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Efficacy of Antimicrobial Mitigation on Escherichia coli CFU and Growth and Development of Hydroponic Leafy Greens

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Efficacy of Antimicrobial Mitigation on *Escherichia coli* CFU and Growth and Development of Hydroponic Leafy Greens

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by

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Abstract

The Food and Drug Administration (FDA) have set new standards that apply to agriculturalists producing crops eaten fresh and/or raw by consumers. This new produce safety rule, known as the Food Safety Modernization Act (FSMA), has established science-based standards for all areas of production in agriculture with regards to microbial contamination. *Escherichia coli* (*E. coli*) O157:H7 is a particular bacterium of concern under FSMA guidelines. Grower compliance is mandatory and therefore vital to the continuation of any farm. Greenhouse hydroponic growers have shown advantages in efficiency when compared to conventional farming methodology. Those, however, with recirculating hydroponic systems face unique challenges when faced with a foodborne bacteria like *E. coli*. Recycling water also recycles pathogens, which can lead to complete crop infection. Traditional methods of eradication are complex and costly. Saponins are natural non-ionic detergents occurring as secondary metabolites in a multitude of plant species. Saponins exhibit many biological properties, one of which is as an antimicrobial. In this study we sought to examine the antimicrobial properties of saponins on *E. coli* and how it relates to plant growth and development of 'Rex' lettuce in a hydroponic NFT system.

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Introduction

A growing concern in agricultural production of raw produce is food safety. The Food and Drug Administration (FDA) has set new standards that apply to all agriculturalists producing crops eaten fresh and/or raw by commercial operations equaling or exceeding $25,000 (USD) per year (FDA, 2011). The FDA has named the new protocol the Food Safety Modernization Act Produce Safety rule (FSMA), which establishes science-based minimum standards for the safe growing, harvesting, packing, and holding of fruits and vegetables grown for human consumption (FDA, 2011). The FDA has put forth these standards in an effort to improve public health while minimizing the onus of responsibility to the American farmer through process validation and infrequent inspections. Signed into law in 2011, it is the first major overhaul of the nation's food safety systems since 1936, shifting the focus of federal regulators toward preventative measures with regards to food safety problems rather than reacting to epidemics after the fact (FDA, 2011).

Fresh, raw produce is part of a healthy diet. Consuming multiple produce items on a daily basis should be promoted as a healthy lifestyle (Ceuppens, 2015; He et al., 2007). In the United States, approximately 50% of all adults (117 million people) have a chronic health condition (CDC, 2018). Many of these conditions are dietary related. Unhealthy eating and physical inactivity have become the leading causes of death in the United States (CSPI, 2018). Between the years 2011 to 2014, greater than 30% of adults were deemed obese, while 15% of children (ages 2-19) were labeled the same (CDC, 2018). In the last three decades, obesity rates have doubled in adults (Fryar, Carroll and Ogden, 2015), tripled in children (CDC, 2018), and quadrupled in adolescents (Fryar, Ogden and Carroll, 2012).

The natural and biological changes related to ageing are manifested by molecular damage incurred upon the cells and organs that make up the human body. Inevitably, the cells' ability to synthesize life sustaining energy is altered (Stanner, 2009). Healthy diets and physical activity are vital to a long and healthy life as they have the potential to shape the rate at which damage to the cells is accumulated, as well as the cells' ability to repair this damage (Stanner, 2009). Consuming a diet high in fresh fruits and vegetables has been shown to reduce the risk for certain chronic diseases like cancer, coronary heart disease, and diabetes (He et al., 2007; WHO, 2002). These commodities should be readily available in every household without fear of foodborne illnesses upon consumption.

Every year 48 million Americans become infected with a foodborne disease causing organism; 128,000 of which require hospitalization and of these 3,000 die (CDC, 2017). These figures do not include undiagnosed cases of infection. *Escherichia coli* (*E. coli*) are one of the most prominent causes of foodborne diarrheal disease in humans. It is also a leading contributor to bacterial infections and extra-intestinal infections in humans and animals alike (Njage and Buys, 2014). *E. coli* bacteria are typically present in the intestines of warm blooded mammals colonizing a newborn's gastrointestinal tract in the first hours after birth (Nataro and Kaper, 1998). There are many serotypes of *E. coli* and most of them are harmless and are a normal inhabitant of the intestinal microflora (Clermont, Bonacorsi, and Bingen, 2000; Rasko et al., 2008). Others, such as Shiga toxin-producing *E. coli* (STEC) O157:H7, are pathogenic and will cause illness upon infection. Under the new FSMA requirements, produce growers using irrigation water from an untreated source can have no detectable generic *E. coli* in their water (FDA, 2011). If an assay for *E. coli* is found to be positive, it is an indication of fecal contamination in the water and a public health concern whether or not the serotype is a STEC.

E. coli are nonsporulating, rod shaped (1 x 3 µm), facultative anaerobic bacterium in the *Enterobactericaceae* family of gamma-proteobacteria (Ray and Bhunia, 2014). The genus *Escherichia* is comprised of mostly gram-negative bacilli that are motile by means of flagella (Nataro and Kaper, 1998). Although *E. coli* evolved millions of years ago, it was first discovered in 1885 by German pediatrician and bacteriologist Theodor Escherich (Hacker and Blum-Oehler, 2007). Sporadic reports have appeared perpetrating *E. coli* in gastroenteritis cases before the 1970's. However, it wasn't until an outbreak linked to cheese imported to the United States in 1971 that drew the attention of many microbiologists as a medical concern (Jay, 1986). By 1982 it became associated as a human pathogen after a multi-state outbreak involving ground beef (Perna et al., 2001).

Phylogenetic analysis has shown that *E. coli* is composed of four main phylogenetic groups: A, B1, B2, and D. Pathogenic varieties contain groups B2 and D (Clermont, Bonacorsi, and Bingen, 2000). At least six different pathotypes of *E. coli* cause a variety of disease (Kaper, Nataro, and Mobley, 2004). These virulent strains (or pathovars) are divided into groups based on mechanisms of infection and the symptoms they produce (Ray and Bhunia, 2014). These groups are: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffuse-adhering *E. coli* (DAEC). Each pathogenic strain has its own unique combination of virulence factors (Kaper, Nataro, and Mobley, 2004), but ultimately all of them cause damage to epithelial cells in the intestinal lining. The intestinal epithelium is an important site of interface between nutrient absorption and intestinal homeostasis (Resta-Lenert and Barrett, 2003). Any disruption to these cells can result in reduced host performance and have potentially fatal ramifications. General symptoms include stomach cramps and diarrhea, low-grade fever, and illness outside of

the intestinal tract, such as urinary tract infections and meningitis. Nonetheless, each case is dependent upon pathotype infection and host resistance.

ETEC pathovars are best known as Traveler's Diarrhea, Montezuma's Revenge, or Delhi Belly (Ray and Bhunia, 2014). They are prevalent in developing nations and the most common cause of diarrheal illness while abroad (Fleckenstein et al., 2010). While general discomfort is experienced by many infected individuals, it is far more severe in areas of the world with poor sanitation. The pathogen is typically spread directly or indirectly by human carriers (Ray and Bhunia, 2014). It is estimated that 400,000,000 children under the age of five in developing nations are affected annually from ETEC (Walker, Steele, and Aguado, 2007). The presence of disease comes from the ability of the bacteria to adhere to the intestinal epithelial cells using fimbriae or pili and subsequently producing either heat labile (LT) and/or heat stable (ST) toxins resulting in increased membrane permeability, electrolyte imbalance, and severe fluid loss (Jay, 1986). The symptom is gastroenteritis and can be fatal to children due to the disproportionate loss of fluid (Ray and Bhunia, 2014).

The EPEC strains are significant in causing infantile diarrhea worldwide, especially in developing countries with poor sanitation (Kenny et al., 1997). Transmitted through human carriers, EPEC do not produce a toxin, but instead form small colonies that attach to the epithelial cells through the use of bundle-forming pili (BFP) and Intimen. This process is called 'localized adherence' (Scaletsky, Silvia, and Trabulsi, 1984). This attachment pattern results in severe lesions on the epithelial layer that destroys microvilli. Lacking the absorptive villi, malabsorption of nutrients and diarrhea ensue. Symptoms may develop in as few as three hours and include profuse watery diarrhea, vomiting, and low grade fever (Ray and Bhunia, 2014).

Pathovars of EIEC cause an infectious diarrhea known as bacillary dysentery (Beutin et al., 1997). The ability to adhere to the mucosal surface has been heralded as a crucial factor for the preservation of bacteria existing within a host organism (Scaletsky, Silvia, and Trabulsi, 1984). EIEC bacterial cells adhere to epithelial cells and invade moving from cell to cell spreading infection through the intestines. Cell damage results in bloody mucoid diarrhea, headache, chills, and fever (Ray and Bhunia, 2014). Interestingly, these strains of *E. coli* have been shown to express virulence regulated by growth temperature. Bacteria incubated at 37° C are virulent and will invade epithelial cells; however the same strains will show a loss of invasive ability when held at 30° C (Maurelli and Sansonetti, 1988). This temperature increase is typical upon EIEC entering a host mammalian body, thus triggering disease.

EAEC was first documented in 1987 when it was isolated in a child from Lima, Peru (Okhuysen and DuPont, 2010). It causes mild, but significant mucosal damage leading to persistent diarrhea (up to 14 days) in children and adults alike (Ray and Bhunia, 2014). They are characterized by their adherence pattern in which they aggregate in a brick-like arrangement on HEP-2 and HeLa cells along the intestinal epithelium (Aslani et al., 2011). A relatively new serovar of EAEC emerged in 2011 known as O104:H4. It was responsible for a large-scale outbreak in Northern Germany that resulted in 3,842 reported illnesses and 53 deaths traced back to consumption of sprouts (Ray and Bhunia, 2014). Resistance to antibiotics has become common to EAEC isolates throughout the world (Aslani et al., 2011). It is now seen as an increasingly recognized cause of enteric disease in the United States, and among the most prevalent causes of persistent diarrhea in developing countries leading to malnutrition and stunted growth due to its ability to cause intestinal dysfunction (Roche et al., 2010).

Isolates of *E. coli* displaying diffuse adhesion (DAEC) were not well documented with regards to pathogenic mechanisms, like other *E. coli* pathovars, for many years (Le Bouguénec, 2006). DAEC has, however, been implicated in diarrheal disease in various studies (Nascimento de Araújo and Giugliano, 2000; Le Bouguénec, 2006). As discussed previously, adhesion is paramount for most pathovars to initiate intestinal infection. EPEC and EAEC display localized adherence and aggregate adherence respectively. The third adherence pattern, diffuse adherence, is characterized by a bacterial colonization that adheres evenly to the whole cell surface (Benz and Schmidt, 1992).

The final of six *E. coli* pathovars discussed herein is of particular importance. It is EHEC and it contains a focal serogroup known as O157:H7, the most frequently reported serotype related to outbreaks in the United States (Kehl et al., 1997). The importance of this pathogenic group stems from the amount of infections and attention it receives every year. It is the leading pathovar associated with foodborne outbreaks in news stories across the United States. Those infected with the EHEC may experience hemorrhagic colitis (bloody diarrhea) and hemorrhagic uremic syndrome (Ray and Bhunia, 2014). Hemolytic uremic syndrome (HUS) is a life threatening illness that causes hemolytic anemia, thrombocytopenia, and renal insufficiency which can lead to kidney failure and death (Gould, 2009). Currently no known treatment exists for HUS. Supportive care and dialysis are the only methods of treatment currently in practice. Every human is susceptible to contracting HUS, but those with compromised immune systems are most at risk. Persons living with HIV/AIDS, leukemia, or viral hepatitis are among these individuals. Age also plays a role in immune system susceptibility. The most common cause of acute renal failure among children in the United States is HUS. However, as age increases, the

likelihood of hospitalization increases with those over the age of 60 being the most likely to have fatal effects (Gould, 2009).

Other serotypes of EHEC, such as those that belong to O5, O26, O55, O91, O111, O113, and O117 are less common, yet remain a serious threat to public health worldwide (Fagen et al., 1999, Ray and Bhunia, 2014). EHEC strains are characterized by their ability to produce a Vero cell cytotoxin, also termed verotoxin or Shiga-like toxin (Jay, 1986; Ray and Bhunia, 2014). Unlike most *E. coli* pathovars, EHEC infection is located in the colon rather than the small intestine (Jay, 1986). From its introduction into the colon, it can cause extra-intestinal illness as shiga-like toxins (Stx) are absorbed into the bloodstream damaging small blood vessels (Ray and Bhunia, 2014). Shiga-like toxins come in two forms: Stx1 and Stx2 based on encoded genes within the bacterial strain (Alperi and Figueras, 2010). A particular EHEC serogroup may produce one or both toxins, although Stx2 is considered the more potent of the two because it is more often associated with HUS (Alperi and Figueras, 2010). Stx binds to the glycolipid receptor globotriaosylceramide in intestinal and kidney cells, blocks protein synthesis, and ultimately causes cell death (Ray and Bhunia, 2014).

The impact of *E. coli* on public health has not been an issue in the past due to effective antibiotics (Njage and Buys, 2014). Recently, there has been an increase in antibiotic resistant *E. coli* strains to which new therapies are required (Njage and Buys, 2014). These drug resistant strains of *E. coli* are being linked to the abuse of antibiotics in food animals, stating that the over use of these antibiotics is promoting the development of resistant bacteria with resistance genes that can be transferred to humans (Njage and Buys, 2014). The potential ramifications of the diseases it causes, the lack of treatment options, and the prospect for an epidemic has led to a rapid increase in research into the fields of pathogenesis, detection, and source contamination

with regards to *E. coli* (Perna et al., 2001). It is now one of the most understood bacterial life forms on the planet due to its popularity among research laboratories around the world (Hacker and Blum-Oehler, 2007).

