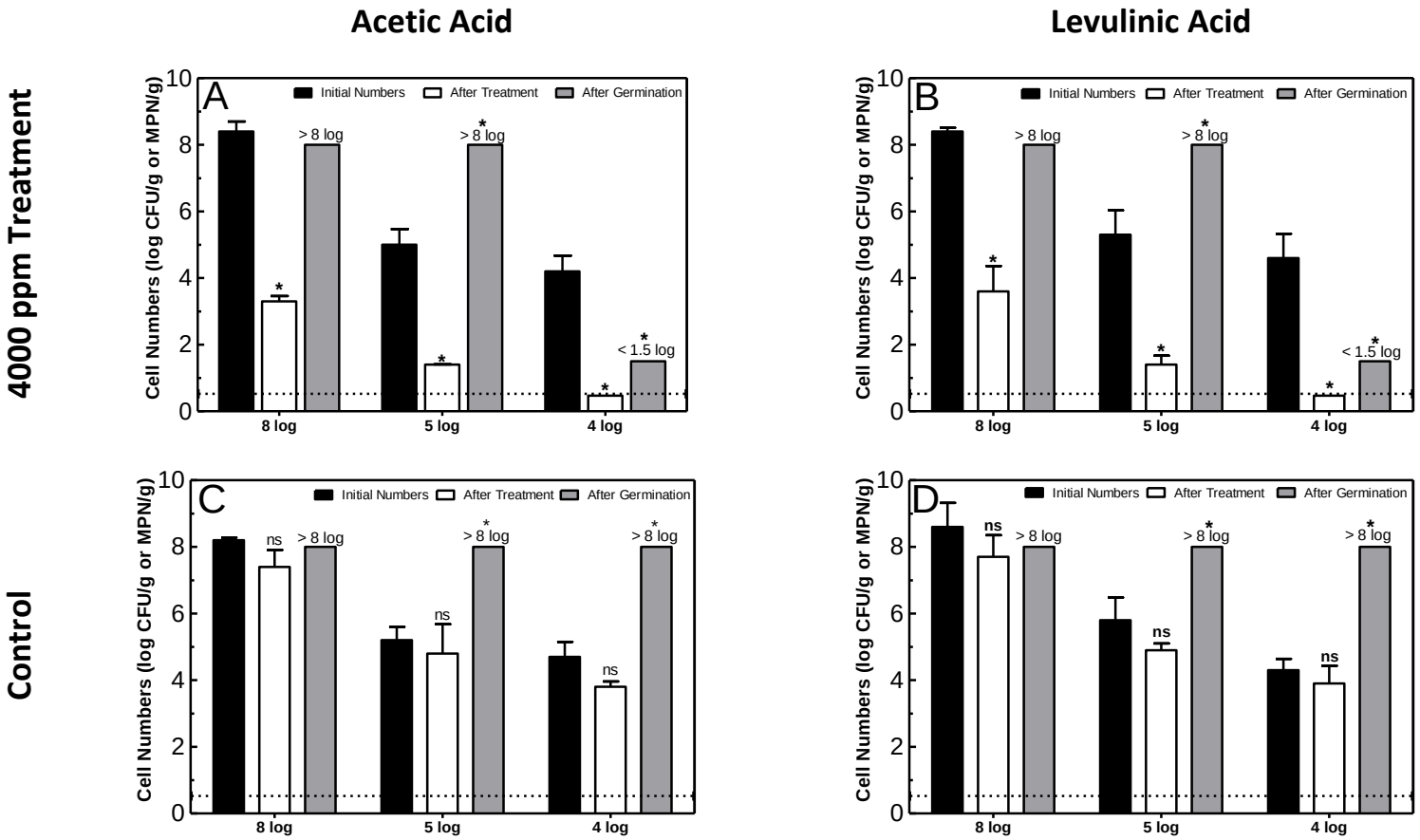


Figure 6.6.Comparison of remaining cell numbers (CFU/g or MPN/g) of pathogen between the control (C or D) and a 30 min 4,000 ppm acidified (50 mM acetic (A) or levulinic acid (B)) nanoemulsion on mung beans. As indicated by an "*", there was a significant difference (un-paired t-test with a 95% confidence interval) between initial cell numbers and cell numbers after treatment and germination when treated with the acidified nanoemulsion. An "ns" indicates that there was no significant difference between samples. All plotted means and standard deviations are from triplicate studies.

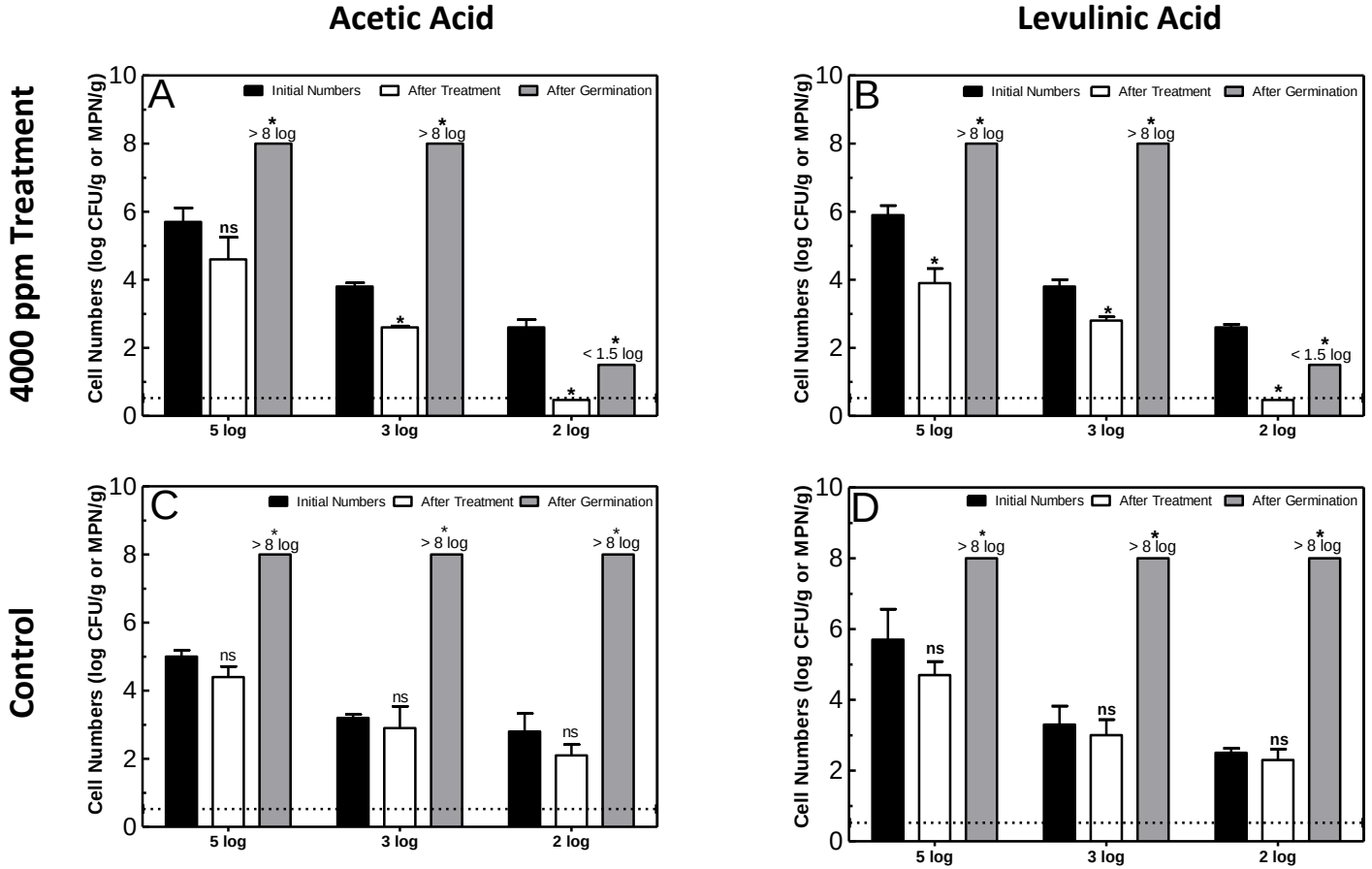


Figure 6.7. Comparison of remaining cell numbers (CFU/g or MPN/g) of pathogen between the control (C) or D) and a 30 min 4,000 ppm acidified (50 mM acetic (A) or levulinic acid (B)) nanoemulsion treatment on broccoli seeds. As indicated by an "*", there was a significant difference (un-paired t-test with a 95% confidence interval) between initial cell numbers and cell numbers after treatment and germination when treated with the acidified nanoemulsion. An "ns" indicates that there was no significant difference between samples. All plotted means and standard deviations are from triplicate studies.

cases of foodborne illness (206). One potential reason for the increase in outbreaks may be linked to the increase production, variety, and use of sprouts in the United States. For example, the per capita demand and consumption of sprouts has dramatically increased over the past decades with millions of pounds being consumed in the United States alone (93, 207).

