ASCAROSIDE #18 AS A PREHARVEST TREATMENT TO REDUCE *ESCHERICHIA COLI* O157:H7 CONTAMINATION ON ROMAINE LETTUCE

by

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(Under the Direction of Laurel L. Dunn)

ABSTRACT

Escherichia coli O157:H7 is a major foodborne pathogen often linked to fresh produce, especially romaine lettuce. This study examined the efficacy of ascaroside #18 (ascr#18) as a pre-harvest treatment to reduce *E. coli* O157:H7 contamination on romaine lettuce. Plants were treated with ascr#18 via foliar spray or soil drench at three concentrations (0.01 µM, 0.1 µM, and 1 µM) before inoculation with *E. coli* O157:H7. Pathogen populations were enumerated on Days 0, 3, and 7. Foliar spray treatments achieved greater reductions than soil drench application, with the highest reduction (2.21 log MPN/head) at 0.1 µM on Day 0 (8-10 days after initial ascr#18 treatment). Although ascr#18 demonstrated significant potential in reducing *E. coli* O157:H7 contamination when compared to the control, differences among concentrations were not significant ($p > 0.05$). Future studies should explore a broader concentration range to optimize its use in commercial leafy green production.

INDEX WORDS: Romaine lettuce, ascr#18, *E. coli* O157:H7, Preharvest treatment, Food safety

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CHAPTER 1

INTRODUCTION

Fruits and vegetables are rich in essential nutrients that support a healthy lifestyle. However, as demand and consumption have risen over the past few decades, they have been frequently associated with outbreaks of foodborne illness (Critzer & Doyle, 2010; Luna-Guevara et al., 2019). Major pathogens like *Salmonella enterica* and *Escherichia coli* O157:H7 significantly contribute to foodborne outbreaks associated with fresh produce, posing a serious public health risk, and contributing to an economic burden estimated at $77.7 billion annually (Olaimat & Holley, 2012; Scharff, 2012). Leafy greens including romaine lettuce are at high risk for contamination with *E. coli* O157:H7, which is frequently implicated in foodborne outbreaks in the United States (Carstens et al., 2019).

Traditional post-harvest washing and sanitizing practices are critical in reducing microbial contamination on fresh produce. Postharvest sanitizers, such as peracetic acid (PAA) and chlorine-based treatments like sodium (NaOCl) or calcium hypochlorite ($Ca(ClO)_2$, are widely employed to prevent cross-contamination through wash water (Allende et al., 2008; Alvaro et al., 2009; Beuchat et al., 2004; Van Haute et al., 2015). However, their efficacy can be affected by factors such as pH, light, and organic matter, and they can form harmful disinfection by-products (Gil et al., 2009; Ramos et al., 2013; Widmer et al., 2025; Zagory, 2000). Additionally, pathogens can adhere to plant tissues or rough surfaces, making them less accessible to conventional sanitizers and difficult to remove or inactivate completely. Therefore, integrating pre-harvest interventions alongside good agricultural practices (GAPs) could help reduce contamination during the growing stage to improve the overall produce safety.

One such strategy is the use of plant immune elicitors. Ascaroside #18 (ascr#18), a major ascaroside pheromone secreted by plant-parasitic nematodes, has been investigated as a natural alternative to prime plant defense mechanisms. Studies have revealed that low concentrations of ascr#18 can trigger plant immune responses and enhance resistance against various pathogens including bacteria, viruses, and nematodes (Klessig et al., 2019; Manohar et al., 2020; Manosalva et al., 2015). Another recent study has shown its efficacy in controlling *Salmonella enterica* on seeds and sprouts (Hu et al., 2023), but its efficacy in reducing foodborne pathogen contamination on leafy greens requires further study.

In this study, we hypothesized that ascr#18 treatment reduces *E. coli* O157:H7 contamination on romaine lettuce. We will examine the efficacy of ascr#18 applied at varying concentrations as either a foliar or soil drench application during different stages as a preharvest treatment.

CHAPTER 2

LITERATURE REVIEW

Foodborne illness in the US

Foodborne illnesses continue to be a major public health challenge in the United States, with millions of cases reported annually. As stated by the Centers for Disease Control and Prevention (CDC), an estimated 48 million people in the US experience foodborne illnesses each year, causing approximately 128,000 hospitalizations and 3,000 deaths (CDC, 2018). These foodborne illnesses have many causes, including pathogens, allergens, and chemical contaminants (Hoffman et al., 2015). Notably, 31 known pathogens have been identified as the primary causes of foodborne illnesses, with nontyphoidal *Salmonella enterica*, *Campylobacter spp*., Norovirus, *Clostridium perfringens*, and *Staphylococcus aureus* being the most significant contributors (Scallan et al., 2011).

Foodborne illnesses not only pose significant health risks but also create a substantial economic burden. The U.S. Government Accountability Office (GAO) reported that six major pathogens, including *Salmonella*, *Listeria monocytogenes*, *Campylobacter*, *Clostridium perfringens*, Shiga toxin-producing *E. coli* (STEC), and norovirus, cause approximately 10 million foodborne illnesses each year in the U.S., leading to 53,300 hospitalizations and more than 900 deaths (U.S Government Accountability Office, 2025). The annual cost of disease caused by these pathogens is estimated to be $14.0 billion, with *Toxoplasma gondii, Campylobacter spp*., *Salmonella, L. monocytogenes*, and norovirus responsible for 90% of the economic and health burden. This estimate covers multiple dimensions, including medical expenditure, productivity losses, and lost utility due to deaths (Hoffmann et al., 2012). According to a 2018 report from the U.S. Department of Agriculture's Economic Research Service, the annual economic burden of 15 leading foodborne pathogens was estimated at $17.6 billion, representing a 13% increase or approximately $2 billion over the 2013 estimate of $15.5 billion for the same pathogens (Hoffmann & Ahn, 2021). A more recent study published in 2024 provided a comprehensive evaluation, estimating the cost of foodborne illness in the U.S. to be $75 billion in 2023, a significant increase due to the expanded scope of 48 million cases from the previously reported 9 million which is caused by 31 major known pathogens and unspecified agents (Hoffmann et al., 2024).

It is estimated that nontyphoidal *Salmonella* causes approximately one million foodborne illnesses annually in the U.S. and two-thirds of these nontyphoidal *Salmonella* infections are attributed to food sources, with an increasing proportion linked to fresh produce (Mitchell Jr et al., 2024). Pathogens like *Salmonella* and *E. coli* also significantly contribute to foodborne outbreaks associated with fresh produce, posing serious public health risks (Olaimat & Holley, 2012). Among these, STEC including *E. coli* O157:H7, causes an estimated 265,000 illnesses annually in the U.S., with costs exceeding $280 million. Leafy greens, a significant source of foodborne STEC outbreaks, are the second most common vehicle for infections, following ground beef (Marshall et al., 2020).

Fresh Produce Related Multistate Outbreaks in the US

In the past few years, consumer behavior has shifted significantly towards healthier lifestyles, which has led to an increased consumption of fruits and vegetables. Fruits and vegetables are important as they are rich in essential nutrients like minerals, vitamins, and phytochemicals, which are vital for a balanced diet. In addition to their nutritional value, they provide several health benefits, including anti-inflammatory and antioxidant effects (Slavin &

Lloyd, 2012). Global fruit and vegetable production nearly doubled from 1980 to 2004, while the United States imports grew to $12.7 billion, with daily sales of pre-cut produce in North America reaching 6 million packages by 2005 (Olaimat & Holley, 2012). The USDA Economic Research Service reports that between 2007 and 2021, U.S. imports of fresh produce increased from 50% to 60% for fruit and 20% to 38% for vegetables; the average American consumed 63.3 kg of fresh vegetables in 2022 (USDA-ERS, 2024; USDA, 2023).

The rising consumer demand for fresh produce has potentially been a contributing factor to an increase in produce-associated foodborne outbreaks (Warriner et al., 2009). Unlike processed foods that are cooked at high temperatures to kill harmful bacteria, fresh produce is generally eaten raw or undergoes minimal processing, which makes it more likely to be a source of foodborne pathogens like *E. coli* O157. Between 1990 and 2003, the Center for Science in the Public Interest (CSPI) recorded 554 produce-related outbreaks that caused 28,315 illnesses. Fruits like cantaloupe and berries were associated with 93 outbreaks, while vegetables like sprouts and mushrooms were linked to 205 outbreaks. Additionally, 256 outbreaks were linked to produce dishes, including lettuce-based salads (DeWaal et al., 2006). Approximately 30% of these cases were attributed to bacteria like *Salmonella*, *E. coli* O157:H7, and *Campylobacter* spp. This trend continued as data from 2004 and 2013 indicated that fresh produce items, including cilantro, cucumbers, cantaloupes, and peppers, were responsible for 629 outbreaks and nearly 20,000 reported illnesses (Martinovic et al., 2022). Table 1 provides examples of several multistate outbreaks associated with fresh produce, showing the associated pathogens, the number of states impacted, and the severity of each outbreak in terms of hospitalizations and deaths.

TABLE 1: Examples of multistate foodborne outbreaks associated with fresh produce consumption in the United States.

Year	Pathogen	Produce involved	Number of cases	Number of states affected	Hospitalizations	Deaths	Reference
2010	*E. coli* O145	Romaine lettuce	26	5	12	0	(CDC, 2010)
2011	*Listeria monocytogenes*	Cantaloupe	147	28	143	33	(Garner & Kathariou, 2016)
2014	*Listeria monocytogenes*	Apples	35	12	34	7	(CDC, 2015)
2015	*Salmonella*	Cucumbers	907	40	204	6	(CDC, 2016)
2018	*E. coli O157:H7*	Romaine lettuce	33	37	104	5	(Bottichio et al., 2020)
2021	*E. coli O157:H7*	Baby spinach	15	10	4	0	(FDA, 2022)
2024	*Salmonella* Africana and *Salmonella* Braenderup	Cucumbers	551	34	155	0	(FDA, 2024a)

Escherichia coli O157:H7

E. coli O157:H7 is a Gram-negative, virulent serotype of Shiga toxin-producing *E. coli* (STEC), also known as enterohemorrhagic *E. coli* (EHEC). These *E. coli* are important food and waterborne pathogens that commonly cause acute bloody diarrhea and are associated with various gastrointestinal illnesses, including diarrhea, abdominal cramps, and in severe cases, hemolytic uremic syndrome (HUS) in humans (Ameer et al., 2023; Bush & Vazquez-Pertejo, 2020; Elias A Rahal et al., 2012). Symptoms usually appear 3 to 4 days following exposure, and most people recover without treatment after 5 to 7 days (CDC, 2024b).