Sources of *E. coli* transmission from one intestinal tract to another can occur through contaminated food and contact with infected animals or people, but contaminated water in fields is commonly suspected as a major factor responsible for outbreaks involving fresh produce (Fonseca, 2011). In 1995, an outbreak in Montana was linked to surface runoff from a cattle ranch leading to contamination of irrigation water on an adjacent, downhill lettuce farm (Fonseca, 2011). Lettuce, like most fresh produce, does not undergo microbial inactivation or preservation treatment, but undergoes only partial interventions like chlorine wash. Washing, even with sanitizers, has been demonstrated to accomplish no more than a 2 log reduction (99%) of bacteria present on fresh produce (Ceuppens, 2015). Due to the lack of pathogen mitigation, viable bacteria, whenever they are present, may persist or increase along the food chain. Consumers could consequently be exposed to virulent strains along this contaminated food chain (Njage, 2014). The use of rapid immunoassay devices in testing for *E. coli* can fail to detect the pathogen if it is present in low numbers (Stewart, 2001). Produce washing can also damage sensitive plant products causing a decrease in marketability and shelf life by increasing the susceptibility to spoilage and mold growth (Ceuppens, 2015).

Beyond human health risks, any business must understand the potential ramifications of purveying contaminated food. For example, in February of 2016, Jack and the Green Sprouts, a farm in River Falls, Wisconsin, was accused of distributing alfalfa sprouts that had been contaminated with *E. coli*. Eleven people were infected in the outbreak; two of these individuals were hospitalized (CDC, 2016). The epidemic was investigated to determine the exact locale of

contamination. Although the evidence was not clear, state, local and federal public health and regulatory officials deemed Jack and the Green Sprouts to be the likely source of contamination (CDC, 2016) and subsequently issued a public notification to avoid eating produce from this particular farm. This negative publicity caused significant economic losses for the business in sales, recalled product, potential lawsuits, and loss of consumer loyalty.

The largest *E. coli* outbreak on record was associated with sprouts. The epidemic affected nearly 6,000 people in Sakai City, Japan (Taormina, 1999). Raw sprouts have a high potential risk of contamination of pathogens due to their unique developmental requirements. The seeds need warm and humid conditions to sprout and grow, which are ideal conditions for bacterial growth and reproduction (USDHHS, 2018). Colonization during germination is not the only means of bacterial internalization of leafy greens. Bacterial contaminants may also enter through the stomata or wounds directly through edible portions, pre-harvest and/or post-harvest (Li, Tajkarimi, and Osburn, 2008). Vacuum cooling is a common post-harvest practice in the leafy green industry. It decreases the amount of time needed to remove field heat from the plants for longer shelf life. It also adds a significant risk on bacterial cell infiltration into lettuce tissue as the vacuum chamber forcibly changes the structure of plant tissue, such as stomata, opening a possible mechanism of *E. coli* internalization (Li, Tajkarimi, and Osburn, 2008). As noted earlier, washing with sanitizers (e.g. sodium hypochlorite) is not deemed effective in eliminating *E. coli* presence. Even the industry standard "triple wash" is not adequate to eliminate internalized bacteria (Li, Tajkarimi, and Osburn, 2008). Detection and treatment methods currently in use are simply not effective enough and are too time consuming to keep pace with a rapidly developing and evolving species like *E. coli* (Gannon et al., 1992).

Despite the contamination risks, leafy greens, particularly lettuce (*Lactuca sativa* L.), constitute a large portion of fresh vegetables consumed in the U.S. In terms of pounds per person, lettuce is the second most consumed vegetable in the U.S., only surpassed by potatoes (AMRC, 2017). In terms of value, it is the leading vegetable crop in the nation, cashing in $1.9 billion in 2015 (AMRC, 2017). California and Arizona were the largest U.S. producers of 2015, contributing 98% of total production (AMRC, 2017).

This valuable and popular crop is attracting increased attention as the nation's population grows ever higher, increasing the demand and profit potential for lettuce grown on a local scale. Ready-to-use salad mixes are a staple component of supermarkets everywhere. These mixes require high quality raw products from an organoleptic and hygienic purveyor (Gonnella et al., 2002). Growers everywhere are noticing this trend and identifying the economic potential behind lettuce and other leafy greens. Greenhouse production, also known as controlled environment agriculture (CEA), is a sector of agriculture that opens possibilities for crop production during times when climate has limited these options (Despommier, 2011; Jensen, 2001). This practice has brought food production sources closer to the consumer. This fact becomes increasingly important when considering post-harvest handling as related to nutrition. Nutrition (and arguably flavor) declines with time. Important vitamins and minerals, such as vitamin C, are degraded post-harvest at an accelerated rate with longer storage periods (Lee and Kader, 2000). The less time it takes food to reach consumers, the more nutritional and marketable value it retains.

Controlled environment agriculture is burgeoning among U.S. growers. The greenhouse industry produced $3 billion in food crop sales in 2013, and agricultural finance professionals project sales to reach $4 billion by 2020 (Rabobank, 2013). Diminishing agricultural land, water

availability and restrictions, market demand for year-round local produce, and a rapidly growing human population have all contributed to the shift from traditional field farming to CEA (Despommier, 2011; Specht et al., 2014). Greenhouse production offers advantages such as reduced water and fertilizer inputs (most notably in hydroponics), but it also offers a reduced potential for food safety issues when compared to soils in open fields (Evans et. al., 2015). Runoff water does not typically affect greenhouse crops because production benches are often elevated off the ground and containerized. A greenhouse structure provides a significant barrier to wildlife making contamination unlikely. Although it is improbable, contamination is not impossible (Orozco-R., 2008).

Every farm is unique and provides its own set of contamination variables with regards to *E. coli* or any other foodborne microbe. As mentioned previously, *E. coli* resides within the gastrointestinal tract of an endotherm host. This environment provides a consistently warm temperature that is favorable to bacterial growth (Winfield and Groisman, 2003). Optimal growth of *E. coli* is reached at 37° C, but grows rapidly from 30° C to 42°C (Ray and Bhunia, 2014). It grows poorly at temperatures between 44° C and 45° C, and discontinues growth at 45.5° C (Doyle and Schoeni, 1984) and experiences death when held at 64.3° C for 9.6 s (Ray and Bhunia, 2014). On the lower end, it does not grow at 10° C or below (Ray and Bhunia, 2014), but will remain alive and without significant population reduction at -20° C (Doyle and Shoeni, 1984). Endotherm intestines offer an environment with a stable pH that is rich in free amino acids and sugars for bacterial consumption (Winfield and Groisman, 2003). It is estimated that half of an *E. coli* cell's life is spent in the intestine of a host before it is excreted to an indeterminate location. This random location is where it spends the second half of its life. The cell will die or, with a small amount of luck, colonize a new host (Savageau, 1983). Once

excreted from a host, *E. coli* struggles for survival facing limited nutrient availability, osmotic stress, large variations in temperature and pH, and predation (Winfield and Groisman, 2003). Livestock are common reservoirs of *E. coli.* The organism's survival outside the host is determined by mere probability of fecal landing zone. Domestic or wild animals within close vicinity to irrigation water used on produce may pose a threat to farmers in the form of *E. coli* contamination (Wachtel et al., 2002). A study by Maule (2000) compared *E. coli* survival in river water, cattle feces, soil cores, and stainless steel work surfaces. Maule concluded that the greatest chance for survival outside of the intestines for *E. coli* was in soil cores containing rooted grass, or pastureland, experiencing only 1 to 2 log reductions over 130 d. More notable to irrigation water sources, survival in river water fell 2 log reductions in 10 d and undetectable levels in 27 d. Other researchers have suggested that *E. coli* can survive up to 91 d in fresh water lakes at cool temperatures (Wang and Doyle, 1998). This is important with regards to irrigating crops for human consumption. Under the FSMA law, growers using untreated water (e.g., well water, river water, rain water) must undergo water quality testing to screen for fecal contaminants. Growers using a treated water source from a city municipality are exempt from this testing procedure (FDA, 2011).

Hydroponics has become an increasingly popular growing system for use in CEA. Many hydroponic systems use a recirculating water technique allowing for more efficient water usage. Barbosa et al. (2015) calculated that a hydroponic lettuce operation uses 12.5 times less water than its modern industrial agriculture (field production) counterpart. A recirculating irrigation system offers growers the opportunity to save on water and fertilizer use and also have a lower environmental impact (Gonnella et al., 2003). *E. coli* exhibits the ability to thrive in fertilized irrigation water in a hydroponic system (Shaw et al., 2016). This presents a unique problem for

farmers that use a recirculating hydroponic system. Since water is collected and recycled through irrigation lines, a microbial contaminant has the potential to infect not some, but the entire crop continually recycling a solution of pathogens (Premuzic et al., 2007).

Though contamination potential remains low in CEA, preventative measures to disinfest water must be a focal point for food safety and public health. Cultural practices, such as personal hygiene and sick employee protocols, will always remain a continually important area in the production of produce, but further disinfestation measures will ensure a crop free of microbial pathogens like *E. coli*. Current techniques used to mitigate microbial pathogens are as follows: Ultraviolet (UV) radiation, biofiltration, heat treatment, ozone injection, chlorination treatment, chlorine dioxide treatment, iodine treatment, and copper or silver ionization (Newman, 2004).

UV radiation is a common method of water disinfestation for numerous microbes. UV light can be partitioned into three categories: UV-A are wavelengths measuring in the range of 315 to 400 nm, UV-B are between 290 and 315 nm, and UV-C are between 220 and 290 nm (Newman, 2004). Using a short wave UV-C wavelength (254 nm), radiation is broadly recognized as an effective means to inactivate pathogens, bacteria protozoa, and viruses (Ehret et al., 2001; Hijnen et al., 2006). The inactivation of microorganisms using UV irradiance is caused by damage to cellular nucleic acids by shortwave UV light rays (Sommer et al., 2000; Zhou and Smith, 2002). Although UV irradiance is widely considered a viable option in water disinfestation, it has limitations that do not make it viable in every circumstance. The light must breach the cell in order to inactivate it. UV light transmittance through an aqueous solution is expressed as a T_{10} value. It is a measurement of the percentage of UV light that passes through 10 mm of a solution. Good T_{10} values are reported at 60% and above to have effective UV

penetration (Pettitt, 2016). In water with poor T_{10} values, small pathogens may pass by the light waves in the shadow of debris and remain active, therefore filtration of the water and cleaning of the UV lamp (Garibaldi et al., 2004; Newman, 2004) are paramount to this method's usefulness. This system also poses problems as it inactivates non-target organisms beneficial to plant growth in a hydroponic system. Lastly, UV light interacts with Fe-chelates causing ion imbalance issues in a hydroponic nutrient solution (Ehret, 2001).

Biofiltration, also known as slow sand filtration (SSF), provides a physical barrier to larger organic pathogens (e.g., fungi and nematodes), but does not eliminate bacteria or viruses. Ultra-filtration or membrane filtration may produce a six log reduction of bacteria, viruses, and parasites (Belbahri et al., 2007). Whereas SSF removal is far less significant, removing about 80% of total bacteria (Wohanka et al., 1999).

Heat pasteurization is reliable and will kill almost any microorganism if held at 95° C for 30 s (Runia, Van Os, and Bollen, 1988). As mentioned previously, *E. coli* is heat labile and will die at 64.3° C in 9.6 s (Ray and Bhunia, 2014). An advantage to heat pasteurization is that it does not add any constituents into the water. Although this treatment technique is common in the Netherlands, high energy costs make this method of disinfestation prohibitive to implement for most commercial growers (Newman, 2004).

Several methods of disinfestation methods use oxidation reactions to disinfect water. Oxidizing chemicals, such as ozone and chlorine, are strong sanitizing treatments. Target and non-target organisms are eradicated through a change in their chemical structure when exposed to oxidizing chemicals (Newman, 2004). Oxidizing compounds lyse pathogens and other

organic material in the water leaving chemical bi-products considered harmless (Newman, 2004).

Ozone injection is a method of using naturally occurring ozone gas (O_3) to mitigate pathogens. When bubbled into water, ozone breaks down into dissolved oxygen (O_2) and hydroxyl ions (OH^-). These hydroxyl free radicals then react with a compound like peptidoglycan in bacterial membranes resulting in membrane permeability and thus cell death (Khadre et al., 2001). Because it is a strong oxidizer, ozone has the ability to have phytotoxic effects on produce as well as cause damage to equipment (Parish et al., 2003). Ozone systems come at a high initial cost as well as to operate and maintain. Ozone is an unstable compound that decomposes quickly making it necessary to produce on site before treatment (Kim et al, 2000, Ray and Bhunia, 2014). Ozone is generated using corona discharge or plasma discharge units. Optimal performance requires clean water with a pH of 4 (Newman, 2004). This is not an optimal pH for plant growth and therefore would require a pressurized holding tank to treat water before it is used for irrigation. High system complexity limits the use of ozone disinfestation. Also of note, ozone may react with some fertilizers oxidizing iron, manganese, and sulfides (Newman, 2004; Pettitt, 2016).

Chlorine treatment is the most commonly used sanitizing agent to prevent, reduce, and eliminate pathogens in water solutions (Braun and Supkoff, 1994; Newman, 2004; Poncet et al., 2001). Disinfectant efficacy is based on concentration and the chlorine form in the solution (hypochlorous acid and hypochlorite ions) (Poncet et al., 2001). It is a fast acting sanitizing agent and kills bacterial pathogens in as little as one to two minutes at a concentration of 50 to 200 ppm (Ray and Bhunia, 2014). Oxidation reactions will decrease the amount of oxidizing compounds and therefore it is important to maintain proper concentration over time (Newman,

2004). Phytotoxicity to plant material is a concern. Premuzic et al. (2007) reported higher amounts of chlorotic and necrotic symptoms on lettuce leaf tissue at higher concentrations (11 ppm) when used as a nutrient water disinfectant in a hydroponic system. As well as plant health implications, the Environmental Protection Agency (EPA) has stated public health concerns with regards to chlorine treatment. According to the EPA, by-products (trihalomethanes) formed when chlorine reacts with organic matter have been classified as potential human carcinogens (Symons et al., 1981; Wang, Deng, and Lin, 2007).