We have previously reported the ability of a spontaneous carvacrol nanoemulsion containing 5 mM sodium citrate to inactivate pathogens on certain sprouting seeds. This formulation produced significant pathogen reductions on contaminated mung bean, radish seed, and alfalfa seeds (2-3 log reduction) but not broccoli seeds (<1 log reduction). In this report, we present data showing that soaking broccoli seeds in an acidified buffer for 60 min increased the pH within the system, with the most significant increase in pH occurring after the first 10 min of soaking. It was found that buffers containing low concentrations of acid could not maintain desired pH levels during treatment. To further investigate this phenomenon, SEM was performed on soaked and un-soaked seeds/beans. It was found that a noticeable change in surface structure on broccoli seeds occurred after a 10 min soak in acidified buffer. This observation, along with the change in buffer color to brown, may be an indication that seed coat material may be removed during soaking, and could possibly sequester protons, resulting in an increase in pH. In addition, any seed coatings or dispersal aids which were added to seeds during commercial production may possibly influence pH during soaking (239, 240). Unlike broccoli seeds, mung beans did not have a significant effect on the pH of the system or have any noticeable change in surface

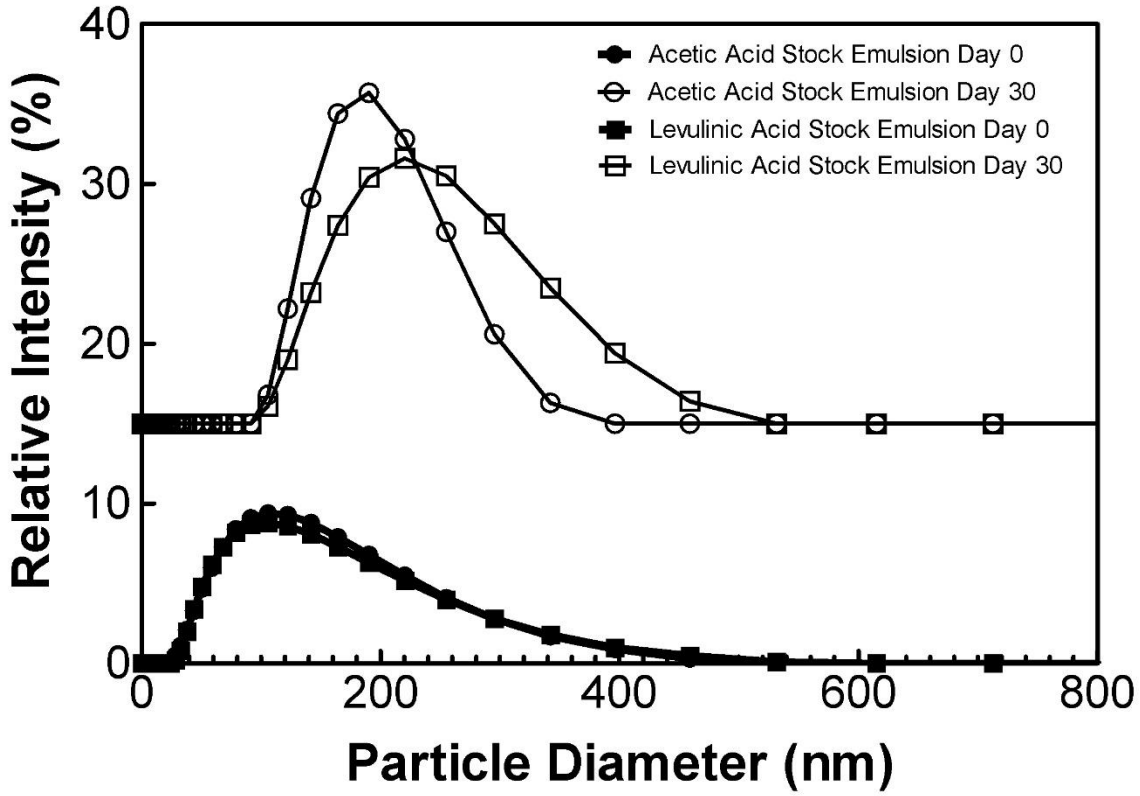


Figure 6.8. Comparison of the mean diameter (Z-average) of freshly prepared and stored (30 days) stock (40,000 ppm) acidified carvacrol nanoemulsion. To distinguish between the samples 15 units were added to the relative intensity of the 30 day sample.

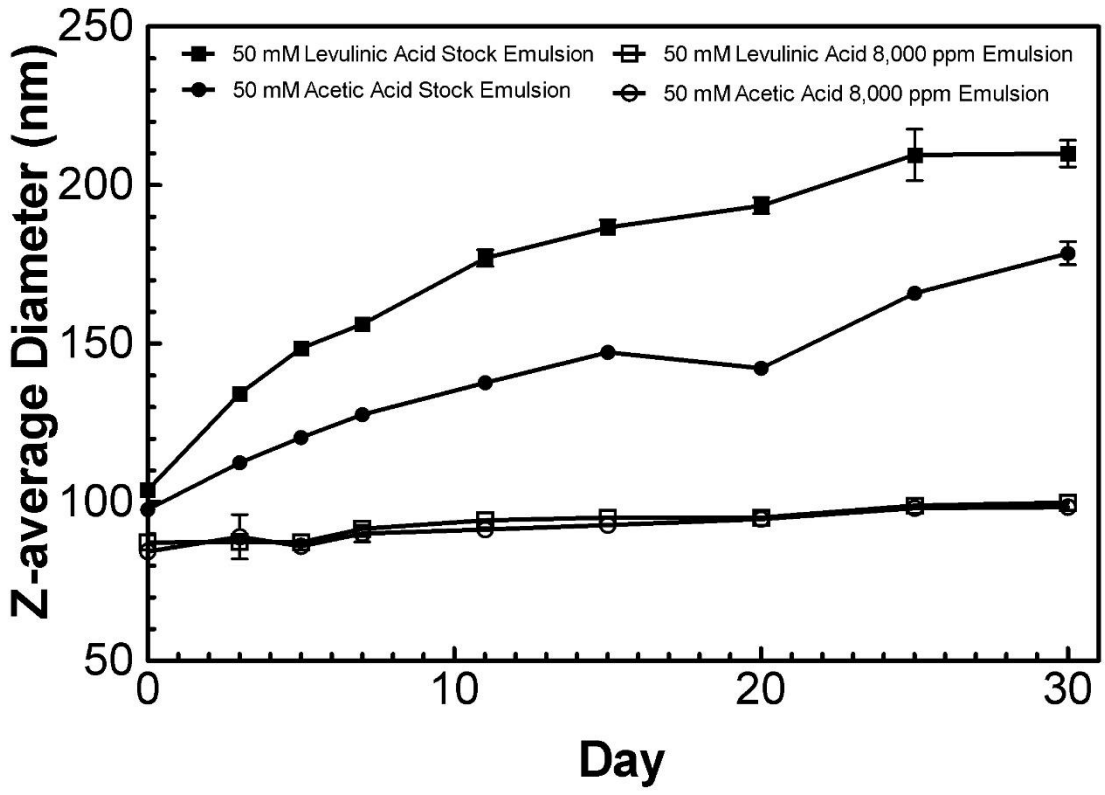


Figure 6.9. Comparison of the increase in mean droplet diameter (Z-average) of undiluted (40,000 ppm) and 5-fold (8,000 ppm) diluted acidified spontaneous carvacrol nanoemulsion when stored for 30 days. All plotted means and standard deviations are from triplicate studies.

