EFFICACIES OF ASCAROSIDE ASCR#18 TREATMENT IN CONTROL OF *SALMONELLA ENTERICA* AND ENTEROHEMORRHAGIC *ESCHERICHIA COLI* ON ALFALFA AND FENUGREEK SEEDS AND SPROUTS

by

XUEYAN HU

(Under the Direction of Jinru Chen)

ABSTRACT

A novel, natural, and effective antimicrobial intervention is in demand for improving the microbial safety of sprouts and sprout seeds. This study aimed to determine the effect of ascaroside ascr#18 treatment on the growth of *Salmonella enterica* and enterohemorrhagic *Escherichia coli* on alfalfa and fenugreek seeds and sprouts. It was found that treatment with 1 mM ascr#18 was more effective than 1 µM treatment for reducing the populations of *Salmonella* and *E. coli* on sprouts, but the level of *Salmonella* reduction was about 1 log level higher than the reduction level of *E. coli*. All four independent variables (seed type, strain type, treatment type, and sprouting time) were significant factors ($P \leq 0.05$) influencing *Salmonella* growth, but strain type was not a significant factor influencing the growth of *E. coli*. The study provides supporting evidence for the potential application of ascr#18 to control pathogen growth on vegetable sprouts.

INDEX WORDS: *Salmonella enterica*, enterohemorrhagic *Escherichia coli*, Ascaroside ascr#18, sprouts, sprout seeds

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A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2022

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December 2022

DEDICATION

I dedicate this work to my beloved grandfather, who showed the most support for my studying abroad and wished me a wonderful life in the world.

ACKNOWLEDGEMENTS

I am sitting in the hall of SLC at Griffin Campus, which has been accompanying me when I am struggling with all the tangled-up stuff over the past several months. I could not have imagined in May that I would finish the whole thesis, but it finally has turned out now coherent and well-organized. It is a great experience for me to pursue my master’s degree, especially when it has happened during the pandemic. I shall express my gratitude to all of the people who have helped and supported me along the journey.

Firstly, I would like to thank my parents for their unconditional support and love all the time. I have shared all my joy and sadness with them and always get courage, comfort, and practical suggestions from them. Also, I shall thank my friends for sticking with me and being my listeners whenever I am down. Secondly, my lab members are always ready to help when I encounter any problems, especially Dr. Seulgi Lee, who has devoted to my project and guided me for several months until I become independent.

Lastly, my major professor, Dr. Jinru Chen, deserves the largest round of applause from me. She is so patient with me helping me to modify every tiny detail of the thesis and has encouraged me throughout the time when I don’t feel well. My committee members, Dr. Koushik Adhikari and Dr. Juan Carlos Díaz Pérez are so nice to me and have provided much advice which has inspired me for my research project.

I do hope I could get closer to the person that I want to be step by step.

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CHAPTER 1

INTRODUCTION

Sprouts are young shoots developed from germinated seeds. During seed germination, many macronutrients and antinutrients are metabolized, and secondary metabolic compounds are synthesized, making sprout consumption much more beneficial health-wise as compared to just vegetable seed consumption (Peñas & Martínez-Villaluenga, 2020). Alfalfa and fenugreek sprouts are commonly consumed in recent years, and they are becoming more and more popular because consumers prefer foods that are nutritious andnutritious foods that require minimal processing (Miyahira & Antunes, 2021; Frías et al., 2007).

However, there are potential risks associated with sprout consumption since sprouts are usually consumed raw or slightly cooked. Germinated seeds contain abundant nutrients to support the growth of microorganisms (Ding et al., 2013), sprouts are, therefore, easily contaminated during the production process. There were at least 64 outbreaks related to vegetable sprout consumption from 1988 to 2020 worldwide, and alfalfa sprouts were involved in more than 55% of these outbreaks (Miyahira & Antunes, 2021). The outbreak caused by enterohemorrhagic *Escherichia coli* (EHEC) O104:H4 occurred in Germany in 2011, and it resulted in 3,842 cases of illness, including 18 deaths. Fenugreek sprouts were identified to be the source of contamination (Beutin & Martin, 2012; Muniesa et al., 2012).

Although in 1999 the U.S. Federal Drug Administration (FDA) recommended using 20,000 ppm calcium hypochlorite to treat sprout seeds before germination to control microbial contamination, outbreaks associated with sprout consumption continue to occur after that guidance was issued (Winthrop et al., 2003; Proctor et al., 2001). Calcium hypochlorite has Category I toxicity, which may cause severe damage to human eyes and skin and is toxic to freshwater fish and invertebrates. It can form potential carcinogens (trihalomethanes) in water, which is a significant environmental concern (Environmental Protection Agency, 1991). Therefore, an alternative intervention that is effective in sanitizing vegetable seeds or sprouts is needed.

Ascarosides are a conserved family of signaling molecules identified exclusively from nematodes. They are not only responsible for regulating the development and social behaviors of nematodes but also trigger plant defense responses. A previous study has shown that low concentrations of ascaroside ascr#18 could boost the resistance of Arabidopsis, tomato, and barley to viral, bacterial, oomycete, fungal, and nematode infections (Manosalva et al., 2015). Another research found that ascarosides can be metabolized by animals, plants, and microorganisms (Yu et al., 2021). Consequently, it could be potentially used as an effective treatment to control pathogen contamination on sprouts.

This project was undertaken for the goal to evaluate whether ascr#18 can be used for controlling human pathogens on alfalfa and fenugreek seeds and sprouts. And it can be divided into two objectives:

1) To determine the effect of ascr#18 treatment on the growth of *Salmonella enterica* on alfalfa and fenugreek seeds and sprouts during sprouting (Chapter 3);

2) To determine the effect of ascr#18 treatment on the growth of enterohemorrhagic *Escherichia coli* on alfalfa and fenugreek seeds and sprouts during sprouting (Chapter 4).

References

Beutin, L., & Martin, A. (2012). Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. *Journal of Food Protection*, *75*(2), 408-418. https://doi.org/10.4315/0362-028x.jfp-11-452

Ding, H., Fu, T. J., & Smith, M. A. (2013). Microbial contamination in sprouts: how effective is seed disinfection treatment? *Journal of Food Science*, *78*(4), R495-R501. https://doi.org/10.1111/1750-3841.12064

Frías, J., Martínez-Villaluenga, C., Gulewicz, P., Pérez-Romero, A., Pilarski, R., Gulewicz, K., & Vidal-Valverde, C. (2007). Biogenic amines and HL60 cytotoxicity of alfalfa and fenugreek sprouts. *Food Chemistry*, *105*(3), 959-967. https://doi.org/10.1016/j.foodchem.2007.04.043

Gensheimer, K., & Gubernot, D. (2016). 20 years of sprout-related outbreaks: FDA's investigative efforts. In Open Forum Infectious Diseases (Vol. 3, No. suppl_1, p. 1438). Oxford University Press. https://doi.org/10.1093/ofid/ofw172.1051

Manosalva, P., Manohar, M., von Reuss, S. H., Chen, S., Koch, A., Kaplan, F., Choe, A., Micikas, R. J., Wang, X., Kogel, K-H., Sternberg, P. W., Williamson, V. M., Schroeder, F. C., & Klessig, D. F. (2015). Conserved nematode signaling molecules elicit plant defenses and pathogen resistance. *Nature Communications*. *6*(1), 1-8. https://doi.org/10.1038/ncomms8795

Miyahira, R. F., & Antunes, A. E. C. (2021). Bacteriological safety of sprouts: A brief review. *International Journal of Food Microbiology*, *352*, 109266. https://doi.org/10.1016/j.ijfoodmicro.2021.109266

Muniesa, M., Hammerl, J. A., Hertwig, S., Appel, B., & Brüssow, H. (2012). Shiga toxin-producing *Escherichia coli* O104:H4: a new challenge for microbiology. *Applied and Environmental Microbiology*, *78*(12), 4065-4073. https://doi.org/10.1128%2FAEM.00217-12

Peñas, E., Gómez, R., Frías, J., & Vidal-Valverde, C. (2008). Application of high-pressure treatment on alfalfa (*Medicago sativa*) and mung bean (*Vigna radiata*) seeds to enhance the microbiological safety of their sprouts. *Food Control*, *19*(7), 698-705. https://doi.org/10.1016/j.foodcont.2007.07.010

Proctor, M. E., Hamacher, M., Tortorello, M. L., Archer, J. R., & Davis, J. P. (2001). Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa sprouts grown from seeds pretreated with calcium hypochlorite. *Journal of Clinical Microbiology*, *39*(10), 3461-3465. https://doi.org/10.1128/jcm.39.10.3461-3465.2001

U. S. Environmental Protection Agency. (1999). *R.E.D. FACTS: Sodium and Calcium Hypochlorite Salts*. https://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/fs_G-77_1-Sep-91.pdf Accessed June 27, 2022.

Winthrop, K. L., Palumbo, M. S., Farrar, J. A., Mohle-boetani, J. C., Abbott, S., Beatty, M. E., Inami, G., & Werner, S. B. (2003). Alfalfa sprouts and *Salmonella* Kottbus infection: A multistate outbreak following inadequate seed disinfection with heat and chlorine. *Journal of Food Protection*. *66*(1): 13-17. https://doi.org/10.4315/0362-028x-66.1.13

Yu, Y., Zhang, Y. K., Manohar, M., Artyukhin, A. B., Kumari, A., Tenjo-Castano, F. J., Nguyen, H., Routray, P., Choe, A., Klessig, D. F., & Schroeder, F. C. (2021). Nematode signaling molecules are extensively metabolized by animals, plants, and microorganisms. *ACS Chemical Biology*. *16*, 6, 1050–1058. https://doi.org/10.1021/acschembio.1c00217

CHAPTER 2

LITERATURE REVIEW

2.1 Consumption of fresh produce, including sprouts

2.1.1 Fresh produce consumption

Fresh fruit and vegetables are important components of a healthy diet due to their rich fiber content and low energy density. They are also associated with decreased chronic disease and optimal weight management (Gustat, O'Malley, Luckett, & Johnson, 2015). Fruit and vegetable consumption in the United States averaged 741 pounds per person per year from 1997 to 1999, up *ca*. 25% compared to twenty years ago. Vegetable consumption increased more rapidly than fruit consumption. The increase in vegetable consumption was attributed to several aspects, such as increased domestic production, availability of diverse products by international trade, and global efforts to promote healthy eating, as well as technological developments to extend the shelf life of fresh produce and modification of product to meet consumers' preferences and provide convenience (Pollack, 2001; Alegbeleye, Singleton & Sant’Ana, 2018). However, Americans’ consumption of fruits and vegetables has declined over the last decade. According to the U. S. Department of Agriculture’s Economic Research Service, loss-adjusted supplies of total fruits and vegetables available to consume in the United States have fallen from 299 to 272 pounds per person from 2003 to 2013 (U. S. Department of Agriculture, Economic Research

Service, 2016). Meanwhile, Americans are consuming other types of nutrient-packed foods. Experts and researchers suggest that consuming more fresh produce could reduce health-related problems in humans (Wang et al., 2021; Bertoia et al., 2016; Hung et al., 2004). Thus, the overall focus of the fresh produce industry is to explore different types of microbiologically safe and wholesome products.

2.1.2 Vegetable sprouts

Sprouts, young shoots from seeds or roots, edible fresh produce. Sprouts have a long history, especially in Asian countries, where they are used as traditional vegetable ingredients for cooking (Benincasa et al., 2019). Mung bean (*Vigna radiata L.*) sprouts have the most extended history and have been consumed by the Chinese starting 5,000 years ago. Initially, more sprouts from legume seeds were consumed than those from cereal grains (Price, 1988). Nowadays, a broad spectrum of sprouts germinated from various seeds, such as alfalfa, broccoli, buckwheat, mung beans, radish, and red cabbage are frequently appearing on daily dining tables (Kumari et al., 2021; Shah et al., 2016; Yilmaz et al., 2020). Due to the rising demand of consumers for healthy and novel foods (Benincasa et al., 2019) and their desire for nutritious food with minimal processing, sprouts, easily cultivated with short production periods and simple steps, have become more and more popular in western countries in the last decades (Peñas & Martínez-Villaluenga, 2020).

Consumers enjoy sprouts not only for their rich nutritional content but also for the therapeutic value they provide. Sprouts have been proven to have a high concentration of bioactive components (Reed et al., 2018), *e.g.*, glucosinolates, isothiocyanates, and phenols in

Brassica sprouts (Márton et al., 2010). Sprouts also have higher percentage of protein and concentration of free amino acids, minerals, vitamins, and unsaturated fatty acids than seeds (Price, 1988; Márton et al., 2010). The reason for the increased nutritional contents is that during the germination process, many macronutrients and antinutrients are metabolized, and secondary metabolic compounds are synthesized (Peñas & Martínez-Villaluenga, 2020), polysaccharides are hydrolyzed into oligosaccharides, fats into free fatty acids, and protein into peptides or free amino acids. These nutrients have much bioavailability in the human body.

Research has explored the various medical functions of sprouts. Lentil (*Lens culinaris*) sprouts can increase plasmatic melatonin concentration of plasmatic melatonin and reduce plasmatic oxidative stress (Rebollo-Hernanz et al., 2020). Pea (*Pisum Sativum*) sprouts contain phenolic phytochemicals which have an antimicrobial effect on the pathogenic microorganism *Helicobacter* (Márton et al., 2010). Sprouted buckwheat (*Fagopyrum esculentum*) has the function to decrease plasma cholesterol levels, improve hypertension conditions, and has neuroprotection, anticancer, anti-inflammatory, and antidiabetic effects (Gimenez-Bastida & Zielinski, 2015). Red cabbage (*Brassica oleracea L. var. capitata f, rubra*) and broccoli (*Brassica oleracea*) sprouts are beneficial to type II diabetes and have antimicrobial and anticancer properties (Shah et al., 2016).

2.1.2 Alfalfa sprouts

Alfalfa (*Medicago sativa* L.) originated from Central Asia and the Middle East and has high protein content and nutritional content (El-Ramady et al., 2020). The alfalfa plant is not typical for food because its leaves have a bitter flavor. However, young alfalfa seedlings have a mild taste. Alfalfa sprouts, always consumed as a garnish, are healthy and highly nutritious. . Alfalfa sprouts contain numerous minerals, such as phosphorus, potassium, calcium, iron, magnesium, copper, manganese, vitamin C, and vitamin A. They have a low-calorie density (7.6 calories in 33g of alfalfa sprouts) and a low glycemic index rating (Hamilton & Vanderstoep, 1979). They are often added to salads, soups, and sandwiches and used as the top or side decoration of other dishes.

Consumption of alfalfa sprouts could help protect against cancers and cerebrovascular diseases (Fan & Thayer, 2001). They can also balance hormones, relieve inflammation, and inhibit the production of cancerous cells (Murray & Pizzorno, 2010). Alfalfa sprouts are rich in vitamin K, and one serving (33 g) of alfalfa sprouts contains almost 13% of the daily recommended intake of vitamin K. The rich fiber in alfalfa sprouts gives them the function to improve the condition of diabetes. Research conducted by Mcintosh and Miller (2001) found that food rich in fiber would improve glycemic control and lipid panels among patients with type II diabetes. Alfalfa sprouts also have the properties of lowing serum cholesterol (Story et al., 1984). Therefore, alfalfa sprouts are good for arteries and blood vessels, preventing coronary heart diseases, atherosclerosis, heart attacks, and strokes (Staughton, 2021).

2.1.3 Fenugreek sprouts

Fenugreek (*Trigonella foenum-graecum* L.) is a traditional food, forage, and medicinal plant. Fenugreek is rich in ascorbic acid, niacin, potassium, iron, and alkaloids (Choudhary, 2022). It can be used as an ingredient for salads, added to cooking sauce and dips because of its unique flavors like mild curry and the scent that is easy to versatile. Fenugreek also contains lysine, L-tryptophan-rich proteins, and mucilaginous fiber as nutritional components (Randhir et al., 2004). Consumption of fenugreek sprouts could increase insulin sensitivity in human bodies and thus improve their tolerance to glucose, especially among people whose glucose responses are impaired (Kiss et al., 2018). That means fenugreek consumption has health benefits for patients with diabetes. Another functional component, trigonelline, in fenugreek sprouts exerts hypoglycemic effects for people who do not have diabetes (Randhir et al., 2004). Fenugreek sprouts also contain saponins, coumarin, phenolics, fenugreekin, and nicotinic acid that have cholesterol-absorbing, antimicrobial, and antioxidant effects by inhibiting the production of reactive oxygen species (Randhir et al., 2004).

2.2 Foodborne outbreaks related to fresh produce and sprouts

2.2.1 Fresh produce and sprouts related outbreaks

Fresh produce is an integral part of a healthy diet (Sivapalasingam, 2004). Recently, as the consumption of fresh produce increased, contaminated fresh produce has been increasingly involved in foodborne outbreaks in many parts of the world. According to the World Health Organization (WHO), at least 1 in 10 people (600 million) is sick annually because of consuming contaminated food, and 420,000 patients died consequently (WHO, 2022). Foodborne diseases have pressured medical services and exacerbated economic and political problems (Alegbeleye, Singleton & Sant'Ana, 2018). The failure to use traditional sanitizing methods to prevent infection and transmission of foodborne pathogens by fresh produce was attributed to the adhesion of pathogens to product surfaces and internalization of pathogens into produce tissues

(Lynch et al., 2009). Consumption of raw produce with no or little processing also contributes to the outbreaks related to fresh produce. Moreover, the higher demand for fresh produce made growers use alternative water sources and increased the use of soil amendments, and these could also increase the chances of fresh produce contamination. Other sources of contamination could be attributed to the increased fresh produce imports, as well as processing, preservation, packaging, and distribution practices (Beuchat, 2002).

The number of foodborne outbreaks linked to fresh produce consumption has increased recently. During 2017-2020, 250 food-related outbreaks were reported, which led to 7,659 illnesses, 2,044 hospitalizations, and 41 deaths. Fresh vegetables were identified as the source of 11% of total outbreaks with known sources. Root/underground vegetables were identified as the source of the most outbreak-associated illnesses (1,400) of any food category (Centers for Disease Control, 2022). The representative species are *Salmonella enterica*, *Escherichia coli*, and *Listeria monocytogenes* (Centers for Disease Control, 2019).

Seed sprouts have been regarded as vehicles of transmission in foodborne outbreaks. Subjective evaluations indicated that the appearance of sprouts was not affected when there were over 10^7 per gram of bacteria growing on the sprouts. Between 2010 and 2017, sprouts accounted for 27.6% of food vehicles within the vegetable category among reported foodborne outbreaks, which was the most frequently identified source (Carstens et al., 2019). From 1988 to 2020, there were at least 64 sprout-related outbreaks globally, with alfalfa sprouts being involved in more than 55% of the outbreaks, followed by beans and clover sprouts. The most common pathogens involved in sprout-associated outbreaks were *Salmonella* and pathogenic *E. coli* (Miyahira & Antunes, 2021).

2.2.2 *Salmonella enterica* and related outbreaks

S. enterica is a gram-negative, rod-shaped, zoonotic facultative intracellular pathogen (Jajere, 2019), and *Salmonella* infection constitutes a significant concern to public health. *S. enterica* can be found in the environment and colonize humans, animals, and plants (Andino, & Hanning, 2015). After being ingested via contaminated food or water or in contact with infected individuals or animals, S. enterica invades the intestinal epithelium in the ileum and colon. This pathogen can cause neutrophilic gastroenteritis, disseminate to systemic sites, and cause sepsis. The virulence factors of S. enterica include flagella, fimbriae, type III secretion systems, toxins, and two-component regulatory systems (Cheng, Eade, & Weidmann, 2019). The common symptoms of enteric salmonellosis are fever, abdominal pain, vomiting, and diarrhea (Knodler & Elfenbein, 2019).