Chlorine dioxide is a very potent disinfectant created by combining hydrochloric acid and sodium chlorite (Van Os, 2008) and has a broader pH range than hypochloric acid alone. It is unstable in gaseous form, but stable and soluble in an aqueous solution (Newman, 2004). Complexities limit this intervention. It must be made on site with specialized equipment. The system itself is complex and appropriate storage facilities must be built to store the chemicals. Implementation costs remain high as well (Pettitt, 2016).

Iodine treatment is an effective bactericide. It acts by inhibiting protein function and is a strong oxidizer (Punyani et al., 2006), but must be monitored closely for phytoxicity dangers as it will react with organic material (Ehret et al., 2001). Higher concentrations of iodine are required to inactivate most microorganisms when compared to chlorine (Punyani et al., 2006). It gives water a foul taste and is costly to maintain (Kim et al., 2000). There is debate over the maximum safe level of iodine dosage in potable water consumed by children (Punyani et al., 2006).

Electrolysis of water by silver and copper electrodes releases positively charged Cu^{+} ions in the water. The free Cu^{+} ions disrupt membrane function in many microorganisms (Van Os, 2008). Copper is an essential plant nutrient, but it is also a heavy metal. Phytoxicity can occur

at high concentrations and must be monitored closely, most notably in closed, recirculating systems (Zheng et al., 2012). The release of heavy metals into water shed is of concern and must be addressed based on regional environmental protection laws (Van Os, 2008). Other limiting factors include system complexity and cost. A medium scale greenhouse operation may spend an initial cost of up to $30,000.00 to purchase a unit and an additional $5.00 per day to operate the unit (Zheng et al., 2008).

All of the methods mentioned hitherto have shown positive results in pathogen management, but none have prevailed as a gold standard. Prohibitive factors have kept growers from using them, often to the detriment of the consumer and grower alike. Therefore, further research is needed in this area of food safety to provide a reliable and affordable solution to reduce foodborne illnesses in produce.

Food scientists have uncovered antimicrobial properties related to natural compounds and their applications to producers. Chemical food preservation has increased in recent years causing an increase in consumer demand for foods that have undergone minimal processing using more naturally based preservatives (Cleveland et al., 2001). Therefore, natural antimicrobials are becoming more prevalent among microbial disinfestation methods in the food industry (Zhu et al., 2015). Human impact on the environment has also become a central theme when discussing pest mitigation of all kinds. Many ancillary products from agriculture (e.g. fertilizer, pesticides, antibiotics, foodborne pathogens) reach local water sheds via runoff causing unintended consequences. Aquatic systems represent a crucial sector for the environmental release, mixing, persistence, and spread of antimicrobial resistant bacteria (Taylor et al., 2011) creating significant unease with regards to public health. For these reasons, a natural antimicrobial

targeting *E. coli* could be investigated to satisfy current trends in the food industry without compromising food safety.

Plant based isolated compounds and essential oils contain secondary metabolites that have been shown to inhibit the growth of bacteria and other microorganisms (Tiwari et al., 2009). Secondary metabolites are a diverse array of organic compounds that appear to have no direct function in the plant's growth and development. Instead, secondary metabolites are synthesized for the purpose of attracting pollinators or defending against herbivores and pathogens (Taiz and Zeiger, 2010). Secondary metabolites fall into one of three categories: terpenes, phenolics, or nitrogen-containing compounds (Taiz and Zeiger, 2010). Terpenes are the largest class of secondary metabolites. They are either synthesized from acetyl-coA via the mevalonic acid pathway, or synthesized from glycolytic intermediates via the methylerythritol phosphate pathway (Taiz and Zeiger, 2010). The majority of terpenes are presumed to be involved in plant defense. Terpenes are toxins that deter herbivores from feeding, but they are also known to have antimicrobial properties (Taiz and Zeiger, 2010). Saponins are terpenes manufactured from glycolytic intermediates. Their name is derived from their ability to foam in water similar to soap (Francis et al., 2002); hence the naming of the compound derived from the Latin word "*sapo*" (Naoumkina et al., 2010). The primary component of a saponin is a steroidal or triterpenoid aglycone skeleton. This skeleton is attached to a moiety of one or more sugar chains (Arabski et al., 2011). The aglycone structure may have multiple unsaturated carbon to carbon covalent bonds. The sugar chain is typically attached at the C_3 position, but it could also have an additional sugar chain attached at the C_{26} or C_{28} position (Francis et al., 2002). The structures of different saponins are diverse and so too are their biological activities as natural nonionic detergents. Biological properties associated with saponins include: cytotoxic,

hemolytic, molluscicidal, anti-inflammatory, antifungal, antiyeast, antibacterial, and antiviral activities (Arabski et al., 2011).

A great deal of the biological effects of saponins has been credited to their action on cellular membranes (Francis et al., 2002). Based on Gram-stain behavior, bacterial cells are allocated as Gram-negative or Gram-positive (Ray and Bhunia, 2014). The primary differences are found in the composition of the cellular membrane that houses the cytoplasmic material. *E. coli*, as mentioned earlier, fall into the Gram-negative category. The cell wall of *E. coli* is a tiered structure consisting of complex layers (Schnaitman, 1970). *E. coli*, like other Gram-negative cells, have an outer membrane, a thin cell wall, and a cytoplasmic membrane (Ray and Bhunia, 2014). The outermost layer contains a complex lipopolysaccharide portion of the envelope (Cronan and Gelmann, 1975; Ray and Bhunia, 2014). The outermost layer also contains two other components, the lipid A portion of the lipopolysaccharide and the murein lipoprotein (Cronan and Gelmann, 1975). The middle membrane, or cell wall, is composed of peptidoglycan and contains mucopeptide layers covalently linked to peptides and lipoproteins (Ray and Bhunia, 2014). The innermost layer, the cytoplasmic membrane, is made up of a phospholipid bilayer where a variety of proteins are located (Ray and Bhunia, 2014). Arabski et al. (2011) postulated that triterpenoid saponins would implant themselves into the lipid bilayer where they would attach to cholesterol. Regions rich with cholesterol-saponin complexes will be formed which will inevitably lyse *E. coli* cells. In nature, cholesterol-free Gram-negative bacteria outer membranes are 90% lipopolysaccharide. The research team hypothesized that the saponins would interact with the lipid A portion of the lipopolysaccharides and increase permeability of the cell membrane. It was observed that saponins increased the amount of clinical *E. coli* in significant numbers with and without antibiotics present, leading the team to

surmise that triterpenoid saponins may increase cell permeability, but ultimately enhance bacterial growth. This evidence is important when understanding the relationship of saponin structure and biological properties related to bacterial cytotoxicity. A comprehensive list of biological activities compiled by Güçlü-Üstündağ and Mazza (2007) can be found in the Appendix.

The previous study extracted triterpenoid saponins from the species *Quillaja saponaria.* It is a tree native to the arid regions of Chile (Cheeke, 2000). It is one of the two leading commercial sources of saponins. The other prominent source is *Yucca shidigera* from the arid desert regions of Mexico and the southwestern United States (Cheeke, 2000). Nonetheless, saponins are found in more than 100 plant families as well as a few marine invertebrates (Güçlü-Üstündağ and Mazza, 2007). Saponins are used in a variety of commercial industries. They serve as vaccine adjuvants (Kensil and Marciani, 1991) and steroid hormones (Balandrin, 1996) in the pharmaceutical industry, foaming agents in beverages, emulsifiers in foods, wetting agents in photography (San Martin and Briones, 1999), fire extinguishers, denatured alcohol (Balandrin, 1996), soaps, and fish poison (Güçlü-Üstündağ and Mazza, 2007) to name a few.

Extraction is the first step in recovering targeted active ingredients from plant material (Wu et al., 2000). Saponin extraction is typically performed using traditional solvent extraction methods (Güçlü-Üstündağ and Mazza, 2007). This technique uses a solvent, like ethanol, combined with the raw plant material. Plant material is usually pretreated (grinding) to reduce particle size in order to increase efficiency of extraction (Wu et al., 2000). The mixture is then agitated for a predetermined amount of time at a predetermined temperature. The mixture is then filtered and allowed time for the solvent to evaporate. The remaining product is the targeted active ingredient (Chen et al., 2007). This process is considered time consuming and less

efficient than more modern extraction methods like ultrasound-assisted extraction (Wu et al., 2000) and microwave-assisted extraction (Chen et al., 2007). Ultrasound-assisted extraction can be carried out in faster times, at lower temperatures (circumventing thermal destruction to extract), use less solvent, and increase extraction yields (Wu et al., 2000). Microwave-assisted extraction has economic advantages in that it requires less time and is more environmentally friendly (Chen et al., 2007).

Apoptosis of bacterial cells is not only inevitable, but beneficial to the population as a whole. Deaths of *E. coli* cells are most likely due to an accumulation of a lethal element (Wang et al., 2010). Programmed cell death of specific subpopulations within a population creates opportunity for more adaptive cells to thrive. Bacterial cells switch among genetic forms in response to cell density, nutrient supply, substratum surface, plasmid burden, incident radiation, viral infection, or the passage of time. This accounts for variation within a bacterial colony trying to overcome a host's immune response (Yarmolinsky, 1995). Simply put: adapt or die. All bacteria reproduce by binary fission. *E. coli* generation time is directly correlated to abiotic factors. It is only natural to suggest that an organism exhibits paramount growth at the temperature it evolved in. Under an optimal temperature of 37° C, a colony of *E. coli* can double its population in 40 min (Plank and Harvey, 1979). This remarkably fast generation time allows *E. coli* to adapt quickly. Colonial cells that can survive within a given environment flourish, while unsuitable cells die. Sublethal stresses may simply cause bacterial injury rather than death. Cellular components such as the cell wall, cytoplasmic membranes, ribosomes and rRNA, structural DNA, and some enzymes have been reported as damaged from such stresses (Ray, 1986; Bozoglu et al., 2004). In many cases this injury is reversible. Straka and Stokes (1959)

demonstrated that thermally injured bacterial cells were able to repair themselves when supplemented with the appropriate nutrients.

As mentioned previously, the presence and persistence of antibiotic resistant bacteria, like *E. coli*, is a growing public health concern. Resistant strains reach the environment through animal and human fecal excrement (Reinthaler et al., 2003). Antibiotic resistance arises through selection of naturally occurring resistant mutants, horizontal gene transfer, and sublethal levels of antibiotics/antimicrobials that induce mutagenesis (Kohanski et al., 2010). Oxidative DNA damage, like that caused by low doses of chlorine treatment, can lead to an accumulation of mutations (Demple and Harrison, 1994). In a hydroponic system, injured *E. coli* cells have the ability to repair themselves and through subsequent generations create a more heterogeneous population that is resistant to oxidative reduction reactions of some chemical treatments. Further research is in need to investigate possible alternative treatment options to mitigate *E. coli* in agricultural irrigation systems that will minimize or alleviate unintended consequences to public safety and the environment.

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Chapter 1

Efficacy of Saponin Mitigation of *E. coli* Under Nonsterile and Sterile Conditions

Abstract

A commercial extract solution of *Yucca*-based saponins was tested for antibiotic effects against *Escherichia coli* (*E.* coli) O157:H7 under nonsterile and sterile conditions. Generic serotypes of *E. coli* (P4, P13, and P68) were combined in equal parts and added to 500 mL of a fertilizer solution to produce a solution with a microbial concentration of 10^6 CFU•mL^{-1}. Under nonsterile conditions without saponin treatment, *E. coli* populations were relatively stable over 144 h. After an initial increase over the first 24 h, saponin concentrations of 25, 50, and 100 μg•mL^{-1} suppressed *E. coli* populations to almost 0 CFU•mL^{-1} by 144 h. The treatment containing a saponin concentration of 200 μg•mL^{-1} also had an initial increase over the first 24 h and subsequently suppressed *E. coli*, but at a slower rate than all other treatments allowing *E. coli* to remain present at 10^3 CFU•mL^{-1} after 144 h. Under sterile conditions without saponin treatment, *E. coli* population increased to 10^7 CFU•mL^{-1} after 24 h and remained at this level for 144 h. Likewise, when saponins were introduced into the solution, *E. coli* concentration increased over the first 24 h period. However after 24 h, increasing levels of saponins increased the rate at which the *E. coli* populations decreased. Saponin rates of 100 and 200 μg•mL^{-1} extirpated *E. coli* completely after 144 h and 120 h respectively. A saponin rate of 50 μg•mL^{-1} suppressed *E. coli* to a population of 10^3 CFU•mL^{-1}. A saponin rate of 25 μg•mL^{-1} suppressed *E. coli* in two out three replications, but overall did not reduce populations significantly. Although saponins decreased *E. coli* populations over time, the effect of saponin concentration on that population decrease was different between sterile and nonsterile conditions.

Introduction

Every year 48 million Americans become infected from a foodborne disease; 128,000 of which require hospitalization and of these 3,000 die (CDC, 2017). *E. coli* are one of the most prominent causes of foodborne diarrheal disease in humans. It is also a leading contributor to bacterial infections and extra-intestinal infections in humans and animals alike (Njage and Buys, 2014). *E. coli* bacteria are typically present in the intestines of warm blooded mammals colonizing the infant gastrointestinal tract in the first hours after birth (Nataro and Kaper, 1998). There are many serotypes of *E. coli* and most of them are harmless and are a normal inhabitant of the intestinal microflora (Clermont, Bonacorsi, and Bingen, 2000). Others, such as Shiga toxin-producing *E. coli* (STEC) O157:H7, are pathogenic and will cause illness upon infection. In 2011, lawmakers instituted a new law known as the Food Safety Modernization Act (FSMA). The law was instated with the intention to modernize the food safety system that had been in place thitherto. The food safety system's update is designed to adapt and face new challenges that exist in the global food system from a preventative stance (FDA, 2011). Through a science-based approach, food producers, shippers, and processors are being held accountable for food safety. To be in compliance of the new law, produce growers using irrigation water from an untreated source can have no detectable generic *E. coli* in their water (FDA, 2011). If an assay for *E. coli* is found to be positive, it is an indication of fecal contamination in the water and a public health concern whether or not the serotype is pathogenic.