More than 100 STEC serogroups are associated with human illness, but *E. coli* O157:H7 is the predominant STEC serotype identified in the United States, responsible for an estimated 73,000 cases annually (Brooks et al., 2005). *E. coli* O157:H7 prevalence in humans is due to its exceptional pathogenicity, which results from multiple virulence factors. This includes genes encoding Shiga toxins (Stx1 and Stx2) and the locus of enterocyte effacement (LEE) pathogenicity island. This region encodes a type III secretion system (T3SS) that allows the bacterium to inject effector proteins into host cells, promoting colonization and evasion of host immune responses (Kaper et al., 2004). Furthermore, *E. coli* O157:H7 carries a 60 MDa virulence plasmid (pO157), which encodes a hemolysin and contributes to its ability to induce attaching and effacing (A/E) lesions on intestinal epithelial cells (Elias A. Rahal et al., 2012).

E. coli O157:H7 is highly virulent due to its extremely low infectious dose, allowing it to cause outbreaks even when only a small number of bacteria are present in contaminated water or food. For healthy adults, as few as 10-100 colony-forming units (CFU) of *E. coli* O157:H7 may be sufficient to cause infection (Elias A. Rahal et al., 2012). However, severe *E. coli* O157:H7 infections are more likely to affect the elderly, children under 5, and immunocompromised individuals. These vulnerable populations may develop serious complications such as HUS, from exposure to even fewer bacterial cells (Ameer et al., 2023; CDC, 2024a). *E. coli* O157:H7 has a high tolerance for acidic environments, allowing it to persist in low pH conditions, including the bovine and human gastrointestinal tracts and certain acidic foods, which increases its potential to cause infection at low doses (Lim et al., 2010; Price et al., 2004). Variations in infectious dose and pathogenicity are influenced by the strain type, the food source, and the host immune status (Teunis et al., 2008). Additionally, some strains of *E. coli* O157:H7 can persist for prolonged periods of time outside its host and on surfaces, primarily because of biofilm formation, which enhances its resistance to sanitizers and contributes to contamination in agricultural and food processing facilities (Vogeleer et al., 2014).

The known distribution of *E. coli* O157:H7 and other STEC strains varies across regions, due to differences in agricultural practices, dietary habits, and surveillance systems. In the U.S., *E. coli* O157:H7 remains the predominant STEC serotype, particularly in outbreaks linked to vegetable row crops, beef, dairy, and fruit (Tack et al., 2021). Leafy greens, especially romaine lettuce, are the most frequent vehicle of *E. coli* O157:H7 contamination, with recurring outbreaks traced to growing regions in California and Arizona (CDC, 2024c). Tack et al. (2021) reported that this serotype accounted for 71% of all STEC outbreaks in the U.S. from 2010 to 2017. Although *E. coli* O157:H7 is the most commonly associated strain in foodborne *E. coli* outbreaks, non-O157 STEC strains are increasingly identified in human infections and are a major public health concern. The most common non-O157 STEC serogroups reported to cause foodborne illness in the U.S. are O26, O111, O103, O121, O45, and O145 (Alharbi et al., 2022; Gould et al., 2013). Gould et al. (2013) found that non-O157 STEC infections were more common among persons of Hispanic ethnicity and were also frequently associated with international travel.

In Europe, non-O157 strains account for a larger proportion of human cases, with serogroup O26 becoming increasingly prevalent (European Centre for Disease Prevention and Control, 2024). A regional survey in England (2013-2017) found that non-O157 serogroups accounted for over 80% of clinical isolates (Hoyle et al., 2021). In South America, studies from countries like Argentina and Chile have identified the presence of all STEC strains, including both non-O157 and O157:H7 variants. The most prevalent non-O157 STEC serogroups identified in Argentina include O145, O26, O174, and O121, with O174 recognized as an emerging pathogen (Torti et al., 2021). Research by Irino et al. (2002) indicates that in Brazil, non-O157 STEC strains have been associated with sporadic cases of nonbloody diarrhea. In Australia and New Zealand, *E. coli* O157 is the most common STEC serogroup associated with human infections, though other serogroups are present. Between 2007 and 2016, *E. coli* O157 accounted for 56% of cases in Australia, followed by O26 (11%) and O111 (7%) (Vally et al., 2012).

In Asia, *E. coli* O157:H7 is the predominant STEC serotype in Japan, while in South Korea, *E. coli* O26 and O111 are the most frequently isolated serotypes in human infections (Furukawa et al., 2018; Jeon et al., 2006). China has reported O157:H7 outbreaks linked to cattle, pigs, and milk, though data on other serotypes remains limited. However, non-O157 STEC strains have been isolated from outpatients with acute diarrhea in southeastern China (Bai et al., 2015; Xu et al., 1999). A study in Calcutta India, found that non-O157 STEC strains are prevalent and more frequently isolated from human cases than O157 (Khan et al., 2002). Both STEC O157 and non-O157 have been isolated in several Middle Eastern countries, including

Iran, but data on STEC prevalence in this region are limited (Tahamtan et al., 2010). In African countries, there is limited data on STEC prevalence, but several studies have identified O157:H7 in various food sources in countries like Nigeria and South Africa (Ateba et al., 2008; Onwumere-Idolor et al., 2024). There are few studies on STEC prevalence in North Africa, but *E. coli* O157 has been detected in food, animal, and human samples from countries like Egypt, Morocco, Tunisia, and Algeria, with limited research on non-O157 STEC serogroups (Ahmed & Van Velkinburgh, 2014; Barka et al., 2014). While *E. coli* O157 is the most commonly identified STEC serotype across all continents, due to its link to severe infections, with improved diagnostics and more robust surveillance systems, the prevalence of non-STEC strains is increasingly significant in many regions worldwide.

E. coli O157:H7 Contamination of Leafy Greens

E. coli O157:H7 outbreaks were historically linked to beef and dairy products, but in recent decades, leafy greens like romaine lettuce have emerged as a common source of infection (Coulombe et al., 2020; Heiman et al., 2015). *E. coli* O157:H7 contamination in romaine lettuce continues to be a recurring food safety issue. Between 2009 and 2018, 40 *E. coli* O157:H7 outbreaks were associated with leafy greens in both Canada and the U.S., leading to 1,212 illnesses, 420 hospitalizations, 77 HUS cases, and 8 deaths. Among these outbreaks, it was reported that romaine lettuce was the most implicated, accounting for 54% of cases, more than any other type of leafy green (Marshall et al., 2020).

Contamination of leafy greens primarily occurs through several routes that are influenced by agricultural practices and environmental factors. Pre-harvest contamination is a major concern with domestic and wild animals being significant sources. Grazing cattle on agricultural lands near leafy green fields is considered a key risk factor, as ruminants, particularly cattle, serve as the primary reservoir for *E. coli* O157:H7, which sheds the bacteria through their feces (Coulombe et al., 2020). For example, a traceback investigation of the 2020 *E. coli* O157:H7 outbreak associated with leafy greens in the Central Coast of California found the outbreak strain in cattle feces collected a mile upslope from a produce farm; this indicates a potential role of nearby livestock in produce contamination (FDA, 2021). Other transmission routes include the use of contaminated irrigation water, soil amendments such as untreated manure, and direct or indirect contact with infected animals (Rangel et al., 2005).

Contaminated irrigation water, especially from surface sources, is a major source of *E. coli* O157:H7 contamination of fresh produce, as it can directly introduce pathogens to the crops (Beuchat, 1996). Surface water is more likely to be contaminated with pathogenic bacteria, parasites, and viruses due to its exposure to environmental contaminants such as municipal waste, livestock runoff, industrial effluents, wildlife, and human pollutants (Alegbeleye et al., 2018; Haldar et al., 2022). Pathogens may be introduced when present in poor-quality irrigation water and adhere to crop surfaces, even penetrating plant tissues under certain conditions. Several studies have shown that this direct introduction increases the risk of internalizing pathogens such as *Salmonella* and Shiga-toxigenic *E. coli* during fruit and vegetable production (Gomes et al., 2009; Guo et al., 2002; Li et al., 2008; Warriner et al., 2003). The ability of pathogens to adhere and internalize makes them harder to remove, reducing the effectiveness of conventional washing and chemical sanitizing methods (Alegbeleye et al., 2018).

Postharvest water is commonly used for washing and cooling leafy greens after harvest, but can also serve as a source of contamination. If not properly sanitized, it can spread pathogens to the fresh produce (Coulombe et al., 2020). Washing contaminated produce may release microorganisms into the water, which can then transfer to other produce when the water is being reused (Possas & Pérez-Rodríguez, 2023).

Poor hygiene practices among workers during post-harvest handling and processing can introduce pathogens to fresh produce (Beuchat, 1996). Workers infected with *E. coli* O157:H7 may unintentionally transfer the pathogen via the fecal-oral route to produce through direct contact during harvesting or packaging if hygiene protocols like proper handwashing are not followed. Poor hygiene practices among workers handling lettuce can be especially problematic when they are asymptomatic carriers of harmful pathogens (Todd et al., 2008).

Leafy greens can also get contaminated by *E. coli* O157:H7 from machinery and equipment. Contaminated tools, bins, or surfaces reused without proper sanitation can spread pathogens across multiple batches. For example, one study found that contaminated processing equipment transferred *E. coli* O157:H7 to multiple batches of fresh-cut lettuce (Buchholz et al., 2012). It is important to use equipment that is easy to clean and sanitize, especially when equipment is in direct contact with fresh produce.

Soil amendments of biological origin like manure, composted agricultural waste, and sewage sludge, are commonly used in crop production to improve soil fertility and boost crop yields. These materials increase nutrient availability and support sustainable agriculture. However, when improperly treated or applied, they present a contamination risk by introducing foodborne pathogens. Raw or improperly treated manure is known to harbor pathogens like *E. coli* O157:H7, which may contribute to the contamination of leafy greens (Alegbeleye et al., 2018; Manyi-Loh et al., 2016). Microorganisms from manure can transfer to crops either through direct contact or indirectly through soil, water, and aerosols. Once introduced to the soil, *E. coli*

O157 can persist under favorable conditions such as moderate temperatures, high moisture, and neutral pH.

Improper storage conditions, especially those with high humidity and inadequate temperature control, can exacerbate contamination by creating favorable environments for pathogen growth. The ability of *E. coli* O157:H7 to persist for prolonged durations under various storage conditions increases the risk of foodborne illness. Luo et al. (2010) observed that *E. coli* O157:H7 could survive on fresh-cut romaine and iceberg lettuce at 5°C, but its growth was restricted, whereas at 12°C storage, bacterial levels increased significantly by over 2.0 log CFU/g within 3 days. Temperature control and humidity management are important to inhibit bacterial growth in fruits and vegetables.