In 1995, an outbreak caused by *S.* Stanly occurred in the U.S. and Finland. The pathogen was transmitted through alfalfa sprouts grown from contaminated seeds (Márton et al, 1997). Canada reported an outbreak in 2005 related to *Salmonella* involving mung bean sprouts, which led to 552 cases (Nesbitt et al., 2010). The largest *S.* Newport outbreak reported in Germany occurred in 2011. Twenty associated cases were reported in the Netherlands. *Salmonella* was detected in mung bean sprouts the patients had consumed previously (Bayer et al., 2014). The outbreaks were traced to seeds that were inadequately disinfected seeds in the U.S. (Winthrop et al., 2003; Proctor et al., 2001).

2.2.3 *E. coli* and related outbreaks

E. coli is a gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium in the genus *Escherichia*. Most *E. coli* strains survive in the gastrointestinal tract of humans and animals as normal flora, causing no harm to the human bodies. Several pathogenic strains have acquired virulence factors from plasmids, transposons, bacteriophages, and/or pathogenicity islands (Lim et al., 2010). One of them is enterohemorrhagic *E. coli* (EHEC) which is a subset that belongs to Shiga-toxin-producing *E. coli* (STEC), whose attachment mechanism is mediated by the Type III secretion system (Cleary, 2004). The symptoms of EHEC infection include abdominal cramps, severe bloody diarrhea (aka hemorrhagic colitis), non-bloody diarrhea, fatigue, nausea, and even life-threatening sequelae hemolytic uremic syndrome (HUS) (Centers for Disease Control, 2021). Among all of the serotypes of EHEC, the most common one is O157:H7, but other serotypes such as O111:NM, O26:H11, O91:H14, and O111:H8, are also frequently associated with EHEC infections (Cleary, 2004).

In the summer of 2011, one of the largest foodborne outbreaks occurred in Germany. EHEC caused the outbreak with the serotype O104:H4 (Buchholz et al., 2011). Another large outbreak caused by *E. coli* O157:H7 occurred in Japan, leading to nearly 10,000 cases. White radish sprouts were identified as a vehicle for transmitting the pathogen (Hara-Kudo et al, 1997). Several multistate *E. coli* O157:H7 outbreaks took place in the United States, such as the one related to alfalfa sprouts in Michigan and Virginia (Taormina & Beuchat, 1999b).

2.3 Seeds contamination during the production process

During commercial production, seeds of fresh produce can be contaminated in some possible routes and sources. Combinations of environmental risk factors influence the frequency and spread of foodborne pathogens, which in turn influence the risk of contamination of agricultural products (Strawn et al., 2013). Sources and routes of produce contamination vary from different production zones due to the unique combination of environmental risk factors of each farm, such as climate, land-use interactions, and topography. Several studies have investigated the potential sources of contamination through production in the supply chain at the pre-harvest and post-harvest stages.

2.3.1 Pre-harvest stage

At the pre-harvest stage, pathogens can grow and proliferate within plants. The risk can be amplified by further direct contamination or proliferation of existing pathogen populations during processing and post-harvest handling (Berger et al., 2010). The contamination sources during the pre-harvest stage include atmospheric deposition, uptake from contaminated soils and groundwater, use of raw or poorly treated manure and compost, contaminated irrigation water or flooding, transfer by insects, or by fecal contamination generated by livestock or wild animals (Alegbeleye, Singleton & Sant'Ana, 2018).

2.3.1.1 Soil and groundwater

The soil ecosystem contains the most significantbiodiversity (Wall, Nielsen, & Six, 2015). Thus, soils usually contain rich microflora, including pathogens for humans and other pathogenic and nonpathogenic organisms (Loynachan, 2013). A more significant number of human pathogens included *S. enterica, Campylobacter* spp., *E. coli, Legionella* spp., *Mycobacterium leprae*, *Shigella* spp., *Bacillus anthracis*, *Clostridium* spp., *Yersinia pestis*, *Burkholderia pseudomallei*, and *Francisella tularensis* (Steffan, Derby, & Brevik, 2020). These pathogens contaminate plants through seeds, roots, or surfaces. Since many soils for agriculture are easily contaminated by pathogenic microorganisms from point and non-point sources, extraneous pathogens may continuously enter the soil environments, some sources of which include contaminated irrigation or wasted water, manure, animal feces, and municipal solid wastes (Santamaria & Toranzos, 2003).

The survival and recalcitrance of pathogens in soil depend on soil type, soil moisture, pH value, temperature, nutrient availability, agronomic practices, and soil biological interactions (Alegbeleye, Singleton & Sant'Ana, 2018). It has been found that cool and moist environments are ideal for the survival of bacteria and viruses, while bacterial and viral populations are usually lower under dry soil conditions (Tate, 1978). Under flooded conditions, *E. coli* survival has been reported to be the highest in organic soils. Some bacterial pathogens such as *Streptococcus faecalis* cannot survive well under low soil moisture conditions (Jamieson et al., 2002). The pH of soil also impacts microbial diversity and biogeochemical processes mediated by them (Wu et al., 2017). For example, the optimal pH for the survival of bacteria is neutral, while fungi can exist under more acidic conditions (Leahy & Colwell, 1990). Groundwater can provide moisture to the soil, which harbors bacterial pathogens such as *S.* Typhimurium, *E. coli*, and *S. faecalis* (Bitton et al., 1983). Compared to surface water, bacteria and viruses survive for a long time in groundwater because groundwater tends to be cooler, offers protection from sunlight, and has less biological activity, making it a significant source of soil contamination (Steele and Odumeru, 2004).

2.3.1.2 Manure

Amended soil with manure is a cost-effective source of nutrients for agricultural purposes and added soil organic materials include livestock excreta, slurries, abattoir wastes, sewage sludge, as well as municipal and industrial waste treatment residuals (Goss, Tubeileh, & Goorahoo, 2013; Levi-Minzi, Riffaldi, & Saviozzi, 1990). Research has shown that raw and improperly treated manure constitute a significant risk of pathogenic contamination for produce (Manyi-Loh et al., 2016). Public health-relevant bacterial and viral pathogens as well as parasites such as *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes*, *Campylobacter* spp., porcine enteroviruses, bovine coronavirus, bovine virus diarrhea *Cryptosporidium parvum*, and *Giardia*, have been isolated from raw/poorly treated manures (Alegbeleye, Singleton & Sant’Ana, 2018).

2.3.1.3 Irrigation water

One of the most frequent pathogens in water-related outbreaks is *E. coli* O157:H7 (Olsen et al., 2002; Saxena, Kaushik, & Mohan, 2015). Depending on ambient temperatures, the organisms can persist in water for quite a long time. *E. coli* O157:H7 can endure extreme environmental conditions such as high acidity and low temperature (Chalmers, Aird, & Bolton, 2000; Islam et al., 2004). The ability of a pathogen to persist/survive in the environment or on produce is an essential determinant in the risk of human infection. The actual risks associated with pathogens occurring in irrigation water are led by various factors such as temperature, pH, oxygen, and UV light (Banach, & Van Der Fels-Klerx, 2020). Since pathogens can persist in water for a long time, regardless of the source or route of exposure, one potentially fatal consequence of pathogen contamination of irrigation water is the repeated inoculation of plants with the pathogens, posing a profound threat to produce safety (Alegbeleye, Singleton & Sant’Ana, 2018). After being introduced into the product, some pathogens can adhere to the surfaces of produce, while others can rapidly internalize into plant tissues under certain conditions, translocate and persist until consumed (Doyle, & Erickson, 2008; Warriner et al., 2003). If this situation happens, many traditional processing and chemical sanitizing methods would be ineffective (Hong, & Moorman, 2005). Although awareness of the potential dangers of using microbiologically compromised water for irrigation has increased recently, microbiological contamination risk has not decreased because some regions that lack water resources use sub-optimal supplementary irrigation water sources.

2.3.1.4 Livestock

It is known that produce fields are often located near animal production zones because agriculture has grown more intensive over the years. The connections between wild animals, livestock, and produce have been strengthened (Strawn et al., 2013), making fruits and vegetables more vulnerable to pre-harvest hazards in many cases. Besides those farm animals that act as reservoirs of enteric pathogens, wild animals such as amphibians, birds, reptiles, rodents, and some insects like flies and beetles also play roles as vehicles of pathogens to contaminate cultivation media and produce (Beuchat, 2006; Lim et al., 2014).

Farm and wild animals can access cultivation areas by intrusion or because of adjacent land use (Jay-Russel, 2013). Birds such as chickens, Canada geese, gulls, migratory ducks, pigeons, and sandhill cranes have been determined to be reservoirs of pathogens such as *E. coli*, *Salmonella*, and *Campylobacter* (Alegbeleye, Singleton & Sant’Ana, 2018). Insects are usually found in manure piles, feedlots, and other habitats near fields, exerting more pressure on farms that practice mixed farming (Martínez-Vaz et al., 2014). Since insects can be found everywhere in cultivation fields, they have access to produce limitlessly. Many bacterial species have evolved to exploit insects as hosts or vectors. Many pathogens use filth flies, fruit flies, cockroaches, and other insects as mechanical and biological vectors to contaminate fruits and vegetables on the field (Alam and Zurek, 2004; Humphrey et al., 2007).

Reptiles, including snakes, lizards, chameleons, turtles, and other ophidians, saurian, and chelonians, have been found harboring enteric bacteria like *Salmonella* (Corrente et al. 2004; Beuchat, 2006). Many wild rodents are asymptomatic carriers of pathogens like *Salmonella* and *Campylobacter* (Alegbeleye, Singleton & Sant’Ana, 2018). They often amplify the number of pathogens in the environment and transfer them to other farm animals and fresh produce (Meerburg & Kijlstra, 2007).

2.3.2 Post-harvest stage

After harvest, fresh produce can also be contaminated with foodborne pathogens during storage, rinsing, and cutting (Berger et al., 2010). Cut surfaces of leaves are a specific target for bacterial pathogens such as *Salmonella*, which shows a specific tropism towards them (Kroupitski et al., 2009). Cutting melons may carry pathogens from the rind onto the edible part of the fruit where pathogens can multiply if the cut melon is not refrigerated (Ukuku, & Sapers, 2007). External sources such as the use of inadequately decontaminated water in hydro coolers, which are used to store and process large quantities of fresh produce, may lead to the contamination of an entire lot of products (Gagliardi, Millner, Lester, & Ingram, 2003).

2.4 Controlling methods for bacterial pathogens on seeds and sprouts

Contaminated seeds and sprouts can be an important source of produce-associated outbreaks of infections. An important strategy to control any disease is eliminating or reducing the number of seed-borne pathogens. For example, it is a critical management practice to use disease-free seeds and transplants to prevent bacterial diseases. Some seed companies even have the resources to produce seeds in areas where these diseases do not occur and to test seeds for pathogens. Manufacturers have utilized 20,000 ppm of calcium hypochlorite solution to decontaminate seeds before germination as a preventative measure (Thomas et al., 2003). Chlorine at up to 200 ppm is a standard method for washing sprouts during or post-production. However, the chlorine-based treatment can only achieve about 1 to 3 log reductions in bacterial population, and the use of the chemical is associated with health and environmental concerns (Sikin, Zoellner, & Rizvi, 2013). Thus, researchers have developed alternative seed decontamination strategies involving physical, chemical, biological, and hurdle interventions that can achieve up to 7-log reductions in bacterial population in some cases (Sikin, Zoellner, & Rizvi, 2013).

2.4.1 Physical interventions

Physical interventions, such as heat, pressure, and irradiation, offer better penetration for internalized or sheltered microorganisms and may be more readily adapted for commercialization than chemical and biological interventions since they are only effective for surface decontamination. In addition, the use of synthetic chemicals does not fit some consumers desires. This attitude is reflected in the ever-increasing consumer preference for the reduced use of synthesized additives and increased use of natural preservatives in foods (Oms-Oliu et al., 2010). Under this circumstance, developing an intervention method that is synthetic chemical free or leaves no residues in treated food becomes a high priority.

2.4.2 Chemical interventions

For now, the clearer promising strategy, is generating antimicrobials that can be easily incorporated into sprout farming systems on-site. Organic acids, chlorine dioxide (ClO_2), electrolyzed oxidizing water, and ozone have many potentials as preferred chemical interventions for ensuring sprout quality and safety (Sikin, Zoellner, & Rizvi, 2013). The main limitations of current chlorine-based washes are the possible hazards associated with producing, transporting, and handling a large amount of chlorine.

2.4.3 Biological interventions

Biological interventions are usually achieved using bacteriophage (live organisms), protective bacterial cultures, yeast, and molds. These organisms may produce antimicrobial substances, such as bacteriocins, organic acid, and quorum-sensing molecules, negatively impacting pathogens' viability (Soltani et al., 2020; Linares-Morales et al., 2018; Zhao, Yu, & Ding, 2020). Biological interventions provide another promising strategy for eliminating synthetic chemicals for sanitization or decreasing their work concentrations that served as preservatives (Gálvez, Abriouel, Cobo, & Pulido, 2011). These alternatives can also be applied to producing organic sprouts for many sprout growers.

2.5 Nematodes, ascarosides, and their functions

2.5.1 Nematodes

Nematodes are small roundworms from the phylum *Nematoda*, believed to be the most abundant organisms on earth (Blaxter et al., 1998). They have existed for an estimated one billion years, making them one of the most ancient and diverse types of animals (Wang, Kumar, & Hedges, 1999). They are thought to have evolved from simple animals about 400 million years before the "Cambrian explosion" of invertebrates able to be fossilized (Holterman, Schratzberger, & Helder, 2019). The two nematode classes, *Chromadorea* and *Enople*a, diverged over 550 million years, so it is difficult to know the age of the two lineages of the phylum accurately (Lambert, & Bekal, 2002). Most nematodes live and sustain themselves by consuming bacteria or other microscopic organisms. Other species are parasites of plants or animals. All plant parasitic nematodes are obligate parasites, feeding exclusively on the cytoplasm of living plant cells (Williamson, & Gleason, 2003). Plant-parasite nematodes can damage many crop plants, causing billions of dollars in agricultural losses every year (Barker, & Koenning, 1998).

Plant-parasite nematodes feed on all parts of the plant, including roots, stems, leaves, flowers, and seeds. In one way, nematodes intake nutrients in plant cells and kill them, thus causing enormous damage to plant tissue. In another way of feeding, nematodes do not kill plant cells but “trick” them into enlarging and grow so that they can produce one or more nutrient-rich feeding cells for the nematode (Lambert, & Bekal, 2002). More plant-parasite nematode species feed on the roots of plants. The feeding process damages the plant’s root system and reduces the plant’s ability to absorb water and nutrients. Typical nematode damage symptoms are the reduction of root mass, a distortion of root structure, and enlargement of the roots (Poveda, Abril-Urias, & Escobar, 2020).

2.5.2 Ascarosides and intra-species interactions in plants

The term “ascarosides” was first introduced to describe a distinct type of lipid detected in parasitic roundworms in the family *Ascaridia* more than 100 years ago. Flury was the first scientist who conducted a detailed chemical analysis of a human intestinal parasite *Ascaris lumbricoides*, which included the isolation of an “unsaponifiable matter” with an unknown composition that accounted for approximately 25% of the total lipid content of the nematode (Ludewig, & Schroeder, 2018). In 1933, Schulz and Becker identified the chemical structures of the Ascaris-derived ascarosides as $C_{33}H_{68}O_4$ (Schulz and Becker, 1933). The exact structure of the ascaroside from the *Ascaris* spp. is defined as glycosides of dideoxysugar ascarylose with long aliphatic side chains attached (Ludewig, & Schroeder, 2013).

After ascaroside ascr#1 was isolated and biochemically defined in 2005, research on ascaroside pheromones in *C. elegans* and other nematodes has significantly progressed (Park, Joo, Park, & Paik, 2019). But it was not until the past decade that the relevance of ascarosides acting as small signaling molecules for many aspects of *Caenorabditis elegans* development and behavior became apparent. It is known now that a group of ascarosides function as pheromone together, and different components act synergistically in dauer induction in nematodes, for example, male attraction, hermaphrodite repulsion, olfactory plasticity, and aggregation (Ludewig, & Schroeder, 2013). Therefore, nematodes secret ascarosides during various activities, including attacking plants.

Immune responses to pathogens attacks in plants and animals are triggered partly by the detection of micro-associated molecular patterns (MAMPs) *via* specific extra- and intra-cellular sensors, including evolutionarily conserved macromolecules specific to different classes of pathogens (Maekawa, Kufer, & Schulze-Lefert, 2011). For example, flagellin, peptidoglycan, and lipopolysaccharide are MAMPs in bacteria (Newman, Sundelin, Nielsen, & Erbs, 2013); chitin is fungi's MAMPs (Eckardt, 2008). Plants and animals can perceive these molecules, and their innate immune systems are activated to combat the pathogens. Similarly, ascarosides, a conserved family of pheromones in parasite nematodes, can be recognized by plants and activate their immune response (Manosalva et al., 2015). Experiments have been done to explore how plants perceive ascarosides and how they trigger plant immune defense. It was found that soybean, rice, maize, wheat, barley, potato, tomato, and *Arabidopsis* can recognize ascaroside ascr#18 and subsequently become resistant to bacterial and viral pathogens and pests (Klessig et al., 2019). The activation mechanisms include upregulation of the salicylic acid and jasmonic acid pathways for signal transduction, activation of certain enzymes for cell responses, and production of immune defense-related substances (Manosalva et al., 2015). The most recent study showed that after contact with ascr#18, plants could metabolize it into short-chain ascarosides through peroxisomal β-oxidation. By secreting metabolized short-chain ascarosides from roots, plants can deter nematodes mixed with ascr#18 at a specific ratio (Manohar et al., 2020).

References

Alam, M. J., & Zurek, L. (2004). Association of *Escherichia coli* O157:H7 with houseflies on a cattle farm. *Applied and Environmental Microbiology*, *70*(12), 7578-7580. https://doi.org/10.1128/AEM.70.12.7578-7580.2004

Alegbeleye, O. O., Singleton, I., & Sant'Ana, A. S. (2018). Sources and contamination routes of microbial pathogens to fresh produce during field cultivation: A review. *Food Microbiology*, *73*, 177-208.