Current methods of pathogen eradication for agricultural water use include UV radiation, biofiltration, oxidation reduction reaction compounds, heat pasteurization, iodine treatment, and electrolysis by silver and copper electrodes (Newman, 2004). All of the aforementioned methods have shown positive results in pathogen management, but each has its limitations.

Prohibitive factors such as cost, maintenance, and system complexity have kept many small to medium level growers from using them, often to the detriment of the consumer and grower alike. Therefore, further research is needed in this area of food safety to provide a reliable and affordable solution to reduce the dissemination of foodborne pathogens through irrigation procedures.

Food scientists have uncovered antimicrobial properties related to natural compounds and their applications for producers. Chemical food preservation has increased in recent years causing an increase in consumer demand for foods that have undergone minimal processing using more naturally based preservatives (Cleveland et al., 2001). Therefore, natural antimicrobials are becoming more prevalent among microbial disinfestation methods in the food industry (Zhu et al., 2015).

Saponins are secondary metabolites found in a variety of plant species. They contain both water-soluble and fat-soluble constituents giving them surfactant characteristics (Cheeke, 2001). Their name resulted from their capability to form soap-like foams in water (Francis et al., 2002); hence the naming of the compound derived from the Latin word "*sapo*" (Naoumkina et al., 2010). The structures of saponins are diverse, depending largely on the source. The main component of a saponin is a steroidal or triterpenoid aglycone skeleton. This aglycone is attached to one or more sugar chains (Arabski et al., 2011). The location of attachment to the sugar moiety is where saponins show great diversity. The varieties of assorted structures have unique biological properties as well. Some saponins act as anti-inflammatory agents, others act as antimicrobial agents (Güçlü-Üstündağ, Ö. and G. Mazza, 2007). A great deal of the biological actions of saponins has been credited to their effects on cellular membranes (Francis et al., 2002). As an antimicrobial, Arabski et al. (2011) postulated that triterpenoid saponins would

"insert themselves into the lipid bilayer of the cellular membrane, bind to cholesterol, form domains enriched with cholesterol-saponin complexes, and inevitably lyse *E. coli* cells." In contrary, what they observed was an increase in *E. coli* populations leading them to conclude that triterpenoid saponins enhanced bacterial growth. Soetan et al. (2006) also reported similar results using a saponin extract derived from sorghum. The saponin extract inhibited growth of *Staphyloccocus aureus,* a Gram-positive bacterium. However, it did not inhibit *E. coli,* possibly due to its more complex cell wall that is found in all Gram-negative bacterium.

The objective of this study was to evaluate the antimicrobial effects of steroidal saponins on *E. coli* in irrigation water under nonsterile and sterile conditions *in vitro.* It was hypothesized that increasing the concentration of saponins in a solution would result in a more rapid decline of *E. coli* populations.

Materials and Methods

Microbial stock culture:

Individual isolates of non-pathogenic *E. coli* (P4, P13, and P68) were obtained from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, Iowa (42.0266° N, 93.6465° W). These isolates were non-verotoxin producing *E. coli* strains, have been classified as a biosafety level 1 contaminant, and recommended for use in antimicrobial susceptibility testing for direct comparison to *E. coli* O157:H7 based on growth and biochemical characteristics (Marshall et al., 2005).

Bacteria were stored at a temperature of -21° C. Bacterial strains were grown and combined under sterile laboratory conditions in the Food Sciences building at Iowa State University, Ames, Iowa using the following procedure: Propagation of strains was quarantined from one another in 10 mL test tubes containing Brain Heart Infusion (BHI) broth (Difco, Becton Dickinson, Sparks, Md.). Cultures were left for 24 h at 37° C. They were then transferred to fresh BHI broth twice more at 24 h intervals at 37° C before the final transfer to BHI broth. Individual strains were then centrifuged at 10,000 rpm for 10 min. The supernatant was poured off and the remaining pellet was suspended in saline. This procedure was repeated for each of the three bacterial isolates. The three individual isolates were then mixed into a single homogenous solution (referred to as the cocktail from here out). This process resulted in a cocktail containing *E. coli* at a concentration of 10^8 colony forming units (CFU)•mL^{-1} and saline to be used in inoculation of the nutrient tank water later in the experiment. A cryoprotective solution was created by adding glycerol to the cocktail to protect bacterial protein layers during freezing. Viable bacterial counts for the cocktail were quantified using serial dilutions and the

standard plate count method on MacConkey agar. The cocktail was frozen in 1 mL micro vials and transported to the University of Arkansas, Fayetteville, Arkansas (36.0678° N, 94.1737° W) where they were stored at -21° C until ready for use.

Inoculation Solution:

Frozen cocktail *E. coli* cultures with a population of 10^8 CFU•mL^{-1} in 1 mL micro vials were removed from -21° C cold storage. Frozen cultures were thawed slowly in a cold water bath. Using sterile technique under a clean hood, 1 mL of thawed cultures were transferred via air displacement micropipette into 9 mL of buffered peptone water (BPW) (Hardy Diagnostics, Santa Maria, Calif.) in 12 mL test tubes and capped. The inoculated test tubes were then incubated at a constant 37° C for a period of 24 h. The resulting solution was 10 mL of *E. coli* cocktail (isolates P4, P13, and P68) at a population of 10^8 CFU•mL^{-1}. This solution constituted the inoculation solution (IS).

Preparation of Agar Media:

Using a digital balance (Ohaus, AP250D), 52.49 g of dehydrated MacConkey agar (Hardy Diagnostics, Santa Maria, Calif.) was weighed out. The dehydrated agar was then added to 1 L of deionized (DI) water in a 2-L Erlenmeyer flask containing a magnetic stir bar. The solution was then heated and mixed on a hotplate stirrer until boiling. At this point, the liquid agar was boiled for an additional 60 s to allow for complete dissolution of the agar. The liquid agar solution was then transferred in equal parts to two 2-L Pyrex autoclave bottles and placed in

an autoclave. Autoclave settings were adjusted to operate a liquid cycle at 121° C with a sterilization time of 15 min at a pressure of 103,422 Pa. Upon completion of the autoclave cycle, the liquid agar was allowed a resting period to cool to a temperature of no more than 60° C and no less than 55° C. Then containment bottles were sterilized by spraying with ammonium chloride (Coverage Plus NPD, Steris, Mentor, Ohio) and moved to a sterile clean hood. To create a selective media, the antimicrobial Rifampicin (Tokyo Chemical Industry, Tokyo) was added to each bottle of media at a concentration of 1 $mL \cdot L^{-1}$ and dissolved by swirling the bottle to create a vortex. This addition of Rifampicin created a selective agar media. Using a graduated macropipette, 18 mL of the selective media was transferred into sterile slippable 100 mm x 15 mm Petri dishes (VWR, Radnor, Pa.) and allowed to cool. Once the agar cooled to 40° C it solidified and was stored in a sterile bag in a refrigerator at 4° C.

Saponin solution

The saponin product used in this study was supplied in a premixed solution (DPI Global, Porterville, Calif.), and contained sapogenin with a steroidal aglycone structure extracted from *Yucca schidigera.* The solution was certified by the Organic Materials Review Institute (OMRI) and contained 14% saponins. Specifications of total dissolved solids and typical analysis are reported in Table 1. The manufacturer conducted a microbiological analysis that did not detect any yeasts or molds, coliforms, *Staphylococcus aureus*, or *Salmonella.* Treatment rates were supplied by amount of active ingredient in the saponin solution.

Inoculation of water flasks

Separate trials were run in two different environmental conditions. A nonsterile trial was conducted in a greenhouse and a sterile trial was carried out under a clean hood. For the trial under nonsterile conditions, 500-mL Erlenmeyer flasks filled with 500 mL of tap water originating from a treated water source (Washington Water Authority, Prairie Grove, Ark.). Each flask was designated a number one through six. Flasks labeled two through six were inoculated with 5 mL of the IS to provide an initial population of 10^6 CFU•mL^{-1} in each flask. Next, all six flasks were given a hydroponic lettuce fertilizer to raise the electrical conductivity (EC) of the solution to 1.4 dS•m^{-1}. The fertilizer formulation was developed by the Evans lab at the University of Arkansas' Department of Horticulture (Table 2). Sulfuric acid was added to lower the pH of the solution to 5.9. Lastly, flasks three, four, five, and six were given saponin treatments of 25, 50, 100, and 200 µg•mL^{-1} respectively (Table 3). This treatment was administered 1 h after inoculation. All flasks were placed on a production bench open to the outside environment in a greenhouse on the campus of the University of Arkansas.

For the trial under sterile conditions, 500-mL Erlenmeyer flasks were sterilized by means of autoclave. Autoclave settings were adjusted to run a gravity cycle at 121° C with a sterilization time of 15 min at a pressure of 103,422 Pa. Tap water (Washington Water Authority) was autoclaved running a liquid cycle at 121° C with a sterilization time of 15 min and a pressure of 103,422 Pa. The sterilized water was allowed to cool to room temperature. All procedures from this point were executed using sterile technique inside of a clean hood in a lab at the University of Arkansas. Flasks were labeled one through six and filled with 500 mL sterilized water. Flasks two through six were inoculated with 5 mL of the IS. University of Arkansas hydroponic lettuce fertilizer and sulfuric acid were placed under UV light for 60 min to

inactivate any potential microorganisms present. Once deemed sterile, it was added to all six flasks. Target EC was 1.4 $dS \cdot m^{-1}$ and target pH was 5.9. Lastly, flasks three, four, five, and six were given saponin treatments of 25, 50, 100, and 200 $\mu g \cdot mL^{-1}$ respectively (Table 4). This treatment was administered 1 h after inoculation. All flasks were covered with petrifilm and left in the clean hood for the duration of the experiment.

Water aliquots and enumeration of bacteria

Aliquots of solution were taken from each flask at specified time points. Before sampling, each flask was swirled to achieve homogeneity. Using a graduated macropipette, 10 mL of solution were sampled at each time point and placed into a sterile 12-mL test tube.

Serial dilutions were made from each aliquot at each time point. A tenfold dilution factor was held constant to create a logarithmic dilution. Each stepwise dilution used BPW as the dilution solution. Dilutions were plated on MacConkey agar using a spread plate technique. Plates were stored inverted in an incubator at 37° C for 24 h. Plates were manually counted post-incubation to obtain *E. coli* population. Only plates with 25 to 250 CFU were used as representative samples. Populations were transposed logarithmically and recorded as $CFU \cdot mL^{-1}$.

Experimental design and statistical analysis

The treatment design established by the investigator to address the hypothesis included flasks of fertilized water inoculated with *E. coli.* Flasks were also supplied with incremental

concentrations of saponin extract (25, 50, 100, and 200 $\mu g \cdot mL^{-1}$) and placed in either a nonsterile environment or a sterile environment. A non-inoculated flask containing fertilized water and no saponin addition served as the negative control. An inoculated flask containing fertilized water and no saponin addition served as the positive control.

A randomized complete block design was used for the experiment. Each environmental condition consisted of three blocks, each containing six subsamples. Subsamples were six flasks containing 500 mL of fertilized water which were randomly assigned to each of the treatment conditions. Experimental units were populations of *E. coli* across several time points. The first time point occurred 1 h after inoculation. This coincided with the addition of the saponin intervention, which was administered immediately after the first aliquot. The second aliquot was taken 1 h after the addition of the saponin intervention. Aliquots were then taken every 24 h for the next 6 d.

Population means were evaluated for each treatment at each time point using a one-way analysis of variance (ANOVA) for each environmental condition. Mean separation was evaluated using a Tukey's Honest Significant Difference (HSD) test. All tests were performed in JMP® Pro Version 13.2.1 (SAS Institute Inc., Cary, NC). All significance levels were set to $\alpha = 0.05$.

Results

At the initiation of the nonsterile experiment, the non-inoculated treatment (negative control) contained no recoverable *E. coli* (data not shown). All inoculated treatments had no saponin addition yet and contained statistically greater populations of *E. coli* ranging from 1.08 x 10^6 CFU•mL^{-1} to 1.56 x 10^6 CFU•mL^{-1}. All inoculated treatments were statistically similar to one another.

After 1 hr from the saponin addition, the non-inoculated treatment without saponin addition had no recoverable *E. coli* (Table 4). This was significantly different from all other treatments that were inoculated with *E. coli*. Inoculated treatments with saponin additions from 0 to 200 μg•mL^{-1} were all statistically similar to one another ranging from 1.09 x 10^6 CFU•mL^{-1} to 1.61 x 10^6 CFU•mL^{-1}.

After 24 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 5). The inoculated treatment without saponin addition was not significantly different from inoculated treatments with saponin additions of 25, 50, and 100 μg•mL^{-1} and contained an *E. coli* population of 2.65 x 10^7 CFU•mL^{-1}. The inoculated treatment with a saponin rate of 200 μg•mL^{-1} contained the lowest population of recovered *E. coli* with 2.74 x 10^6 CFU•mL^{-1} which was significantly less than treatments with saponin additions of 25 and 50 μg•mL^{-1}, but not significantly different than the inoculated treatment containing a saponin addition of 100 μg•mL^{-1}.

After 48 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 6). The inoculated treatment without

saponin addition had the highest population of *E. coli* with 2.67 x 10^7 CFU•mL^{-1}. However, it was not significantly different from any of the inoculated treatments with saponin additions.