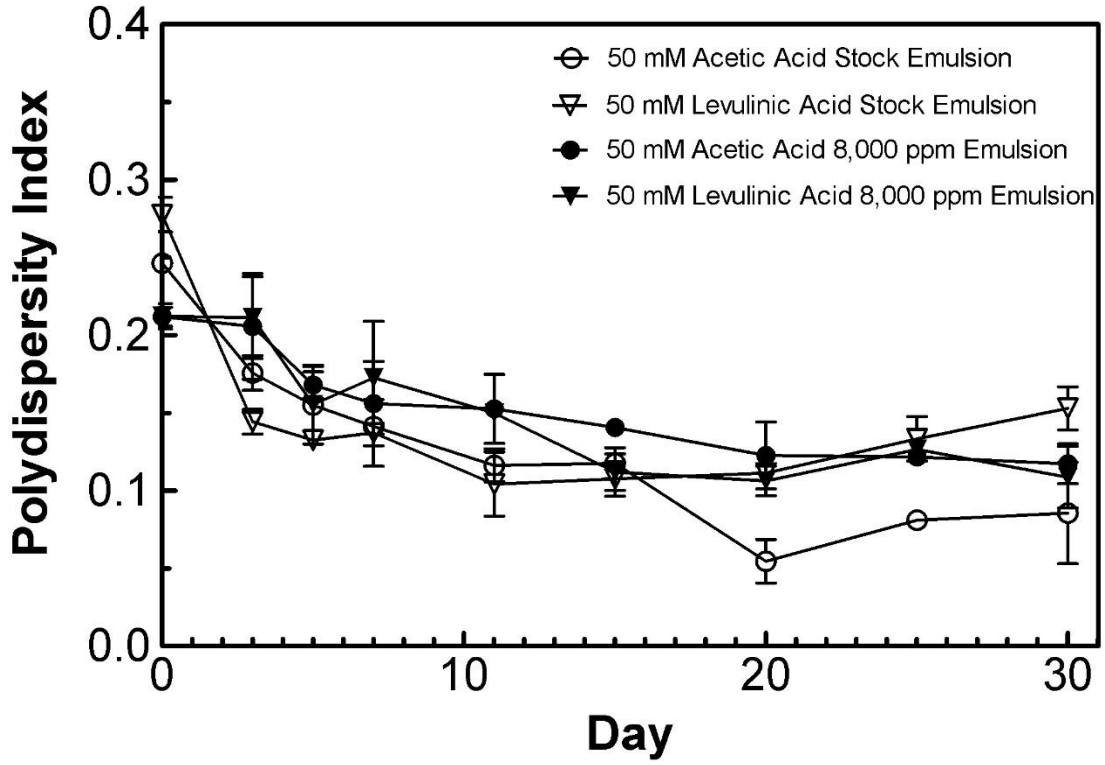


Figure 6.10. Comparison of the change in polydispersity index of undiluted (40,000 ppm) and 5-fold diluted (8,000 ppm) acidified spontaneous carvacrol nanoemulsion when stored for 30 days. All plotted means and standard deviations are from triplicate studies.

characteristics after soaking. The antimicrobial effectiveness of the proposed acidified carvacrol nanoemulsion is sensitive to pH and loses potency with an increase in pH. This may be why treatments with the original and acidified carvacrol nanoemulsion resulted in greater log reductions on mung beans than on broccoli seeds.

Charged or reactive compounds such as cellulose, lignin, peptides, glycosides, and other reactive substances found in seeds can also have an effect on the nanoemulsion droplets as well (153, 154). Various mechanisms, such as electrostatic screening, bridging, and binding effects can decrease the solubility and overall effectiveness of the nanoemulsion. Once the solubility and stability of an emulsion is disrupted, rapid phase separation, as a result of droplet aggregation and creaming occurs. It has been demonstrated that the antimicrobial properties of the carvacrol nanoemulsion is greatly reduced following destabilization and creaming. It is believed that the reduction in efficacy is due to the inability of the carvacrol droplets to interact with the bacterial cells in the aqueous phase.

We have observed optimal antimicrobial activity and overall stability of the carvacrol nanoemulsion was obtained at a pH ≤ 4.0 (41). Thus, changes in pH during treatment may explain why lower antimicrobial activity was observed during the treatment of contaminated broccoli seeds with a 5 mM sodium citrate spontaneous carvacrol nanoemulsion. It was found that 50 mM of either levulinic or acetic acid prevented the pH of the system from surpassing pH 3.5 for broccoli seeds. Mung beans did not exhibit a dramatic change in the pH levels for either 5 mM sodium citrate, or

any organic acids tested in this manuscript. Control of the pH during antimicrobial treatments may be essential for consistency in results.

The addiction of these acids improved the effectiveness and maintained the stability of the carvacrol nanoemulsion. They are also proven antimicrobial compounds that have wide acceptability in the food safety community (200, 251). Organic acids have a direct impact on the intracellular pH of pathogens. Protonated organic acids can pass through the outer membrane of bacteria and once in the cytoplasm, dissociate, releasing protons and anions inside the cell. This sudden influx of charged compounds disrupts a cells homeostasis by acidifying the cytoplasm. If acid levels are high enough, functional enzyme denaturation will occur ultimately leading to cell death (32, 156). The development of spontaneous nanoemulsions in 50 mM acetic and levulinic acids increased the effectiveness of carvacrol nanoemulsions on both mung bean and broccoli seeds, while the acid alone (used as controls) did not provide significant reductions of *Salmonella* on either seed type (**Figure 6.6** and **6.7**). We believe the concentrations used in this study were not high enough to demonstrate any strong antimicrobial activity.

There is a need to find an effective way to prevent, or at the very least reduce the incidence of foodborne illness from sprout based products. The presented treatment technique could be a food-grade, GRAS alternative to current caustic calcium hypochlorite or expensive pasteurization units. The use of a natural active ingredient, such as carvacrol, is an attractive alternative to traditional caustic treatments since many consumers are now concerned with the use of synthetic compounds within the

food industry (28). Sprout producers would be able to purchase or produce their own concentrated carvacrol nanoemulsion and dilute as needed based on their production volume. Based on the storage stability data, a concentrated stock solution could be stored for up to 30 days without any decrease in antimicrobial activity. Using the acidified spontaneous carvacrol nanoemulsion system, we were able to reduce 4 log CFU/g of a *S.* Enteritidis cocktail on mung bean seeds and produce a final sprout product that did not have any detectable (≤ 1.5 log CFU/g) levels of pathogen (**Tables 6.1** and **6.2**). Since no *Salmonella* was detected on the final sprout product it can be assumed that the initial inoculum of *Salmonella* was inactivated with the treatment.

Treatment of contaminated broccoli seeds consistently produced a 1.6 – 2.1 log reduction of *S.* Enteritidis with no detectable (≤ 1.5 log CFU/g) pathogens on sprouts from seeds initially contaminated with 2 log CFU *S.* Enteritidis per gram of broccoli seed. Based on these results, the proposed acidified emulsion treatment may supplement or replace the currently recommended treatment of sprouting seeds.

CHAPTER 7

INFLUENCE OF SPROUTING ENVIRONMENT, ANTIMICROBIAL TREATMENTS, AND PATHOGENS ON THE MICROBIOTA OF SPROUTS

7.1 Abstract

Sprouts are popular for health conscious and vegetarian consumers. Unfortunately, consumers are constantly being reminded about the potential risk of foodborne illness from eating bean sprouts. It has been shown that sprouting seeds in recycled germination water may limit the level of pathogen contamination on germinated sprouts. The presence or absence of certain organisms may influence the perishability and susceptibility for post-germination contamination. The microbial populations of both sprouting seeds and sprouts were studied using the terminal fragment length polymorphism (T-RFLP) analysis. The microbial population over the course of germination in a controlled setting was analyzed and compared to the final sprout microbiota of commercially grown sprouts using seeds from the same distributer. The influence of *Salmonella spp.* and treatment with an acidified carvacrol nanoemulsion on the final microbiota was also studied. Similar organismal families were found across the varieties, regardless of the initial population on each sprouting seed. The microbiota for each variety following aseptic germination was primarily composed of Pseudomonadaceae. Commercially germinated sprout varieties had significantly lower abundances of Pseudomonadaceae for all three varieties. Commercial sprouts also housed more diverse microbial families than aseptically grown sprouts, such as Bacillaceae, Sphingomonadaceae, Phyllobacteriacae, and Lactobacillaceae. When

sprouting seeds were inoculated with 1 log CFU/g *S.* Enteritidis, after germination the microbiota consisted of predominantly Pseudomonadaceae and Enterobacteriaceae for all varieties. Seeds that were treated with the antimicrobial nanoemulsion prior to germination had no detectable *S.* Enteritidis TRFs or viable cell counts as determined by plate counts, suggesting complete inactivation.

7.2 Introduction

The continued promotion of sprouts as a natural superfood has moved sprout based products to the forefront of the health movement. The constant threat of foodborne illness has also illuminated the apparent health risks associated with sprouts. The majority of sprout related foodborne disease has been linked to contaminated sprouting seeds, which can yield final sprout products with pathogen levels >8 log CFU/g (86). This has resulted in the implementation of numerous governmental polices, both domestic and abroad, that are designed to mitigate the health risks of minimally processed sprouts (70, 76). Yet, the occurrence of sprout related illness continues.

Recently, there has been a revived interest on the topic surrounding the microbiota of sprouting seeds and the environment in which they are sprouting. Organisms such as *Bacillus spp.*, *Pseudomonas spp.*, *Lactococcus spp.,* and indicator organisms like coliforms have been found to be part of the sprout microbiota (8, 30, 182, 257). The presence of potential antagonistic organisms, such as *Lactococcus spp.* and *Pseudomonas spp.*, may actually aid in the limitation or even prevent the growth of pathogenic organisms and indicator organisms may elude to the presence of a “dirty”

sprouting environment or inadequate Good Manufacturing Practices (GMPs) (151, 262, 263).

Studies have demonstrated that introducing sprouting seeds to bacterial communities derived from used sprout water can hinder the growth of foodborne pathogens (151, 257). Sprout-derived organisms may be better suited for the sprouting environment and limit the presence of foodborne pathogens through competitive exclusion or through the production of antagonistic compounds. However, environmental factors such as climate, water supply, and variations in sprouting seeds can have an effect on the final microbiota (8, 194, 257).