Andino, A., & Hanning, I. (2015). *Salmonella enterica*: survival, colonization, and virulence differences among serovars. *The Scientific World Journal*, *2015*. https://doi.org/10.1155%2F2015%2F520179

Banach, J. L., & Van Der Fels-Klerx, H. J. (2020). Microbiological reduction strategies of irrigation water for fresh produce. *Journal of Food Protection*, *83*(6), 1072-1087. https://doi.org/10.4315/JFP-19-466

Barker, K. R., & Koenning, S. R. (1998). Developing sustainable systems for nematode management. *Annual Review of Phytopathology*, *36*(1), 165-205. https://doi.org/10.1146/annurev.phyto.36.1.165

Bayer, C., Bernard, H., Prager, R., Rabsch, W., Hiller, P., Malorny, B., Pfefferkorn, B., Frank, C., Jong, A. de., Friesema, I., Stark., K., & Rosner, B. M. (2014). An outbreak of *Salmonella* Newport associated with mung bean sprouts in Germany and the Netherlands, October to November 2011. *Eurosurveillance*, *19*(1), 20665. https://doi.org/10.2807/1560-7917.ES2014.19.1.20665

Benincasa, P., Falcinelli, B., Lutts, S., Stagnari, F., & Galieni, A. (2019). Sprouted grains: A comprehensive review. *Nutrients*, *11*(2), 421. https://doi.org/10.3390/nu11020421

Berger, C. N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P., & Frankel, G. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environmental Microbiology*, *12*(9), 2385-2397. https://doi.org/10.1111/j.1462-2920.2010.02297.x

Bertoia, M. L., Mukamal, K. J., Cahill, L. E., Hou, T., Ludwig, D. S., Mozaffarian, D., Willett, W. C., Hu, F. B., & Rimm, E. B. (2016). Correction: Changes in intake of fruits and vegetables and weight change in United States men and women followed for up to 24 years: Analysis from three prospective cohort studies. *PloS Medicine*, *13*(1), e1001956. https://doi.org/10.1371/journal.pmed.1001956

Beuchat, L. R. (2002). Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes and Infection*, *4*(4), 413-423. https://doi.org/10.1016/S1286-4579(02)01555-1

Beuchat, L. R. (2006). Vectors and conditions for preharvest contamination of fruits and vegetables with pathogens capable of causing enteric diseases. *British Food Journal*, *108*(1), 38-53. https://doi.org/10.1108/00070700610637625

Bitton, G., Farrah, S. R., Ruskin, R. H., Butner, J., & Chou, Y. J. (1983). Survival of pathogenic and indicator organisms in groundwater. *Groundwater*, *21*(4), 405-410. https://doi.org/10.1111/j.1745-6584.1983.tb00741.x

Blaxter, M. L., De Ley, P., Garey, J. R., Liu, L. X., Scheldeman, P., Vierstraete, A., Vanfleteren, J. R., Mackey, L. Y., Dorris, M., Frisse, L. M., Vida, J. T., & Thomas, W. K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature*, *392*(6671), 71–75. https://doi.org/10.1038/32160

Bora, K. S., & Sharma, A. (2011). Phytochemical and pharmacological potential of *Medicago sativa*: A review. *Pharmaceutical Biology*, *49*(2), 211-220. https://doi.org/10.3109/13880209.2010.504732

Buchholz, U., Bernard, H., Werber, D., Böhmer, M. M., Remschmidt, C., Wilking, H., Deleré, Y., an der Heiden, M., Adlhoch, C., Dreesman, J., Ehlers, J., Ethelberg, S., Faber, M., Frank, C., Fricke, G., Greiner, M., Höhle, M., Ivarsson, S., Jark, U., Kirchner, M., Koch, J., Krause, G., Luber, P., Rosner, B., Klaus, S., & Kühne, M. (2011). German outbreak of *Escherichia coli* O104:H4 associated with sprouts. *New England Journal of Medicine*, *365*(19), 1763–1770. https://doi.org/10.1056/nejmoa1106482

Centers for Disease Control. (2019). *CDC and Food Safety: Fruit and Vegetable Safety*. https://www.cdc.gov/foodsafety/newsletter/fruit-veggie-3-20-19.html Accessed June 29, 2022.

Centers for Disease Control. (2021). *Escherichia coli*. https://www.cdc.gov/ecoli/ecoli-symptoms.html Accessed Oct. 15, 2022.Accessed June 29, 2022.

Centers for Disease Control. (2022). *Summary of Possible Multistate Enteric (Intestinal) Disease Outbreaks*. https://www.cdc.gov/foodsafety/outbreaks/lists/annual-summaries.html Accessed Jun. 29, 2022.

Chalmers, R. M., Aird, H., & Bolton, F. J. (2000). Waterborne *Escherichia coli* O157. *Journal of Applied Microbiology*, *88*(S1), 124S-132S. https://doi.org/10.1111/j.1365-2672.2000.tb05340.x

Cheng, R. A., Eade, C. R., & Wiedmann, M. (2019). Embracing diversity: differences in virulence mechanisms, disease severity, and host adaptations contribute to the success of nontyphoidal *Salmonella* as a foodborne pathogen. *Frontiers in Microbiology*, *10*, 1368. https://doi.org/10.3389/fmicb.2019.01368

Choudhary, T. (2022). *14 amazing benefits and uses of fenugreek sprouts for skin, hair and health*. STYLECRAZE. https://www.stylecraze.com/articles/benefits-of-fenugreek-sprouts-for-skin-hair-and-health/ Accessed Jun. 28, 2022.

Cleary, T. G. (2004). The role of Shiga-toxin-producing *Escherichia coli* in hemorrhagic colitis and hemolytic uremic syndrome. *Seminars in Pediatric Infectious Diseases*, *15*(4), 260–265. https://doi.org/10.1053/j.spid.2004.07.007

Corrente, M., Madio, A., Friedrich, K. G., Greco, G., Desario, C., Tagliabue, S., D'Incau, M., Campolo, M., & Buonavoglia, C. (2004). Isolation of *Salmonella* strains from reptile faeces and comparison of different culture media. *Journal of Applied Microbiology*, *96*(4), 709–715. https://doi.org/10.1111/j.1365-2672.2004.02186.x

Doyle, M. P., & Erickson, M. C. (2008). Summer meeting 2007–the problems with fresh produce: an overview. *Journal of Applied Microbiology*, *105*(2), 317-330. https://doi.org/10.1111/j.1365-2672.2008.03746.x

Eckardt, N. A. (2008). Chitin signaling in plants: insights into the perception of fungal pathogens and rhizobacterial symbionts. *Plant Cell*, *20*(2), 241. https://doi.org/10.1105/tpc.108.058784

El-Ramady, H., Abdalla, N., Kovacs, S., Domokos-Szabolcsy, E., Bákonyi, N., Fari, M., & Geilfus, C. M. (2020). Sustainable biorefinery and production of alfalfa (*Medicago sativa L.*). *Egyptian Journal of Botany*, *60*(3), 621-639. https://dx.doi.org/10.21608/ejbo.2020.37749.1532

Fan, X., & Thayer, D. W. (2001). Quality of irradiated alfalfa sprouts. *Journal of Food Protection*, *64*(10), 1574-1578. https://doi.org/10.4315/0362-028X-64.10.1574

Gagliardi, J. V., Millner, P. D., Lester, G., & Ingram, D. (2003). On-farm and postharvest processing sources of bacterial contamination to melon rinds. *Journal of Food Protection*, *66*(1), 82-87. https://doi.org/10.4315/0362-028X-66.1.82

Gálvez, A., Abriouel, H., Cobo, A., & Pulido, R. P. (2011). Natural Antimicrobials for Biopreservation of Sprouts. In T. Ng (Eds.). *Soybean-Biochemistry, Chemistry and Physiology* (pp. 67-82). IntechOpen. http://doi.org/10.5772/15746

Gimenez-Bastida, J. A., & Zielinski, H. (2015). Buckwheat as a functional food and its effects on health. *Journal of Agricultural and Food Chemistry*, *63*(36), 7896-7913. https://doi.org/10.1021/acs.jafc.5b02498

Goss, M. J., Tubeileh, A., & Goorahoo, D. (2013). A review of the use of organic amendments and the risk to human health. *Advances in Agronomy*, *120*, 275-379. https://doi.org/10.1016/B978-0-12-407686-0.00005-1

Gustat, J., O'Malley, K., Luckett, B. G., & Johnson, C. C. (2015). Fresh produce consumption and the association between frequency of food shopping, car access, and distance to supermarkets. *Preventive Medicine Reports*, *2*, 47-52. https://doi.org/10.1016/j.pmedr.2014.12.009

Hamilton, M. J., & Vanderstoep, J. (1979). Germination and nutrient composition of alfalfa seeds. *Journal of Food Science*, *44*(2), 443-445. https://doi.org/10.1111/j.1365-2621.1979.tb03807.x

Hara-Kudo, Y., Konuma, H., Iwaki, M., Kasuga, F., Sugita-Konishi, Y., Ito, Y., & Kumagai, S. (1997). Potential hazard of radish sprouts as a vehicle of *Escherichia coli*

O157:H7. *Journal of Food Protection*, *60*(9), 1125-1127. https://doi.org/10.4315/0362-028X-60.9.1125

Holterman, M., Schratzberger, M., & Helder, J. (2019). Nematodes as evolutionary commuters between marine, freshwater and terrestrial habitats. *Biological Journal of the Linnean Society*, *128*(3), 756-767. https://doi.org/10.1093/biolinnean/blz107

Hong, C. X., & Moorman, G. W. (2005). Plant pathogens in irrigation water: challenges and opportunities. *Critical Reviews in Plant Sciences*, *24*(3), 189-208. https://doi.org/10.1080/07352680591005838

Humphrey, T., O'Brien, S., & Madsen, M. (2007). Campylobacters as zoonotic pathogens: a food production perspective. *International Journal of Food Microbiology*, *117*(3), 237-257. https://doi.org/10.1016/j.ijfoodmicro.2007.01.006

Hung, H. C., Joshipura, K. J., Jiang, R., Hu, F. B., Hunter, D., Smith-Warner, S. A., Colditz, G. A., Rosner, B., Spiegelman, D., & Willett, W. C. (2004). Fruit and vegetable intake and risk of major chronic disease. *Journal of the National Cancer Institute*, *96*(21), 1577-1584. https://doi.org/10.1093/jnci/djh296

Islam, M., Doyle, M. P., Phatak, S. C., Millner, P., & Jiang, X. (2004). Persistence of enterohemorrhagic *Escherichia coli* O157: H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *Journal of Food Protection*, *67*(7), 1365-1370. https://doi.org/10.4315/0362-028X-67.7.1365

Jajere, S. M. (2019). A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. *Veterinary World*, *12*(4), 504. https://doi.org/10.14202%2Fvetworld.2019.504-521

Jamieson, R. C., Gordon, R. J., Sharples, K. E., Stratton, G. W., & Madani, A. (2002). Movement and persistence of fecal bacteria in agricultural soils and subsurface drainage water: A review. *Canadian Biosystems Engineering*, *44*(1), 1-9.

Jay-Russell, M. T. (2013). What is the risk from wild animals in food-borne pathogen contamination of plants? *CAB Reviews*, *8*(040), 1-16. http://dx.doi.org/10.1079/PAVSNNR20138040

Kiss, R., Szabó, K., Gesztelyi, R., Somodi, S., Kovács, P., Szabó, Z., Németh, J., Priksz, D., Kurucz, A., Juhász, B., & Szilvássy, Z. (2018). Insulin-sensitizer effects of fenugreek seeds in parallel with changes in plasma MCH levels in healthy volunteers. *International Journal of Molecular Sciences*, *19*(3), 771. https://doi.org/10.3390%2Fijms19030771

Klessig, D. F., Manohar, M., Baby, S., Koch, A., Danquah, W. B., Luna, E., Park, H. J., Kolkman, J. M., Turgeon, B. G., Nelson, R., Leach, J. E., Williamson, V. M., Kogel, K. H., Kachroo, A., & Schroeder, F. C. (2019). Nematode ascaroside enhances resistance in a broad spectrum of plant-pathogen systems. *Journal of Phytopathology*, *167*(5), 265–272. https://doi.org/10.1111/jph.12795

Knodler, L. A., & Elfenbein, J. R. (2019). *Salmonella enterica*. *Trends in Microbiology*, *27*(11), 964-965. https://doi.org/10.1016/j.tim.2019.05.002

Kroupitski, Y., Pinto, R., Brandl, M. T., Belausov, E., & Sela, S. (2009). Interactions of *Salmonella enterica* with lettuce leaves. *Journal of Applied Microbiology*, *106*(6), 1876–1885. https://doi.org/10.1111/j.1365-2672.2009.04152.x

Kumari, S., Phogat, D., Sehrawat, K. D., Choudhary, R., Rajput, V. D., Ahlawat, J., Karunakaran, R., Minkina, T., & Sehrawat, A. R. (2021). The effect of *Ascophyllum* *nodosum* extract on the nutraceutical antioxidant potential of *Vigna radiata* sprout under salt stress. *Plants*, *10*(6), 1216. https://doi.org/10.3390/plants10061216

Lambert, K., & Bekal, S. (2002). Introduction to plant-parasitic nematodes. *The Plant Health Instructor*, *10*, 1094-1218. http://dx.doi.org/10.1094/PHI-I-2002-1218-01

Leahy, J. G., & Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiological Reviews*, *54*(3), 305-315. https://doi.org/10.1128/mr.54.3.305-315.1990

Lethaby, A., Marjoribanks, J., Kronenberg, F., Roberts, H., Eden, J., & Brown, J. (2007). Phytoestrogens for vasomotor menopausal symptoms. *Cochrane Database of Systematic Reviews*, *4*, CD001395-CD001395. https://doi.org/10.1002/14651858.CD001395.pub3

Levi-Minzi, R., Riffaldi, R., & Saviozzi, A. (1990). Carbon mineralization in soil amended with different organic materials. *Agriculture, Ecosystems & Environment*, *31*(4), 325-335. https://doi.org/10.1016/0167-8809(90)90231-2

Lim, J. A., Lee, D. H., & Heu, S. (2014). The interaction of human enteric pathogens with plants. *The Plant Pathology Journal*, *30*(2), 109. https://doi.org/10.5423%2FPPJ.RW.04.2014.0036

Lim, J. Y., Yoon, J. W., & Hovde, C. J. (2010). A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. *Journal of Microbiology and Biotechnology*, *20*(1), 5. PMID: 20134227; PMCID: PMC3645889. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3645889/

Linares-Morales, J. R., Gutiérrez-Méndez, N., Rivera-Chavira, B. E., Pérez-Vega, S. B., & Nevárez-Moorillón, G. V. (2018). Biocontrol processes in fruits and fresh produce, the use of lactic acid bacteria as a sustainable option. *Frontiers in Sustainable Food Systems*, *2*, 50. https://doi.org/10.3389/fsufs.2018.00050

Loynachan, T. E. (2013). Human disease from introduced and resident soilborne pathogens. In E. C. Brevik & L. C. Burgess (Eds). *Soils and Human Health* (pp. 107-136). Boca Raton, Florida: CRC Press.

Ludewig, A. H., & Schroeder, F. C., Ascaroside signaling in *C. elegans* (January 18, 2013), *Wormbook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.155.1, http://www.wormbook.org.

Lynch, M. F., Tauxe, R. V., & Hedberg, C. W. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiology & Infection*, *137*(3), 307-315. https://doi.org/10.1017/S0950268808001969

Maekawa, T., Kufer, T. A., & Schulze-Lefert, P. (2011). NLR functions in plant and animal immune systems: so far and yet so close. *Nature Immunology*, *12*(9), 817-826. https://doi.org/10.1038/ni.2083

Mahon, B. E., Pönkä, A., Hall, W. N., Komatsu, K., Dietrich, S. E., Siitonen, A., Cage, G., Hayes, P. S., Lambert-Fair, M. A., Bean, N. H., Griffin. P. M., & Slutsker, L. (1997). An international outbreak of *Salmonella* infections caused by alfalfa sprouts grown from contaminated seeds. *Journal of Infectious Diseases*, *175*(4), 876-882. https://doi.org/10.1086/513985

Manohar, M., Tenjo-Castano, F., Chen, S., Zhang, Y. K., Kumari, A., Williamson, V. M., Wang, X., Klessig, D. F., & Schroeder, F. C. (2020). Plant metabolism of nematode pheromones mediates plant-nematode interactions. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-019-14104-2

Manosalva, P., Manohar, M., von Reuss, S. H., Chen, S., Koch, A., Kaplan, F., Choe, A., Micikas, R. J., Wang, X., Kogel, K-H., Sternberg, P. W., Williamson, V. M., Schroeder, F. C., & Klessig, D. F. (2015). Conserved nematode signaling molecules elicit plant defenses and pathogen resistance. *Nature Communications*, *6*(1), 1-8. https://doi.org/10.1038/ncomms8795

Manyi-Loh, C. E., Mamphweli, S. N., Meyer, E. L., Makaka, G., Simon, M., & Okoh, A. I. (2016). An overview of the control of bacterial pathogens in cattle manure. *International Journal of Environmental Research and Public Health*, *13*(9), 843. https://doi.org/10.3390/ijerph13090843

Martínez-Vaz, B. M., Fink, R. C., Diez-Gonzalez, F., & Sadowsky, M. J. (2014). Enteric pathogen-plant interactions: molecular connections leading to colonization and growth and implications for food safety. *Microbes and Environments*, *29*(2), 123-135. https://doi.org/10.1264/jsme2.me13139

Márton, M., Mándoki, Z. S., Csapó-Kiss, Z. S., & Csapó, J. (2010). The role of sprouts in human nutrition. A review. *Acta Universitatis Sapientiae: Alimentaria*, *3*, 81-117. http://www.acta.sapientia.ro/acta-alim/C3/alim3-5.pdf

Mcintosh, M., & Miller, C. (2001). A diet containing food rich in soluble and insoluble fiber improves glycemic control and reduces hyperlipidemia among patients with type 2 diabetes mellitus. *Nutrition Reviews*, *59*(2), 52-55. https://doi.org/10.1111/j.1753-4887.2001.tb06976.x

Meerburg, B. G., & Kijlstra, A. (2007). Role of rodents in transmission of *Salmonella* and Campylobacter. *Journal of the Science of Food and Agriculture*, *87*(15), 2774-2781. https://doi.org/10.1002/jsfa.3004

Miyahira, R. F., & Antunes, A. E. C. (2021). Bacteriological safety of sprouts: A brief review. *International Journal of Food Microbiology*, *352*, 109266. https://doi.org/10.1016/j.ijfoodmicro.2021.109266

Murray, M. T., & Pizzorno, J. (2010). *The encyclopedia of healing foods*. Simon and Schuster.

Nesbitt, A., Ravel, A., Murray, R., McCormick, R., Savelli, C., Finley, R., Parmley, J., Agunos, A., Gilmour, M., & Canadian Public Health Laboratory Network. (2012). Integrated surveillance and potential sources of *Salmonella* Enteritidis in human cases in Canada from 2003 to 2009. *Epidemiology & Infection*, *140*(10), 1757-1772. https://doi.org/10.1017/S0950268811002548

Newman, M. A., Sundelin, T., Nielsen, J. T., & Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Frontiers in Plant Science*, *4*, 139. https://doi.org/10.3389/fpls.2013.00139

Nguyen, Y., & Sperandio, V. (2012). Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *Frontiers in Cellular and Infection Microbiology*, *2*, 90. https://doi.org/10.3389%2Ffcimb.2012.00090

Olsen, S. J., Miller, G., Breuer, T., Kennedy, M., Higgins, C., Walford, J., Mckee, G., Fox, K., Bibb, W., & Mead, P. (2002). A waterborne outbreak of *Escherichia coli* O157:H7 infections and hemolytic uremic syndrome: implications for rural water systems. *Emerging Infectious Diseases*, *8*(4), 370. https://doi.org/10.3201%2Feid0804.000218

Oms-Oliu, G., Rojas-Graü, M. A., González, L. A., Varela, P., Soliva-Fortuny, R., Hernando, M. I., Munuera, I. P., Fiszman, S., & Martín-Belloso, O. (2010). Recent approaches using chemical treatments to preserve quality of fresh-cut fruit: A Review. *Postharvest Biology and Technology*, *57*(3), 139–148. https://doi.org/10.1016/j.postharvbio.2010.04.001

Park, J. Y., Joo, H. J., Park, S., & Paik, Y. K. (2019). Ascaroside pheromones: chemical biology and pleiotropic neuronal functions. *International Journal of Molecular Sciences*, *20*(16), 3898. https://doi.org/10.3390/ijms20163898

Peñas, E., & Martínez-Villaluenga, C. (2020). Advances in production, properties and applications of sprouted seeds. *Foods*, *9*(6), 790. https://doi.org/10.3390/foods9060790

Price, T. V. (1988). Seed sprout production for human consumption—a review. *Canadian Institute of Food Science and Technology Journal*, *21*(1), 57-65. https://doi.org/10.1016/S0315-5463(88)70718-X

Pollack, S. L. (2001). Consumer demand for fruit and vegetables: the US example. In A. Regmi (Eds). *Changing Structure of Global Food Consumption and Trade* (pp.49-54). Darby, Pennsylvania: Diane Publishing.