After 72 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 7). The inoculated treatment without saponin addition had the highest population of *E. coli* with 1.30 x 10^7 CFU•mL^{-1} and was statistically similar to the inoculated treatments with saponin additions of 50, 100, and 200 μg•mL^{-1}. The inoculated treatment containing the least amount of saponin addition (25 μg•mL^{-1}) had no recoverable *E. coli* and was similar to the non-inoculated treatment and significantly different than all other inoculated treatments.

After 96 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 8). The inoculated treatment without saponin addition was similar to the inoculated treatment containing a saponin rate of 200 μg•mL^{-1} which contained *E. coli* populations of 1.79 x 10^5 CFU•mL^{-1} and 2.70 x 10^5 CFU•mL^{-1} respectively. These two treatments were significantly higher than any of the other treatments with the saponin addition. The inoculated treatments with saponin additions of 25, 50, and 100 μg•mL^{-1} had no significant differences in recovered *E. coli* populations.

After 120 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 9). The inoculated treatment without saponin addition had an *E. coli* population of 7.42 x 10^6 CFU•mL^{-1} and was significantly higher than the population of any of the other inoculated treatments with saponin addition. There were no significant differences in *E. coli* populations recovered from the inoculated treatments with saponin rates of 25, 50 or 100 μg•mL^{-1}. However, the inoculated solution treated with 200

$\mu g \cdot mL^{-1}$ had a recoverable population of 8.18×10^3 $CFU \cdot mL^{-1}$ which was higher than the inoculated treatments containing the lower saponin concentrations.

After 144 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no detectable *E. coli* (Table 10). All inoculated treatments with and without saponin additions were similar and ranged from 0 $CFU \cdot mL^{-1}$ to 1.8×10^6 $CFU \cdot mL^{-1}$.

At the initiation of the sterile experiment, the non-inoculated treatment contained no recoverable *E. coli* (data not shown). All inoculated treatments had no saponin addition yet and contained statistically greater populations of *E. coli* ranging from 2.00×10^6 $CFU \cdot mL^{-1}$ to 6.32×10^6 $CFU \cdot mL^{-1}$. All inoculated treatments were statistically similar to one another.

After 1 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 11). This was significantly different from all other treatments that were inoculated with *E. coli*. Inoculated treatments with saponin additions from 0 to 200 $\mu g \cdot mL^{-1}$ were all statistically similar to one another ranging from 3.73×10^6 $CFU \cdot mL^{-1}$ to 1.11×10^7 $CFU \cdot mL^{-1}$.

After 24 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 12). The inoculated treatment without saponin addition was similar to the inoculated treatment with a saponin addition of 200 $\mu g \cdot mL^{-1}$ which had *E. coli* populations of 3.12×10^7 $CFU \cdot mL^{-1}$ and 3.06×10^7 $CFU \cdot mL^{-1}$ respectively. The highest populations were recovered in the inoculated treatments with saponin additions of 25, 50, and 100 $\mu g \cdot mL^{-1}$. These treatments were statistically similar and contained *E. coli* populations ranging from 7.24×10^7 $CFU \cdot mL^{-1}$ to 2.79×10^8 $CFU \cdot mL^{-1}$.

After 48 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 13). The inoculated treatment without saponin addition had a similar *E. coli* population to the inoculated treatment with a saponin addition of 25 $\mu g \cdot mL^{-1}$. These two were the greatest recovered *E. coli* populations containing 6.70×10^7 $CFU \cdot mL^{-1}$ and 7.30×10^7 $CFU \cdot mL^{-1}$ respectively. The inoculated treatments with saponin additions of 50 and 100 $\mu g \cdot mL^{-1}$ were statistically similar containing 2.60×10^7 $CFU \cdot mL^{-1}$ and 2.48×10^7 $CFU \cdot mL^{-1}$ respectively. The inoculated treatment with a saponin addition of 200 $\mu g \cdot mL^{-1}$ contained significantly less *E. coli* than all other inoculated treatments with 5.78×10^6 $CFU \cdot mL^{-1}$.

After 72 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 14). The inoculated treatment without saponin addition contained 2.98×10^7 $CFU \cdot mL^{-1}$ and was statistically similar to the inoculated treatment with a saponin addition of 25 $\mu g \cdot mL^{-1}$ which had an *E. coli* population of 6.54×10^7 $CFU \cdot mL^{-1}$. These two treatments contained significantly higher *E. coli* populations than all other treatments. The inoculated treatment with a saponin addition of 200 $\mu g \cdot mL^{-1}$ had an *E. coli* population of 1.54×10^5 $CFU \cdot mL^{-1}$ which was significantly less than all other inoculated treatments.

After 96 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 15). The inoculated treatment without saponin addition contained the highest population of *E. coli* with 4.82×10^7 $CFU \cdot mL^{-1}$. However, it was not significantly different from the inoculated treatments with saponin additions of 25, 50, and 100 $\mu g \cdot mL^{-1}$. The inoculated treatment with a saponin addition of 200 $\mu g \cdot mL^{-1}$

contained the lowest *E. coli* population recovered at 2.43 x 10^3 CFU•mL^{-1} which was not significantly different from all other treatments with saponin additions.

After 120 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 16). The inoculated treatment without saponin addition contained the highest population of *E. coli* with 4.03 x 10^7 CFU•mL^{-1}. However, it was not significantly different from the inoculated treatments with saponin additions of 25 and 50 μg•mL^{-1}. The inoculated treatment with a saponin addition of 100 μg•mL^{-1} had the lowest amount of recovered *E. coli* with 8.77 x 10^2 CFU•mL^{-1}. This treatment was not significantly different from the inoculated treatment with a saponin addition of 200 μg•mL^{-1} which no longer had an *E. coli* population that was recoverable.

After 144 h from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 17). The inoculated treatment without saponin addition continued to have the highest *E. coli* population at 3.62 x 10^7 CFU•mL^{-1} which was not significantly different from the inoculated treatments containing saponin additions of 25 and 50 μg•mL^{-1}. Inoculated treatments with saponin additions of 100 and 200 μg•mL^{-1} did not have recoverable *E. coli* populations. However, they were statistically similar to the inoculated treatments with saponin additions of 25 and 50 μg•mL^{-1}.

Discussion

The two environmental conditions produced somewhat contradictory results. Under sterile conditions, increasing saponin concentrations were correlated with the rate of decline seen in *E. coli* populations. Only the inoculated treatment without any saponin addition maintained a stable population of *E. coli* through 144 h (Fig. 3). In this regard, saponins in a sterile environment behave much like other antimicrobials. When concentrations of antimicrobials are increased, its efficacy becomes more potent (Poncet et al., 2001). Previous research by Aboaba, Smith, and Olude (2006) support these results. Their research was also performed *in vitro* where the team observed antibacterial action against *E. coli* using saponins extracted from *Entanda africana* (*E. africana).* A phytochemical analysis was performed on the *E. africana* extract which revealed mostly saponins, but also phenolic compounds like tannins, which have been shown to have some bactericidal effects (Taguri, Tanaka, and Kouno, 2004). The team, however, concluded that the overwhelming percentage of saponins found in the extract were to be contributed to the antimicrobial properties.

In this study it is important to note that not all saponin levels suppressed *E. coli* to the point of non-recoverable numbers after 144 h. The two highest levels of saponin concentration (100 and 200 μg•mL-1) eliminated the *E. coli* to the point of no recovery. The treatments containing 25 $\mu g \cdot mL^{-1}$ and 50 $\mu g \cdot mL^{-1}$ of saponins had populations of 10^7 and 10^3 $CFU \cdot mL^{-1}$ respectively. Without carrying the experiment out further in time, it is hard to surmise whether these treatments would have suppressed *E. coli* to non-recoverable amounts, or lead to resurgence in population.

Under nonsterile conditions, however, efficacy of saponins as an antimicrobial was more complicated. All treatments containing a saponin addition suppressed *E. coli* populations, but rates of suppression differed from sterile conditions (Fig. 2). Even the inoculated treatment without a saponin addition had a net loss in *E. coli* population. The inoculated treatments with saponin additions of 25, 50, and 100 $\mu g \cdot mL^{-1}$ all suppressed *E. coli* at relatively similar rates. However, the treatment with the highest level of saponin addition (200 $\mu g \cdot mL^{-1}$) suppressed *E. coli* at a much slower rate. This slower rate never completely eradicated the *E. coli* population to a non-recoverable amount. No treatment actually suppressed *E. coli* to non-recoverable amounts after 144 h. The inoculated treatment with a saponin addition of 25 $\mu g \cdot mL^{-1}$ decreased the population to non-recoverable amounts after 72 h, but was followed by a population resurgence over the next 72 h. The lack of complete die back is consistent with previous saponin research on *E. coli* (Soetan et al., 2006; Arabski, 2011). Arabski et al. (2011) postulated that saponins do in fact cause cell permeability, but instead of having a lysing effect, allow nutrients to readily pass through *E. coli* membranes. However, this does not explain why the *E. coli* were suppressed to non-recoverable populations under sterile conditions with increased saponins.

Performing this experiment under nonsterile conditions led to results that were difficult to draw inferences from. By creating a sterile environment, the potential for unwanted microbial interaction to confound the results of *E. coli* growth was eliminated. It is postulated that another microorganism impacted the mortality of *E. coli* in the nonsterile environment. Under sterile conditions, *E. coli* was the only microbe that had the potential to be lysed by the addition of saponins, and thus was the case. Under nonsterile conditions, a diverse collection of microorganisms were vying to survive. Microbes are constantly in close competition with other microbes for the same resources and space. Bacteria can weaken or eradicate other bacterial

species through diverse mechanisms (Hibbing, Fuqua, and Peterson, 2010). Without the addition of saponins, *E. coli* and presumably a host of other microbes maintained a status quo in which all microbes remained at stable populations in a natural balance of ecology. When saponins were added at high concentrations, the death rate of *E. coli* occurred at a slow pace. This can be explained through the previously mentioned postulate of Arabski (2011) and the findings from Soetan et al. (2006), who described saponin's antimicrobial effect as being limited to Gram-positive bacteria only. As saponins increased, the ecology within each flask broke down as natural Gram-positive bacterial populations were lysed by saponins. However, the debris of these lysed cells created an abundance of nutrients available to Gram-negative bacteria, like *E. coli.* Newly developed pores in *E. coli* membranes, created by saponins, passed these nutrients readily, maintaining a high population. Inevitably the food source waned, leading to a decline in *E. coli* population. Lower concentrations of saponins in turn lysed Gram-positive cells less quickly. This lower yield of decaying nutrients to feed upon caused a faster decline in *E. coli* populations as ecological homeostasis broke down. It is hypothesized that an unknown Gram-negative bacteria outcompeted *E. coli* for the limited nutrients available.

It should be noted in this experiment that several time points had vast numerical differences that were not statistically different (i.e. Table 6). Reproducing comparable data was difficult throughout the course of this experiment and led to high variance between replications. Previous research with saponin extracts has reported similar difficulties. Oleszek (1996) stated that saponin extractions from the same plant material, at the same plant location, using the same extraction techniques could not produce similar results from one replication to the next.

Conclusions

Overall, steriodal saponins extracted from *Yucca schidigera* exhibited inhibitory effects on *E. coli* in both nonsterile and sterile conditions. However, environmental conditions influenced the ability of the saponin compound to inhibit the mortality of *E. coli.* Increasing the concentration of saponins increased the rate of death for *E. coli* populations under sterile conditions. *E. coli* without saponins in fertilized irrigation water survived at significant populations for up to 144 h under nonsterile and sterile conditions. Overall, the investigated saponin does not appear to be an acceptable additive as a mitigation tool for *E. coli* in a hydroponic nutrient solution to meet the zero-tolerance threshold of FSMA compliance.

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Tables

Table 1. Saponin solution content specifications.

Typical analysis of saponin solution[z]:	
Moisture, %	50.00
Acid Detergent Fiber, %	0.50
Ash, %	2.61
Sulfur, %	0.07
Phosphorus, %	0.12
Sodium, %	0.09
Copper, %	<0.01
Zinc, %	<0.01
Crude Protein, %	1.49
Acid Hydrolysis Fat, %	3.28
Magnesium, %	0.21
Potassium, %	0.68
Calcium, %	0.31
Iron, %	<0.01
Manganese, %	<0.01
Estimated ME, kcal/kg	1,805.15

[z]Total dissolved solids: 50%

Table 2. University of Arkansas greenhouse lettuce fertilizer formulation[zy]

Fertilizer source[x]	Stock A	Stock B
$Ca(NO_3)_2\ 3(H_2O)$	90 g•L^{-1}	
KNO_3	40 g•L^{-1}	
10% Fe-DTPA	4.11 g•L^{-1}	
K_2SO_4		17 g•L^{-1}
KH_2PO_4		15 g•L^{-1}
$MgSO_4\ 7(H_2O)$		60 g•L^{-1}
$MnSO_4H_2O$		310 mg•L^{-1}
$ZnSO_4\ 7(H_2O)$		30 mg•L^{-1}
H_3BO_3		275 mg•L^{-1}
$CuSO_4\ 5(H_2O)$		39 mg•L^{-1}
$(NH_4)_6\ Mo_7O_{24}\ 4(H_2O)$		11.1 mg•L^{-1}

[z]Does not account for mineral elements present in water

[y]Stock A and B must be added at equal rates

[x] $Ca(NO_3)_2\ 3(H_2O)$ = calcium nitrate, KNO_3 = potassium nitrate, 10% Fe-DTPA = 10% iron chelate, K_2SO_4 = potassium sulfate, KH_2PO_4 = monopotassium phosphate, $MgSO_4\ 7(H_2O)$ = magnesium sulfate heptahydrate, $MnSO_4H_2O$ = manganese sulfate monohydrate, $ZnSO_4\ 7(H_2O)$ = zinc sulfacte heptrahydrate, H_3BO_3 = boric acid, $CuSO_4\ 5(H_2O)$ = copper sulfate pentahydrate, $(NH_4)_6\ Mo_7O_{24}\ 4(H_2O)$ = ammonium molybdate tetrahydrate.