Outbreaks associated with romaine lettuce tend to follow a seasonal pattern, with most cases occurring in the months of spring and fall rather than in the summer and winter (Coulombe et al., 2020). These periods align with transitions in U.S. lettuce-growing regions, which may influence pathogen prevalence. *E. coli* O157:H7 survives significantly longer on romaine harvested in the fall compared to late spring, due to environmental factors such as cooler storage temperatures, higher moisture levels, and reduced microbial competition during this season (USDA Agricultural Research Service, 2024). This may help explain the increased incidence of outbreaks linked to lettuce cultivated and harvested toward the end of the growing seasons in California and Arizona.

Several factors influence the ability of *E. coli* O157:H7 to adhere to and proliferate on leafy green surfaces, including leaf surface characteristics, leaf age, nutrient availability, and environmental conditions (Brandl & Amundson, 2008). Leaf surface topography plays a key role in bacterial colonization, as microbes tend to cluster in specific microsites rather than spreading uniformly. Common colonization sites include rough areas, veins, trichomes, epidermal cell wall junctions, and stomata. These areas harbor higher concentrations of water and nutrients, creating favorable microenvironments for microbial growth and attachment (Beattie & Lindow, 1999; Brandl & Amundson, 2008; Doan et al., 2020). Doan et al. (2020) examined the surface structure of leafy greens and discovered that *E. coli* O157:H7 adhered more readily to leaves with prominent veins compared to those with smoother surfaces. These vein structures provided protective niches, aiding bacterial persistence. These surface features also increased *E. coli* O157:H7 resistance to chlorine washes, reducing the effectiveness of post-harvest sanitization.

Leaf age also significantly influences *E. coli* O157:H7 contamination risk on leafy greens. Younger leaves are more prone to bacterial colonization due to their structural and chemical composition. Brandl and Amundson (2008) reported that *E. coli* O157:H7 populations were as much as ten times greater on younger, inner lettuce leaves than on middle leaves, largely due to differences in nutrient availability. Their analysis showed that young leaf exudates contained 2.9 times more nitrogen and 1.5 times more carbon than those from middle-aged leaves. These nutrients enhance bacterial growth and colonization. The study also showed that adding ammonium nitrate to middle leaves boosted *E. coli* O157:H7 growth, indicating that lower nitrogen levels naturally limit its growth on the older leaves.

Postharvest Handling of Fresh Produce

The Food Safety Modernization Act (FSMA) was enacted in 2011, shifting the FDA’s focus from addressing food contamination to emphasizing its prevention (FDA, 2011). Under FSMA, the Standards for the safe Growing, Harvesting, Packing, and Holding of fresh fruits and vegetables intended for human consumption, also known as the Produce Safety Rule, were established in 2016. This rule introduced basic requirements for the safe production and handling of fresh fruits and vegetables, aiming to reduce the risk of foodborne illnesses (FDA, 2016). Recognizing the ongoing challenges of foodborne illnesses associated with fresh produce, the FDA implemented stricter regulations. Subpart E of the revised FSMA Produce Safety Rule (21 CFR 112.44) now mandates that agricultural water used in washing or cooling processes must not contain any detectable levels of generic *E. coli* per 100 mL sample (FDA, 2024b). However, traditional washing techniques and post-harvest sanitizers, while helpful in removing surface contaminants, may not effectively eliminate all microbial risks.

Washing fresh produce is important as it removes some dirt, debris, and surface pathogens. Therefore, properly treated wash water can reduce the load of surface pathogens but does not result in complete decontamination. One significant challenge with wash water is the potential for cross-contamination, where pathogens from contaminated produce can spread to clean produce during washing, although the addition of antimicrobial substances, such as chlorine or peracetic acid, can result in microbial inactivation and reduce subsequent cross-contamination of produce (Gil et al., 2009). However, pathogen adherence on fresh produce surfaces poses a major challenge. Pathogens can attach to rough surfaces, crevices, or cut edges of produce, making them difficult to remove or inactivate with wash water. Furthermore, the presence of organic matter in wash water may reduce the effectiveness of certain antimicrobial agents, requiring higher concentrations or more frequent replenishment to maintain effectiveness (Gil et al., 2009).

Chlorine washes such as sodium hypochlorite (NaOCl) are widely used post-harvest sanitizers due to their strong antimicrobial properties. They are commonly used at a concentration ranging from approximately 25 to 200 ppm for a contact time of 1 to 2 minutes when washing fresh produce. This process works by oxidizing microbial cell components, which helps to reduce populations of pathogens such as *Salmonella, Listeria, and E. coli* on the surface of fruits and vegetables (Beuchat, 1998). Chlorine washes can reduce microbial levels by 2 -3 log CFU/g, but do not fully eliminate them as bacteria can adhere in tiny crevices on the surface (Gross et al., 2016). Its efficacy can also be significantly affected by the pH level of the water and the presence of organic matter, which can neutralize chlorine and reduce its antimicrobial activity (Zagory, 2000). Additionally, chlorine can produce potentially harmful by-products, like haloacetic acids and trihalomethanes, when reacting with organic matter in water, posing health and environmental concerns (Gil et al., 2009).

Beyond chlorine, other postharvest sanitizers such as peracetic acid (PAA) have gained popularity for produce washing in the fresh produce industry. Under 21 CFR 173.315(a), PAA is generally recognized as safe (GRAS) chemical that can be utilized for fruits and vegetables, with guidelines recommending that its concentration in wash water should not exceed 80 ppm to ensure safety (USDA, 2024). Several authors have demonstrated that PAA effectively reduces microbial contamination on various fresh produce surfaces and has the advantage of maintaining its effectiveness even in the presence of organic matter, unlike chlorine (Huang et al., 2018; Van Haute et al., 2015). Its strong oxidizing properties provide extensive antimicrobial activity to effectively combat a broad spectrum of foodborne pathogens and spoilage organisms. Additionally, PAA produces minimal to no toxic by-products such as carboxylic acids and does not produce halogen-containing disinfection by-products (DBPs), making it a more environmentally friendly option for sanitizing produce (Kitis, 2004). While PAA offers several advantages over chlorine, it also has its limitations. One major disadvantage associated with the use of PAA is its reduced antimicrobial efficacy at the allowed concentrations for vegetables, which can be a significant limitation in effectively reducing pathogen loads (Ramos et al., 2013).

Its effectiveness can be limited by its corrosive nature, as exposure to high concentrations may cause respiratory irritation, damage to the eyes, and other health issues for workers (Kitis, 2004).

Other alternative sanitizers include ozone, electrolyzed water, and ultraviolet (UV) light. Ozone is a powerful oxidant that effectively reduces microbial loads and degrades pesticide residues. However, ozone treatment can potentially deteriorate the flavor and color of fresh produce, impacting its sensory quality (Ramos et al., 2013). Electrolyzed water is highly effective against a wide range of foodborne pathogens and reduces microbial counts in fruits and vegetables due to its high oxidation-reduction potential (over 1000 mV), low pH (2-4), and active oxidizers like hypochlorous acid (Graca et al., 2011). UV light, used commonly for surface decontamination, disrupts microbial DNA and prevents replication. Its use is limited to surface applications and requires direct exposure, making it less effective for complex surfaces or internalized pathogens; moreover, in some specific conditions, microorganisms may repair UV-C light-induced damage, which may significantly reduce the safety of produce (Alexandre et al., 2012; Ramos et al., 2013). These sanitizers have limitations that require careful consideration of their use. Following FDA regulatory guidelines is important for safe use, ensuring concentrations and application methods are optimized to enhance efficacy while minimizing potential risks. A summary of the limitations of these conventional sanitizers is presented in Table 2.

TABLE 2: Some limitations of chlorine and other chemical sanitizers used as a wash solution for fresh produce (adapted with minor adjustments from Ramos et al. (2013)).

Sanitizer type	Advantages	Limitations
Chlorine (hypochlorite)	Low cost	- Formation of harmful disinfection by-products (DBPs) - Efficacy depends on pH, organic matter, temperature, light and air exposure - Only 1 to 2 log CFU/g bacterial inactivation
Chlorine dioxide	Higher efficacy in a wider pH range than chlorine	- It is not effective at the allowed concentrationss for fresh produce
Peroxyacetic acid	No harmful DBP formation	- Limited antimicrobial efficacy at approved concentrations on vegetables
Hydrogen Peroxide	No harmful residues or by-products	- Low efficacy on fresh produce at permitted concentrations - It is less effective against fungi, yeasts, and viruses
Acidified sodium chlorite	More effective than hypochlorite due to its low pH	- There is little data available on chlorinated by-products
Quaternary Ammonium Compounds (QACs)	Both colorless and odorless	- Ineffective at low pH (< 6)

Natural antimicrobial compounds

Natural antimicrobial compounds are bioactive substances obtained from diverse sources, such as animals, plants, bacteria, and fungi, which can inhibit foodborne pathogens (Gyawali & Ibrahim, 2014). These compounds, including essential oils (EO), plant extracts, and nematode-derived molecule (ascaroside), are increasingly studied as alternatives to conventional chemical sanitizers due to their efficacy and their ability to reduce drawbacks related to using conventional sanitizers. This shift is driven by growing consumer concerns regarding the safety of synthetic antimicrobials (Lucera et al., 2012). Many of these compounds are recognized as GRAS by the FDA, which allows their inclusion in food-related applications, provided they meet the required safety standards (ASM, 2024).

The mechanisms of action of natural antimicrobials include disruption of microbial cell membranes, affecting nucleic acid mechanisms, causing the decay of the proton motive force, and depleting adenosine triphosphate (ATP). They also inhibit enzymatic activity and interfere with membrane function by interacting with bacterial membrane proteins (Burt, 2004; Quinto et al., 2019). These antimicrobial properties have been demonstrated in vitro against a range of foodborne pathogens, including *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 (Mith et al., 2014). However, the successful application of these natural compounds is influenced by factors such as the type of compound, concentration, pH, and the target microorganism (ASM, 2024). Applying natural antimicrobials in the fresh produce industry requires careful implementation of tested methods to maintain their effectiveness and reduce contamination risks. Ongoing studies continue to explore how these compounds perform under various environmental conditions and how they can be incorporated into post-harvest practices.