Poveda, J., Abril-Urias, P., & Escobar, C. (2020). Biological control of plant-parasitic nematodes by filamentous fungi inducers of resistance: *Trichoderma*, mycorrhizal and endophytic fungi. *Frontiers in Microbiology*, *11*, 992. https://doi.org/10.3389/fmicb.2020.00992

Proctor, M. E., Hamacher, M., Tortorello, M. L., Archer, J. R., & Davis, J. P. (2001). Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa sprouts grown from seeds pretreated with calcium hypochlorite. *Journal of Clinical Microbiology*, *39*(10), 3461-3465. https://doi.org/10.1128/JCM.39.10.3461-3465.2001

Randhir, R., Lin, Y. T., Shetty, K., & Lin, Y. T. (2004). Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asia Pacific Journal of Clinical Nutrition*, *13*(3), 295-307. PMID: 15331344.

Rebollo-Hernanz, M., Aguilera, Y., Herrera, T., Cayuelas, L. T., Dueñas, M., Rodríguez-Rodríguez, P., Ramiro-Cortijo, D., Arribas, S. M., & Martín-Cabrejas, M. A. (2020). Bioavailability of melatonin from lentil sprouts and its role in the plasmatic antioxidant status in rats. *Foods*, *9*(3), 330. https://doi.org/10.3390/foods9030330

Santamaría, J., & Toranzos, G. A. (2003). Enteric pathogens and soil: a short review. *International Microbiology*, *6*(1), 5-9. https://doi.org/10.1007/s10123-003-0096-1

Saxena, T., Kaushik, P., & Mohan, M. K. (2015). Prevalence of *E. coli* O157:H7 in water sources: an overview on associated diseases, outbreaks and detection methods. *Diagnostic Microbiology and Infectious Disease*, *82*(3), 249-264. https://doi.org/10.1016/j.diagmicrobio.2015.03.015

Schulz, F. N., & Becker, M. (1933). Ascaryl alcohol. *Biochemistry Ztschr*, *265*, 253-259.

Shah, M. A., Sarker, M. M. R., & Gousuddin, M. (2016). Antidiabetic potential of *Brassica Oleracea* Var. *Italica* in type 2 diabetic sprague dawley (sd) rats. *International Journal of Pharmacognosy Phytochemical Research*, *8*(3), 462-469. http://dx.doi.org/10.13140/RG.2.1.4509.3529

Sikin, A. M., Zoellner, C., & Rizvi, S. S. (2013). Current intervention strategies for the microbial safety of sprouts. *Journal of Food Protection*, *76*(12), 2099-2123. https://doi.org/10.4315/0362-028x.jfp-12-437

Sivapalasingam, S., Friedman, C. R., Cohen, L., & Tauxe, R. V. (2004). Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through

1997. *Journal of Food Protection*, *67*(10), 2342-2353. https://doi.org/10.4315/0362-028X-67.10.2342

Soltani, S., Hammami, R., Cotter, P. D., Rebuffat, S., Said, L. B., Gaudreau, H., Bédard, F., Biron, E., Drider, D., & Fliss, I. (2020). Bacteriocins as a new generation of antimicrobials: Toxicity aspects and regulations. *FEMS Microbiology Reviews*, *45*(1). https://doi.org/10.1093/femsre/fuaa039

Staughton, J. (2021). *11 wonderful benefits of alfalfa sprouts*. Organic Facts. https://www.organicfacts.net/alfalfa-sprouts.html Accessed Jun. 28, 2022.

Steele, M., & Odumeru, J. (2004). Irrigation water as source of foodborne pathogens on fruit and vegetables. *Journal of Food Protection*, *67*(12), 2839-2849. https://doi.org/10.4315/0362-028X-67.12.2839

Steffan, J. J., Derby, J. A., & Brevik, E. C. (2020). Soil pathogens that may potentially cause pandemics, including severe acute respiratory syndrome (SARS) coronaviruses. *Current Opinion in Environmental Science & Health*, *17*, 35-40. https://doi.org/10.1016%2Fj.coesh.2020.08.005

Story, J. A., LePage, S. L., Petro, M. S., West, L. G., Cassidy, M. M., Lightfoot, F. G., & Vahouny, G. V. (1984). Interactions of alfalfa plant and sprout saponins with cholesterol in vitro and in cholesterol-fed rats. *The American Journal of Clinical Nutrition*, *39*(6), 917-929. https://doi.org/10.1093/ajcn/39.6.917

Strawn, L. K., Fortes, E. D., Bihn, E. A., Nightingale, K. K., Gröhn, Y. T., Worobo, R. W., Wiedmann, M., & Bergholz, P. W. (2013). Landscape and meteorological factors affecting prevalence of three food-borne pathogens in fruit and vegetable farms. *Applied and Environmental Microbiology*, *79*(2), 588–600. https://doi.org/10.1128/aem.02491-12

Taormina, P. J., Beuchat, L. R., & Slutsker, L. (1999). Infections associated with eating seed sprouts: an international concern. *Emerging Infectious Diseases*, *5*(5), 626. https://doi.org/10.3201%2Feid0505.990503

Tate III, R. L. (1978). Cultural and environmental factors affecting the longevity of *Escherichia coli* in histosols. *Applied and Environmental Microbiology*, *35*(5), 925-929. https://doi.org/10.1128/aem.35.5.925-929.1978

Thomas, J. L., Palumbo, M. S., Farrar, J. A., Farver, T. B., & Cliver, D. O. (2003). Industry practices and compliance with US Food and Drug Administration guidelines among California sprout firms. *Journal of Food Protection*, *66*(7), 1253-1259. https://doi.org/10.4315/0362-028x-66.7.1253

Ukuku, D. O., & Sapers, G. M. (2007). Effect of time before storage and storage temperature on survival of *Salmonella* inoculated on fresh-cut melons. *Food Microbiology*, *24*(3), 288-295. https://doi.org/10.1016/j.fm.2006.04.007

U. S. Department of Agriculture, Economic Research Service. (2016). *A Closer Look at Declining Fruit and Vegetable Consumption Using Linked Data Sources*. https://www.ers.usda.gov/amber-waves/2016/july/a-closer-look-at-declining-fruit-and-vegetable-consumption-using-linked-data-sources/ Accessed Oct. 14, 2022.

Vermeer, C. V. (2012). Vitamin K: the effect on health beyond coagulation–an overview. *Food & Nutrition Research*, *56*(1), 5329. https://doi.org/10.3402%2Ffnr.v56i0.5329

Wall, D. H., Nielsen, U. N., & Six, J. (2015). Soil biodiversity and human health. *Nature*, *528*(7580), 69-76. https://doi.org/10.1038/nature15744

Wang, D. D., Li, Y., Bhupathiraju, S. N., Rosner, B. A., Sun, Q., Giovannucci, E. L., Rimm, E. B., Manson, J. E., Willett, W., C., Stampfer M., J., & Hu, F. B. (2021). Fruit and vegetable intake and mortality: results from 2 prospective cohort studies of US men and women and a meta-analysis of 26 cohort studies. *Circulation*, *143*(17), 1642-1654. https://doi.org/10.1161/CIRCULATIONAHA.120.048996

Wang, D. Y. C., Kumar, S., & Hedges, S. B. (1999). Divergence time estimates for the early history of animal phyla and the origin of plants, animals and fungi. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *266*(1415), 163-171. https://doi.org/10.1098/rspb.1999.0617

Warriner, K., Ibrahim, F., Dickinson, M., Wright, C., & Waites, W. M. (2003). Internalization of human pathogens within growing salad vegetables. *Biotechnology and Genetic Engineering Reviews*, *20*(1), 117-136. https://doi.org/10.1080/02648725.2003.10648040

Williamson, V. M., & Gleason, C. A. (2003). Plant–nematode interactions. *Current Opinion in Plant Biology*, *6*(4), 327-333. https://doi.org/10.1016/S1369-5266(03)00059-1

Winthrop, K. L., Palumbo, M. S., Farrar, J. A., Mohle-Boetani, J. C., Abbott, S., Beatty, M. E., Inami, G., & Werner, S. B. (2003). Alfalfa sprouts and *Salmonella* Kottbus infection: a multistate outbreak following inadequate seed disinfection with heat and chlorine. *Journal of Food Protection*, *66*(1), 13-17. https://doi.org/10.4315/0362-028x-66.1.13

World Health Organization. (2022). *Food Safety*. https://www.who.int/en/news-room/fact-sheets/detail/food-safety Accessed Sep. 28, 2022.

Wu, Y., Zeng, J., Zhu, Q., Zhang, Z., & Lin, X. (2017). pH is the primary determinant of the bacterial community structure in agricultural soils impacted by polycyclic aromatic hydrocarbon pollution. *Scientific Reports*, *7*(1), 1-7. https://doi.org/10.1038%2Fsrep40093

Yilmaz, H. Ö., Ayhan, N. Y., & Meriç, Ç. S. (2020). Buckwheat: a useful food and its effects on human health. *Current Nutrition & Food Science*, *16*(1), 29-34. https://doi.org/10.2174/1573401314666180910140021

Zhao, X., Yu, Z., & Ding, T. (2020). Quorum-sensing regulation of antimicrobial resistance in bacteria. *Microorganisms*, *8*(3), 425. https://doi.org/10.3390%2Fmicroorganisms8030425

CHAPTER 3

EFFICACY OF ASCAROSIDE ASCR#18 TREATMENT IN CONTROL OF *SALMONELLA ENTERICA* ON ALFALFA AND FENUGREEK SEEDS AND SPROUTS[1]

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Has been submitted to *Food Research International*

3.1 Introduction

Sprouts are young shoots that are germinated from seeds. Commonly consumed sprouts include those produced from alfalfa or fenugreek seeds. Alfalfa (*Medicago sativa*) is a flowering plant in the pea family (*Fabaceae* or *Leguminosae*) (Hong et al., 2011), and its sprouts are a common food for humans. The consumption of alfalfa sprouts has increased in recent years because modern consumers prefer foods that are more nutritious and need less processing (Miyahira & Antunes, 2021; Frías et al., 2007). Fenugreek (*Trigonella foenum-graecum*) is also from the *Fabaceae* family. It has been used as a food source and therapeutic agent since fenugreek contains active substances such as alkaloids, flavonoids, and steroids. Thus, fenugreek sprouts are considered healthy and disease-preventing (Snehlata & Payal, 2012). During the germination of seeds, many macronutrients and antinutrients are metabolized, and secondary metabolic compounds are synthesized, making germinated sprouts have potential health benefits (Peñas & Martínez-Villaluenga, 2020).

The germination process provides favorable environmental conditions and abundant nutrition to support the growth of foodborne pathogens (Ding et al., 2013). However, sprouts have risks since they are usually consumed raw or lightly cooked (Proctor et al., 2001). Thus, leafy sprouts are easily contaminated by them during the production process. Therefore, sprout-related outbreaks of gastrointestinal infection have occurred quite frequently. According to the Centers for Disease Control and Prevention, there were at least 64 outbreaks related to sprouts from 1988 to 2020 worldwide, including 46 outbreaks in the United States (Miyahira & Antunes, 2021). Alfalfa sprouts were involved in more than 55% of these outbreaks. Although in 1999, the U.S. Food and Drug Administration recommended using 20,000 ppm calcium hypochlorite to decontaminate sprout seeds before germination, sprout-associated outbreaks continued to occur after the guidance was issued (Winthrop et al., 2003; Proctor et al., 2001). Thus, more effective alternative treatments are needed.

Low concentrations of ascr#18, a major component of the ascarosides secreted by plant-parasite nematodes could act as a pheromone to boost plant resistance to viral, bacterial, oomycete, fungal, and nematode infections by triggering their immune responses (Manosalva et al., 2015). This study aimed to investigate the effectiveness of ascr#18 treatment against *Salmonella enterica* on alfalfa and fenugreek sprouts during the sprouting process.

3.2 Materials and methods

3.2.1 Sprout seeds and bacterial strains

Alfalfa (*Medicago sativa*, cv. unidentified) and fenugreek (*Trigonella foenum-graecum*, cv. unidentified) seeds were obtained from a commercial source (Otis S. Twilley Seed Co., Inc., Hodges, SC USA) and stored at 10ºC before being used in experiments. *Salmonella enterica* subspecies *enterica* serotype Stanley and Cubana were used in the study, and these two *Salmonella* strains have been previously linked to sprout-associated outbreaks of infections (Mahon et al., 1997; Mohle-Boetani et al. 2001). Nalidixic acid (NA) resistant derivates of the bacterial strains were grown on tryptic soy agar (TSA) supplemented with 50 µg/mL of NA (MP Biomedicals Santa Ana, CA USA) at 37ºC for 16 h. The obtained cultures were purified on bismuth sulfite agar (BSA) (Becton, Dickinson, and Company [BD] Sparks, MD USA) under the same experimental conditions.

Freeze-dried ascroside#18 extracted from plant-parasite nematodes was provided by Ascribe Biosciences Inc. The product was dissolved in 100% ethanol to make a 10 mM stock solution kept at -20ºC before use. The stock solution was diluted using sterile distilled water to working concentrations of 1 mM or 1 µM for seed treatments.

3.2.2 Preparation of lyophilized bacterial inoculums in sandy soil

Purified *S.* Stanley and *S.* Cubana were inoculated on TSA plates and incubated for 24 h at 37˚C. Single colonies were selected and transferred to tryptic soy broth (TSB) and incubated under the same condition. The resulting cultures were centrifuged at 5,000 *g* for 5 min (Brinkmann instruments. Inc., Westbury, NY, USA), and the supernatants were discarded. Cell pellets were washed twice with sterilized phosphate-buffered saline (PBS; pH 7.4). After the final wash, cells were resuspended in 10% sterilized skim milk (Walmart Inc., Bentonville, AR, USA) to a final concentration of *ca*. 10^9 CFU/ml. The cell suspensions were transferred into a glass test tube and kept at -20˚C for 24 h. A Benchtop Freeze Dry system (Labcono, Kansas City, MO USA) was used to freeze-dry the cell cultures. Lyophilized cells of *S*. Stanley and *S*. Cubana (10^8 CFU/g) were then mixed with sterilized sandy soil (Mosser Lee Co. Millston, WI USA), respectively to make the pathogen population in the soil at 10^4 to 10^5 CFU/g. The inoculated soil was hand-massaged for 3 min, followed by agitation on an orbital shaker (model 3520, Lab-Line Instruments, Melrose Park, IL USA) at 250 rpm and room temperature for 18 h to ascertain that the inoculated bacterial cells were evenly distributed in the soil.

3.2.3 Decontamination of sprout seeds

A commercial sodium hypochlorite solution (6%; The Clorox Company Oakland, CA USA) was diluted in sterile water at a ratio of 1:2 to reach a concentration of 20,000 ppm. Purchased alfalfa and fenugreek seeds were treated with the diluted sodium hypochlorite (5 ml per gram of seeds) with agitation on a platform shaker (Model 421105, Nutator, Sparks, MD USA) for 15 min at room temperature. Spent sanitizer solution was aspirated, and residual sodium hypochlorite on alfalfa or fenugreek seeds was neutralized with Dey-Engley neutralization broth (BD; 5 ml per gram of seeds) for 10 min with agitation. The seeds were then rinsed twice with 5 ml of sterilized deionized water before being dried on a piece of sterilized weighing paper (Fisher Scientific, Pittsburgh, PA USA) for 1 h in a biological safety cabinet (class II type A/B 3, Nuaire, Plymouth, MN USA) at room temperature.

3.2.4. Seed treatment with ascr#18

Sanitized alfalfa and fenugreek seeds were treated with 1 mM or 1 µM ascr#18 (0.8 ml per gram of seeds). The control groups of seeds were treated with the same volumes of 10% or 0.01% ethanol that were in the working concentrations of ascr#18 solutions. Seeds in both experimental and control groups were treated at room temperature for 20 min. In the following, treatment solutions were removed, and treated seeds were dried on sterile weighing paper for 1 h at room temperature.

Dried alfalfa and fenugreek seeds were then mixed with inoculated sandy soil in a Whirl-Pak bag (1oz or 18oz; Nasco Fort Atkinson, WI USA) and agitated on an orbital shaker（Lab-Line Instruments) at 250 rpm for 1 h. The sample bags were flipped over after the first 30 min of agitation. The seeds and soil were then separated using a sterile sieve, and *Salmonella* cells loosely associated with the inoculated seeds were removed by rinsing twice with sterilized DI water. Subsequently, the seeds were used for sprouting and to determine the precise levels of bacterial inoculation.

3.2.5 Sprouting

Seeds for the sprouting experiments were planted on 1% water agar (BD) in squared Petri-dishes with a 6*6 grid (Simport Beloeil, QC, Canada). Seeds treated with ascr#18 were placed on 1% water agar containing 10 µM of ascr#18, while the control seeds were planted on water agar without the ascr#18. The Petri-dishes were placed in transparent plastic containers (66Qt. Steritile, Townsend, MA USA) with damp paper towels at the bottom of the containers for 7 days at 25°C in the dark. On days 1, 3, 5, and 7, five sprouts of each kind were collected and placed into a Whirl-Pak bag (1 oz. Nasco). Collected sprouts were mixed with either 2.5 or 5 ml of PBS, depending on the weight of the sprout samples. Samples were homogenized for 1 min using a pestle. *Salmonella* populations in the resulting homogenates were subsequently determined using both NA-TSA and BSA plates.

When *Salmonella* population dropped to below the detectable level of the plate count assay (detection limits in log CFU/g vary depending on the weight of sprout samples at each sampling point), sprout seeds (0.5 g) were aseptically mixed with 4.5 mL of lactose broth (BD), and the mixture was vortexed for 1 min and left at room temperature for 60 min. The pH of the mixture was adjusted to 6.8 when necessary. The samples were incubated for 24 h at 37°C; Into a sprout sample, universal pre-enrichment broth (M188) (BD) in 9 times volume was added, and the mixture was vortexed for 1 min before being incubated for 24 h at 37°C. Pre-enriched mixture (0.1 ml) was transferred to 10 ml Rappaport-Vassiliadis (RV) broth (BD) and another 1 ml mixture to 10 ml tetrathionate (TT) broth (BD). The RV broth was incubated for 24 h at 42°C, while TT broth was for 24 h at 37˚C. Subsequently, a loopful (10 µl) of enriched cultures from the RV and TT broth was inoculated on NA-TSA and BSA. The plates were incubated for 24 h at 37˚C.

3.2.6 Statistical analysis

The experiment included two replications and each sample had two duplicates. The ANOVA procedure of SAS (SAS, Inst., Cary, NC) was used to analyze the data. Fisher's least significant different test separated the means. Significant differences in pathogen cell populations ($P \leq 0.05$) from seed types, treatment types, and treatment times, as well as bacterial strains, were compared using R Studio Version 1.3.1073 (Free Software Foundation, Inc., MA, USA). The independent variables were seed type, strain type, treatment type and sprouting time, the dependent variable was the population of bacterial cells on seeds and sprouts.

3.3 Results

Results of statistical analysis showed that the four defined independent variables (seed type, strain type, treatment type, and sprouting time) were all significant ($P \leq 0.05$) factors influencing the growth of *Salmonella* on sprouts (Table 3.1). The mean populations of *Salmonella* on fenugreek seeds and sprouts were significantly higher than those on alfalfa seeds and sprouts (Table 3.2). *S*. Stanly grew better than *S*. Cubana on seed and sprout samples. The group treated with 0.01% ethanol had a significantly higher cell population than those treated with 10% ethanol. Treatment with 1 mM ascr#18 reduced the mean pathogen population by about 4.0 log CFU/g compared to the group with 10% ethanol. However, treatment with 1 µM of ascr#18 only reduced the mean *Salmonella* population by *ca.* 1 log CFU/g. The mean population of *Salmonella* on sprouts increased with sprouting time and reached the peak population on day 5 (Table 3.2, Fig. 3.1 and Fig. 3.2).