Table 3. Nonsterile and sterile flask analysis by treatment

Treatment	*E. coli*[z]	Saponin addition[y]
1	-	-
2	+	-
3	+	+25 µg•mL^{-1}
4	+	+50 µg•mL^{-1}
5	+	+100 µg•mL^{-1}
6	+	+200 µg•mL^{-1}

[z]Positive (+) indicates presence of *E. coli* at a population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.

[y]Positive (+) indicates presence of saponins followed by specified rate, negative (-) indicates saponin intervention not present.

Table 4. Comparison of colony forming units (CFU) means by treatment under nonsterile conditions 1 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 b
+	0	13.94654	1.14 x 10^6 a
+	12.5 µg•mL^{-1}	14.22762	1.51 x 10^6 a
+	25 µg•mL^{-1}	14.29175	1.61 x 10^6 a
+	50 µg•mL^{-1}	13.99783	1.20 x 10^6 a
+	100 µg•mL^{-1}	13.90169	1.09 x 10^6 a

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 5. Comparison of colony forming units (CFU) means by treatment under nonsterile conditions 24 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 b
+	0	17.09266	2.65 x 10^7 a
+	12.5 µg•mL^{-1}	17.42101	3.68 x 10^7 a
+	25 µg•mL^{-1}	17.10391	2.68 x 10^7 a
+	50 µg•mL^{-1}	16.75995	1.90 x 10^7 ab
+	100 µg•mL^{-1}	14.82347	2.74 x 10^6 b

[z]Positive (+) indicates presence of *E. coli* at a population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 6. Comparison of colony forming units (CFU) means by treatment under nonsterile conditions 48 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 a
+	0	17.10017	2.67 x 10^7 a
+	12.5 µg•mL^{-1}	16.51014	1.48 x 10^7 a
+	25 µg•mL^{-1}	16.04875	9.33 x 10^6 a
+	50 µg•mL^{-1}	16.68908	1.77 x 10^7 a
+	100 µg•mL^{-1}	16.72786	1.84 x 10^7 a

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 7. Comparison of colony forming units (CFU) means by treatment under nonsterile conditions 72 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 b
+	0	16.38046	1.30 x 10^7ab
+	12.5 µg•mL^{-1}	0	0 b
+	25 µg•mL^{-1}	3.06805	2.05 x 10^1 ab
+	50 µg•mL^{-1}	14.11562	1.35 x 10^6 ab
+	100 µg•mL^{-1}	15.53470	5.58 x 10^6 a

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 8. Comparison of colony forming units (CFU) means by treatment under nonsterile conditions 96 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 b
+	0	12.09515	1.79 x 10^5 a
+	12.5 µg•mL^{-1}	1.38629	3 b
+	25 µg•mL^{-1}	2.83321	16 b
+	50 µg•mL^{-1}	0	0 b
+	100 µg•mL^{-1}	12.50618	2.70 x 10^5 a

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 9. Comparison of colony forming units (CFU) means by treatment under nonsterile conditions 120 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 c
+	0	15.81969	7.42 x 10^6 a
+	12.5 µg•mL^{-1}	1.25276	2.5 c
+	25 µg•mL^{-1}	0.91629	1.5 c
+	50 µg•mL^{-1}	0.69315	1 c
+	100 µg•mL^{-1}	9.00957	8.18 x 10^3 b

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[z]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 10. Comparison of colony forming units (CFU) means by treatment under nonsterile conditions 144 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 a
+	0	14.41435	1.82 x 10^6 a
+	12.5 µg•mL^{-1}	7.02198	1.12 x 10^3 a
+	25 µg•mL^{-1}	3.01062	19.3 a
+	50 µg•mL^{-1}	0.69315	1 a
+	100 µg•mL^{-1}	6.14847	4.67 x 10^2 a

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 11. Comparison of colony forming units (CFU) means by treatment under sterile conditions 1 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 b
+	0	15.44475	5.10 x 10^6 a
+	12.5 µg•mL^{-1}	16.22246	1.11 x 10^7 a
+	25 µg•mL^{-1}	16.02817	9.14 x 10^6 a
+	50 µg•mL^{-1}	15.75425	6.95 x 10^6 a
+	100 µg•mL^{-1}	15.13192	3.73 x 10^6 a

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[z]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 12. Comparison of colony forming units (CFU) means by treatment under sterile conditions 24 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 c
+	0	17.25593	3.12 x 10^7 b
+	12.5 µg•mL^{-1}	19.44672	2.79 x 10^8 a
+	25 µg•mL^{-1}	18.20996	8.10 x 10^7 ab
+	50 µg•mL^{-1}	18.09772	7.24 x 10^7 ab
+	100 µg•mL^{-1}	17.23651	3.06 x 10^7 b

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 13. Comparison of colony forming units (CFU) means by treatment under sterile conditions 48 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 e
+	0	18.02020	6.70 x 10^7 ab
+	12.5 µg•mL^{-1}	18.10597	7.30 x 10^7 a
+	25 µg•mL^{-1}	17.07361	2.60 x 10^7 bc
+	50 µg•mL^{-1}	17.02635	2.48 x 10^7 c
+	100 µg•mL^{-1}	15.56991	5.78 x 10^6 d

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 14. Comparison of colony forming units (CFU) means by treatment under sterile conditions 72 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 d
+	0	0	2.98 x 10^7 a
+	12.5 µg•mL^{-1}	17.99603	6.54 x 10^7 a
+	25 µg•mL^{-1}	14.73579	2.51 x 10^6 b
+	50 µg•mL^{-1}	13.63279	8.33 x 10^5 b
+	100 µg•mL^{-1}	11.94471	1.54 x 10^5 c

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 15. Comparison of colony forming units (CFU) means by treatment under sterile conditions 96 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 c
+	0	17.69087	4.82 x 10^7 a
+	12.5 µg•mL^{-1}	17.36800	3.49 x 10^7 ab
+	25 µg•mL^{-1}	12.85318	3.82 x 10^5 abc
+	50 µg•mL^{-1}	10.90230	5.43 x 10^4 abc
+	100 µg•mL^{-1}	7.79606	2.43 x 10^3 bc

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 16. Comparison of colony forming units (CFU) means by treatment under sterile conditions 120 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 b
+	0	17.51186	4.03 x 10^7 a
+	12.5 µg•mL^{-1}	17.27183	3.17 x 10^7 ab
+	25 µg•mL^{-1}	11.00045	5.99 x 10^4 ab
+	50 µg•mL^{-1}	6.77765	8.77 x 10^2 b
+	100 µg•mL^{-1}	0	0 b

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Table 17. Comparison of colony forming units (CFU) means by treatment under sterile conditions 144 h after saponin intervention.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 b
+	0	17.40457	3.62 x 10^7 a
+	12.5 µg•mL^{-1}	17.19990	2.95 x 10^7 ab
+	25 µg•mL^{-1}	8.79800	6.62 x 10^3 ab
+	50 µg•mL^{-1}	0	0 b
+	100 µg•mL^{-1}	0	0 b

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^6 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a Tukey's honest significant difference test ($\alpha = 0.05$).

Figures

Fig.1. *E. coli* inoculated irrigation water trials with saponin treatments from (a) a nonsterile greenhouse environment and (b) a sterile clean hood. Treatments begin left to right, starting with one and ending with six. See table 3 for treatment analysis.

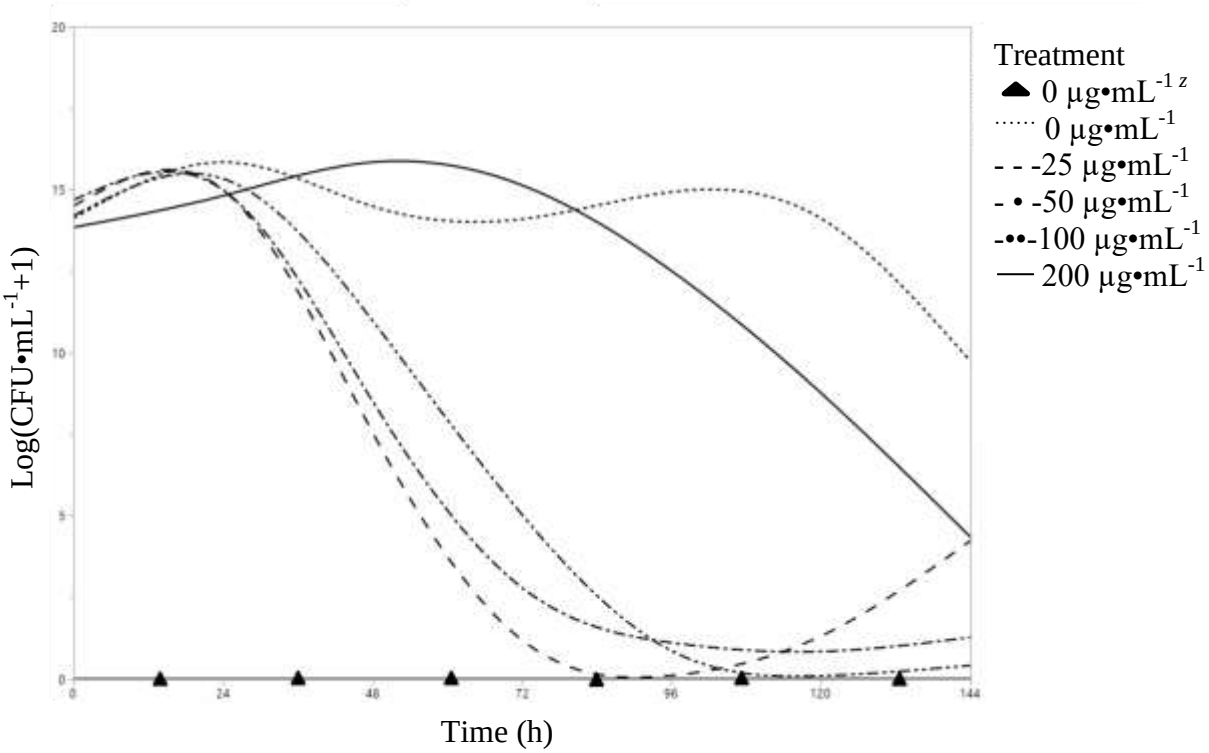

Fig. 2. LS means plot of *E. coli* concentration by treatment over time under nonsterile conditions.
[z]Indicates the non-inocultaed treatment.

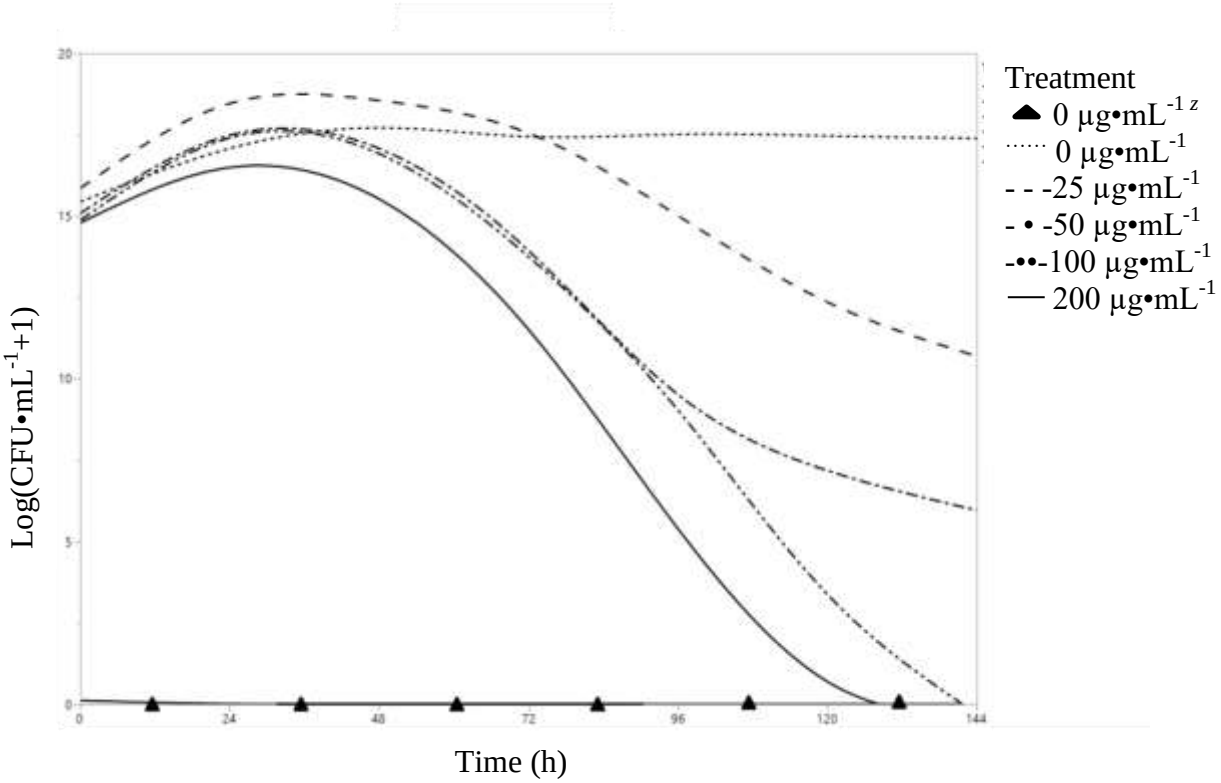

Fig. 3. LS means plot of *E. coli* concentration by treatment over time under sterile conditions.
[z]Indicates the non-inocultaed treatment.