Essential Oils and Plant Extracts

Essential oils (EOs), extracted from various parts of plants like leaves, flowers, roots, seeds, and bark, have long been valued and recognized for their antioxidant, antimicrobial, and anti-inflammatory properties (Bakkali et al., 2008; Burt, 2004). These oils are natural mixtures of volatile compounds, each adding to their antimicrobial effects, making them effective for pathogen control. Their active components, including thymol, eugenol, and carvacrol, contribute to their efficacy in inactivating foodborne pathogens like *E. coli* O157:H7 and *Salmonella spp* (Pandey et al., 2017). Research shows that EOs demonstrate greater antimicrobial effects on Gram-positive bacteria compared to Gram-negative bacteria. This difference is due to structural differences in their cell walls, which influence EO penetration and efficacy (Gurtler & Garner, 2022). This mode of action inhibits bacterial growth and reduces the potential for resistance development, which is common with synthetic sanitizers.

Several studies have highlighted the potential of some types of EOs as effective post-harvest wash sanitizers for fresh produce. Dunn et al. (2019) evaluated clove bud oil (0.2% and 0.5%) and thyme oil (0.2% and 0.5%) emulsions as sanitizing agents for produce washing, specifically targeting a five-serovar *S. enterica* cocktail. Their findings showed that these essential oil emulsions performed better than chlorine. Additionally, their strong antimicrobial activity, even in the presence of organic matter, indicates they could serve as viable replacements for chlorine in postharvest produce sanitation. Karagözlü et al. (2011) also demonstrated that mint and basil oils, even at low concentrations (0.01 ml/L, 0.032 ml/L, or 0.08 ml/L) significantly reduced *Salmonella* Typhimurium and *E. coli* O157:H7 populations on contaminated fresh-cut lettuce and purslane. Their results showed a reduction of up to 2 log CFU/g in *S.* Typhimurium with a 10-minute basil oil wash at 0.08 ml/L, highlighting the ability of EO to reduce microbial loads. Additional studies have also evaluated the use of oregano oil to decrease *E. coli* O157:H7 and *Salmonella* Typhimurium populations on iceberg lettuce. Gündüz et al. (2010) evaluated the effectiveness of oregano oil at three different concentrations (25, 40, and 75 ppm), along with four different treatment times (5, 10, 15, and 20 min) to inactivate *S.* Typhimurium at 20°C. The results showed that washing lettuce with oregano oil at 75 ppm gave a better decontamination result when compared to washing with 50 ppm of chlorine in the inactivation of *S*. Typhimurium.

Further studies have reinforced the antimicrobial effects of plant extracts, particularly garlic and onions against various foodborne pathogens. A study conducted by Benkeblia (2004) showed that essential oils from different onions and garlic inhibited the growth of *Salmonella* Enteritidis and *Staphylococcus aureus*. The antimicrobial efficacy of these extracts was found to increase with higher concentrations of essential oil. For example, garlic oil at high concentrations (200–500 ml/L) demonstrated a stronger antibacterial effect than onion extracts, with *S.* Enteritidis being more sensitive than *S. aureus*. The findings indicate that garlic oil may be an effective natural antibacterial agent in food safety, especially when used at higher concentrations to enhance microbial reduction in contaminated food. In organic farming, onion and garlic essential oils have potential applications as natural wash solutions to inhibit the growth of foodborne pathogens. *Moringa oleifera* extract has also demonstrated significant antibacterial activity against various pathogens, including *E. coli* O157:H7, and *S. enterica*, which are frequently linked to fresh produce (Abdallah et al., 2023).

Bacteriophage

Bacteriophages or phages are viruses that specifically infect and lyse bacterial cells, thereby providing a natural way to control bacterial contamination in food (Loc Carrillo et al., 2005). Phages are increasingly recognized as a proposed alternative to chemical treatment and antibiotics due to their target specificity, eco-friendliness, and potential as biocontrol agents for detecting pathogenic bacteria within the food industry (García et al., 2008). Their selective action on bacterial pathogens, including *E. coli* O157:H7, makes them useful in fresh produce.

Recent studies have shown the efficacy of the use of bacteriophage in reducing foodborne pathogens in fresh produce. Sharma et al. (2009) reported that a mixture of three *E. coli* O157:H7 specific bacteriophages known as ECP-100 achieved significant reductions in the number of viable *E. coli* O157:H7 populations on experimentally fresh-cut iceberg lettuce and cantaloupe, particularly under modified atmosphere conditions and storage at lower temperatures. ECP-100 treatment on lettuce resulted in a 1.57 to 2.42 log CFU/cm^2 reduction compared to controls, while phage-treated cantaloupes stored at 4°C showed a 2.57 log CFU/ml decrease compared to untreated samples, indicating high efficacy of phage across different fresh produce types. Some of the findings on the use of bacteriophages to reduce pathogen populations on fresh produce suggest that phages can also serve as an alternative antimicrobial agent to control bacterial contamination of agricultural produce in vitro (Wang et al., 2017).

Organic Acids

Organic acids like acetic, propionic, sorbic, lactic, malic, and citric acids are natural antimicrobial agents widely used for their well established efficacy, cost-effectiveness, and ability to prevent the growth of foodborne pathogens (Lucera et al., 2012). They elicit antibacterial activity by lowering environmental and cellular pH, promoting anion accumulation that disrupts microbial cell functions, and inhibiting enzymatic activity and cellular metabolism (Wang et al., 2019). Their antimicrobial properties have led to their recognition as GRAS, making them increasingly valuable as alternatives to traditional chemical sanitizers in fresh produce safety. Combinations of different organic acids have the potential to provide broader antimicrobial activity than relying on a single acid alone (Lucera et al., 2012).

Several studies have highlighted the efficacy of organic acids in reducing bacterial contamination in fresh fruits and vegetables. Akbas and Ölmez (2007) investigated the effectiveness of organic acids (citric and lactic acids), ozonated water, and chlorine in reducing bacteria growth and population, while still maintaining fresh-cut iceberg lettuce storage quality. Lettuce samples were treated with 0.5% citric acid, 0.5% lactic acid, 4 mg/L ozonated water, or 100 mg/L chlorine for 2 minutes. During storage for 12 days, organic acid treatments resulted in lower mesophilic and psychrotrophic bacterial counts compared to ozonated water and chlorine treatments. Lactic acid dipping effectively reduced *Enterobacteriaceae* populations by 2.2 log CFU/g and maintained low levels for the first 6 days of storage. The moisture content, color, β-carotene, texture, and vitamin C levels of the lettuce samples during storage were not shown to have significant changes due to treatment. This study concluded that lactic and citric acid treatments, as well as ozonated water, could serve as alternative treatments to chlorine for extending the shelf life of lettuce.

Similarly, the antimicrobial effects of citric, malic, propionic, lactic, and acetic acid against *E. coli* O157:H7, *L. monocytogenes,* and *S.* Typhimurium, on organic lettuce and apples were also clearly demonstrated by (Park et al., 2011). Lettuce and apples were inoculated and treated with 1% and 2% organic acid solutions for 0, 0.5, 1, 5, and 10 minutes. After 10 minutes of treatment, significant reductions in pathogen populations were observed compared to the control (distilled water). For lettuce, the reductions ranged from 0.93 to 2.98 log CFU/g, depending on the type of acid and its concentration, with citric acid achieving the highest reduction (1.85 to 2.86 log CFU/g). No significant changes were observed in the color of the lettuce samples during storage. These results confirm the efficacy of organic acids in reducing microbial contamination and significantly mitigating pathogen levels when used as part of post-harvest sanitation protocols.

Ascaroside #18 (ascr#18)

While many natural compounds are employed as produce washes to achieve surface decontamination, certain antimicrobials take a different approach by focusing on stimulating the innate immune response of the host plant, rather than surface decontamination. One such novel treatment is ascaroside #18 (ascr#18), a nematode-derived molecule that has gained attention as a pre-harvest treatment that activates plant defense mechanisms. By triggering the immune response of the plant, ascr#18 offers a novel strategy that distinguishes it from other external wash sanitizers while providing a long-term solution for microbial inactivation in fresh produce.

Ascr#18 is an important component of the ascaroside family, an evolutionarily conserved group of signaling molecules secreted by nematodes, particularly plant-parasitic nematodes. It is the most abundant ascaroside and functions effectively even at picomolar to micromolar concentrations (Manosalva et al., 2015). The structure of these ascarosides includes dideoxysugar ascarylose, in addition to lipophilic chains derived from fatty acids, and other components from primary metabolism. They function as molecular pheromones unique to nematodes (Klessig et al., 2019). It was first recognized as a small component in the ascaroside profile of *Caenorhabditis elegans* but has since emerged as the most abundant ascaroside in three different plant-parasitic nematode species, including *Meloidogyne* spp., as demonstrated by Manosalva et al. (2015). They reported that the ascr#18 in these species of nematodes is characterized by an 11-carbon side chain and typically accompanied by compounds with longer carbon chains.

The specific efficacy of ascr#18 in priming plant defenses against pathogens has received much interest in recent research. Its primary mode of antimicrobial action involves metabolism within plants through peroxisomal β-oxidation, generating shorter-chained ascarosides that activate the defense response (Manohar et al., 2020). This activation involves the upregulation of mitogen-activated protein kinases (MAPKs) and modulates defense signaling pathways regulated by salicylic acid (SA) and jasmonic acid (JA), thereby inducing the expression of defense-related genes. Furthermore, plant cells perceive ascr#18 as nematode-associated molecular patterns (NAMP) or pathogen-associated molecular patterns (PAMP), activating immune responses in plant-nematode interaction. At low concentrations, ascr#18 triggers plant immune responses against bacterial, viral, and nematode infections (Manosalva et al., 2015).

Ascr#18 has shown promise as an antimicrobial agent against soilborne pathogens. A study on ascr#18 found that plant treatment with ascr#18 inhibited *Pseudomonas syringae pv.* Tomato (*Pst*) on the *Arabidopsis* roots, highlighting its antibacterial activity against the soilborne pathogen (Manosalva et al., 2015). Recent studies have expanded to explore the effectiveness of ascr#18 in controlling foodborne pathogens in agricultural settings. It has effectively reduced *S. enterica* levels in fenugreek and alfalfa sprouts and seeds. However, its efficacy has not yet been thoroughly evaluated under field conditions, which presents a gap in understanding its potential at a commercial scale. This study highlights the potential of ascr#18 as an effective treatment to improve the microbial safety of vegetable seeds and sprouts (Hu et al., 2023).