Under the treatments with 1 mM ascr#18 and 0.01% ethanol, the mean cell populations of *Salmonella* on fenugreek sprouts were significantly higher than those on alfalfa sprouts (Table 3). However, opposite results were noticed when 1 µM ascr#18 was used. Furthermore, there was no significant population difference ($P > 0.05$) between the two types of sprouts treated with 10% ethanol.

The mean cell populations of the two *Salmonella* strains in the two ascr#18 treated groups were significantly lower ($P \leq 0.05$) than the control groups regardless of the type of growth media and seeds used in the experiment (Table 3.3). Alfalfa sprout samples from the two controls had similar mean cell populations when enumerated on both NA-TSA and BSA, while the fenugreek sprouts from the control group with 0.01% ethanol had a higher cell population than samples from the other control group. The average reduction in the mean *Salmonella* population resulting from the 1 mM treatments was 5.71 log CFU/g on alfalfa sprouts and 2.24 log CFU/g on fenugreek sprouts according to the enumeration results from BSA. As for the 1 µM treatment, the level reductions from alfalfa and fenugreek sprouts were 0.45 and 1.94 log CFU/g, respectively.

Like the results of the overall statistical analysis (Table 3.2), *S*. Stanley in general had a significantly higher ($P \leq 0.05$) cell population than *S*. Cabana in individual treatment groups (Table 3.4). However, in the control group with 0.01% ethanol, the population of the two *Salmonella* strains was similar ($P > 0.05$).

Populations of the two individual *Salmonella* strains were significantly lower ($P < 0.05$) in ascr#18 treated groups than in the control groups (Table 3.4). The 1 mM treatment was more effective than the 1 µM treatment. *S*. Stanley had a higher cell population in the control group with 0.01% ethanol compared to the group with 10% ethanol. However, *S*. Cubana population was significantly higher in the group with 10% ethanol. The 1 mM treatments reduced the populations of *S*. Stanley and *S*. Cubana by 4.24 and 3.71 log CFU/g, respectively. The 1 µM treatment reduced the population of *S*. Stanley by 1.08 log CFU/g and *S*. Cubana by 1.31 log CFU/g according to the enumeration results from BSA.

All alfalfa samples treated with 1 mM ascr#18 and inoculated with either *S*. Cubana or *S*. Stanley tested negative for the pathogen in the plate count assay. The detection limit for *S*. Cubana contaminated samples were calculated as 1.00 log CFU/g on Day 0, 1.92 log CFU/g on Day 1, 2.05 log CFU/g on Day 3, 2.02 log CFU/g on Day 5, and 2.00 log CFU/g on Day 7, depending on the weights of the seed/sprout samples. Results showed that only the samples on Day 5 tested positive for the pathogen. All samples that tested negative for *Salmonella* in the plate count assay were subjected to enrichment.

The detection limit for *S*. Stanley contaminated samples were 1.00 log CFU/g on Day 0, 1.96 log CFU/g on Day 1, 2.00 log CFU/g on Day 3, 1.92 log CFU/g on Day 5, and 1.89 log CFU/g on Day 7. With enrichment, the alfalfa seed samples treated with 1 mM ascr#18 tested positive for the pathogen on Day 0, but the Day 1 to Day 7 samples treated with the same concentration of ascr#18 were all negative for the pathogen in the enrichment assay.

3.4 Discussion

The current study found that the mean population of *Salmonella* on fenugreek seeds and sprouts was significantly higher ($P < 0.05$) than the population on alfalfa seeds and sprouts (Table 3.2). Using the same seed inoculation approach with contaminated sandy soil, a similar observation was made by Cui, Liu, & Chen (2020). Another study examined the growth rate of *E. coli* in the extracts of alfalfa and fenugreek sprouts, and it was noticed that all tested bacterial strains including *E. coli* MG1655 (OR:H48), JHI5025 (unknown serotype), JHI5039 (unknown serotype), Sakai (O157:H7), and ZAP1589 (O157:H7) grew faster in fenugreek extract than in alfalfa extract, especially at 25°C (Merget et al., 2019).

Bacterial colonization on seeds is determined by many factors that can impact the probability and suitability of bacterial growth. The population difference of *Salmonella* in the two types of sprouts may be attributed to their nutrition compositions. A previous study examined the contents of five different polyamines, including agmatine (AGM), putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM), before and after alfalfa and fenugreek seed germination. The mass of PUT and CAD in a single fenugreek sprout (1,079 mg/kg and 3,563 mg/kg) were both higher than in alfalfa sprouts (1,015 and 1,910 mg/kg) (Cigić et al., 2020). During the germination process, the total mass of the five polyamines in fenugreek sprouts increased by 4,808 mg/kg, while in alfalfa sprouts the mass of total polyamines increased by 3,051 mg/kg. Polyamines are organic compounds with more than two amino groups, and they are necessary for the physiological functions of bacteria, including growth, and biofilm formation, and the production of products vital for their survival, such as siderophores (Michael, 2018).

The minimum nutritional requirements for the growth of bacteria are water, carbon source, nitrogen source, and some inorganic, including phosphate and sulfate (Taylor, 2019). A previous study found that seeds with larger mass had more mineral nutrients and carbon-based reserves than those with smaller mass (Milberg et al., 1998). A single alfalfa seed is much smaller than a fenugreek seeds. As a relative small seed, the average weight of an alfalfa is 2 mg, while that for a fenugreek seed is 16 mg (Rankin, 2008). A study by Vaughton & Ramsey (2001) found that the nitrogen and phosphorus concentrations in large seeds were over 10-fold higher than in small seeds. According to the study's relationships of seed mass and nutrient content, the nitrogen and phosphorus contents in a single alfalfa seed were *ca*. 100 µM and 10 µM, respectively. At the same time, the concentrations of the two nutrient components per fenugreek seed are 1,300 µM and 120 µM (Altuntaş et al., 2005). Nitrogen is an essential element for life since it is a component of amino acids and nucleotides necessary for synthesizing proteins and nucleic acids (Howarth, 2022). Phosphorus is also essential for life because it composes the backbone of DNA and RNA, the energy carrier ATP, and the cell membrane (Elser, 2012).

Results of the current study showed that *S*. Stanley has a significantly higher ($P \leq 0.05$) population on seeds and sprouts than *S*. Cubana (Table 3.2), which is consistent with the result from Cui, Liu & Chen (2020). The growth of *Salmonella* was serotype-dependent (Oscar, 2000; Juneja, Marks, & Huang, 2003). A previous study compared the growth kinetics of some *Salmonella* serotypes and found that different serotypes had different growth paces. Among the tested strains, *Salmonella* serotype Paratyphi B reached the stationary phase within 24 h and had a peak population of 9.68 log CFU/ml at the stationary phase. In comparison, while *Salmonella* serotype Typhi did not reach the stationary phase even after 48 h of inoculation and the population at 48 h was only 9.18 log CFU/ml (Díez-García, Capita, & Alonso-Calleja, 2012).

Salmonella populations increased about 3 log CFU/g on both sprouts from day 0 to day 1 (Fig. 3.1B). This change is consistent with a previous study that used bacteriophages to control *Salmonella* growth (Pao et al., 2004). After day 1, the mean populations of the pathogen became stable at *ca*. 5 log CFU/g with a slight drop after that. Similar results have also been observed by Fong et al. (2017). This phenomenon could be caused by the depletion of available seed exudates, limiting the growth of bacterial pathogens on sprouts after the first day of germination (Howard & Hutcheson 2003).

Ascarosides are derivatives of dideoxy sugar combined with fatty acid-derived lipophilic side chains or other primary metabolism-derived moieties (Klessig et al., 2019). Ascarosides cannot be defined as pesticides or pathogen-cides because they do not kill pests and pathogens (Bouchie, 2019). Results of initial study confirmed these findings, as ascr#18 failed to demonstrate any inhibitory activities against *Salmonella* in the agar diffusion assay and minimal inhibitory concentration test (data not shown). Ascr#18 is a major component of a conserved family of nematode pheromones. It is perceived by plant cells, when encountered, as pathogen invasion, triggered by something like the so-called “microbe or pathogen-associated molecular patterns” (MAMPs or PAMPs), subsequently leading to the activation of plant immune responses. MAMPs or PAMPs are specific molecules or parts of certain molecules that have conserved structures or chemical patterns unique to a specific pathogenic microorganism. Lipopolysaccharides, peptidoglycan, and flagellin of bacterial pathogens are MAMPs to human cells during infection (Erbs & Newman, 2012). These molecules or patterns can be recognized by specific pattern recognition receptors (PRRs) on the surface of host cells. Ascarosides could trigger a similar immune response by the “nematode-associated molecular patterns” (NAMPs) (Choi & Klessig, 2016). The activated characteristic plant responses include 1). The onset of conserved signal transduction through upregulation of the salicylic acid and jasmonic acid pathways (Pieterse, van der Does, Zamioudis, Reyes, & Van Wees, 2012); 2). Activation of certain enzymes, such as mitogen-activated protein kinase and calcium-dependent protein kinase, which are important actors of plant signaling that can elicit a wide range of physiological responses in the cells (Ichimura, et al., 2002; Yip Delormel & Boudsocq, 2019), and 3). Production of reactive oxygen species during the process, which makes plants maintain normal growth and improve tolerance to stress (Huang, Ullah, Zhou, Yi, & Zhao, 2019).

The chemical structure indicates that ascaroside molecules are highly lipophilic, which means they have a low solubility in aqueous media (Choe et al., 2012). For this reason, freeze-dried ascr#18 was first dissolved in 100% ethanol before being diluted to work concentrations using sterile distilled water. According to a previous study, certain ethanol concentrations affected plants' development (Sako et al., 2018). For example, 10% ethanol projected stress to, and 25% ethanol could kill, plant cells (Nicholson, 2019). Ethanol can also inhibit the germination of plant seeds. Ethanol at 1 to 10 mM of inhibited the germination of tomato seeds (Chen et al., 2020). To avoid any probable impact of ethanol on seed germination, sprout development, and *Salmonella* growth, treatments with 10% and 0.01% ethanol, the concentrations of ethanol in the 1 mM and 1 µM ascr#18 working solutions were included in the current studies as the control groups.

The results of this study reveal that low concentrations of ascr#18 can reduce the populations of two *Salmonella* strains artificially inoculated on alfalfa and fenugreek According to the results in Table 3.1, treatment with ascr#18 reduces the population of *Salmonella* on alfalfa and fenugreek sprouts. According to our knowledge, ascr#18 has not been used to control human pathogen growth on sprouts, but it has been tested against plant pathogens. In a study by Manosalva et al. (2015), 1 µM of ascr#18 was used to treat the roots of *Arabidopsis* for 24 h before inoculation with *Pseudominas syringae* pv. tomato (*Pst*). The treatment decreased the population of *Pst* from 6.70 to 5.60 log CFU/cm^2 of tomato leaves compared to the control group. In this study, treatment with 1 µM ascr#18 reduced the population of *Salmonella* by *ca*. 1 log CFU/g of sprout (Table 3.2). It was found in the current study that treatment with 1 mM ascr#18 was more effective than the 1 µM treatment. However, it was indicated that increasing the ascaroside concentration to 5 µM in the study by Manosolva et al. (2015) was less effective than the 1 µM treatment. It is still not known whether a dose-response could be observed after more concentrations with larger concentration difference are used.

Although 1 mM ascr#18 treatment was effective in control of *Salmonella* on alfalfa sprouts (Tables 3.2 and 3.3), *S*. Cubana was detected on Day 5 through the enrichment assay. This finding emphasizes the challenges of controlling pathogen growth on sprouts and sprout-associated outbreaks of infections. In commercial sprout production, large number of vegetable seeds are used. When a single seed evades sanitizing treatment or plant immune response, the consequence could be devastating.

3.5 Conclusion

The results of this study reveal that low concentrations of ascr#18 can reduce the populations of two *Salmonella* strains artificially inoculated on alfalfa and fenugreek seeds during sprouting. Treatment with 1 mM of ascr#18 was more effective than the 1 µM treatment. The mean populations of *Salmonella* on the seeds and sprouts are influenced by seed type, *Salmonella* strain type, treatment type, and sprouting time. The study provides supporting evidence for the potential use of ascr#18 for the control of pathogen contamination on edible sprouts.

References

Altuntaş, E., Özgöz, E., & Taşer, Ö. F. (2005). Some physical properties of fenugreek (*Trigonella foenum-graceum L.*) seeds. *Journal of Food Engineering*, *71*(1), 37-43. https://doi.org/10.1016/j.jfoodeng.2004.10.015

Bouchie, A. J. (2019). *Worm pheromones protect major crops*. Phys.org. Retrieved July 2, 2022, from https://phys.org/news/2019-07-worm-pheromones-major-crops.html

Chen, Y., Almasaud, R. A., Carrie, E., Desbrosses, G., Binder, B. M., & Chervin, C. (2020). Ethanol, at physiological concentrations, affects ethylene sensing in tomato germinating seeds and seedlings. *Plant Science*, *291*, 110368. https://doi.org/10.1016/j.plantsci.2019.110368

Choe, A., von Reuss, S. H., Kogan, D., Gasser, R. B., Platzer, E. G., Schroeder, F. C., & Sternberg, P. W. (2012). Ascaroside signaling is widely conserved among nematodes. *Current Biology*, 22(9), 772-780. https://doi.org/10.1016/j.cub.2012.03.024

Choi, H. W., & Klessig, D. F. (2016). DAMPs, MAMPs, and NAMPs in plant innate immunity. *BMC Plant Biology*, *16*(1), 1-10. https://doi.org/10.1186%2Fs12870-016-0921-2

Cigić, K. I., Rupnik, S., Rijavec, T., Ulrih, P. N., & Cigić, B. (2020). Accumulation of agmatine, spermidine, and spermine in sprouts and microgreens of alfalfa, fenugreek, lentil, and daikon radish. *Foods*, *9*(5), 547. https://doi.org/10.3390%2Ffoods9050547

Cui, Y., Liu, D., & Chen, J. (2020). Fate of *Salmonella enterica* and enterohemorrhagic *Escherichia coli* on vegetable seeds contaminated by direct contact with artificially inoculated soil during germination. *Journal of Food Protection*, *83*(7), 1218-1226. https://doi.org/10.4315/JFP-20-021

Díez-García, M., Capita, R., & Alonso-Calleja, C. (2012). Influence of serotype on the growth kinetics and the ability to form biofilms of *Salmonella* isolates from poultry. *Food Microbiology*, *31*(2), 173-180. https://doi.org/10.1016/j.fm.2012.03.012

Ding, H., Fu, T. J., & Smith, M. A. (2013). Microbial contamination in sprouts: how effective is seed disinfection treatment? *Journal of Food Science*, *78*(4), R495-R501. https://doi.org/10.1111/1750-3841.12064

Elser, J. J. (2012). Phosphorus: a limiting nutrient for humanity? *Current Opinion in Biotechnology*, *23*(6), 833-838. https://doi.org/10.1016/j.copbio.2012.03.001

Erbs, G., & Newman, M. A. (2012). The role of lipopolysaccharide and peptidoglycan, two glycosylated bacterial microbe-associated molecular patterns (MAMPs), in plant innate immunity. *Molecular Plant Pathology*, *13*(1), 95-104. https://doi.org/10.1111/j.1364-3703.2011.00730.x

Fong, K., LaBossiere, B., Switt, A. I., Delaquis, P., Goodridge, L., Levesque, R. C., Danyluk, M. D., & Wang, S. (2017). Characterization of four novel bacteriophages isolated from British Columbia for control of non-typhoidal *Salmonella in vitro* and on sprouting alfalfa seeds. *Frontiers in Microbiology*, 2193. https://doi.org/10.3389/fmicb.2017.02193

Frías, J., Martínez-Villaluenga, C., Gulewicz, P., Perez-Romero, A., Pilarski, R., Gulewicz, K., & Vidal-Valverde, C. (2007). Biogenic amines and HL60 cytotoxicity of alfalfa and fenugreek sprouts. *Food Chemistry*, *105*(3), 959-967. https://doi.org/10.1016/j.foodchem.2007.04.043

Hong, Y. H., Wang, S. C., Hsu, C., Lin, B. F., Kuo, Y. H., & Huang, C. J. (2011). Phytoestrogenic compounds in alfalfa sprout (*Medicago sativa*) beyond coumestrol. *Journal of Agricultural and Food Chemistry*, *59*(1), 131-137. https://pubs.acs.org/doi/10.1021/jf102997p

Howard, M. B., & Hutcheson, S. W. (2003). Growth dynamics of *Salmonella enterica* strains on alfalfa sprouts and in waste seed irrigation water. *Applied and Environmental Microbiology*, *69*(1), 548-553. https://doi.org/10.1128/AEM.69.1.548-553.2003

Howarth, R. W. (2022). Nitrogen. In Mehner, T., Tockner, K. (ed.). *Encyclopedia of Inland Waters* (Second Edition, pp. 155-162). Amsterdam, Netherland: ELSEVIER.

Huang, H., Ullah, F., Zhou, D. X., Yi, M., & Zhao, Y. (2019). Mechanisms of ROS regulation of plant development and stress responses. *Frontiers in Plant Science*, 10, 800. https://doi.org/10.3389/fpls.2019.00800

Ichimura, K. & Mapk Group. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends in Plant Science*, *7*(7), 301-308. https://doi.org/10.1016/s1360-1385(02)02302-6

Juneja, V. K., Marks, H. M., & Huang, L. (2003). Growth and heat resistance kinetic variation among various isolates of *Salmonella* and its application to risk assessment. *Risk Analysis 23*(1), 199-213. https://doi.org/10.1111/1539-6924.00300

Klessig, D. F., Manohar, M., Baby, S., Koch, A., Danquah, W. B., Luna, E., Park, H. J., Kolkman, J. M., Turgeon, B. G., Nelson, R., Leach, J. E., Williamson, V. M., Kogel, K. H., Kachroo, A., & Schroeder, F. C. (2019). Nematode ascaroside enhances resistance in a broad spectrum of plant–pathogen systems. *Journal of Phytopathology*, *167*(5), 265-272. https://doi.org/10.1111/jph.12795

Mahon, B. E., Pönkä, A., Hall, W. N., Komatsu, K., Dietrich, S. E., Siitonen, A., Cage, G., Hayes, P. S., Lambert-Fair, M. A., Bean, N. H., Griffin, P. M., & Slutsker, L. (1997). An international outbreak of *Salmonella* infections caused by alfalfa sprouts grown from contaminated seeds. *Journal of Infectious Diseases*. *175*(4), 876-882. https://doi.org/10.1086/513985

Manosalva, P., Manohar, M., von Reuss, S. H., Chen, S., Koch, A., Kaplan, F., Choe, A., Micikas, R. J., Wang, X., Kogel, K-H., Sternberg, P. W., Williamson, V. M., Schroeder, F. C., & Klessig, D. F. (2015). Conserved nematode signaling molecules elicit plant defenses and pathogen resistance. *Nature Communications*. *6*(1), 1-8. https://doi.org/10.1038/ncomms8795

Merget, B., Forbes, K. J., Brennan, F., McAteer, S., Shepherd, T., Strachan, N. J., & Holden, N. J. (2019). Relating growth potential and biofilm formation of Shigatoxigenic *Escherichia coli* to in planta colonisation and the metabolome of ready-to-eat crops. *BioRxiv*, 523175. https://doi.org/10.1101/523175

Michael, A. J. (2018). Polyamine function in archaea and bacteria. *Journal of Biological Chemistry*, *293*(48), 18693-18701. https://doi.org/10.1074/jbc.TM118.005670

Milberg, P., Pérez-Fernández, M. A., & Lamont, B. B. (1998). Seedling growth response to added nutrients depends on seed size in three woody genera. *Journal of Ecology*, *86*(4), 624-632. https://doi.org/10.1046/j.1365-2745.1998.00283.x

Miyahira, R. F., & Antunes, A. E. C. (2021). Bacteriological safety of sprouts: A brief review. *International Journal of Food Microbiology*, 352, 109266. https://doi.org/10.1016/j.ijfoodmicro.2021.109266

Mohle-Boetani, J. C., Farrar, J. A., Werner, S. B., Minassian, D., Bryant, R., Abbott, S. , Slutsker, L., & Vugia, D. J. (2001). *Escherichia coli* O157 and *Salmonella* infections associated with sprouts in California, 1996–1998. *Annals of Internal Medicine*, *135*(4), 239-247. https://www.acpjournals.org/doi/10.7326/0003-4819-135-4-200108210-00008.