A recent study demonstrated that the microbial populations of radish sprouts was dependent on season fluctuations (8). They found that both aerobic plate counts (APCs) and coliform levels were higher during the summer months than winter months and that there was a significant difference in both variables between different farms (8). The use of various antimicrobial treatments, such as calcium hypochlorite, electrolyzed water, and organic acids have also been shown to be effective against foodborne pathogens but also effect the sprout microbiome (66, 167, 231, 257).

Seed treatments, environmental factors, and/or slight changes during the germination process have an influence on the final sprout microbiota. Therefore, the purpose of this study is to analyze the microbial population over the course of germination in a controlled setting, and compare the final sprout microbiota to that of commercial sprouts grown from the same seed distributer. The influence of *Salmonella*

spp. and treatment with an acidified carvacrol nanoemulsion on the final microbiota will also be studied. Sprout based microbial communities would be profiled using Terminal Fragment Length Polymorphisms (T-RFLP) (**Diagram 7.1**). This technique fingerprints microbial communities by analyzing the lengths of restricted 16s rDNA sequences from microbial communities. Each fragment (TRF) will be identified by matching the length of the restricted fragment to hypothetical restricted fragments generated from a 16s rDNA library. Though not as deep as current next-generation sequencing technologies, this type of community analysis has be used with much success through out a variety of disciplines.

7.3 Materials and Methods

7.3.1 Bacterial strains and culture conditions

The bacterial culture used in the presented experiment was *S.* Enteritidis strains 1045 (ATCC BAA-1045). Stock cultures of the organism were stored at -80 ^{o}C in tryptic soy broth (TSB; BD Diagnostic Systems, Cat# DF0064-07-6) containing 25% (v/v) glycerol. Monthly, frozen stock cultures were transferred to working cultures by plating on tryptic soy agar (TSA; BD Diagnostic Systems, Cat# DF0370-075) slants/plates and incubating at 37 ^{o}C for 24 hrs.

Periodically, working cultures were streaked on differential media to ensure purity. For *S.* Enteritidis, cultures were spread on xylose, lysine, deoxycholate (XLD) agar (Remel Cat# R459902). Cultures were incubated overnight in TSB at 37 ^{o}C on a rotary shaker set at 150 RPM. All cultures were diluted with TSB to the desired cell numbers.

7.3.2 Formation of antimicrobial nanoemulsions

Carvacrol (4 g) (Sigma-Aldrich, Cat# W224502-100G-K) was added to 6 g of medium chain triglyceride (MCT) oil (Miglyol 812, Witten, Germany) and thoroughly mixed with a magnetic stir bar for 5 min at 125 RPM. Once mixed, Tween 80® (10 g) (Sigma-Aldrich, Cat# P1754-500ml) was added to the oil mixture and mixed with a magnetic stir bar for another 5 min at 125 RPM. The oil/Tween 80® mixture (20 g) was titrated, at a rate of 2 mL/min, into 80 mL of 50 mM acetic acid buffer (pH 2.5; Macron chemical, Cat# V196-05) containing a magnetic stirring bar set to 600 RPM and allowed to mix for an additional 15 min.The emulsion was filter sterilized through a sterile 0.45 µm syringe filter (Fisher Scientific Cat# 09-719-005) and stored in sterile 50 mL conical tubes at 2 - 5 ^{o}C for up to 3 weeks. Droplet size was measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK).

7.3.3 Sample preparation and DNA extraction for the native seed and sprout microbiota during germination

All beans/seeds and sprouts were generously donated by Jonathan's Organics (Rochester, MA). Microbial population samples were taken at various time points during the germination process. For initial microbial populations on sprouting seeds, 100 g of seeds were added to a sterile 250 mL Whirl-Pack bag containing 200 mL of maximum recovery diluent (MRD :1 g/L peptone, 8.5 g/L sodium chloride, pH 7.0) with 0.025% sodium dodecyl sulfate (SDS) and vigorously shaken for 1 min (132). The cell suspension was then transferred to sterile 50 mL centrifuge tubes and centrifuged for 10 min at 12,000 RPM. Following centrifugation, the supernatant from each tube was removed

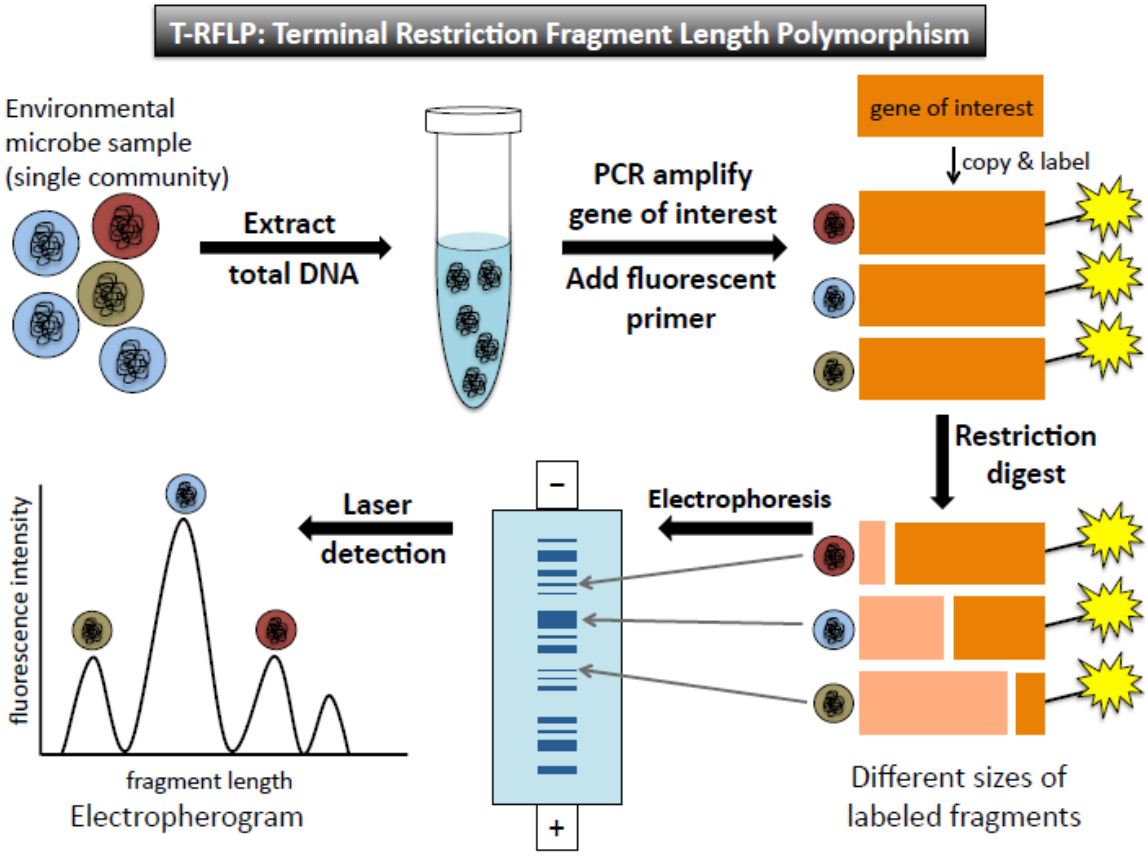


Diagram 7.1 – Typical workflow for T-RFLP analysis (1).

and the pellets suspended in 10 mL of sterile saline. Suspended pellets were pooled together and centrifuged for another 10 min at 12,000 RPM. Again, the supernatant was removed and the final pellet suspended in 10 mL of sterile saline. The final cell suspension was stored at -80 ^{o}C in 25% glycerol until needed.

For microbial population sampling during the germination process and of commercial sprout samples, 25 g of sprouting seeds/sprouts were added to a sterile 250 mL Whirl-Pack bag containing 200 mL of MRD (1 g/L peptone, 8.5 g/L sodium chloride, pH 7.0) with 0.025% SDS and vigorously shaken for 1 min (132). The cell suspension was then transferred to sterile 50 mL centrifuge tubes and centrifuged for 10 min at 12,000 RPM. Following centrifugation, the supernatant from each tube was removed and the pellets suspended in 10 mL of sterile saline. The final cell suspension was stored at -80 ^{o}C in 25% glycerol until needed. DNA extracts from all samples were obtained using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Cat# 12888-50) as directed by the manufacturer.

7.3.4 Sample preparation and DNA extraction for contaminated, treated, and commercially germinated sprout microbiotas

DNA extracts from all samples were obtained using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Cat# 12888-50) as directed by the manufacturer. Preparation for each sample was performed as described below.