The efficacy of ascr#18 as a preharvest treatment is highly dependent on its concentration, with low doses often being more effective in priming plant defenses against pathogens. A study by Manosalva et al. (2015) showed that while ascr#18 enhanced *Arabidopsis* resistance to *Pst* and provided protection against *Phytophthora infestans* in tomato at lower concentrations, its effectiveness decreased at higher concentrations. Such dose-response effects are commonly observed in other plant signaling compounds. The optimal concentration of ascr#18 for plant treatments is important to maximize its protective effects.

Ascr#18 can be applied to plants through soaking, soil drenching, or foliar spraying. Soil drench involves applying the compound directly to the root zone, allowing for systemic distribution, while foliar application involves spraying the compound on the leaves, potentially enabling more immediate, localized responses (Schroeder, 2014). Ascr#18 activates defense mechanisms by triggering MAPKs and modulating hormone signaling pathways, which could potentially lead to metabolic costs for the plant if not optimally applied (Manosalva et al., 2015). The optimal application method may vary depending on the crop type and target pathogen.

Conclusion

Contamination of leafy greens with human pathogens continues to cause foodborne illnesses due to its cultivation practices and surface characteristics, despite current agricultural practices and regulations. Conventional sanitizers have shown limitations in efficacy and raise environmental concerns, prompting the need for natural alternatives.

Ascr#18, a novel intervention, has demonstrated the potential to elicit plant defenses and reduce pathogen contamination in numerous studies. However, there remains a gap in research specifically addressing the efficacy of ascr#18 in protecting leafy greens against foodborne pathogens. This study seeks to evaluate ascr#18 efficacy as a pre-harvest treatment against *E. coli* O157 on romaine lettuce and relate these findings to its potential as a natural antimicrobial agent. The second objective is to investigate the optimal method of ascr#18 application on plants through foliar spray and soil drench techniques. We will also use this experiment to determine the best method of applying the ascr#18 treatment to other leafy greens. The result of this study could be crucial to developing more effective food safety interventions and enhancing the microbial safety of fresh produce when combined with good agricultural practices.

CHAPTER 3

MATERIALS AND METHODS

Bacterial cultures

A four-strain cocktail of pathogenic *E. coli* O157:H7 was obtained from -80°C glycerol stocks from the isolate collection of the Department of Food Science and Technology, University of Georgia, Athens. The strains included CDC 658 (human feces, cantaloupe-associated outbreak disease), F4546 (human feces, alfalfa sprout-associated disease), H1730 (human feces, lettuce-associated disease) (Megan M Lang et al., 2004) and K3995 (clinical isolate, spinach-associated outbreak) (Zangari et al., 2014).

E. coli O157:H7 strains were streaked on CHROMagar O157 (CHROMagar Microbiology, Paris, France) and then cultured overnight in Tryptic Soy Broth (TSB; Difco, Sparks, MD) at 35°C. Rifampicin resistance was developed by transferring 10 µL of culture to fresh TSB and spreading 0.1 mL aliquots on Tryptic Soy Agar (TSA; Difco, Sparks, MD) plates with increasing rifampicin concentrations, starting at 12.5 mg/mL and doubling until resistance was achieved at 50 mg/mL (Weinstein & Zaman, 2019). Rifampicin-resistant strains were stored with 50% glycerol in 2 ml microcentrifuge tubes (ThermoFisher Scientific, Waltham, MA) at −80 until use.

A growth curve assay was performed using a microplate reader (Biotek, Winooski, VT) to test for growth inhibition in each of the four *E. coli* O157:H7 strains and evaluate differences between the wild-type and rifampicin-adapted strains. Cultures were grown overnight in TSB plate with 50 mg/mL of rifampicin (TSB-R) at 35°C. Diluted cultures (200 µL) were added to individual wells of a 96-well plate (Falcon). OD600 readings were taken every 60 minutes for 24 hours at 35°C with continuous shaking between readings. Growth curve data showed no difference in the growth of the different strains.

To prepare the inoculum, frozen glycerol stock cultures were revived by transferring them into TSB-R and incubating overnight at 35°C. The overnight cultures were then streaked onto a TSA plate supplemented with 50 mg/ml rifampicin (TSA-R) and incubated again at 35°C overnight. Colonies were picked with a sterile loop onto 10 ml of TSB-R and incubated for 24h at 35°C. Before use, 1 ml of each of the cultures was transferred to 2 ml microcentrifuge tubes and centrifuged at 12,000 RCF for 2 minutes. Supernatant was decanted and pellets were washed with 1 ml of 0.1% peptone. Cultures were washed a total of three times and resuspended in 9 ml of 0.1% peptone for a total volume of 10 ml. A 1 ml aliquot from each tube was combined with 36 ml of 0.1% peptone for an approximate final concentration of 7 log CFU/mL. The final suspension was serially diluted and enumerated using an Eddy Jet 2 spiral plater (Neutec, Farmingdale, NY, USA) on TSA-R plates and incubated for 24 hours at 35°C.

Lettuce seeds

Romaine lettuce (*Lactuca sativa* L, var. Dragoon, cv. Green mini) seeds were purchased from Johnny's Selected Seeds (Winslow, ME) and stored at 4 °C before use for this experiment. The Dragoon variety is a mini-romaine well suited to greenhouse production due to its compact and uniform size, with a seed to harvest cycle of 43 days. Plants were grown in a climate-controlled greenhouse at the University of Georgia Horticulture Farm (Athens, GA), with temperature setpoints of approximately 20 °C at night and 27 °C during the day. Relative humidity averaged around 65% during the day and 83% at night, though actual conditions varied slightly depending on weather and time of day. Seeds were sown in cell trays filled with Miracle-Gro potting mix and irrigated every other day. After 4 weeks, seedlings were transplanted into ½ gallon square pots (5.5 in x 5.5 in x 6 in). Ascr#18 plant treatments were applied after transplanting when the plants were about 6 weeks old. Lettuce heads were randomly assigned to three different ascr#18 concentration treatments and control groups. Each treatment had three independent replicates with three independent samples (n=9).

Ascr#18 preparation

Ascr#18 stock solutions (10 µM and 100 µM) were provided by Ascribe Bioscience Inc. and stored at 4°C. Working solutions of 0.01 µM, 0.1 µM, and 1 µM were prepared by diluting the stock in 0.1% (v/v) Tween-20, a non-ionic surfactant. For the 0.1 µM and 1 µM concentrations, 1 mL of the stock was diluted in 99 mL Tween-20 solution, while the 0.01 µM solution was prepared by further diluting 1 mL of the 0.1 µM solution (1:10). All working solutions were stored at 4°C before plant treatment.

Plant treatment

Lettuce plants were treated with the ascr#18 formulation via foliar spray or soil drench (3 mL per plant), except for the control groups. The formulation was evenly applied on the leaf surfaces using a spray bottle for the foliar spray. For the soil drench application, it was applied directly to the plant root zone. The treatments were conducted three times; 7-10 days after the plants were transplanted, 2 days pre-inoculation, and 2 days post-inoculation (Figure 1). Before inoculation, plants were moved from the horticulture greenhouse to an environmental chamber (24°C, 70% RH) located in the Department of Food Science and Technology, University of Georgia, Athens BSL-2 lab. A water treatment control was also included to ensure physical removal is not the cause of reduction of *E. coli* O157:H7 populations inoculated onto romaine lettuce.

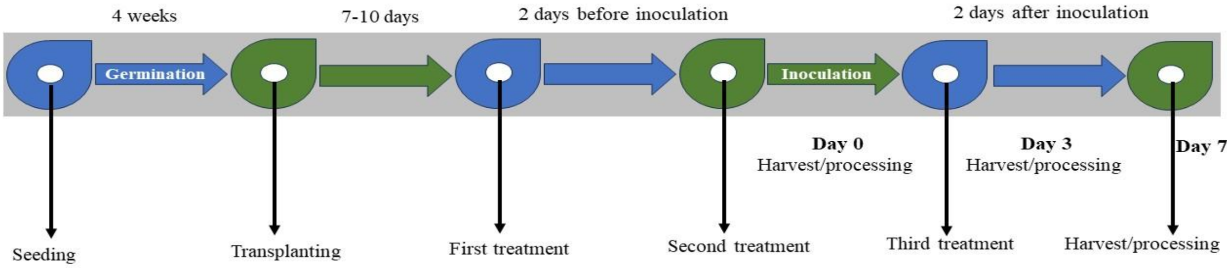


Figure 1: Diagram illustrating the application schedule for the ascr#18 plant-based treatment and *E. coli* O157:H7 inoculation onto romaine lettuce plants.

Lettuce inoculation

Romaine lettuce heads were individually placed inside the biosafety cabinet. A 100 µL aliquot of the inoculum was spot inoculated onto 10 different locations on each head using a micropipette (Corning) to ensure consistent application of the inoculum and allow more accurate determination of the reduction in the pathogen population (M. M. Lang et al., 2004). The lettuce heads were allowed to air dry for 1 h inside the biosafety cabinet. Three biological replicates from each treatment method and control group were harvested and processed on Day 0, with the remaining lettuce heads returned to the environmental chamber (Percival Scientific Inc., Perry, IA) for later processing on Days 3 and 7.