Nicholson, J. (2019). *The effect of alcohol on plants*. Sciencing. Retrieved July 9, 2022, from https://sciencing.com/effect-alcohol-plants-8006187.html

Oscar, T. P. (2000). Variation of lag time and specific growth rate among 11 strains of *Salmonella* inoculated onto sterile ground chicken breast burgers and incubated at 25◦C. *Journal of Food Safety*, *20*(4), 225-236. https://doi.org/10.1111/j.1745-4565.2000.tb00301.x

Pao, S., Rolph, S. P., Westbrook, E. W., & Shen, H. (2004). Use of bacteriophages to control *Salmonella* in experimentally contaminated sprout seeds. *Journal of Food Science*, *69*(5), M127-M130. https://doi.org/10.1111/j.1365-2621.2004.tb10720.x

Peñas, E., & Martínez-Villaluenga, C. (2020). Advances in production, properties and applications of sprouted seeds. *Foods*, 9(6), 790. https://doi.org/10.3390/foods9060790

Pieterse, C. M., Van der Does, A., Zamioudis, C., Leon-Reyes, L. H. A., & Van Wees, S. C. (2012). Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology*, 28, 489-521. https://doi.org/10.1146/annurev-cellbio-092910-154055

Proctor, M. E., Hamacher, M., Tortorello, M. L., Archer, J. R., & Davis, J. P. (2001). Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa sprouts grown from seeds pretreated with calcium hypochlorite. *Journal of Clinical Microbiology, 39*(10), 3461-3465. https://doi.org/10.1128/JCM.39.10.3461-3465.2001

Rankin, M. (2008). *Determining the Optimum Alfalfa Seeding Rate*. Team Forage. Retrieved July 13, 2022, from https://fyi.extension.wisc.edu/forage/

Sako, K., Sunaoshi, Y., Tanaka, M., Matsui, A., & Seki, M. (2018). The duration of ethanol-induced high-salinity stress tolerance in Arabidopsis thaliana. *Plant Signaling & Behavior*, *13*(8), e1500065. https://doi.org/10.1080/15592324.2018.1500065

Snehlata, H. S., & Payal, D. R. (2012). Fenugreek (*Trigonella foenum-graecum L.*): an overview. *International Journal of Current Pharmaceutical Review Research*, *2*(4), 169-187. http://impactfactor.org/PDF/IJCPR/2/IJCPR,Vol2,Issue4,Article1.pdf

Taylor, S. (2019). *What three conditions are ideal for bacteria to grow?* Sciencing. Retrieved July 13, 2022, from https://sciencing.com/three-conditions-ideal-bacteria-grow-9122.html

Vaughton, G., & Ramsey, M. (2001). Relationships between seed mass, seed nutrients, and seedling growth in *Banksia cunninghamii* (*Proteaceae*). *International Journal of Plant Sciences*, *162*(3), 599-606. https://doi.org/10.1086/320133

Winthrop, K. L., Palumbo, M. S., Farrar, J. A., Mohle-boetani, J. C., Abbott, S., Beatty, M. E., Inami, G., & Werner, S. B. (2003). Alfalfa sprouts and *Salmonella* Kottbus infection: A multistate outbreak following inadequate seed disinfection with heat and chlorine. *Journal of Food Protection*. *66*(1), 13-17. https://doi.org/10.4315/0362-028X-66.1.13

Yip Delormel, T., & Boudsocq, M. (2019). Properties and functions of calcium-dependent protein kinases and their relatives in *Arabidopsis thaliana*. *New Phytologist*, *224*(2), 585-604. https://doi.org/10.1111/nph.16088

Table 3.1

Type III tests for fixed effects by the statistical model of sprout sampling of *Salmonella*(α=0.05).

NATSA

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Seed	1	42.30221	42.30221	164.79	<.0001
Strain	1	35.42865	35.42865	138.01	<.0001
Treatment	3	500.4879	166.8293	649.88	<.0001
Time	4	170.7495	42.68738	166.29	<.0001
Replicate	1	0.089776	0.089776	0.35	0.556
Seed*Treatment	3	108.2037	36.06789	140.5	<.0001
Strain*Treatment	3	22.71778	7.572594	29.5	<.0001

BSA

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Seed	1	47.07815	47.07815	192.11	<.0001
Strain	1	40.35077	40.35077	164.66	<.0001
Treatment	3	467.2838	155.7613	635.61	<.0001
Time	4	222.5714	55.64284	227.06	<.0001
Replicate	1	0.459031	0.459031	1.87	0.175
Seed*Treatment	3	108.8928	36.2976	148.12	<.0001
Strain*Treatment	3	20.64559	6.881862	28.08	<.0001

Table 3.2
Overall mean total aerobic and *Salmonella* counts at different sampling points and from alfalfa and fenugreek sprouts developed from seeds underwent different treatments.

		Salmonella population (Log CFU/g)	
		NATSA	BSA
Sprout seeds			
	Fenugreek (n = 80)	5.49A	5.43A
	Alfalfa (n = 80)	4.46B	4.34B
Bacterial strain			
	S. Stanley (n = 80)	5.45A	5.39A
	S. Cubana (n = 80)	4.51B	4.38B
Seed treatment			
	EtOH (0.01%) (n = 40)	6.46A	6.36A
	EtOH (10%) (n = 40)	6.16B	6.00B
	Ascr#18 (1 µM) (n = 40)	5.27C	5.17C
	Ascr#18 (1 mM) (n = 40)	2.01D	2.02D
Sprout time			
	5 (n = 32)	5.81A	5.76A
	3 (n = 32)	5.56B	5.63AB
	7 (n = 32)	5.50B	5.45B
	1 (n = 32)	5.04C	5.01C
	0 (n = 32)	2.97D	2.58D
Replicate			
	One (n = 80)	5.00A	4.94A
	Two (n = 80)	4.95A	4.83A

Values of the same variables in each column followed by the same letters are not significantly different ($P > 0.05$). EtOH: ethanol; Ascr#18: an $(\omega-1)$-hydroxy fatty acid ascaroside excreted by plant-parasitic nematodes; NATSA: tryptic soy agar supplemented with nalidixic acid; BSA: bismuth sulfite agar.

Table 3.3

Mean total aerobic and *Salmonella* counts in samples collected from alfalfa or fenugreek sprouts developed from seeds underwent different treatments.

		Cell population (Log CFU/g)			
Seed treatment		EtOH (10%)	EtOH (0.01%)	Ascr#18 (1 µM)	Ascr#18 (1 mM)
NATSA (n = 160)					
	Alfalfa (n = 80)	6.04Aa	6.07Ba	5.60Ab	0.14Bc
	Fenugreek (n =80)	6.28Ab	6.86Aa	4.95Bc	3.88Ad
BSA (n = 160)					
	Alfalfa (n = 80)	5.81Aa	5.94Ba	5.49Ab	0.13Bc
	Fenugreek (n =80)	6.18Ab	6.78Aa	4.84Bc	3.92Ad

Values within the same column followed by the same uppercase letters are not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letters from the same growth medium are not significantly different ($P > 0.05$). EtOH: ethanol; Ascr#18: An (ω−1)-hydroxy fatty acid ascaroside excreted by plant-parasitic nematodes; NATSA: tryptic soy agar supplemented with nalidixic acid; and BSA: Bismuth sulfite agar.

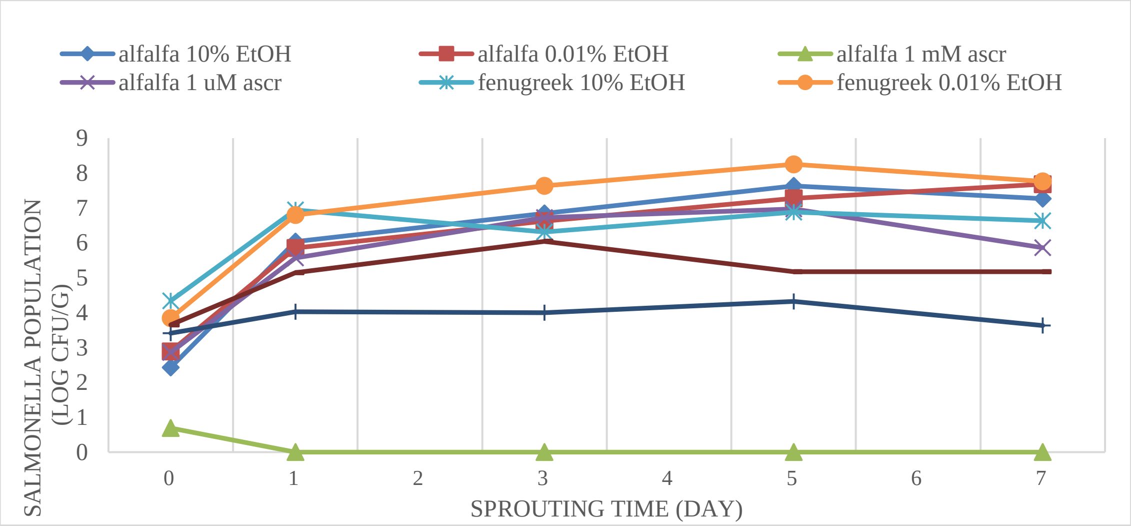
Table 3.4
Mean *S.* Cubana and *S.* Stanley counts in samples collected from both types of sprouts developed from seeds underwent different treatments.

Seed treatment		Cell population (Log CFU/g)			
		EtOH (10%)	EtOH (0.01%)	Ascr#18 (1 μM)	Ascr#18 (1 mM)
NATSA (n = 160)					
	S. Cubana (n = 80)	5.24Bb	6.42Aa	5.12Bb	1.24Bc
	S. Stanley (n =80)	7.08Aa	6.51Ab	5.43Ac	2.77Ad
BSA (n = 160)					
	S. Cubana (n = 80)	5.02Bb	6.26Aa	4.95Bb	1.31Bc
	S. Stanley (n =80)	6.98Aa	6.46Ab	5.38Ac	2.74Ad

Values within the same column followed by the same uppercase letters are not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letters from the same growth medium are not significantly different ($P > 0.05$). EtOH: ethanol; Ascr#18: An (ω−1)-hydroxy fatty acid ascaroside excreted by plant-parasitic nematodes; NATSA: tryptic soy agar supplemented with nalidixic acid; and BSA: Bismuth sulfite agar.

Figure 3.1
Mean population of cells from different treatment groups on alfalfa and fenugreek seeds or sprouts during the germination process. A. mean cell populations from NATSA plates. B. mean cell populations from BSA plates.

A



B

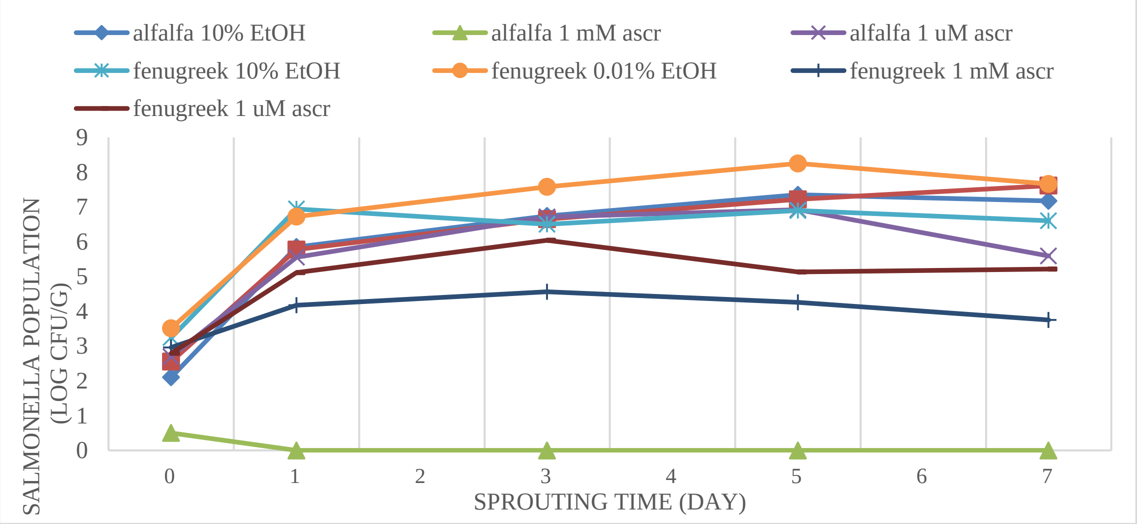
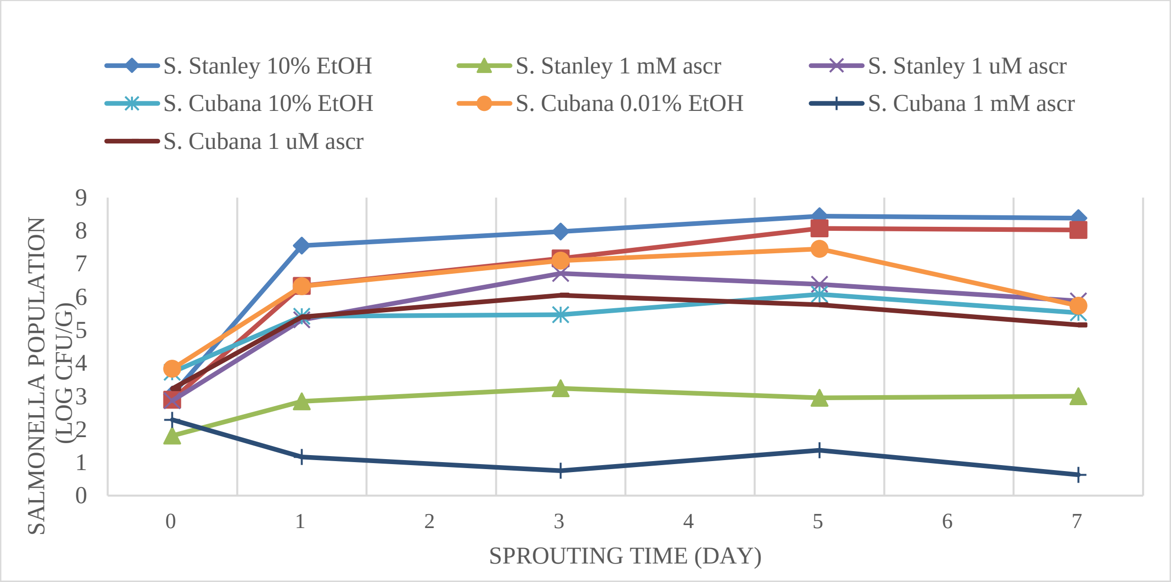
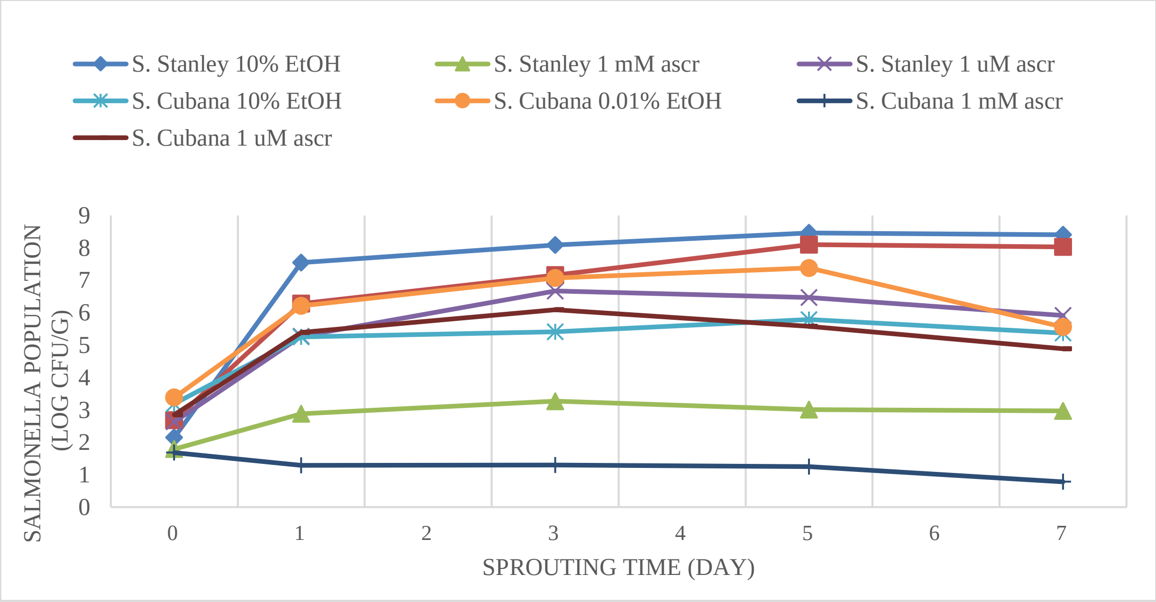


Figure 3.2
Mean population of cells of different strains from different treatment groups during the germination process. A. mean cell populations from NATSA plates. B. mean cell populations from BSA plates.

A



B



CHAPTER 4

EFFICACY OF ASCAROSIDE ASCR#18 TREATMENT IN CONTROL OF *SALMONELLA ENTERICA* ON ALFALFA AND FENUGREEK SEEDS AND SPROUTS[2]

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To be submitted to *Food Control*

4.1 Introduction

Nematode, commonly known as roundworm, includes any worms in the *Nematoda* phylum. They are among the most abundant animals on Earth (Williamson & Gleason, 2003). Although about 30,000 species of nematodes have been described, it represents only a small proportion of the free-living forms that have been identified. The number of extant nematode species is estimated to be a million or more (Kiontke & Fitch, 2013).

The nematode has parasitic, pathogenic, and/or bacterivorous life stages (Yu et al., 2021). During the infection process, most nematode species secret a type of pheromone, ascarosides, which is a conserved family of organic substances with a similar chemical structure. Ascraosides are signaling molecules in the model organism *C. elegans*, which act as a population density signal for intraspecies communication (Jeong et al., 2005; Butcher et al., 2007). A recent study revealed that micromolar or picomolar concentrations of ascaroside ascr#18, a major ascaroside of plant-parasitic nematodes, could be recognized by plants, *via* a mechanism like the microbe-associated molecular patterns (MAMPs), activating specific immune system defense signaling pathways (Choi & Klessig, 2016). During this process, the genes associated with MAMPs and signaling pathways are upregulated to facilitate immune activation. Another study showed that the immune systems of both monocot and dicot plants could be activated by ascr#18. However, different concentrations are needed to trigger the immune systems of different plants and combating different pathogens (Manosalva et al., 2015).

Both alfalfa and fenugreek are dicotyledonous plants, (Wu et al., 1988; Sakhare et al., 2015), and their sprouts are typical food for humans. Alfalfa sprouts, always consumed as a garnish, are considered healthy and nutritious since they contain several vitamins and antioxidants. Some believe that consuming alfalfa sprouts could protect against cancers and cerebrovascular diseases (Fan & Thayer, 2001). Fenugreek sprouts contain lysine, L-tryptophan-rich proteins, mucilaginous fiber, and some unique substances such as saponins, coumarin, fenugreekine, and nicotinic acid that have therapeutic functions (Randhir et al., 2004).