7.3.4.1 Contaminated Mung Bean and Broccoli Samples

All beans/seeds used in this study were generously provided by Jonathan's Organics (Rochester, MA). *S.* Enteritidis 1045 was inoculated in TSB (9 mL) and

incubated overnight at 37 ^{o}C. Batches (20g) of beans/seeds were soaked in 50 mL of diluted *S.* Enteritidis cocktail at a concentration of 2 log CFU/mL for 20 min to achieve an inoculation level on beans/seeds of 1 log CFU/g.

The inoculated beans/seeds were then transferred to a sterile glass petri dish containing sterile filter paper (Fisher Scientific Cat# 09-803-6D) within a biological safety cabinet, and allowed to dry overnight at ambient temperature. Samples that were not subjected to the carvacrol nanoemulsion treatment were germinated as described below. For treatment samples, artificially contaminated beans/seeds (20 g) were placed in sterile 250 mL beakers and soaked in the acidified carvacrol nanoemulsion (50 mM acetic acid, 8,000 ppm carvacrol) with agitation (125 RPM) for 30 min. For negative controls, beans/seeds not inoculated with *S.* enterica were also subjected to the same treatment conditions. After treatment, the batches were rinsed once with 50 mL of sterile deionized water and sprouted.

Mung Bean Sprouting: Mung beans (20 g) were transferred to a sterile 1000 mL bottle and soaked in 150 mL of distilled water at 20 ^{o}C for 24 hrs. The water was removed, and sprouting continued for 4 days at 20 ^{o}C, with daily water by a 5-min soak in 150 mL of distilled water. After four days, 200 mL of MRD (1 g/L peptone, 8.5 g/L sodium chloride, pH 7.0) with 0.025% SDS was added to the 1 L bottle and vigorously shaken for 1 min (132). The final cell suspension was stored at -80 ^{o}C in 25% glycerol until needed.

Broccoli Seed Sprouting: Batches (10 g) of broccoli seeds were transferred to a sterile 250 mL beaker and soaked in 150 mL of sterile distilled water at 20 ^{o}C for 24 hours. The water was removed, and the seeds transferred to 3 pieces of sterile filter paper (Fisher

Scientific Cat# 09-803-6D) on top of a sterile plastic test tube rack in a sterile stainless steel container with a lid. The seeds were sprouted in the dark at 20 ^{o}C for 3 days. The seeds were watered with 15 mL of sterile distilled water every 8 hrs. After sprouting, sprouts were aseptically transferred to a sterile 500 mL Whirl-Pack bag containing 200 mL of MRD (1 g/L peptone, 8.5 g/L sodium chloride, pH 7.0) with 0.025% SDS and was vigorously shaken for 1 min (132). The final cell suspension was stored at -80 ^{o}C in 25% glycerol until needed.

Total aerobic and viable *S.* Enteritidis counts were performed by serially diluting the rinseate and plating on either TSA or XLD agar plates. Inoculated plates were allowed to incubate for 24 hr at 37 ^{o}C.

7.3.4.2 Commercially Germinated Sprout Samples

Jonathan's Organic mung bean and broccoli sprouts were obtained directly from the processing plant. Sprouts (35 g) were aseptically transferred to a sterile 500 mL Whirl-Pack bag containing 200 mL of MRD (1 g/L peptone, 8.5 g/L sodium chloride, pH 7.0) with 0.025% SDS and was vigorously shaken for 1 min (132). The final cell suspension was stored at -80 ^{o}C in 25% glycerol until needed.

7.3.5 Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis

Nearly full length portions of the 16s rDNA were amplified using 20 µL Intron Hi-Fidelity Maxime PCR pre-mix tubes (Bulldog Bio Cat# 25185) with VIC labeled (Applied Biosystems) primers 8f (5'-AGAGTT TGA TCC TGG CTC AG-3') and 1492R (5'-ACG GCT ACC TTG TTACGA CTT-3'), using the manufactures recommended procedure (157, 256).

Amplification was performed with a C1000 Thermal Cycler (Bio Rad, Hercules, CA) using a cycle program consisting of a 3 min initial denaturation step (95 ^{o}C); 34 cycles of 95 ^{o}C for 60 sec, 54 ^{o}C for 45 sec, and 70 ^{o}C for 60 sec; and an 8 min final extension step at 70 ^{o}C (157). Amplification products were separated on a 1% agarose gel and visualized under UV light using Midori Green nucleic acid stain (Bulldog Bio, Cat #MG06).

Restriction digestions were performed in 96-well microtiter plates. The PCR reaction products (7 µL) was combined with 10 U of *MspI* (New England Bio Labs) for a total reaction mixture of 30 µL. The reaction mixtures were incubated at 37 ^{o}C for 3 hr and then stored at -20 ^{o}C. DNA fragment analysis was performed with an AB3100 sequencer (Applied Biosciences) at the University Of Massachusetts Genomics Resource Center.

Initial analysis of terminal restriction fragments (TRFs) was performed using Peak Scanner v2.0 software (Applied Biosystems). Further analysis and cleanup was performed using the T-RFLP analysis package for R statistical analysis software with a cut-off between ≥50 and ≤500 bp (2). Clustering, and multi-dimensional scaling was performed using the Paleontological Statistics (PAST) software with a bootstrap sample value of 9999 (104). Any potential TRFs were identified by comparing TFRs to bacterial 16s and soil databases using the MiCA T-RFLP Analysis APLAUS+ platform(216).

7.4 Results

7.4.1 The influence of environment on the sprout microbiota

The comparison between the microbiota of each variety prior to sprouting can be seen in **Figure 7.1**. Though similar organismal families can be found across the

varieties, their clustering reveals little similarity between them. The family Peptococcaceae makes up the majority (52%) of the population on mung beans. Whereas, Peptococcaceae was found to have a lesser presence on broccoli seeds (24%) and no presence on alfalfa seeds (**Figure 7.1**). The presence of Sphingomonadaceae was comparable between mung bean (29%) and broccoli seeds (32%). Alfalfa seeds was also found to house Sphingomonadaceae, making up 13% of the microbiota.

Broccoli and alfalfa seeds housed microbial families not present on mung beans. Lactobacillaceae (22%) and Nocardiaceae (14%) were present on alfalfa seeds but not mung beans. Oxalobacteraceae was found to make up 13% and 2% of the microbial abundance on broccoli and alfalfa seeds respectively, with no detectable abundance on mung beans (**Figure 7.1**).

The influence of sprouting conditions/environment for mung bean, broccoli, and alfalfa sprouts can been seen in **Figures 7.2, 7.3, and 7.4** respectively. Regardless of the initial population on each sprouting seed, the microbiota for each variety following aseptic germination was primarily composed of Pseudomonadaceae (≥94% for all varieties).

Commercially germinated sprout varieties had significantly lower abundances of Pseudomonadaceae for all three varieties. Commercial sprouts also housed more diverse microbial families than aseptically grown sprouts (**Figure 7.5**). Bacillaceae, Sphingomonadaceae, Phyllobacteriacae, and Lactobacillaceae were detected on

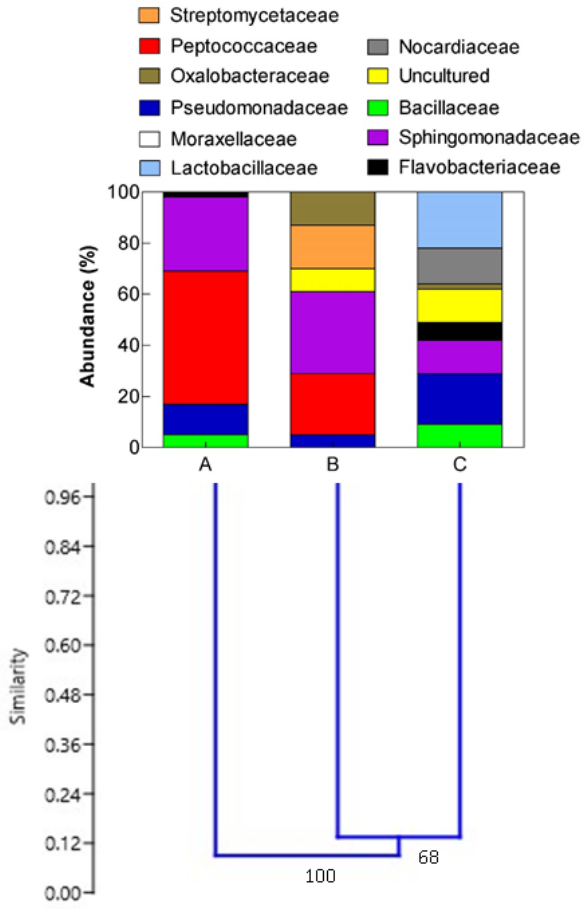


Figure 7.1 Comparison of the initial microbiota on mung beans (A), broccoli seeds (B), and alfalfa seeds (C). Hierarchical clustering was performed using the Bray-Curtis similarity index based on the paired grouping (UPGMA) algorithm. Bootstrap values were determined based on a sample value of 9999.