Microbial analysis

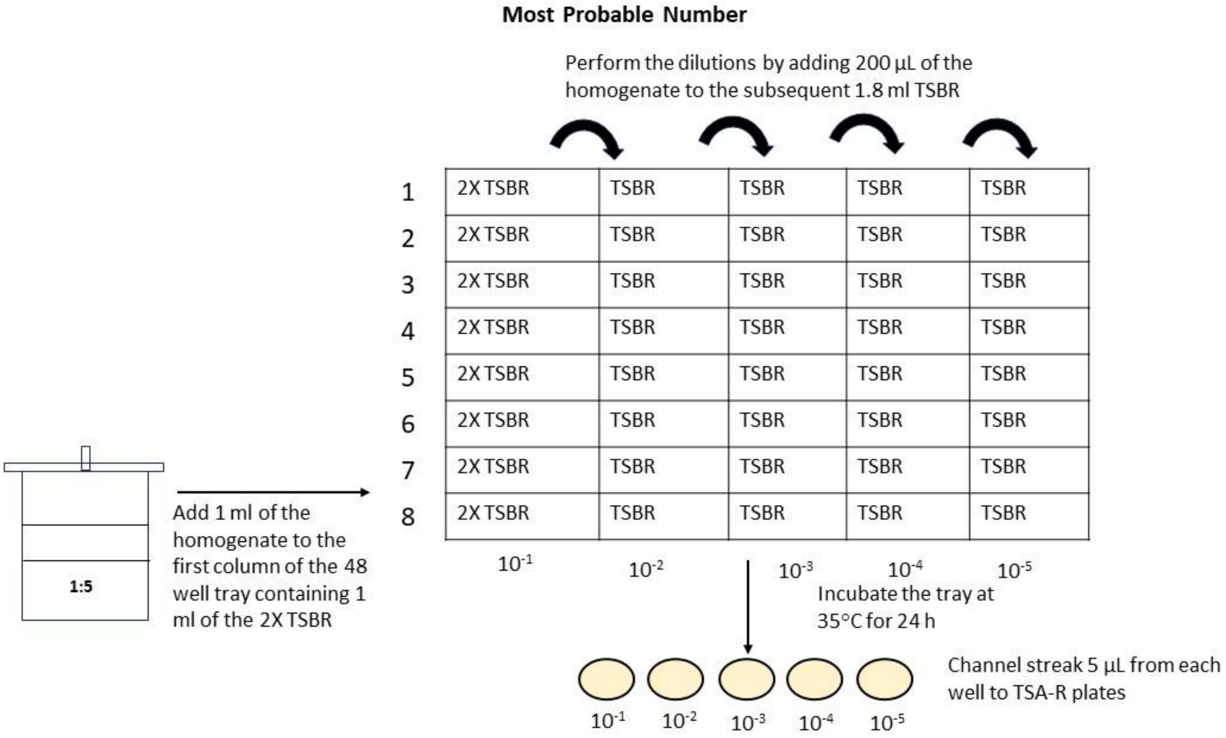
To evaluate ascr#18 efficacy, lettuce heads were harvested using pruning shears decontaminated between cuttings with 70% ethanol. Harvested leaves were carefully placed in a sterile Whirl-pak® (VWR International, Radnor, PA). Each sample was rinsed with 250 ml of 0.1% peptone and Tween-80 (Sigma-Aldrich Co, St. Louis, MO) solution. Samples were hand massaged for 60 seconds, after which the rinsate was spiral plated on TSA-R plates using an Eddy Jet 2 spiral plater (exponential-50 µL setting). Colonies on plates were counted after incubating for 24 h at 35°C.

Recovered bacterial populations were estimated using the Most Probable Number (MPN) procedure with 48 deep-well plates when counts fell under the plating limit of detection (Figure 2). Wells were filled with TSB-R, comprising five dilutions and eight replicates. The first row contained 1 ml double-strength TSB-R, while others had 1.8 ml single-strength TSB-R. A 1ml aliquot of the rinsate was added to double-strength wells, followed by four tenfold serial dilutions (0.2 ml into 1.8 ml). Plates were sealed with adhesive film (allowing air exchange) and incubated at 35°C for 24 h, then 5 µL from each well was channel streaked onto TSA-R plate and incubated again at 35°C for 24 h. Positive and negative wells were recorded and the MPN results were calculated using an online calculator (EPA, 2020). The results were expressed as the amount of *E. coli* O157:H7 per lettuce head (MPN/Head) with 95% confidence intervals. The raw MPN/mL values were converted to per head estimates by multiplying by the total rinsate volume (250 mL). These were then log transformed and reported as log MPN/Head. The detection limit was 1.45 log MPN/Head of *E. coli* O157:H7. Samples for days 3 and 7 were harvested and processed as previously described.

Statistical Analysis

The experiment consisted of two application methods, four treatment groups with three biological replicates each, three sampling points (days 0, 3, and 7), and three independent experimental replications, resulting in a total sample size of 216. For each treatment group, nine samples were analyzed per experimental replication, with three samples collected per sampling day (n = 9). Data collected were analyzed using the mixed model ANOVA in JMP Pro 17 (SAS

Institute Inc., Cary, NC). Tukey’s test was used for pairwise comparisons when significant differences were detected. Analyses assessed differences ($P < 0.05$) between treatment and control groups.



1	2X TSBR	TSBR	TSBR	TSBR	TSBR
2	2X TSBR	TSBR	TSBR	TSBR	TSBR
3	2X TSBR	TSBR	TSBR	TSBR	TSBR
4	2X TSBR	TSBR	TSBR	TSBR	TSBR
5	2X TSBR	TSBR	TSBR	TSBR	TSBR
6	2X TSBR	TSBR	TSBR	TSBR	TSBR
7	2X TSBR	TSBR	TSBR	TSBR	TSBR
8	2X TSBR	TSBR	TSBR	TSBR	TSBR
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}

FIGURE 2. Schematic diagram of MPN procedure (Bardsley et al., 2021)

CHAPTER 4

RESULTS

Reduction of *E. coli* O157:H7 on ascr#18-treated Romaine lettuce

The fixed effects of ascr#18 on *E. coli* O157:H7 populations on romaine lettuce were analyzed using ANOVA (Table 1). Application method (foliar spray), treatment concentration, and sampling day each had a significant impact on *E. coli* O157:H7 populations, although their interactions were not significant ($P > 0.05$). Similarly, when ascr#18 treatment was applied as a soil drench, neither treatment concentration, sampling day, nor their interaction had a significant effect on *E. coli* O157:H7 populations ($P > 0.05$). Overall analysis considering both foliar spray and soil drench methods revealed that application method, treatment concentrations, and the interaction between treatment concentration and application method were all significant ($P \leq 0.05$) factors influencing *E. coli* O157:H7 reductions on romaine lettuce in this study.

Effect of application method

Figure 3 presents the mean populations of *E. coli* O157:H7 on romaine lettuce following treatment with ascr#18 at different concentrations, comparing foliar spray and soil drench application methods. A consistent trend is evident across all tested concentrations (0.01 µM, 0.1 µM, and 1 µM). The foliar spray application resulted in significantly higher *E. coli* O157:H7 reduction compared to the soil drench application method ($P < 0.05$). On average across all days (0, 3, and 7), foliar spray at 0.01 µM, reduced *E. coli* O157:H7 populations to 3.5 log MPN/Head, compared to 5.5 log MPN/Head population for the soil drench treatment. At 0.1 µM, foliar spray-treated lettuce had populations of 2.9 log MPN/Head, while soil drench-treated lettuce had populations of 5.3 log MPN/Head. Similarly, at 1 µM, populations decreased to 3.2

log MPN/Head for foliar spray, whereas soil drench remained at 5.4 log MPN/Head ($P < 0.05$). Although increases in ascr#18 concentration from 0.01 µM to 1 µM resulted in slight reductions in pathogen populations for both application methods, the differences between concentrations within each method were not statistically significant ($P > 0.05$). The foliar spray consistently reduced *E. coli* O157:H7 to below or near 3.5 log MPN/Head across all tested concentrations, while the soil drench method averaged approximately 5 log MPN/Head final populations.

Table 3. Fixed effect of ascr#18 in reducing *E. coli* O157:H7 populations on romaine lettuce when applied as a foliar spray or soil drench ($\alpha = 0.05$).

Source	**Nparm**	**DF**	**Sum of Squares**	**F ratio**	**Prob > F**
Foliar spray					
Treatment	3	3	56.06	7.88	<.0001
Day	2	2	27.47	5.79	0.0042
Treatment*Day	6	6	5.36	0.38	0.8925
Soil drench					
Treatment	3	3	7.06	2.54	0.0590
Day	2	2	1.00	0.55	0.5810
Treatment*Day	6	6	4.51	0.81	0.5617
Overall analysis (Foliar + Soil Drench)					
Method	1	1	223.87	140.73	<.0001
Treatment	3	3	54.69	11.46	<.0001
Method*Treatment	3	3	15.42	3.23	0.0230

DF: Degree of freedom, Nparm: Number of Parameters.

Pr > F: *P* value, Effects are considered significant when *P* value ≤ 0.05.

Sample size: 216 =2 application method × 4 treatment groups × 3 time points (day 0, 3, 7) × 3 biological replicates × 3 independent experiments

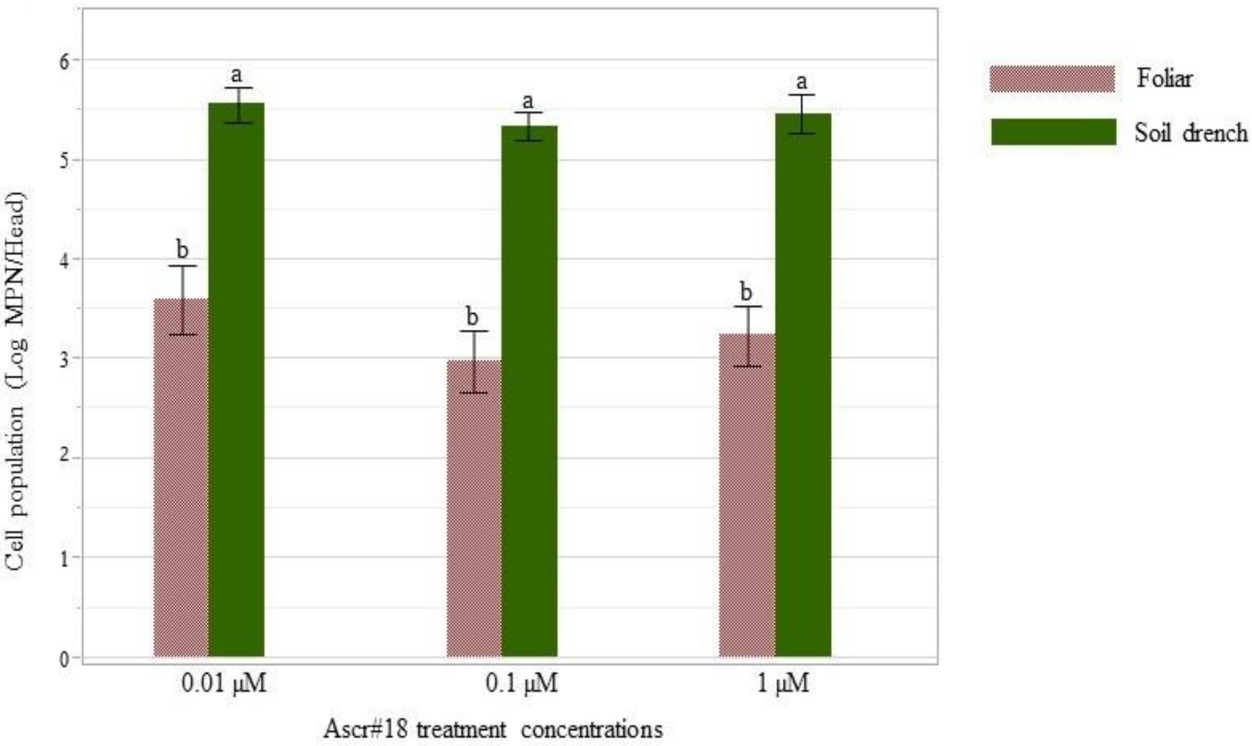


Figure 3. Mean populations of *E. coli* O157:H7 on romaine lettuce treated with different ascr#18 concentrations (0.01 µM, 0.1 µM, 1 µM) using foliar spray and soil drench application methods. Means values with different letters were significantly different (P < 0.05; limit of detection = 1.45 log MPN/Head). Error bars indicate standard error.