However, consuming these healthy foods contaminated with pathogenic microorganisms has been linked to multiple outbreaks of gastrointestinal infections. The U.S. Food and Drug Administration (FDA) stated that contaminated seed was the likely source of most sprout-related outbreaks (FDA, 2019). To prevent sprout seed contamination, the agency recommended, in 1999, the use of 20,000 ppm calcium hypochlorite as a seed sanitization treatment (FDA, 2017). Despite the tireless effort of the sprout industry to adhere to the government's recommendation, sprout-associated infection outbreaks continued to occur. From 2000 to 2011, there were 23 outbreaks associated with sprout consumption, according to the U.S. Centers for Disease Control and Prevention (CDC), and most of the outbreaks were attributed to alfalfa sprouts (Beutin & Martin, 2012). In 2011, an Escherichia coli O104:H4 infection outbreak was linked to fenugreek sprouts. The outbreak caused 3,842 cases, including 53 deaths in Germany (Beutin & Martin, 2012).

This study aimed to assess whether the growth of enterohemorrhagic *Escherichia coli* strains on alfalfa and fenugreeks seeds and sprouts could be effectively controlled by the treatment with ascr#18.

4.2 Materials and methods

4.2.1 Materials

Alfalfa (*Medicago sativa*, cv. unidentified) seeds and fenugreek (*Trigonella foenum-graecum*, cv. unidentified) seeds were obtained from a commercial source (Otis S. Twilley Seed Co., Inc., Hodges, SC USA). The purchased seeds were stored at 10℃ before use. The study used two E. coli strains, E. coli O157:H7 F4546 and E. coli O104:H4 BAA-2326, and the two bacterial strains were previously linked to alfalfa- (Zansky et al., 2002) or fenugreek-associated (European Food Safety Authority, 2011) outbreaks of infections. Stock cultures of nalidixic acid (NA) resistant derivates of the two bacterial strains were grown on tryptic soy agar (TSA) supplemented with 50 µg/mL of NA (MP Biomedicals Santa Ana, CA USA) at 37ºC for 16 h. Grown colonies were purified on MacConkey (MAC) or sorbitol MacConkey (SMAC) agar plates. Purified cultures were cultivated in Luria-Bertani broth under the same growth condition. The resulting bacterial cultures were washed with sterile water and then mixed with 10% sterilized skim milk (Walmart Inc., Bentonville, AR USA) to a final concentration of *ca*. 10^9 CFU/ml, before being frozen at -20ºC for 24 h. Free Zone Benchtop Freeze Dry system (Labcono, Kansas City, MO USA) was used to freeze-dry the cell cultures, and dehydrated bacterial cells were stored at -20ºC until use. The cell population in the freeze-dried culture was *ca*. 10^8 CFU/g.

Freeze-dried ascr#18 was provided by Ascribe Biosciences Inc (Ithaca, NY USA). The product was first dissolved in a specific volume of 100% (v/v) ethanol to make a 10 mM stock solution which was kept at -20ºC before use. The stock solution was diluted with sterile deionized water to working concentrations of 1 mM and 1 µM for sprout seed treatments in the study.

4.2.2 Preparation of inoculating sandy soil

Sterilized sandy soil (Mosser Lee Co. Millston, WI, USA) was mixed with each of the freeze-dried bacterial cultures at 10^4 to 10^5 CFU/g. The sandy soil and bacterial cell mixtures were rotated for 18 h on an orbital shaker (model 3520, Lab-Line Instruments, Melrose Park, IL USA) at 250 rpm and room temperature to ensure the pathogen cells were evenly distributed.

4.2.3 Seed decontamination and treatment with ascr#18

Alfalfa and fenugreek seeds were decontaminated with 20,000 ppm sodium hypochlorite (The Clorox Company Oakland, CA USA) with agitation on a platform shaker (Model 421105, Nutator, Sparks, MD USA) for 15 min at room temperature. Residual sodium hypochlorite was neutralized using Dey-Engley broth (Becton Dickenson, Sparks, MD USA) for 10 min under the same condition. The seeds were rinsed twice with sterilized deionized water before being dried on a sterilized weighing paper (Fisher Scientific, Pittsburgh, PA USA) at room temperature for 1 h in a biological safety cabinet (class II type A/B 3, Nuaire, Plymouth, MN USA).

Dried seeds were then treated with 1 mM or 1 µM ascr#18 at 800 µl per gram of seeds. Since the 1 mM and 1 µM of ascr#18 working solutions contained either 10% or 0.01% ethanol, seeds treated with the two ethanol concentrations were included in the study as controls. Seeds in the experimental and the control groups were kept at room temperature for 20 min. The treatment solutions were subsequently removed, and treated seeds were dried on sterile weighing paper for 1 h at room temperature in the biosafety cabinet

4.2.4 Seed inoculation and sprouting

Seeds from each treatment group were collected after drying and mixed with inoculated sandy soil described above in Whirl-Pak bags (1 oz or 18 oz, Nasco Fort Atkinson, WI USA) at 10 g soil per gram of seeds. Seed and soil mixtures were first rotated on an orbital shaker（Lab-Line Instruments Inc, IL USA) at 250 rpm for 30 min, and then the bags were flipped over and rotated for an additional 30 min. After the inoculation, seeds were separated from the soil using sterile sieves, and collected seeds were used in the sprouting experiment and to determine precise levels of pathogen inoculation on sprout seeds.

Pathogen-inoculated seeds were then planted on 1% (m/m) water agar (Becton Dickenson) in squared Petri dishes with a 6*6 grid (Simport Beloeil, QC, Canada). Specifically, seeds treated with ascr#18 were planted on 1% water agar containing 10 µM of ascr#18, while the control seeds were planted on water agar without ascr#18. The Petri dishes were placed in transparent plastic containers (66Qt. Steritile, Townsend, MA, USA) with damp paper towels at the bottom of the containers for 7 days at 25ºC in the dark.

4.2.5. Microbiological analysis

On days 1, 3, 5, and 7 of the sprouting, five sprouts were collected from each treatment group and placed in a Whirl-Pak bag (1 oz., Nasco). Whereas on day 0, 0.5 g of sprout seeds were collected. The seeds or sprouts were mixed with appropriate volumes of phosphate-buffered saline (pH 7.4), and the samples were then ground using pestles for 1 min. Samples contaminated with *E. coli* F4546 were plated on SMAC agar plates, while those with *E. coli*

BAA-2326 were on MAC plates. Both sets of samples were also plated on NA-TSA plates. Inoculated plates were incubated at 37°C for 18 h before colony enumeration.

4.2.6 Statistical analysis

The study's independent variables were identified as seed type, strain type, treatment type, and sprouting time. The dependent variable was the cell populations on alfalfa and fenugreek seeds or sprouts. There were two replications included in experiment and each sample had two duplicates. The ANOVA linear model of the Statistical Analysis Software (SAS version 9.4; SAS, Institute, Cary, NC USA) was used to analyze the data. Fisher's least significant difference was used to separate the means. Significant differences in the pathogen cell populations ($P \leq 0.05$) resulting from each independent variable were compared in the analysis.

4.3 Results

Except for strain type ($P > 0.05$), the other three independent variables (seed type, treatment type, and sprouting time) had a significant influence ($P \leq 0.05$) on EHEC growth on sprouts (Table 4.1). Furthermore, there are significant interactions between seed type and treatment type, as well as strain type and treatment type.

The mean cell populations on alfalfa and fenugreek sprouts were significantly different ($P < 0.05$), varying by 1.55 log CFU/g based on data collected from MAC/SMAC plates (Table 2). However, there was no significant difference ($P > 0.05$) between the mean cell populations of the two *E. coli* strains. Among the four treatment groups, the mean cell population from the group treated with 10% ethanol was not significantly different from the other control group using 0.01% ethanol. However, both control groups had significantly higher cell populations than those treated with 1 µM ascr#18. The lowest cell population was from the group treated with 1 mM ascr#18, which was 3.30 log CFU/g lower than the group treated with 10% ethanol. Cell populations increased significantly during the first day of sprouting, but the population plateaued on day 3 and forward (Table 4.2, Fig. 4.1 and Fig. 4.2).

Table 4.3 and Table 4.4 illustrated detailed comparisons between each set of the two significant interactions shown in Table 4.1. When seed type and treatment type (Table 4.3) or strain type and treatment method (Table 4.4) were split from all the other independent variables, similar results were noticed, *i.e.,* the cell populations from samples treated with 10% and 0.01% ethanol were similar ($P > 0.05$), and the cell population from these two treatment groups were significantly higher ($P \leq 0.05$) than the group treated with 1 µM ascr#18. The group treated with 1 mM ascr#18 had the lowest cell population, significantly lower than the groups treated with 1 µm ascr#18 and two concentrations of ethanol.

The population differences between the 1 mM ascr#18 group and 10% ethanol groups were 2.95 and 3.66 log CFU/g on alfalfa and fenugreek sprouts, respectively based on the results from MAC/SMAC (Table 4.3). While between the groups treated with 1 µM ascr#18 and 0.01% ethanol, the population differences were 1.37 and 1.74 log CFU/g, respectively. For the two types of seeds, fenugreeks seeds and sprouts had significantly higher cell populations than alfalfa sprouts regardless of treatment groups.

Compared to its control, treatment with 1 mM ascr#18 reduced the cell population of F4546 and BAA-2326 by 3.51 and 3.10 log CFU/g, respectively, based on data collected from

MAC/SMAC agar plates (Table 4.4). The 1 µm ascr#18 treatment reduced the population of BAA-2326 by 0.76 log CFU/g and the F4546 population by 2.35 log CFU/g.

The populations of F4546 were significantly higher ($P \leq 0.05$) than BAA-2326 in samples treated with the two concentrations of ethanol and significantly lower than the those treated with 1 µM ascr#18. However, the populations of the two strains were not significantly different ($P > 0.05$) in samples treated with 1 mM of ascr#18.

4.4 Discussions

Ascarosides are the glycosides of dideoxysugar ascarylose (Park et al., 2019). They have long aliphatic side chains and are very lipophilic (Ludewig & Schroeder, 2013), which is why ascr#18 used in the study was first dissolved and stored in 100% ethanol. With the use of this solvent, the potential impact of ethanol in ascaroside treatment solutions has to be considered, as previous studies showed that ethanol concentration higher than 10% could be toxic to plant cells, and concentrations at 4 - 6% can stunt plant growth (Miller & Finan, 2006). The current study, thus, included two ethanol treatment controls in the study.

Not only lipophilic, but ascr#18 is also sensitive to visible light according to a commercial source (MedChemExpress, n.d.). The recommended storage condition is under -20°C for a month. The chemical in a solid form should also be kept away from moisture. It is unclear whether these stringent handling and storage conditions will challenge its use as seed and sprout treatment interventions.

It is known that animals' and plants' immune response to pathogen attack is triggered by detecting conserved molecules on the pathogen surface, such as lipopolysaccharide, flagellin, and peptidoglycan, *via* intra- and extra-cellular sensors (Roudaire et al., 2021; Newman Sundelin, Nielsen, & Erbs, 2013). These pathogen-specific surface molecules are known as microbe-associated molecular patterns (MAMPs). Research showed that ascarosides secreted by plant-parasitic nematodes function similarly to the MAMPs. Plant cells perceive ascarosides as signals to activate immune responses, combat nematode attacks, and reduce the severity of infection (Manohar et al., 2020). Ascr#18, a major component of ascarosides, triggers plant defenses by activating of mitogen-activated protein kinases, upregulation of salicylic acid- and jasmonic acid-mediated defense signaling pathways, and expression of defense genes. Previous research has shown that ascr#18 can enhance the resistance of different dicot and monocot plants, including tomato, potato, rice, wheat, soybean, and *Arabidopsis* to nematodes, bacterial, and viral infections (Klessig et al., 2019).

The concentration of ascr#18 needed to achieve the desired level of protection depends on several factors, including the host type and pathogen/pest types, as well as the treatment methods used (Klessig et al., 2019; Manosalva et al., 2015). In the current study, the more effective concentration of ascr#18 treatment is 1 mM, higher than the known optimal working concentrations against non-bacterial pathogens in non-sprout plant tissues identified by Klessig et al. (2019). Ascr#18 has not been previously tested for control of human pathogen growth on vegetable sprouts based on our knowledge. The current study only tested the effect of two different concentrations of ascr#18 which were selected based on a small-scale preliminary study. The two concentrations varied 1,000 times. To identify the optimal concentration for seed and sprout treatment, perhaps other concentrations, especially those between 1 mM and 1 µM should be tested in future studies.

Except for treatment with 1 μm ascr#18, *E. coli* cells grew better (*P* < 0.05) on fenugreek than on alfalfa sprouts (Table 4.2). A similar observation was made with the same two *Salmonella* strains, Stanley and Cubana, growing on alfalfa and fenugreek sprouts under similar sprouting conditions (Hu, Lee, Manohar & Chen, Unpublished). The observed cell population difference between the two types of sprouts could be attributed to the variations in individual seed mass, and the nutrient composition of alfalfa *vs*. fenugreek seeds. Previous studies have shown that individual fenugreek seeds have a much larger mass and contain a greater amount of nutrients such as polyamine which could serve as both carbon and nitrogen source for bacterial growth (Milberg, Pérez-Fernández, & Lamont, 1998; Vaughton & Ramsey, 2001; Cigić et al., 2020).

Compared with the results of a previous study (Hu, Lee, Manohar & Chen, Unpublished), it was noticed that *E. coli* strains had different growth rates than *S. enterica* on the same types of sprouts under similar growth conditions. According to the study, the mean population of the two *Salmonella* strains from the two types of sprouts on day 3 of sprouting was 5.55 log CFU/g, over 1 log CFU/g higher than the population of *E. coli* observed in this study (Table 4.2). A similar result was observed in a previous study by Charkowski et al. (2002), and over a 2-day spouting period, the average cell population of *S. enterica* was 3.7 log CFU/sprout, while *E. coli* population was 2.3 log CFU/sprout. The authors believed the growth difference between the two pathogens could be attributed to the better ability of *S. enterica* to utilize the nutrients in sprouts exudates and to adhere to alfalfa roots and seed coats compared to *E. coli* O157:H7 (Charkowski et al., 2002). This argument seems valid since the current study observed that the cell population of *E. coli* peaked after the 1st day of sprouting and stayed relatively steady for the rest of the sprouting process (Figure 3.1). Nevertheless, the cell population of *Salmonella* continued to increase after the 1st day of sprouting and did not peak until day 3 (Hu, Lee, Manohar, & Chen, Unpublished).

The current study found no significant difference in the overall mean population of the two EHEC strains (Table 4.1). Several previous studies have been performed focusing on the growth of different *E. coli* strains during sprouting. Different observations have been made in these studies. Without any chemical treatment intervention, Cui, Liu & Chen (2020) found that *E. coli* F4546 grew significantly better on vegetable sprouts than *E. coli* BAA-2326 when they were introduced to the surface of sprout seeds with accompanying contamination through contact with contaminated sandy soil. However, when the pathogens were introduced to seeds *via* vacuum infiltration, no significant difference in the cell populations of these two strains was noticed (Liu, Cui, Walcott, & Chen, 2018).

4.5 Conclusions

This study found that treatment of sprout seeds with ascr#18 before and during sprouting could control the growth of EHEC on alfalfa and fenugreek sprouts. The most effective concentration was 1 mM of ascr#18. Seed type, EHEC strain type, and sprouting times were significant factors influencing the growth of EHEC on sprouts. These results provide supporting evidence for the possible use of ascarosides to treat sprout seeds or sprouts before or during the sprouting process. However, the treatment did not consistently eliminate EHEC from sprouts, revealing the challenge of controlling pathogen growth on sprouts.

References

Beutin, L., & Martin, A. (2012). Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. *Journal of Food Protection*, *75*(2), 408-418. https://doi.org/10.4315/0362-028X.JFP-11-452

Butcher, R. A., Fujita, M., Schroeder, F. C., & Clardy, J. (2007). Small-molecule pheromones that control dauer development in *Caenorhabditis elegans*. *Nature Chemical Biology*, *3*(7), 420-422. https://doi.org/10.1038/nchembio.2007.3

Charkowski, A. O., Barak, J. D., Sarreal, C. Z., & Mandrell, R. E. (2002). Differences in growth of *Salmonella enterica* and *Escherichia coli* O157:H7 on alfalfa sprouts. *Applied and Environmental Microbiology*, *68*(6), 3114-3120. https://doi.org/10.1128%2FAEM.68.6.3114-3120.2002

Choi, H. W., & Klessig, D. F. (2016). DAMPs, MAMPs, and NAMPs in plant innate immunity. *BMC Plant Biology*, *16*(1), 1-10. https://doi.org/10.1186/s12870-016-0921-2

Cui, Y., Liu, D., & Chen, J. (2020). Fate of *Salmonella enterica* and enterohemorrhagic *Escherichia coli* on vegetable seeds contaminated by direct contact with artificially inoculated soil during germination. *Journal of Food Protection*, *83*(7), 1218-1226. https://doi.org/10.4315/JFP-20-021

European Food Safety Authority. (2011). Shiga toxin-producing *E. coli* (STEC) O104:H4 2011 outbreaks in Europe: Taking Stock. *EFSA Journal*, *9*(10), 2390. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2011.2390

Fan, X., & Thayer, D. W. (2001). Quality of irradiated alfalfa sprouts. *Journal of Food Protection*, *64*(10), 1574-1578. https://doi.org/10.4315/0362-028X-64.10.1574

Hu, X., Lee, S., M. Manohar, & Chen, J. Unpublished. Efficacy of ascaroside ascr#18 treatments in control of *Salmonella enterica* on alfalfa and fenugreek seeds and sprouts.

Jeong, P. Y., Jung, M., Yim, Y. H., Kim, H., Park, M., Hong, E., Lee, W., Kim, Y. H., Kim, K., & Paik, Y. K. (2005). Chemical Structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. *Nature*, *433*(7025), 541-545. https://doi.org/10.1038/nature03201

Kiontke, K., & Fitch, D. H. (2013). Nematodes. *Current Biology*, *23*(19), R862-R864. https://doi.org/10.1016%2Fj.cub.2013.08.009

Klessig, D. F., Manohar, M., Baby, S., Koch, A., Danquah, W. B., Luna, E., Park, H., Kolkman, J. M., Turgeon, B. G., Nelson, R., Leach, J. E., Williamson, V. M., Kogel, K., Kachroo, A., & Schroeder, F. C. (2019). Nematode ascaroside enhances resistance in a broad spectrum of plant–pathogen systems. *Journal of Phytopathology*, *167*(5), 265-272. https://doi.org/10.1111/jph.12795

Liu, D., Cui, Y., Walcott, R., & Chen, J. (2018). Fate of *Salmonella enterica* and enterohemorrhagic *Escherichia coli* cells artificially internalized into vegetable seeds during germination. *Applied and Environmental Microbiology*, *84*(1), e01888-17. https://doi.org/10.1128/AEM.01888-17

Ludewig, A. H., & Schroeder, F. C. Ascaroside signaling in *C. elegans* (January 18, 2013), *Wormbook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.155.1, http://www.wormbook.org.