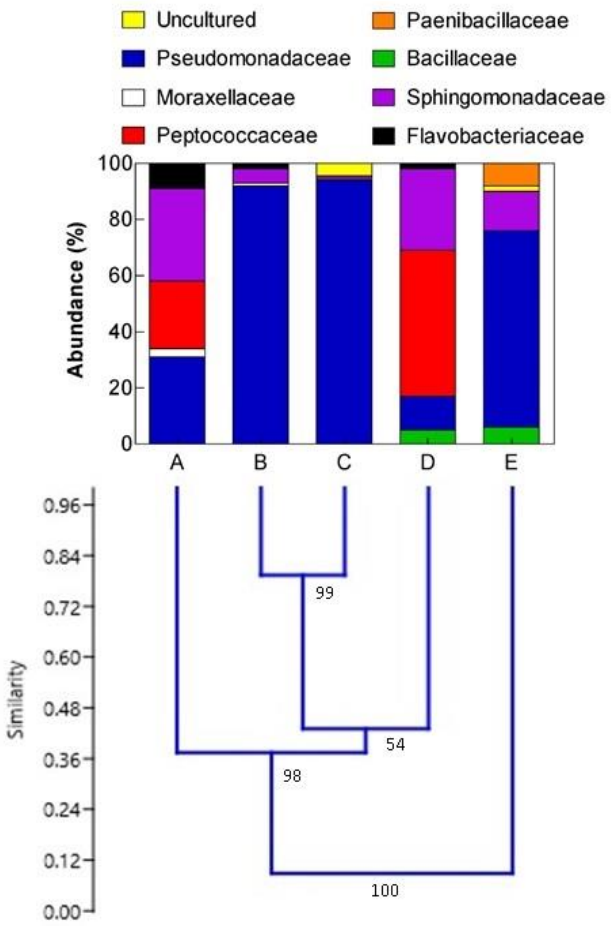


Figure 7.2 Comparison of the microbiota of mung bean sprouts germinated under sterile conditions and commercially grown mung bean sprouts from the same distributer. Mung beans were germinated in sterile containers and watered daily using sterile distilled water. Microbial samples were collected from the initial seeds (D) and after 1 day (A), 4 days (B), and 5 days (C) of germination. Laboratory grown samples were compared to commercial germinated mung bean sprouts (E). Hierarchical clustering was performed using the Bray-Curtis similarity index based on the paired grouping (UPGMA) algorithm. Bootstrap values were determined based on a sample value of 9999.

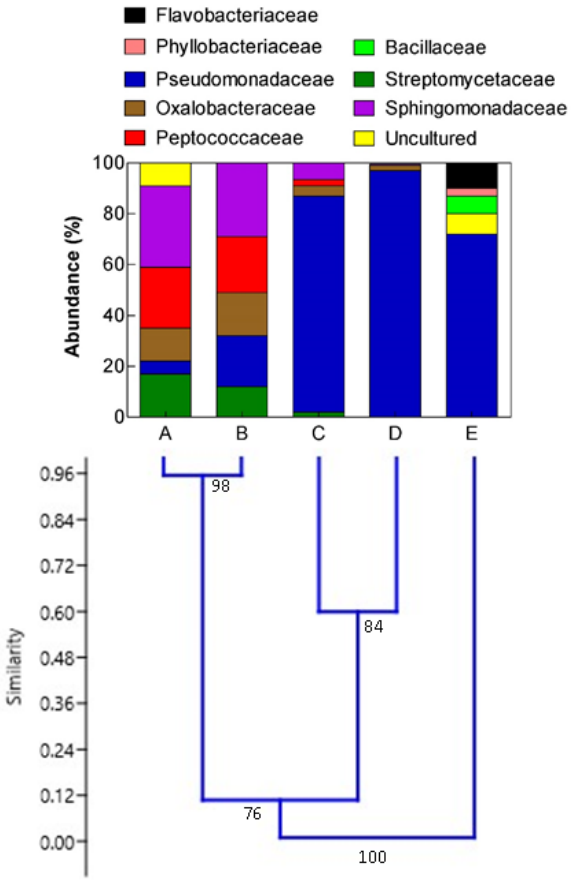


Figure 7.3 Comparison of the microbiota of broccoli sprouts germinated under sterile conditions and commercially grown broccoli sprouts from the same distributer. Broccoli sprouts were germinated in sterile containers and watered daily using sterile distilled water. Microbial samples were collected from the initial seeds (A) and after 1 day (B), 4 days (C), and 5 days (D) of germination. Laboratory grown samples were compared to commercial germinated broccoli sprouts (E). Hierarchical clustering was performed using the Bray-Curtis similarity index based on the paired grouping (UPGMA) algorithm. Bootstrap values were determined based on a sample value of 9999.

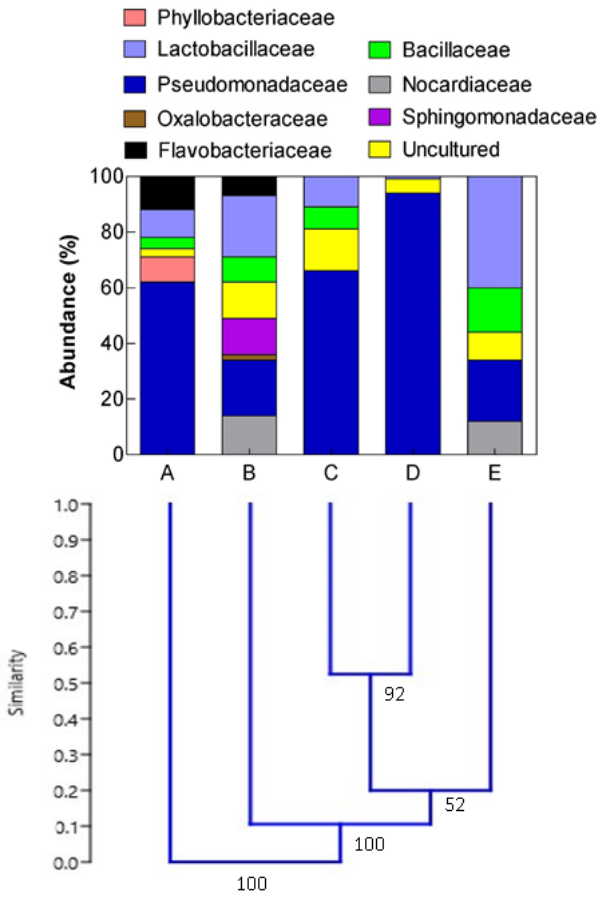


Figure 7.4 Comparison of the microbiota of alfalfa sprouts germinated under sterile conditions and commercially grown alfalfa sprouts from the same distributer. Alfalfa sprouts were germinated in sterile containers and watered daily using sterile distilled water. Microbial samples were collected from the initial seeds (B) and after 1 day (E), 4 days (C), and 5 days (D) of germination. Laboratory grown samples were compared to commercial germinated alfalfa sprouts (A). Hierarchical clustering was performed using the Bray-Curtis similarity index based on the paired grouping (UPGMA) algorithm. Bootstrap values were determined based on a sample value of 9999.

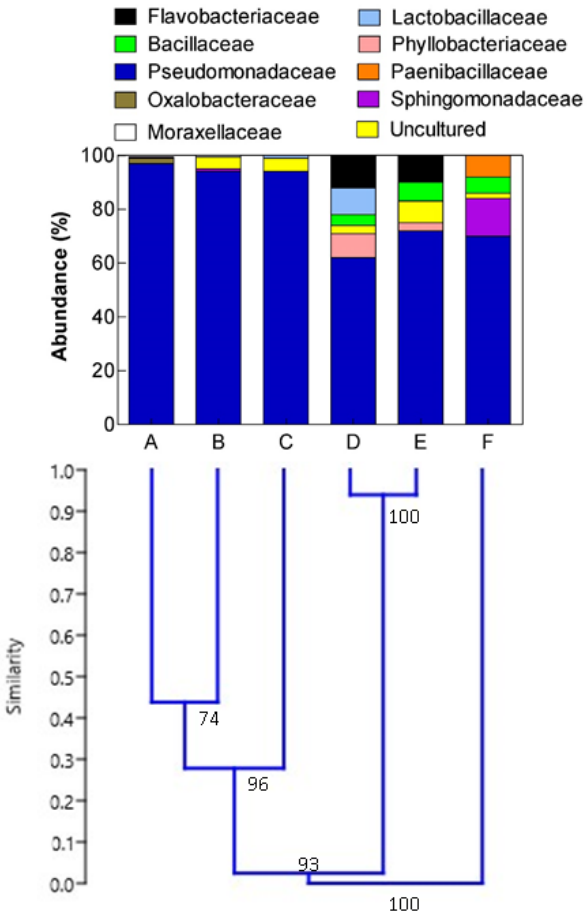


Figure 7.5. Comparison of the microbiota of sprouts germinated under sterile conditions and commercial sprouts grown from the same distributer. The microbiota of aseptically germinated sprouts (broccoli sprouts (A), mung bean sprouts (B), alfalfa sprouts (C)) were compared to commercial grown varieties (alfalfa sprouts (D), broccoli sprouts (E), and mung bean sprouts (F)).Hierarchical clustering was performed using the Bray-Curtis similarity index based on the paired grouping (UPGMA) algorithm. Bootstrap values were determined based on a sample value of 9999.

commercially grown sprouts but not laboratory grown, even though those families were initial found on the sprouting seeds.