Chapter 2

Efficacy of Antimicrobial Mitigation on *Escherichia coli* CFU and Growth and Development of Hydroponic 'Rex' Lettuce

Abstract

A commercial saponin extract solution derived from *Yucca schidigera* was evaluated for bactericidal effects against *Escherichia coli* (*E. coli*) and toxicity to lettuce grown in a hydroponic system. Isolates of *E. coli* (P4, P13, and P68) were combined in equal parts and added to 130 L of a fertilized solution recirculating in an NFT system growing 'Rex' lettuce. After 5 weeks in the NFT system, *E. coli* populations were lowest in the inoculated treatment that did not contain any saponin addition when compared to all other inoculated treatments. All treatments containing saponins had *E. coli* populations significantly higher than the treatments without saponins. The treatment containing 100 $\mu g \cdot mL^{-1}$ saponin extract had an *E. coli* population of 10^4 $CFU \cdot mL^{-1}$ after 5 weeks which was significantly more than all other treatments. Increasing *E. coli* populations were directly correlated with increasing saponin concentration. Plant growth was also stymied by increasing saponin concentrations. Fresh shoot weight and dry shoot weight were both significantly greater in treatments without the saponin addition after 5 weeks in the NFT system. Lettuce head diameter was significantly reduced by saponin treatments with concentrations of 50 and 100 $\mu g \cdot mL^{-1}$. Saponins, a nonionic surfactant, elicited a toxicity effect on plant growth and development most likely through root membrane permeation. Lettuce leaves were also tested for the potential of *E. coli* to travel systemically to the edible portions of the plant. No *E. coli* was found to travel in this manner. It was concluded that steroidal saponins extracted from *Yucca schidigera* are not an acceptable compound for use

in mitigation of *E. coli* in hydroponic irrigation water due to its ineffectiveness as a bactericide and toxicity to lettuce.

Introduction

Every year 48 million Americans become infected from a foodborne disease; 128,000 of which require hospitalization and of these 3,000 die (CDC, 2017). These figures do not include undiagnosed cases of infection. *E. coli* are one of the most prominent causes of foodborne diarrheal disease in humans. It is also a leading contributor to bacterial infections and extra-intestinal infections in humans and animals alike (Njage and Buys, 2014). *E. coli* bacteria are typically present in the intestines of warm blooded mammals colonizing the infant gastrointestinal tract in the first hours after birth (Nataro and Kaper, 1998). There are many serotypes of *E. coli* and most of them are harmless and normally inhabit the intestinal tract (Clermont, Bonacorsi, and Bingen, 2000). Others, such as Shiga toxin-producing *E. coli* (STEC) O157:H7, are pathogenic and will cause illness upon infection.

Phylogenetic analysis has shown that *E. coli* is composed of four main phylogenetic groups: A, B1, B2, and D. Pathogenic varieties contain groups B2 and D (Clermont, Bonacorsi, and Bingen, 2000). At least six different pathotypes of *E. coli* have been identified to cause a variety of disease symptoms (Kaper, Nataro, and Mobley, 2004). These virulent strains (or pathovars) are divided into groups based on mechanisms of infection and the symptoms they produce (Ray and Bhunia, 2014): enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffuse-adhering *E. coli* (DAEC). Each pathogenic strain has its own unique combination of virulence factors (Kaper, Nataro, and Mobley, 2004), but ultimately all of them cause damage to epithelial cells in the intestinal lining.

An intensely monitored serotype of *E. coli* is O157:H7, which belongs to the pathovar group EHEC. The infamous serotype O157:H7 is the most frequently reported serotype related to foodborne illness outbreaks in the United States (Kehl et al., 1997). Persons infected with O157:H7 may experience hemorrhagic colitis (bloody diarrhea) and hemorrhagic uremic syndrome (Ray and Bhunia, 2014). Hemorrhagic uremic syndrome (HUS) is a life threatening illness that causes hemolytic anemia, thrombocytopenia, and renal insufficiency which can lead to kidney failure and death (Gould, 2009). Every human is susceptible to HUS, but those with compromised immune systems are most at risk. A primary source of *E. coli* infection in the United States is through contaminated agricultural products. These products serve as the vehicle *E. coli* utilizes to transfer from one intestinal tract to another.

The Food and Drug Administration (FDA) has made a complete overhaul of the nation's food safety procedures in order to address new challenges imposed by a global food system. The system revamp was signed into law in 2011 and is known as the Food Safety Modernization Act (FSMA). FSMA establishes science-based minimum standards for the safe growing, harvesting, packing, and holding of fruits and vegetables grown for human consumption (FDA, 2011). The law shifts the focus of federal regulators to prevent foodborne outbreaks rather than respond to outbreaks after they have already happened.

The FDA has put forth these standards in an effort to improve public health while minimizing the burden of responsibility to the American farmer through process validation and infrequent inspections. To be in compliance, farmers must undergo irrigation water testing that screens for microbiological activity. Under the new FSMA requirements, produce growers using irrigation water from an untreated source can have no detectable generic *E. coli* in their water

(FDA, 2011). If an assay for *E. coli* is found to be positive, it is an indication of fecal contamination in the water and a public health concern whether or not the serotype is a STEC.

Preventative measures to disinfest water must be a focal point for food safety and public health. Cultural practices, such as personal hygiene and sick employee protocols, will always remain a continually important area in the production of produce, but further disinfestation measures will ensure a crop free of microbial pathogens like *E. coli*. Current techniques used to mitigate microbial pathogens can be effective, but have cost and complexity limitations that prohibit their use to many farmers. UV radiation is effective and widely used, however small pathogens may pass by the light waves in the shadow of debris and remain active, therefore filtration of the water and cleaning of the UV lamp (Garibaldi et al., 2004) are paramount to this method's usefulness. Biofiltration may reduce pathogens, but does not eliminate them (Wohanka et al., 1999; Belbahri et al., 2007). Heat pasteurization requires large energy inputs making this a non-viable method on a commercial scale (Newman, 2004). Oxidizing agents, such as ozone injection and chlorination, may eliminate pathogens in water at proper doses, but have adverse effects when used in crop irrigation water. Ozone injection may react with some fertilizers oxidizing iron, manganese, and sulfides (Newman, 2004). Chlorination may cause phytotoxic symptoms to plants (Premuzic et al., 2007) and produce the by-product trihalomethane which is classified by the Environmental Protection Agency (EPA) as potential human carcinogen (Symons et al., 1981). Iodine is a strong oxidizer, but similarly to chlorination treatments must be monitored for plant phytotoxicity. Iodine also gives off a foul odor and is costly to maintain (Kim et al., 2000). Electrolysis of water by silver and copper electrodes release heavy metals into the water and therefore cause environmental concerns with regards to local watersheds (Van Os., 2008).

Food scientists have uncovered antimicrobial properties related to natural compounds and their applications to producers. Chemical food preservation has increased in recent years causing an increase in consumer demand for foods that have undergone minimal processing using more naturally based preservatives (Cleveland et al., 2001). Therefore, natural antimicrobials are becoming more prevalent among microbial disinfestation methods in the food industry (Zhu et al., 2015). Plant based isolated compounds contain secondary metabolites that are known to retard or inhibit the growth of bacteria, yeasts, and molds (Tiwari et al., 2009). Saponins are secondary metabolites found in numerous plant species. Their name is derived from their ability to foam in water similar to soap (Francis et al., 2002); hence the naming of the compound derived from the Latin word "*sapo*" (Naoumkina et al., 2010). Saponins are nonionic detergents that have an assortment of biological properties. Their structure is comprised of a steroidal or triterpenoid aglycone skeleton attached to one or more sugar chains (Arabski et al., 2011). This diversity in structure is what leads to the great diversity in biological properties. Saponin properties are known to be: antibacterial, antifungal, hemolytic, membrane depolarizing, ammonia binding, antiyeast, antimold (Oleszek, 1996; Arabski et al., 2011), and many others. Their effects are generally credited to their ability to permeate cellular membranes (Francis et al., 2002).

This study had three objectives. The first objective was to evaluate the antimicrobial effects of a saponin solution extracted from *Yucca schidigera* on *E. coli* in a recirculating hydroponic NFT system. The second objective was to evaluate the effects of the same saponin extract on plant growth and development traits and how this will affect the decision making process of whether or not to use the extract in question as a mitigation tool for *E. coli* contaminated water. The third objective was to test the postulate that *E. coli* can move

systemically from contaminated water, through the root system, and into the edible portions of lettuce plants. It was hypothesized that increasing the concentration of saponins would decrease viable *E. coli* population as well as decrease yields of 'Rex' lettuce heads in a greenhouse hydroponic system. It was also hypothesized that *E. coli* would not travel systemically through the plants vascular system to edible portions of 'Rex' lettuce.

Materials and Methods

Microbial stock culture

Individual isolates of non-pathogenic *E. coli* (P4, P13, and P68) were obtained from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, Iowa (42.0266° N, 93.6465° W). These isolates were non-verotoxin producing *E. coli* strains, have been classified as a biosafety level 1 contaminant, and recommended for use in antimicrobial susceptibility testing for direct comparison to *E. coli* O157:H7 based on growth and biochemical characteristics (Marshall et al., 2005).

Bacteria were stored at a temperature of -21° C. Bacterial strains were grown and combined under sterile laboratory conditions in the Food Sciences building at Iowa State University, Ames, Iowa using the following procedure: Propagation of strains was quarantined from one another in 10 mL test tubes containing Brain Heart Infusion (BHI) broth (Difco, Becton Dickinson, Sparks, Md.). Cultures were left for 24 h at 37° C. They were then transferred to fresh BHI broth twice more at 24 h intervals at 37° C before the final transfer to BHI broth. Individual strains were then centrifuged at 10,000 rpm for 10 min. The supernatant was poured off and the remaining pellet was suspended in saline. This procedure was repeated for each of the three bacterial isolates. The three individual isolates were then mixed into a single homogenous solution (referred to as the cocktail from here out). This process resulted in a cocktail containing *E. coli* at a concentration of 10^8 colony forming units (CFU)•mL^{-1} and saline to be used in inoculation of the nutrient tank water later in the experiment. A cryoprotective solution was created by adding glycerol to the cocktail to protect bacterial protein layers during freezing. Viable bacterial counts for the cocktail were quantified using serial dilutions and the

standard plate count method on MacConkey agar. The cocktail was frozen in 1 mL micro vials and transported to the University of Arkansas, Fayetteville, Arkansas (36.0678° N, 94.1737° W) where they were stored at -21° C until ready for use.

Inoculation solution

Frozen cocktail *E. coli* cultures with a population of 10^8 CFU•mL^{-1} in 1 mL micro vials were removed from -21° C cold storage. Frozen cultures were thawed slowly in a cold water bath. Using sterile technique under a clean hood, 1 mL of thawed cultures were transferred via air displacement micropipette into 9 mL of buffered peptone water (BPW) (Hardy Diagnostics, Santa Maria, Calif.) in 12 mL test tubes and capped. The inoculated test tubes were then incubated at a constant 37° C for a period of 24 h. The resulting solution was 10 mL of *E. coli* cocktail (isolates P4, P13, and P68) at a population of 10^8 CFU•mL^{-1}. This solution constituted the inoculation solution (IS).

Preparation of agar media

Using a digital balance (Ohaus, AP250D), 52.49 g of dehydrated MacConkey agar (Hardy Diagnostics, Santa Maria, Calif.) was weighed out. The dehydrated agar was then added to 1 L of deionized (DI) water in a 2-L Erlenmeyer flask containing a magnetic stir bar. The solution was then heated and mixed on a hotplate stirrer until boiling. At this point, the liquid agar was boiled for an additional 60 s to allow for complete dissolution of the agar. The liquid agar solution was then transferred in equal parts to two 2-L Pyrex autoclave bottles and placed in

an autoclave. Autoclave settings were adjusted to operate a liquid cycle at 121° C with a sterilization time of 15 min at a pressure of 103,422 Pa. Upon completion of the autoclave cycle, the liquid agar was allowed a resting period to cool to a temperature of no more than 60° C and no less than 55° C. Then containment bottles were sterilized by spraying with ammonium chloride (Coverage Plus NPD, Steris, Mentor, Ohio) and moved to a sterile clean hood. To create a selective media, the antimicrobial Rifampicin (Tokyo Chemical Industry, Tokyo) was added to each bottle of media at a concentration of 1 $mL \cdot L^{-1}$ and dissolved by swirling the bottle to create a vortex. This addition of Rifampicin created a selective agar media. Using a graduated macropipette, 15 mL of the selective media was transferred into sterile slippable 100 mm x 15 mm Petri dishes (VWR, Radnor, Pa.) and allowed to cool. Once the agar cooled to 40° C it solidified and was set aside. At this point, 20 g of dehyrdrated tryptic soy agar (Difco, Becton Dickinson, Sparks, Md.) was weighed out on an electronic balance. Using a graduated cylinder, 500 mL of DI water was measured out and added to a 1 L Erlenmeyer flask containing a magnetic stir bar along with the dehydrated tryptic soy agar (TSA). The flask was placed on a hotplate stirrer until boiling. Once boiling, the agar was removed and the dissolved agar solution was transferred in equal parts into two 1-L Pyrex autoclave bottles and placed in an autoclave. Autoclave settings were adjusted to run a liquid cycle at 121° C with a sterilization time of 15 min and a pressure of 103,422 Pa. Upon completion of the autoclave cycle, the liquid agar was allowed a resting period to cool to a temperature of no more than 60° C and no less than 55° C. Then containment bottles were sterilized by spraying with ammonium chloride (Coverage Plus NPD, Steris, Mentor, Ohio) and moved to a sterile clean hood. Using a graduated macropipette, 6 mL of TSA media was transferred to each of the previously prepared MacConkey plates to

create an overlay. Once the agar cooled to 40° C it solidified and the TSA overlay plates were then stacked and packaged in sterile bags and placed in a refrigerator at 4° C.

Saponin solution

The saponin product used in this study was supplied in a premixed solution (DPI Global, Porterville, Calif.), and contained sapogenin with a steroidal aglycone structure extracted from *Yucca schidigera.* The solution was certified by the Organic Materials Review Institute (OMRI) and contained 14% saponins. Specifications of total dissolved solids and typical analysis are reported in Table 1. The manufacturer conducted a microbiological analysis that did not detect any yeasts or molds, coliforms, *Staphylococcus aureus*, or *Salmonella.* Treatment rates were supplied by amount of active ingredient in the saponin solution.