Manohar, M., Tenjo-Castano, F., Chen, S., Zhang, Y. K., Kumari, A., Williamson, V. M., Wang, X., Klessig, D. F., & Schroeder, F. C. (2020). Plant metabolism of nematode pheromones mediates plant-nematode interactions. *Nature Communications*, *11*(1), 1-11. https://doi.org/10.1038/s41467-019-14104-2

Manosalva, P., Manohar, M., von Reuss, S. H., Chen, S., Koch, A., Kaplan, F., Choe, A., Micikas, R. J., Wang, X., Kogel, K-H., Sternberg, P. W., Williamson, V. M., Schroeder, F. C., & Klessig, D. F. (2015). Conserved nematode signaling molecules elicit plant defenses and pathogen resistance. *Nature Communications*, *6*(1), 1-8. https://doi.org/10.1038/ncomms8795

MedChemExpress. (n.d.). *Ascr#18*. https://www.medchemexpress.com/ascr-18.html Accessed Aug. 22, 2022.

Milberg, P., Pérez-Fernández, M. A., & Lamont, B. B. (1998). Seedling growth response to added nutrients depends on seed size in three woody genera. *Journal of Ecology*, *86*(4), 624-632. https://doi.org/10.1046/j.1365-2745.1998.00283.x

Miller, W. B., & Finan, E. (2006). Root-zone alcohol is an effective growth retardant for paperwhite narcissus. *HortTechnology*, *16*(2), 294-296. https://doi.org/10.21273/HORTTECH.16.2.0294

Newman, M. A., Sundelin, T., Nielsen, J. T., & Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Frontiers in Plant Science*, *4*, 139. https://doi.org/10.3389%2Ffpls.2013.00139

Park, J. Y., Joo, H. J., Park, S., & Park, Y. K. (2019). Ascaroside pheromones: chemical biology and pleiotropic neuronal functions. *International Journal of Molecular Sciences*, *20*(16), 3898. https://doi.org/10.3390/ijms20163898

Randhir, R., Lin, Y. T., Shetty, K., & Lin, Y. T. (2004). Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asia Pacific Journal of Clinical Nutrition*, *13*(3).

Roudaire, T., Héloir, M. C., Wendehenne, D., Zadoroznyj, A., Dubrez, L., & Poinssot, B. (2021). Cross kingdom immunity: The role of immune receptors and downstream signaling in animal and plant cell death. *Frontiers in Immunology*, *11*, 612452. https://doi.org/10.3389%2Ffimmu.2020.612452

Sakhare, S. D., Inamdar, A. A., & Prabhasankar, P. (2015). Roller milling process for fractionation of fenugreek seeds (*Trigonella foenumgraecum*) and characterization of milled fractions. *Journal of Food Science and Technology*, *52*(4), 2211-2219. https://doi.org/10.1007/s13197-014-1279-9

U.S. Food and Drug Administration. (2017). *Guidance of Industry: Sprouts*. https://www.fda.gov/files/food/published/Draft-Guidance-for-Industry--Compliance-with-and-Recommendations-for-Implementation-of-the-Standards-for-the-Growing--Harvesting--Packing--and-Holding-of-Produce-for-Human-Consumption-for-Sprout-Operations-%28PDF%29.pdf Accessed May 29, 2022.

U.S. Food and Drug Administration. (2019). *FDA Issues Draft Guidance for Reducing Food Safety Hazards in the Production of Seed for Sprouting*. https://www.fda.gov/food/cfsan-constituent-updates/fda-issues-draft-guidance-reducing-food-safety-hazards-production-seed-sprouting Accessed: July 26, 2022.

Vaughton, G., & Ramsey, M. (2001). Relationships between seed mass, seed nutrients, and seedling growth in *Banksia cunninghamii* (*Proteaceae*). *International Journal of Plant Sciences*, *162*(3), 599-606. https://doi.org/10.1086/320133

Williamson, V. M., & Gleason, C. A. (2003). Plant–nematode interactions. *Current Opinion in Plant Biology*, *6*(4), 327-333. https://doi.org/10.1016/S1369-5266(03)00059-1

Wu, S. C., Bögre, L., Vincze, É., Kiss, G. B., & Dudits, D. (1988). Isolation of an alfalfa histone H3 gene: structure and expression. *Plant Molecular Biology*, *11*(5), 641-649. https://doi.org/10.1007/BF00017464

Yu, Y., Zhang, Y. K., Manohar, M., Artyukhin, A. B., Kumari, A., Tenjo-Castano, F. J., Nguyen, H., Routray, P., Choe, A., Klessig, D. F., & Schroeder, F. C. (2021). Nematode signaling molecules are extensively metabolized by animals, plants, and microorganisms. *ACS Chemical Biology*, *16*(6), 1050–1058. https://doi.org/10.1021/acschembio.1c00217

Zansky, S., Wallace, B., Schoonmaker-Bopp, D., Smith, P., Ramsey, F., Painter, J., Gupta, A., Kalluri, P., & Noviello, S. (2002). Outbreak of multi-drug resistant *Salmonella* Newport--United States, January-April 2002. *Journal of the American Medical Association*. *288*(8), 951-953. https://doi.org/10.1001/jama.288.8.951

Table 4.1

Type III tests for fixed effects by the statistical model of sprout sampling of *Escherichia coli*(α=0.05).

NATSA

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Seed	1	75.076	75.076	91.24	<.0001
Strain	1	0.65025	0.65025	0.79	0.3767
Treatment	3	299.9766	99.99221	121.52	<.0001
Time	4	67.11549	16.77887	20.39	<.0001
Replicate	1	0.00001	0.00001	0	0.9972
Seed*Treatment	3	13.02367	4.341222	5.28	0.0023
Strain*Treatment	3	15.60521	5.201735	6.32	0.0007

MAC/SMAC

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Seed	1	95.80573	95.80573	107.85	<.0001
Strain	1	0.102516	0.102516	0.12	0.735
Treatment	3	322.3082	107.4361	120.94	<.0001
Time	4	113.8194	28.45485	32.03	<.0001
Replicate	1	0.419226	0.419226	0.47	0.4941
Seed*Treatment	3	10.544	3.514667	3.96	0.0111
Strain*Treatment	3	15.52965	5.176551	5.83	0.0012

Table 4.2
Overall mean total aerobic and enterohemorrhagic *Escherichia coli* counts at different sampling points and from alfalfa and fenugreek sprouts developed from seeds underwent different treatments.

		Escherichia coli population (Log CFU/g)	
		NATSA	MAC/SMAC
Sprout seed			
	Fenugreek (n = 80)	4.44A	4.20A
	Alfalfa (n = 80)	3.07B	2.65B
Bacterial strain			
	E. coli F4546 (n = 80)	3.82A	3.40A
	E. coli BAA-2326 (n = 80)	3.69A	3.45A
Seed treatment			
	EtOH (0.01%) (n = 40)	5.02A	4.79A
	EtOH (10%) (n = 40)	4.78A	4.49A
	Ascr#18 (1 µM) (n = 40)	3.66B	3.23B
	Ascr#18 (1 mM) (n = 40)	1.55C	1.18C
Sprouting time			
	Day 3 (n = 32)	4.29A	3.93A
	Day 5 (n = 32)	4.21A	4.08A
	Day 7 (n = 32)	4.08AB	3.88AB
	Day 1 (n = 32)	3.67B	3.43B
	Day 0 (n = 32)	2.53C	1.79C
Replicate			
	Two (n=80)	3.76A	3.47A
	One (n=80)	3.75A	3.37A

Values of the same variables in each column followed by the same letters are not significantly different ($P > 0.05$). EtOH: ethanol; Ascr#18: An (ω−1)-hydroxy fatty acid ascaroside excreted by plant-parasitic nematodes; NATSA: tryptic soy agar supplemented with nalidixic acid; MAC: MacConkey agar; and SMAC: Sorbitol MacConkey agar.

Table 4.3

Mean total aerobic and *Escherichia coli* counts in samples collected from alfalfa or fenugreek sprouts developed from seeds underwent different treatments.

		Cell population (Log CFU/g)			
Seed treatment		EtOH (10%)	EtOH (0.01%)	Ascr#18 (1 µM)	Ascr#18 (1 mM)
NATSA (n = 160)					
	Alfalfa (n = 80)	3.67Bab	4.46Ba	3.35Ab	0.80Bc
	Fenugreek (n =80)	5.88Aa	5.60Aa	3.98Ab	2.30Ac
MAC/SMAC (n = 160)					
	Alfalfa (n = 80)	3.32Bab	4.13Ba	2.76Bb	0.37Bc
	Fenugreek (n =80)	5.65Aa	5.44Aa	3.70Ab	1.99Ac

Values within the same column followed by the same uppercase letters are not significantly different (*P* > 0.05); values within the same row followed by the same lowercase letters from the same growth medium are not significantly different (*P* > 0.05). EtOH: ethanol; Ascr#18: An (ω−1)-hydroxy fatty acid ascaroside excreted by plant-parasitic nematodes; NATSA: tryptic soy agar supplemented with nalidixic acid; MAC: MacConkey agar; and SMAC: Sorbitol MacConkey agar.

Table 4.4

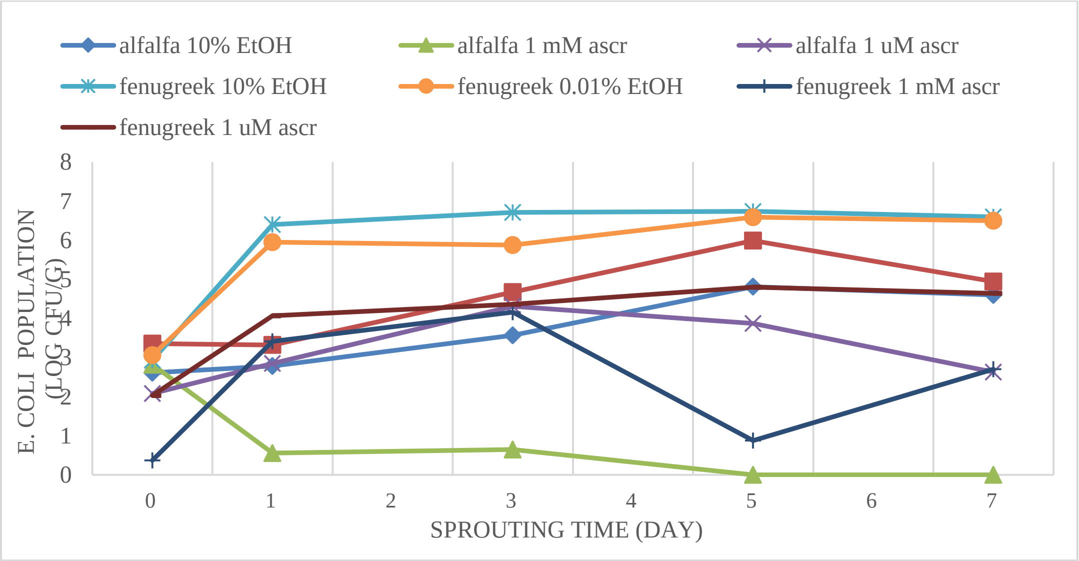
Mean *E. coli* O157:H7 F4546 and *E. coli* O104:H4 BAA-2326 counts in samples collected from both types of sprouts developed from seeds underwent different treatments.

		Cell population (Log CFU/g)			
Seed treatment		EtOH (10%)	EtOH (0.01%)	Ascr#18 (1 µM)	Ascr#18 (1 mM)
NATSA (n = 160)					
	E. coli O157:H7 F4546 (n = 80)	5.04Aa	5.25Aa	3.18Bb	1.80Ac
	E. coli O104:H4 BAA-2326 (n =80)	4.51Ba	4.80Ba	4.14Ab	1.31Ac
MAC/SMAC (n = 160)					
	E. coli O157:H7 F4546 (n = 80)	4.67Aa	5.05Aa	2.70Bb	1.16Ac
	E. coli O104:H4 BAA-2326 (n =80)	4.30Aa	4.52Ba	3.76Ab	1.20Ac

Values within the same column followed by the same uppercase letters are not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letters from the same growth medium are not significantly different ($P > 0.05$). EtOH: ethanol; Ascr#18: An (ω−1)-hydroxy fatty acid ascaroside excreted by plant-parasitic nematodes; NATSA: tryptic soy agar supplemented with nalidixic acid; MAC: MacConkey agar; and SMAC: Sorbitol MacConkey agar.

Figure 4.1
Mean cell populations from different treatment groups on alfalfa and fenugreek seeds or sprouts during the germination process. A. *Escherichia Coli* mean cell populations shown on NATSA plates. B. *Escherichia Coli* mean cell populations shown on MAC or SMAC plates.

A



B

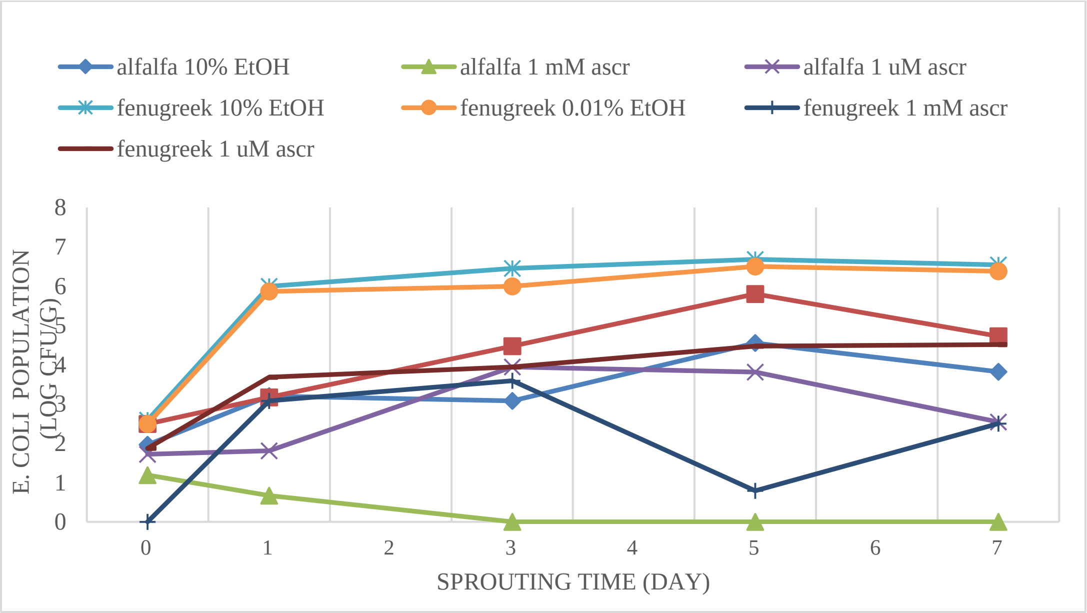
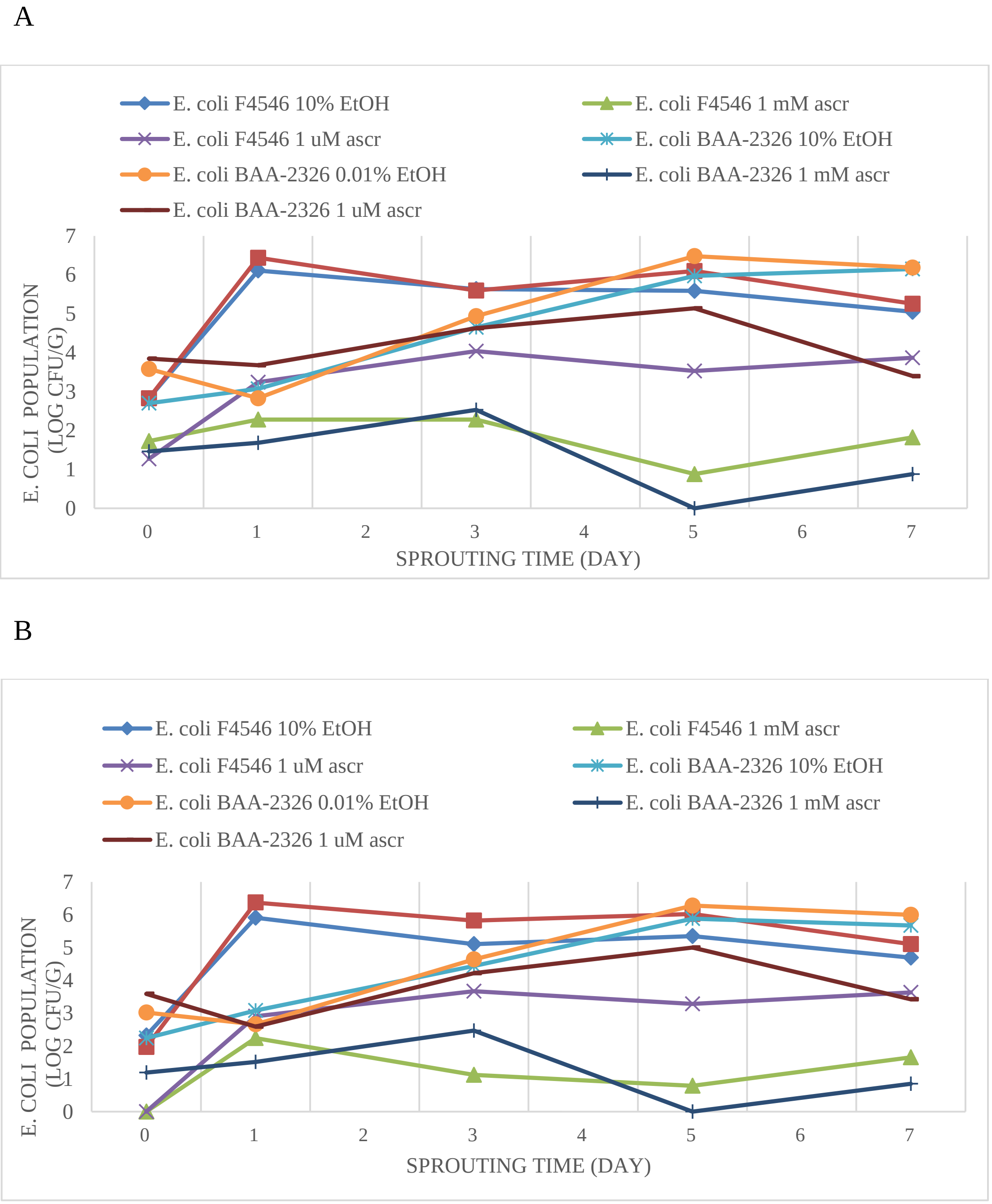


Figure 4.2
Mean cell populations of two *Escherichia Coli* strains from different treatment groups during the germination process. A. *Escherichia Coli* mean cell populations shown on NATSA plates. B. *Escherichia Coli* mean cell populations shown on MAC or SMAC plates.



CHAPTER 5

CONCLUSIONS

The conclusions of the study are as follows:

1). Treatment with low concentrations of ascaroside ascr#18 reduced the populations of two *Salmonella* strains on alfalfa and fenugreek seeds and sprouts during sprouting. Treatment with 1 mM of ascr#18 was more effective than the 1 µM treatment. The mean populations of *Salmonella* on seeds and sprouts were influenced by seed type, *Salmonella* strain type, and sprouting time besides treatment type. The populations of *Salmonella* were significantly higher on fenugreek than on alfalfa sprouts. *S*. Stanley grew better than *S*. Cubana on sprouts. The population of *Salmonella* increased from day 0 to day 3 and reached the peak population on Day 5.

2). Ascr#18 treatment of sprout seeds before germination could control the growth of enterohemorrhagic *E. coli* on alfalfa and fenugreek seeds and sprouts. Higher treatment efficacy was observed when 1 mM of ascr#18 was used. *E. coli* population was reduced by 3.54 and 1.15 log CFU/g by treatment with 1 mM and 1 µM ascr#18, respectively. Moreover, seed type and sprouting times were also the main factors affecting the mean populations on sprouts. Similar to *Salmonella,* the populations of *E. coli* were significantly higher on fenugreek than on alfalfa sprouts. The population of *E. coli* peaked on day 1 and decreased slightly from day 3 to day 7.

The study's findings provide supporting evidence for the potential application of ascr#18 to control pathogen contamination on edible sprouts.