Impact of ascr#18 treatment concentrations.

In this study, all ascr#18 treatments (0.01 µM, 0.1 µM, and 1 µM) resulted in reductions of *E. coli* O157:H7 populations when compared to its untreated controls over a 7-day sampling period (0 d, 3 d, and 7 d). The extent of reduction of ascr#18 treatment concentrations varied across the sampling period (Table 4).

Significant reductions in *E. coli* O157:H7 were observed on Days 0 and 3 for the foliar spray application method ($P < 0.05$; Table 4). In comparison to the untreated control, *E. coli* O157:H7 reductions ranged from 1.11-2.21 log MPN/head on Day 0. Treatment at 0.1 µM ascr#18 showed the highest reduction of *E. coli* O157:H7, with a decrease of 2.21 log MPN/head. However, ascr#18 treatments at 0.01 µM and 1 µM resulted in *E. coli* O157:H7 reductions of 1.11 and 1.58 log MPN/head, respectively. By Day 3, all foliar treatments demonstrated reductions between 1.86-2.05 log MPN/head. While the 0.1 µM treatment maintained a relatively higher microbial reduction (2.05 log MPN/head) than other treatments, differences between treatment concentrations were not significantly different ($P > 0.05$). By Day 7, microbial reductions decreased, suggesting a potential decline in antimicrobial effectiveness over time. The 0.01, 0.1, and 1 µM ascr#18 treatment reduced the population of *E. coli* O157:H7 by 0.72, 1.35, and 1.41 log MPN/head, respectively. The microbial reduction obtained was greater for 0.1 µM ascr#18 treatment across Days 0 and 3. However, no significant differences ($P > 0.05$) were observed among the ascr#18 treatment concentrations or between the treatments and the control used in the study on Day 7 (Fig. 4).

Ascr#18 treatment applied through soil drench showed smaller reductions in *E. coli* O157:H7 across the sampling period (Fig. 5); the population differences between the ascr#18 treatment and the untreated controls ranged from 0.22 to 0.77 log MPN/head on Day 0. The 0.1 µM treatment exhibited the highest reduction (0.77 log MPN/head), followed closely by the 1 µM treatment (0.72 log MPN/head), while the 0.01 µM treatment showed the least reduction (0.22 log MPN/head). However, there were no significant differences in reductions among the treatment concentrations ($P > 0.05$). By Day 3, reductions remained within a similar range (0.48–0.76 log MPN/head), with the highest reduction observed at 1 µM treatment, followed by 0.1 µM treatment, which showed a reduction of 0.58 log MPN/head. By Day 7, reductions generally decreased further among all the ascr#18 treatment concentrations tested, ranging from -0.07–0.41 log MPN/head. The 1 µM treatment showed a slight rebound in bacterial populations, resulting in a small increase in *E. coli* O157:H7 compared to the untreated control (-0.07 log MPN/head). Overall, the greatest reduction in ascr#18 treated inoculated romaine lettuce was observed with 0.1 µM treatment on Day 0. No significant differences ($P > 0.05$) were observed between the soil drench treatments and the control on Days 0, 3, and 7 (Fig. 5).

Table 4. Microbial reduction (Log MPN/Head) on romaine lettuce in comparison to ascr#18 treatment concentration and sampling time (*n* = 9)

		Log *E. coli* O157:H7 reductions (Log MPN/Head)		
Treatment		**Day 0**	**Day 3**	**Day 7**
Foliar				
	0.01 µM	1.11	1.95	0.72
	0.1 µM	2.21	2.05	1.35
	1 µM	1.58	1.86	1.41
Soil drench				
	0.01 µM	0.22	0.48	0.41
	0.1 µM	0.77	0.58	0.41
	1 µM	0.72	0.76	-0.07

The log reduction represents the log difference between the mean viable cell counts of the control and treatment groups (Log MPN/Head). Control and treatments were compared separately for each sampling point (Days 0, 3 & 7). Control, inoculated but not treated; ascr#18 treatment concentration, 0.01 µM, 0.1 µM, 1 µM.

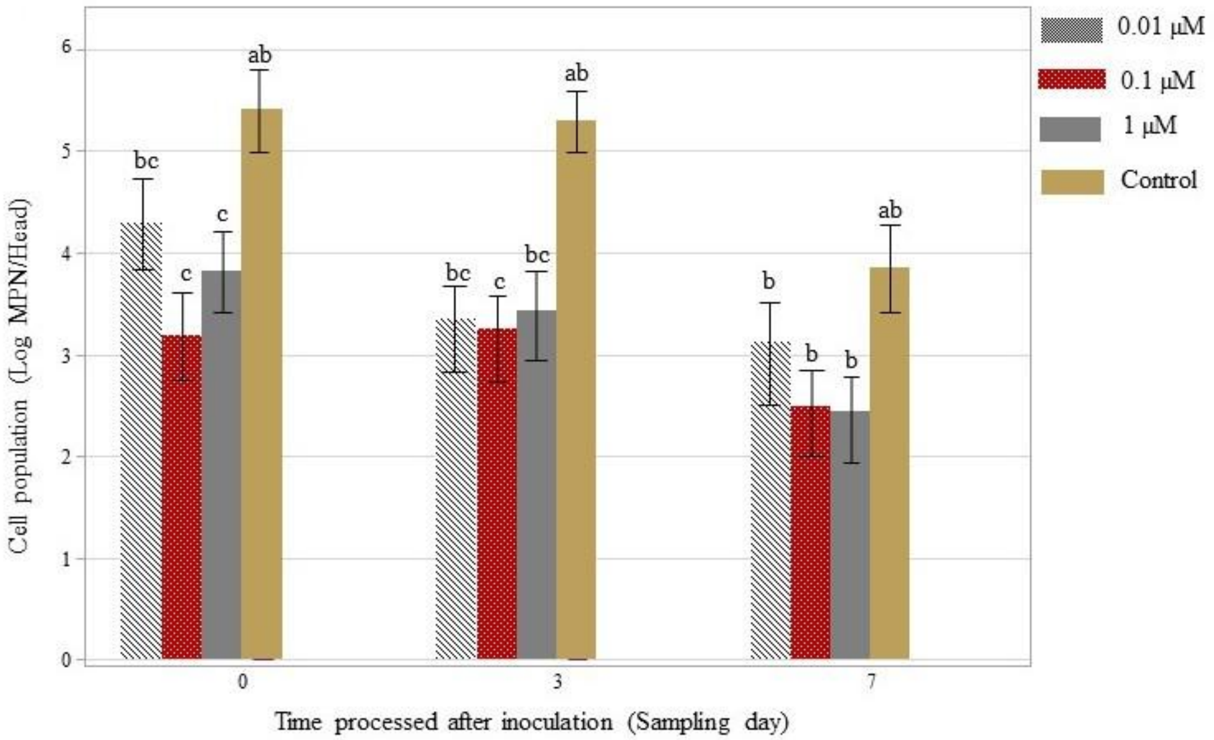


Figure 4. Mean cell populations of *E. coli* O157:H7 inoculated on romaine lettuce (*n* = 9) treated via foliar application across different ascr#18 concentration treatments (0.01, 0.1, 1 µM) at each sampling point (Days 0, 3, & 7). Limit of detection = 1.45 log MPN/Head. Each bar diagram represents average counts and error bars indicate standard error.

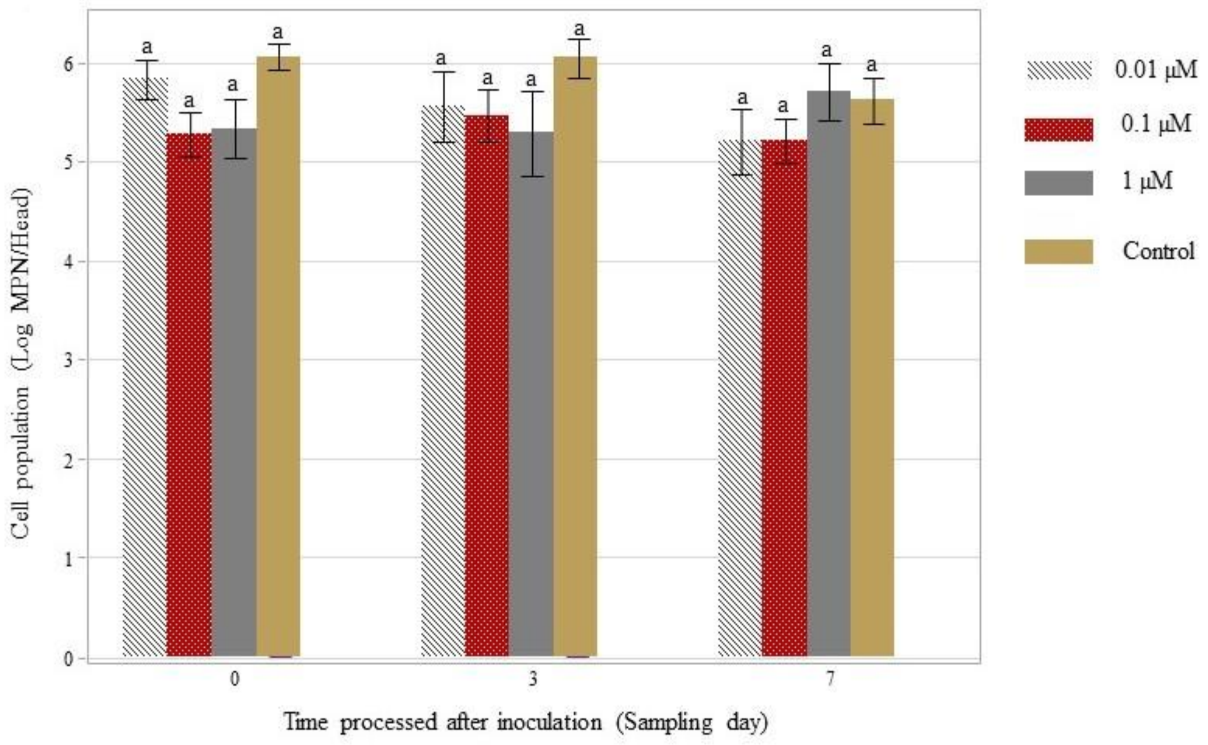


Figure 5. Mean cell populations of *E. coli* O157:H7 inoculated on romaine lettuce (*n* = 9) treated via soil drench application across different ascr#18 concentration treatments (0.01, 0.1, 1 μM) at each sampling point (Days 0, 3, & 7). Limit of detection = 1.45 log MPN/Head. Each bar diagram represents average counts and error bars indicate standard error.