7.4.2 Effect of an antimicrobial carvacrol nanoemulsion treatment and the presence of S. Enteritidis on the microbiota of sprouts

The effect of the presence of *S.* Enteritidis on the final microbiota of germinated sprouts, and the microbiota of sprouts whose seeds were treated with an antimicrobial carvacrol nanoemulsion prior to germination can be seen in **Figures 7.6** and **7.7**. When mung beans were inoculated with 1 log CFU/g *S.* Enteritidis, after germination the microbiota consisted of Pseudomonadaceae and Enterobacteriaceae (**Figure 7.6 lane B**). Similar results were found with inoculated (1 log CFU/g *S.* Enteritidis) broccoli seeds; Pseudomonadaceae and Enterobacteriaceae were the dominate family of organisms (**Figure 7.7 lane B**). It was assumed that *S.* Enteritidis was the only organism represented within the Enterobacteriaceae classification since no other samples were found to have TRFs within that family. Sprouts that were germinated from non-treated contaminated mung beans and broccoli seeds resulted final *S.* Enteritidis counts of ≥ 8 log CFU/g of sprouts (**Figure 7.8**).

Artificially contained and unadulterated mung beans and broccoli seeds that were treated with the carvacrol nanoemulsion had a final population that was ≥90% Pseudomonadaceae and overall Bray-Curtis similarity indices of 0.72 and 0.48 respectively (**Figure 7.6** and **7.7**). The lack of TRFs representative of Enterobacteriaceae, along with no detectable *S.* Enteritidis using plate counts supports previous findings

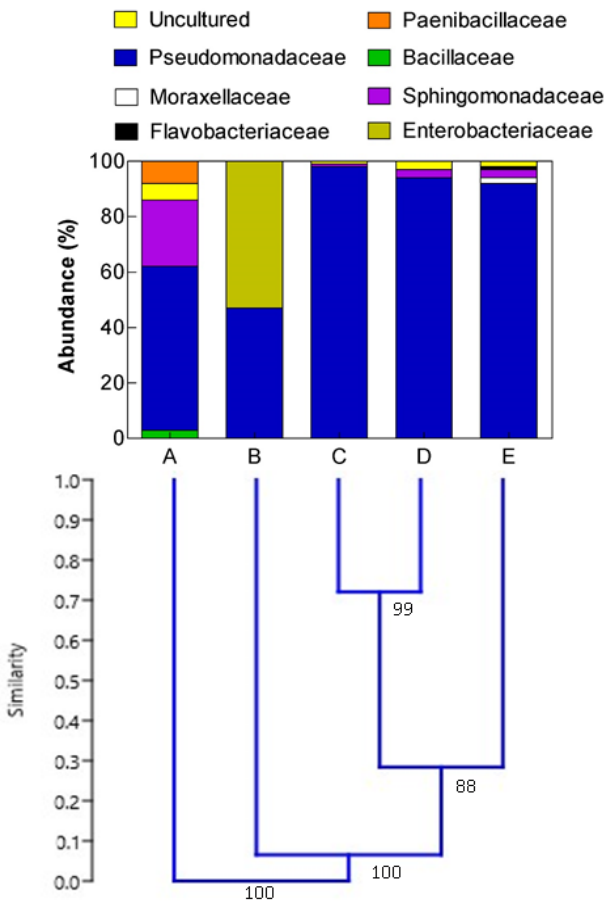


Figure 7.6. Comparison of the microbiota of mung beans that were treated with a carvacrol nanoemulsion and/or contaminated with *S.* Enteritidis (1 log CFU/g of seed) germinated under sterile conditions. The microbiota of germinated sprouts (contaminated beans (B), treated beans (C), treated contaminated beans (D), and unadulterated beans (E)) were compared to commercial grown sprouts (A).Hierarchical clustering was performed using the Bray-Curtis similarity index based on the paired grouping (UPGMA) algorithm. Bootstrap values were determined based on a sample value of 9999.

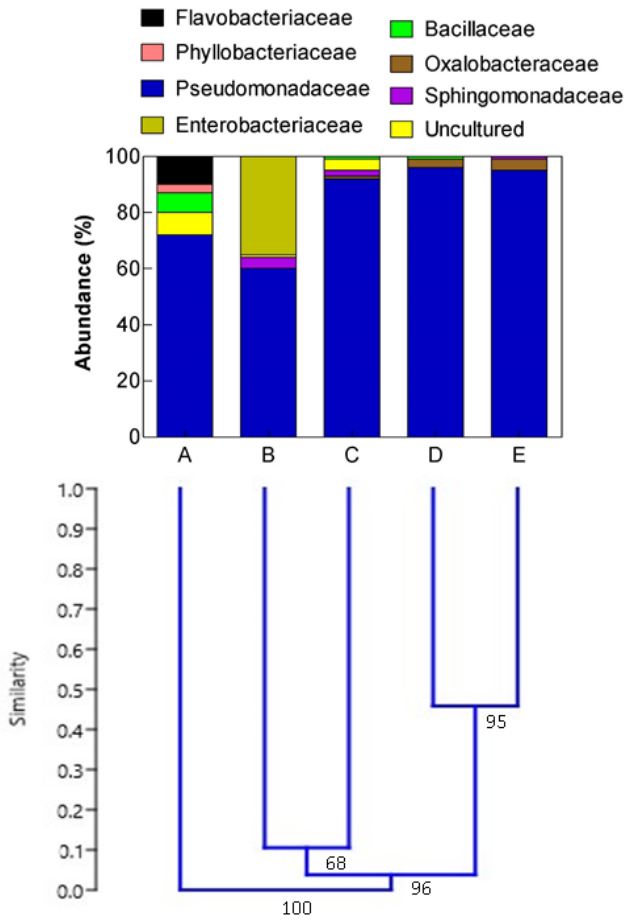


Figure 7.7. Comparison of the microbiota of broccoli seeds that were treated with a carvacrol nanoemulsion and/or contaminated with *S.* Enteritidis (1 log CFU/g of seed) germinated under sterile conditions. The microbiota of germinated sprouts (contaminated seeds (B), treated seeds (D), treated contaminated seeds (E), and unadulterated seeds (C)) were compared to commercial grown sprouts (A).Hierarchical clustering was performed using the Bray-Curtis similarity index based on the paired grouping (UPGMA) algorithm. Bootstrap values were determined based on a sample value of 9999.

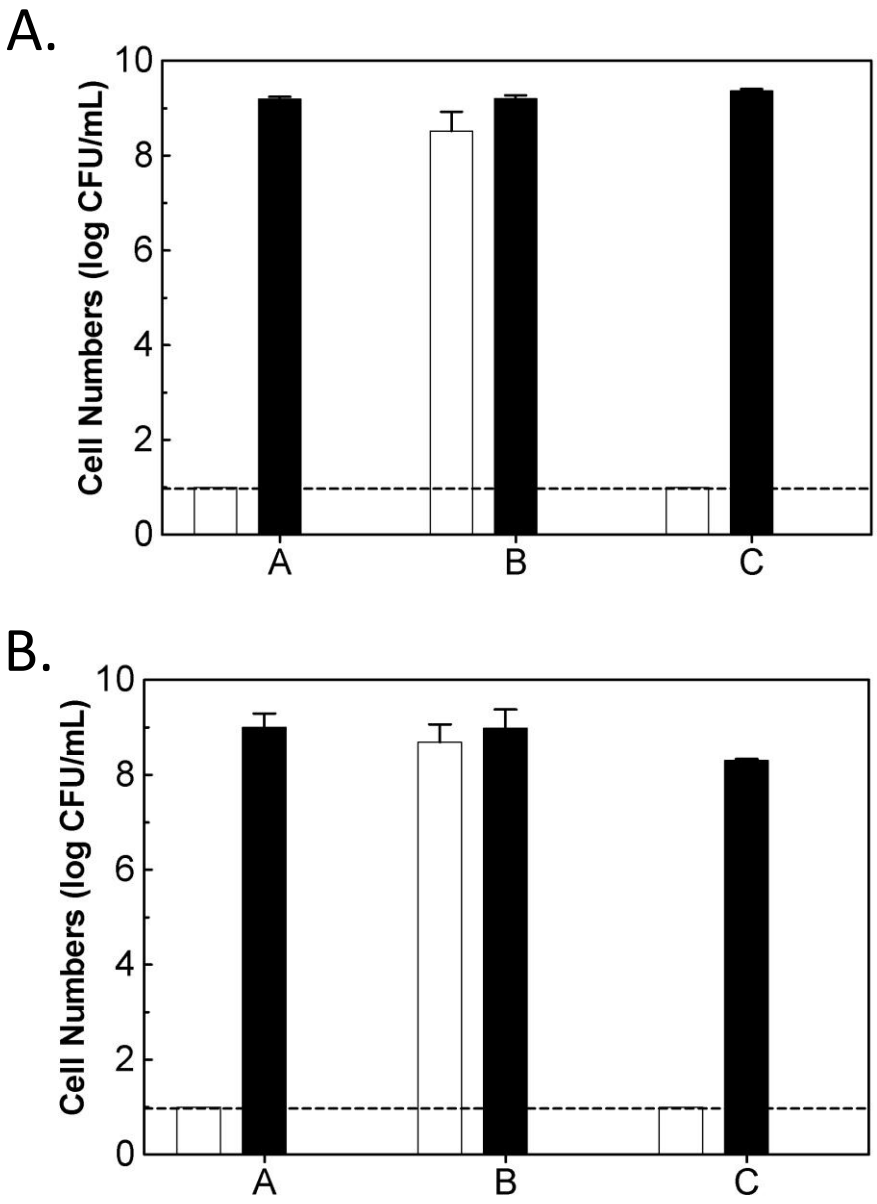


Figure 7.8. The total aerobic (□) and *S.* Enteritidis (■) counts of germinated mung bean (A) and broccoli (B) sprouts. The samples are as follows: (A) negative control, (B) contaminated sprouting beans, and (C) contaminated beans treated with a carvacrol nanoemulsion.

regarding the effectiveness of the acidified carvacrol nanoemulsion (**Figure 7.8**).

7.5 Discussion

All varieties of sprouts are grown under conditions ideal for microbial growth. Coupled with the fact that sprouts are minimally processed and often consumed raw, the correlation between foodborne disease and sprouts should not be a surprise. The native microbiota of sprouting seeds and their sprouting environment may play a pivotal role in minimizing or preventing foodborne pathogen contamination on sprouts. It has been known for some time that the most common vector for foodborne pathogens are the sprouting seeds themselves. Microbial community fingerprinting of three sprouting seed varieties failed to indicate the presence of any foodborne pathogen, though a variety of microbial families commonly associated with soil and produce were noted.

The overall microbial populations on the final aseptically germinated sprouts varied greatly from the initial microbiota of the sprouting seeds. After 4 days of germination, the majority of the microbiota was comprised of Pseudomonadaceae, a family of ubiquitous organisms commonly associated with soil and produce. Other studies have also confirmed that ≥ 90% of the microbial population on sprouts were found to be Pseudomonadaceae (8, 257). However, one study found that Enterobacteriaceae was dominate on soy bean sprouts (167). The shift in microbial diversity from seed/bean to sprout was quite dramatic. The majority of populations present on seeds were not detectable on the final germinated product. For example, Lactobacillaceae (22%) was found on alfalfa seeds but went undetected on the

aseptically germinated alfalfa sprouts. Interestingly, Lactobacillaceae was present on commercially grown sprouts. Bacillaceae, Sphingomonadaceae, and Phyllobacteriacae all demonstrated a similar pattern of being present on commercially germinated sprouts and not laboratory grown.

The actual sprouting environment may play a role in this observation. Organisms within the families Lactobacillaceae, Bacillaceae, Sphingomonadaceae, and Phyllobacteriacae may not be able to grow or may be subject to competitive exclusion when grown under sterile conditions in the laboratory. On the other hand, these organisms may thrive and be part of the microbiota of the commercial sprouting environment. In this case, seeds may be exposed to high enough levels to minimize competitive exclusion or fully germinated sprouts may be exposed to these organisms, encouraging their presence on sprouts.

The classifications of detected organisms may allude to the presence of beneficial organisms both on sprouts and in the germinating environment. Organisms such as *Lactobacillus* spp. (Lactobacillaceae), *Pseudomonas* spp. (Pseudomonadaceae), *Paenibacillus* spp. (Paenibacillaceae), and *Sphingomonas* spp. (Sphingomonadaceae) have all been shown to demonstrate antagonistic activity against foodborne pathogens associated with bean sprouts (89, 125, 205). The presence of antagonistic organisms may explain way used sprout water can hinder the growth of foodborne pathogens (151, 257).

The use of a T-RFLP analysis has been use to study gut, produce, plant roots, and soil microbial communities (52, 53, 223). The cost, time, and high degree of specificity and reproducibility are the main attractors to this method of community analysis. Even though T-RFLP analyses provide relative abundance values for each TRF, it suffers from low discriminatory power and limited depth when compared to next-generation sequencing technologies (235). Other limitations such as, amplification and labeled-probe annealing biases have an influence on T-RFLP analyses, as they do in all methodologies utilizing the PCR (181). Unfortunately, the identification of individual organisms could not be achieved using this type of community fingerprinting.

Based on the findings of this study, it can be concluded that environmental factors from the sprouting environment can influence the microbiota of sprouts. The germination of sprouting seeds in a sterile environment promoted the growth of organisms adapted for that type of environment, as indicated by the dramatic shift in microbial population during the germination process for tested sprout varieties. When sprouted in a commercial setting, organisms that are present and thriving in the sprouting environment may the microbial community of sprouts. This was supported by the degree of similarity and node separation between the microbial communities of sprouts germinated in the laboratory and sprouts from a commercial sprout farm. With more understanding about how outside factors can influence sprouts microbial diversity, new and improved practices can be developed to help minimize the incidence of foodborne illness from the consumption of raw sprouts.

CHAPTER 8

CONCLUSION

The paradoxical nature of sprouts continues to place them in the spotlight for both health benefits and foodborne illness. Throughout the world, governing agencies continue to implement regulations and practices designed to combat the biological issues often linked to sprouts. The purpose of the research presented in this dissertation was to explore creative approaches to this pressing issue. The utilization of either antagonist organisms or emulsified essential oils were found to potential alternatives to traditional techniques.

The novel *S. plymuthica* EJ was found quite effective against both *S.* Enteritidis and *E. coli* O157:H7 in both *in vitro* and *in vivo* settings. Unfortunately, the practical application of this technique is limited by a variety of factors. Extensive survivability, both on the seed and on the germinated sprout, studies need to be performed to determine if seeds stored for greater the 30 days would continue to have high enough levels to retard pathogenic growth. From this study, the novel isolate was able to survive for 30 days on sprouting seeds, with a final viable cell count of ~5 log CFU/g. The present findings have shown that number of ≥ 8 log CFU/g of *S. plymuthica* EJ are need to limit or prevent the proliferation of pathogens. Studies exploring the lower inoculum levels need to be completed before any practical applications can be discussed.

Second, *S. plymuthica* is considered an opportunistic pathogen. A number of topical infections have been linked to *S. plymuthica*, some with severe complications (33, 146). This dramatically decreases the chances of this organism being considered a

potential biological control agent for produce systems. The results of this study do demonstrate the concept of biocontrol agents.

Chemical sanitizing techniques are the most common and, more often than not, the most economical treatment method. The gold standard seed/bean disinfection is 20,000 ppm calcium hypochlorite. A less caustic, more “natural” alternative to calcium hypochlorite may be the use of antimicrobial essential oils. These compounds are considered natural and have a better standing with consumers than hypochlorites. The use of spontaneously emulsified essential oil nanoemulsions were examined as a potential alternative treatment for sprouting seeds/beans. The essential oil used during these studies was carvacrol, an essential oil found in a variety of herbs. The antimicrobial activity of carvacrol has been linked to its ability to disrupt cellular membranes and effect pH gradients within cells. Using spontaneous emulsification, a carvacrol nanoemulsion was developed and tested on a variety of sprout based systems. This food-grade and GRAS antimicrobial treatment was able to inactivate both *S.* Enteritidis and *E. coli* O157:H7 on all tested sprout varieties. With a treatment per kg seed cost three-times that of bleach, this system is not the most economical treatment available but it is less expensive than premium commercial peroxyacetic acid products. Scale-up manufacturing process and packaging would also have to be optimized.

The final aspect of this research was to see if the final microbiota on sprouts reliant on the initial microbial community on sprouting seeds/beans or is influenced on environmental factors. It was determined that the final microbiota of sprouts is different than sprouting seeds/beans and the introduction of pathogenic or

environmental organisms will affect the observable microbiota of germinated sprouts. These findings further emphasize the need for effective seed disinfectants and proper GMPs throughout the whole seed – market process.

Sprouts will continue to a food for health conscious consumers. They will also be scrutinized for their chronic correlation with foodborne pathogens such as *Salmonella* spp. and *E. coli* O157:H7. It is vital that research continues in the areas of prevention, disinfection, and detection of pathogens on produce. Hopefully, the results found in this dissertation will help the advance towards safer produce.

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