CHAPTER 5

DISCUSSION

Ascarosides, including ascr#18, function similarly to microbe-associated molecular patterns (MAMPs) by triggering plant immune responses, which help reduce the impact of pathogen attacks and nematode infections (Manohar et al., 2020). The immune-stimulating property of ascr#18 has been shown to enhance the resistance of various plants such as tomato, potato, rice, wheat, soybean, and *Arabidopsis*, against nematodes, bacteria, fungi, and viruses (Klessig et al., 2019). Recent studies have further demonstrated the antimicrobial potential of ascr#18 in food safety applications. For instance, Hu et al. (2023) reported that ascr#18 treatment at 1 µM applied as a seed treatment or immersion, reduced *Salmonella* populations on alfalfa and fenugreek sprouts. Similarly, the efficacy of ascr#18 treatment for controlling enterohemorrhagic *E. coli* (EHEC) growth on sprouts has also been established (Hu et al., 2024). In the current study, we extend these findings to romaine lettuce, where foliar application of ascr#18 reduced *E. coli* O157:H7 populations by 1 - 2 log MPN/head.

According to the results in Figure 3, ascr#18 method of application is a significant factor in reducing the population of *E. coli* O157:H7 populations on contaminated romaine lettuce. While previous studies have primarily focused on root treatment, soil drench, or immersion methods for ascr#18 to control both soilborne and foodborne pathogens, foliar spray applications also show promise. Foliar sprays are widely used in leafy green production for various treatments, including nutrient supplementation and fertilizer application (Kathi et al., 2024; Nayak et al., 2020). Treatment with ascr#18 using foliar spray application was more effective than the soil drench in all tested concentrations in this present study. Interestingly, using the foliar application approach, Klessig et al. (2019) made a similar observation that foliar applications of ascr#18 at concentrations ranging from 0.01–10 µM effectively controlled plant pathogens in non-sprout plant tissues such as maize, rice, wheat, and soybean. The superior performance of foliar application in this current study may be attributed to the primary occurrence of *E. coli* O157:H7 contamination on leaf surfaces. In a study by Manosalva et al. (2015), leaf infiltration of ascr#18 at 0.3 µM or 1 µM activated both local and systemic defenses in *Arabidopsis,* demonstrating that plants respond to ascr#18 via their leaves. This response was comparable to that observed with root application. Our results are also consistent with previous research on the efficacy of the application methods for natural antimicrobials. It was found that spraying lytic bacteriophages onto lettuce after hypochlorite washing resulted in greater immediate reductions of *E. coli* O157:H7 populations (2.22 log CFU/cm^2 on day 0) compared to immersion method (Ferguson et al., 2013). Similarly, the current study showed that foliar sprays of ascr#18 achieved reductions of 1.5 - 2 log MPN/head reduction compared to less than 1 log MPN/head reduction for soil drench applications. Additional work is needed to evaluate whether combining both application methods could achieve a greater reduction of *E. coli* O157:H7 populations on leafy greens.

All tested concentrations of ascr#18 (0.01 µM, 0.1 µM, and 1 µM) significantly decreased the *E. coli* O157:H7 populations on romaine lettuce for the foliar spray application. No clear dose-response relationship was observed, as all ascr#18 treatments were significantly effective when compared to the control treatment (Fig. 4). Ascr#18 has been shown to effectively reduce pathogens across *Arabidopsis* roots (Manohar et al., 2020), and fenugreek and alfalfa sprouts (Hu et al., 2023), targeting soilborne and human pathogens respectively, at lower concentrations. These findings show ascr#18 efficacy at low doses, achieving pathogen reductions comparable to a postharvest wash with chlorine or PAA. However, there exists no clear dose-response, preventing the determination of an optimal dose for pre-harvest pathogen control in fresh produce. The lack of a dose-response relationship in this study may also have been due to the narrow range of concentrations tested. A previous study found that a 1 µM ascr#18 treatment on *Arabidopsis* roots significantly reduced virulent *Pseudomonas syringae* populations, while higher concentrations (5 µM) were less effective (Manosalva et al., 2015). The results of this study align with previous studies demonstrating that ascr#18 activates plant immunity, enabling protection against pathogens even at very low doses.

The current study found that ascr#18-induced reduction of *E. coli* O157:H7 on romaine lettuce was most pronounced on Day 0. Foliar spray application achieved up to 2.21 log MPN/head with the 0.1 µM concentration on Day 0, outperforming soil drench treatments, which achieved maximum reductions of 0.77 log MPN/head on Day 0 (Table 3). Over time, the efficacy of both application methods diminished, with no significant differences detected between the treatments and the control by Day 7. Other studies on plant defense elicitors have also demonstrated a temporal decline in efficacy when used to inhibit soilborne pathogens. For instance, Cohen et al. (2011) observed that resistance induced by DL-3-amino-butyric acid (BABA) against *Bremia lactucae* in lettuce declined over time, with treatment failing to prevent sporulation 5 days post-inoculation. Manosalva et al. (2015) also demonstrated that ascr#18-induced defenses peak within 24–72 hours post-treatment, with diminishing systemic effects beyond this window. In *Arabidopsis*, root pretreatment with ascr#18 induced significant upregulation of defense-related genes (FRK1, PHI1, PR-4, and LOX2) within 6–24 hours, but these responses waned by 48-96 hours, suggesting transient activation of plant immunity which is common in pattern-triggered immunity (PTI), where plants respond to pathogen-associated molecular patterns (PAMPs). While the current study used both preventive (applied two days before inoculation) and curative (applied two days after inoculation) treatments, the highest reduction in *E. coli* O157:H7 populations was observed on Day 0, following preventive treatment alone, highlighting that ascr#18 is better in priming the plant defenses prior to challenge from inoculation. The observed decline in efficacy at later sampling points may reflect the rapid metabolism and degradation of ascr#18 in planta, as studies have shown that approximately 50% of ascr#18 is metabolized within 12 hours in *Arabidopsis* leaves, with further conversion into shorter-chain ascarosides (e.g., ascr#9) and eventual dissipation by 96 hours (Manohar et al., 2020). Further study should be done using curative treatment alone, particularly when inoculation precedes ascr#18 application to better understand whether plants can still activate defensive response or exhibit immunity to control pathogens under these conditions.

Although ascr#18 treatment at the tested concentrations reduced *E. coli* O157:H7 populations on romaine lettuce (Table 4), the observed reductions varied across application methods. These findings emphasize the complexity of controlling pathogen growth in produce (Alvaro et al., 2009; Aryal et al., 2024). Chlorine-based treatments remain the standard for reducing microbial loads on fresh produce surfaces by inactivating pathogens to prevent cross-contamination, concerns over chemical residues and byproduct formation limit their widespread acceptance. For ascr#18 to emerge as a practical alternative, it must demonstrate consistent efficacy across varying conditions while offering benefits like ease of use and minimal impact on produce quality. Unlike other novel interventions such as bacteriophages (García et al., 2008) and essential oils (Dunn et al., 2016), ascr#18 stands out for its ability to prime plant immune defenses. Its application as a pre-harvest treatment could add an extra layer of protection, reducing pathogen risks early rather than relying solely on post-harvest interventions to control contamination. As the demand for safer and more sustainable food safety solutions grows, ascr#18 may offer a promising option for leafy green growers. Future studies are needed to evaluate the long-term benefits of ascr#18 in commercial settings.

CHAPTER 6

CONCLUSION

With the persistent challenge of foodborne outbreaks associated with fresh produce, particularly leafy greens, ongoing development and research into preharvest interventions along with GAPs are important to minimize microbial contamination. This study demonstrated that ascr#18 effectively reduced *E. coli* O157:H7 levels on romaine lettuce, providing strong evidence for its potential as a pre-harvest treatment for growers. Unlike post-harvest washing methods, which primarily remove surface pathogens, ascr#18 targets contamination at its source in the field and reduces risk before harvest. However, ascr#18 is not intended to replace post-harvest washing but rather to serve as an additional layer of protection.

While these results also highlight the greater efficacy of foliar spray applications compared to soil drench methods, further studies are required to determine the optimal concentrations for maximum pathogen reduction. Expanding studies to assess a broader concentration range is necessary for its application in the fresh produce industry. In the future, ascr#18 could be a valuable preharvest strategy to improve the safety of fresh produce and reduce the risk of foodborne illness.

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APPENDIX

FIGURE 6: Image of romaine lettuce (var. Dragoon) plants growing in the University of Georgia Horticulture Farm greenhouse.

TABLE 5. The connecting letters report for application methods at different ascr#18 treatment concentrations. Mean cell population values with different letters indicate significant differences ($p<0.05$).

Treatment	Level			Mean
0.01 µM	Soil drench	A		5.5397
	Foliar		B	3.5775
0.1 µM	Soil drench	A		5.3231
	Foliar		B	2.9647
1 µM	Soil drench	A		5.4441
	Foliar		B	3.2208

TABLE 6. The connecting letters report for foliar spray treatments. Mean cell population values with different letters indicate significant differences ($p<0.05$).

Day	Level			Mean
0	Control	A		5.3929
	0.01 µM	A	B	4.2773
	1 µM		B	3.8124
	0.1 µM		B	3.1763
3	Control	A		5.2898
	1 µM	A	B	3.4261
	0.01 µM		B	3.3378
	0.1 µM		B	3.2360
7	Control	A		3.8388
	0.01 µM	A		3.1174
	0.1 µM	A		2.4818
	1 µM	A	B	2.4241

TABLE 7. The connecting letters report for soil drench treatments. Mean cell population values with different letters indicate significant differences ($p<0.05$).

Day	Level		Mean
0	Control	A	6.0645
	0.01 µM	A	5.8395
	1 µM	A	5.3375
	0.1 µM	A	5.2867
	Control	A	6.0547
3	0.01 µM	A	5.5670
	0.1 µM	A	5.4690
	1 µM	A	5.2908
	1 µM	A	5.7039
7	Control	A	5.6261
	0.1 µM	A	5.2136
	0.01 µM	A	5.2125