Propagation of seedlings and transplant to nutrient film technique (NFT) system

In a greenhouse at the University of Arkansas, Oasis Horticube (Smithers Oasis, Kent, Ohio) hydroponic grow media (276 cell) was placed in ebb and flow hydroponic propagation trays (American Hydroponics, Arcata, Calif.). The media was then leached with fresh water in order to saturate completely and expel any contaminants acquired from manufacturing. Individual seed of 'Rex' lettuce (Johnny's Selected Seeds, Winslow, Maine) was sown in each Oasis cell at a rate of one seed per cell. Seeds were then top watered with fertilized water using the University of Arkansas greenhouse lettuce formulation (Table 2; Chapter 1) at an EC of 1.0 $dS \cdot cm^{-1}$ and pH 5.9. Ebb and flow trays were then covered with white plastic sheeting to

increase the humidity at the site of germination. Subsequent watering was administered by sub irrigation using the same fertilizer recipe and concentration as previously mentioned; frequency was dependent upon abiotic conditions present in the greenhouse. Greenhouse thermostatic controls were set to heat at 18.3° C and cool at 21.1° C. Seedlings remained in the nursery until developing four true leaves which took on average 19 d. All seedling populations were screened for *E. coli,* and since no *E. coli* populations were recovered seedlings were deemed to be *E coli* free at this stage.

Hydroponic NFT systems (American Hydroponics, Arcata, Calif.) were prepared by adding 130 L of county water (Washington Water Authority, Prairie Grove, Ark.) to the nutrient tank and beginning the flow of water. Each of the NFT systems utilized a recirculating irrigation system fed by a submersible pump in a reservoir (nutrient tank) pumping water to each trough at a flow rate of 275 $mL \bullet min^{-1}$. The NFT troughs were positioned to maintain a 2.5% slope in order to keep the flow of solution through the trough continuous and never stagnant. Nutrient tanks that received *E. coli* treatment were inoculated at this time with 20 mL of the IS. Next, nutrient minerals were provided using the University of Arkansas greenhouse lettuce fertilizer formulation (Table 2; Chapter 1) with an EC of 1.4 $dS \bullet cm^{-1}$. Sulfuric acid was added to lower the pH to 5.9. The previously mentioned water chemistry and water volume was maintained throughout the duration of the experiment. Next, the saponin solution was added at varying levels to each nutrient tank. Lastly, seedlings were transplanted into the NFT systems. Plant density was spaced so that each lettuce head was 20.32 cm x 20.32 cm apart from one another. Seedlings were selected to best represent a uniform distribution at the time of transplanting. Automated greenhouse controls were set to heat the greenhouse at 18.3° C and cool at 21.1° C. Only ambient light was used.

Inoculation of NFT systems and saponin intervention

Each individual NFT system was designated a treatment at random by rolling a six sided die. Treatment 1 served as the negative control, containing no *E. coli* and no saponins. Treatments 2 through treatment 6 were all inoculated with 20 mL of the IS to give an initial population of 10^4 CFU•mL^{-1} per reservoir. Treatment 2 served as the positive control. It contained *E. coli,* but did not contain any saponin intervention. Treatements 3 through treatment 6 all contained a saponin intervention at increasing levels. Treatment 3 was given saponins at a rate of 12.5 μg•mL^{-1}, the lowest level of intervention. Treatment 4 was given saponins at a rate of 25 μg•mL^{-1}. Treatment 5 was given saponins at a rate of 50 μg•mL^{-1}. Treatment 6 was given saponins at a rate of 100 μg•mL^{-1}.

Data collection

Water temperature, EC, and pH of the nutrient water flowing through the NFT system was monitored and recorded daily with a combination EC/pH/temperature probe (Bluelab, Tauranga, New Zealand). Weekly average water temperatures can be found in Table 1.

Water samples of 25 mL were taken at various time points throughout the duration of the experiment. Each NFT system was sampled individually. To ensure a homogenous sample, 5 mL aliquots were taken using a graduated macropipette from five separate locations within each system: top half of the nutrient reservoir, lower half of the nutrient reservoir, drain collector, NFT channel, and dripper emitter. These samples were used to evaluate living *E. coli* populations in each NFT system.

Enumeration of *E. coli* populations was determined by creating serial dilutions for each 25 mL aliquot. A tenfold dilution factor was held constant to create a logarithmic dilution. Each stepwise dilution used BPW as the dilution solution. Dilutions were plated on TSA overlay plates using a spread plate technique. Plates were stored inverted in an incubator at 37° C for 24 h. Plates were manually counted post-incubation. Only plates with 25 to 250 CFU were used as representative samples. Counts were transposed logarithmically and recorded as $CFU \bullet mL^{-1}$.

Equal amounts of heads of lettuce were collected for analysis from each NFT system at various time points throughout the course of this experiment. Plants were evaluated for growth characteristics, or tested for the presence of *E. coli*. All plants were pulled from the NFT channels simulating a hydroponic grower's harvest technique with intact roots. The root system was then removed by cutting the stalk level with the remaining Oasis grow media.

Plants that had been designated for measuring growth characteristics were weighed immediately to determine fresh shoot weight (g) on a digital balance (Ohaus, AP250D). Lettuce head diameter was then measured (cm) using a standard ruler. Lettuce heads were then placed inside a paper sack and into an oven. Heads were heated at moderate temperature for a minimum of 2 d to become devoid of any water content. The remaining contents were weighed on a digital balance to determine dry shoot weight (g).

Plants that had been designated to be tested for *E. coli* presence were harvested as described earlier and then immediately transferred into 35.56 cm x 48.26 cm sterile sample bags (Nasco, Fort Atkinson, Wis.). Plant weight was determined using a digital balance (Ohaus, AP250D). Plant weight was multiplied by nine; the product yielded the amount of DI water (mL) to be added to the sample bag. The bag contents were then manually stomached.

Stomaching is a homogenization technique used in microbiological examination of various foods in which solids are ground together with liquids in order to release viable microorganisms (Tuttlebee, 1975). The resulting solution was used to determine presence or absence of *E. coli* on or within the edible portions of the lettuce leaves by the enumeration techniques described previously.

Experimental design and statistical analysis

The treatment design established by the investigator to address the hypothesis included six individual hydroponic NFT systems located side by side in the same greenhouse space. All NFT systems maintained 130 L of fertilized water. Of the six NFT systems, five were inoculated with *E. coli* leaving a non-inoculated systems serving as the negative control. The remaining five systems were each supplied with incremental concentrations of saponin extract (0, 12.5, 25, 50, and 100 $\mu g \bullet mL^{-1}$). The inoculated system that did not contain a saponin addition served as the positive control.

A randomized complete block design was used for this experiment. Each of three replications was treated as individual blocks, all of which contained all six treatment levels in randomized order. Treatment locations were chosen for each block by rolling a six sided die. Data collection was dictated by a routine time schedule. Water aliquots were taken from all treatments 1 h after inoculation, 1 h after the addition of saponins, and then once every week for five weeks. Plant collections for growth characteristics and *E. coli* presence were taken after 1 week of growth in the NFT system and every week after for 5 weeks.

For *E. coli* enumeration, data was analyzed as a repeated measure to evaluate changes over time to each treatment level. A full factorial using response variable $CFU \bullet mL^{-1}$ and factor levels treatment, time, and water temperature was performed to examine interaction effects and main effects. Means for treatment effects at each time interval were separated using a pairwise student's t-test (LSD). Average water temperature was calculated as a pooled average from all NFT nutrient reservoirs for each time point between sampling. An equivalence test was performed to validate mean differences of weekly water temperatures were in fact not significantly different from one another (Table 1).

Plant growth and development data were normalized by examining each measurement as a percentage of the negative control. Not all replications could be run simultaneously; therefore this technique was used to eliminate the changing abiotic factors (i.e. ambient light, temperature) throughout the course of each replication. A one-way analysis of variance (ANOVA) was used at each time point to evaluate mean differences of three growth characteristics: shoot fresh weight, shoot dry weight, and fresh head diameter. Mean separation was determined using Tukey's HSD for each characteristic.

Statistical analyses were performed using JMP® Pro Version 13.2.1 (SAS Institute Inc., Cary, NC). All significance levels were set to $\alpha = 0.05$.

Results

Part 1:

Effects of saponins on growth of E. coli

The three way interaction of time*treatment*water temperature was not significant, nor were any of the two way interaction effects (Table 2). Water temperature and time were not significant as main effects, however treatment was significant (p-value = 0.0073).

After 1 h from inoculation of the nutrient water reservoirs, the non-inoculated treatment contained no recoverable *E. coli* (Table 3). All inoculated treatments had no saponin addition at this time point and contained trace amounts of *E. coli*.

After 1 h from the saponin addition, the non-inoculated treatment without saponin addition had no recoverable *E. coli* (Table 4). The inoculated treatment without saponin addition contained trace amounts of *E. coli* and was statistically similar to all of the inoculated treatments containing a saponin addition.

After 168 h (1 week) from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 5). All of the inoculated treatments increased *E. coli* CFUs by at least 2 logs. The inoculated treatment without saponin addition had numerically the fewest *E. coli* CFUs of all the inoculated treatments with 3.06×10^2 CFU•mL^{-1} and was statistically similar to the inoculated treatment with a saponin concentration of 12.5 μg•mL^{-1} which had 3.69×10^3 CFU•mL^{-1}. The inoculated treatments with saponin concentrations of 25, 50, and 100 μg•mL^{-1} were all statistically similar and had the greatest amount of *E. coli* ranging from 2.87×10^4 CFU•mL^{-1} to 1.89×10^5 CFU•mL^{-1}.

After 336 h (2 weeks) from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 6). The inoculated treatment without saponin addition had 1.47 x 10^2 CFU•mL^{-1} which was similar to the inoculated treatment with a saponin concentration of 12.5 μg•mL^{-1}. The inoculated treatment with a saponin concentration of 25 μg•mL^{-1} contained 1.91 x 10^4 CFU•mL^{-1} and was statistically similar to all inoculated treatments with saponin additions. The inoculated treatments containing saponin concentrations of 50 and 100 μg•mL^{-1} had the highest numerical values of *E. coli* populations with 1.05 x 10^5 CFU•mL^{-1} and 4.07 x 10^5 CFU•mL^{-1} respectively and were significantly different from the treatment with a saponin concentration of 12.5 μg•mL^{-1}.

After 504 h (3 weeks) from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 7). The inoculated treatment without saponin addition experienced a reduction in *E. coli* population from the previous week to 3.26 x 10^1 CFU•mL^{-1} which was significantly less than all other inoculated treatments. The inoculated treatment containing a saponin concentration of 12.5 μg•mL^{-1} had 3.01 x 10^3 CFU•mL^{-1} and was similar to the inoculated treatments with saponin additions of 25 and 50 μg•mL^{-1}, but not to the treatment with a saponin concentration of 100 μg•mL^{-1}. The inoculated treatment with a saponin concentration of 100 μg•mL^{-1} had numerically the highest *E. coli* population containing 1.71 x 10^5 CFU•mL^{-1} which was not significantly different from the treatments containing 25 and 50 μg•mL^{-1} of saponins.

After 672 h (4 weeks) from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 8). The inoculated treatment without saponin addition reduced in population to 6.69 x 10^0 CFU•mL^{-1} which was now statistically similar to the non-inoculated treatment. The inoculated treatments with saponin

additions of 12.5 and 25 $\mu g \cdot mL^{-1}$ contained *E. coli* populations of 6.94×10^2 $CFU \cdot mL^{-1}$ and 1.94×10^3 $CFU \cdot mL^{-1}$ respectively which were similar to each other. The inoculated treatment with a saponin addition of 100 $\mu g \cdot mL^{-1}$ had the highest numerical population of *E. coli* at 5.85×10^4 $CFU \cdot mL^{-1}$ which was similar to the treatment containing saponins with a concentration of 50 $\mu g \cdot mL^{-1}$ and significantly different from treatments containing saponins at a concentration of 25 $\mu g \cdot mL^{-1}$ or less.

After 840 h (5 weeks) from the saponin addition, the non-inoculated treatment without saponin addition continued to have no recoverable *E. coli* (Table 9) and was not different from the inoculated treatment without saponin addition. The inoculated treatments containing saponin concentrations of 12.5, 25, and 50 $\mu g \cdot mL^{-1}$ were all similar and contained *E. coli* populations that ranged from 2.09×10^2 $CFU \cdot mL^{-1}$ to 1.62×10^3 $CFU \cdot mL^{-1}$. The inoculated treatment with a saponin addition of 100 $\mu g \cdot mL^{-1}$ had significantly more *E. coli* than any other treatment with 4.09×10^4 $CFU \cdot mL^{-1}$.

Lettuce heads that were evaluated for the presence of *E. coli* every week were not found to have any recoverable populations compartmentalized within the edible portions of the plant (data not shown). Occasional contamination occurred, but only from experimenter error.

Part 2:

Effects of saponins on plant growth and development

Plant growth characteristics were measured every week for 5 weeks throughout the course of this experiment and can be found in Tables 10 to 12. However, this section will focus on the measurements from week 5, which most accurately depicts a fully mature head of 'Rex' lettuce at time of harvest in an industry scenario.

After 840 h (5 weeks) in the NFT system, the non-inoculated treatment that did not contain saponins and the inoculated treatment that did not contain saponins were numerically the largest in terms of fresh shoot mass and dry shoot mass and were only similar to the inoculated treatment with a saponin addition of 25 $\mu g \cdot mL^{-1}$ (Tables 10 and 11). The inoculated treatment containing a saponin concentration of 12.5 $\mu g \cdot mL^{-1}$ was statistically similar in fresh and dry shoot mass to the treatment with 25 $\mu g \cdot mL^{-1}$ of saponins. The inoculated treatment with a saponin addition of 50 $\mu g \cdot mL^{-1}$ had significantly less fresh and dry shoot mass than the lower concentrations of saponins, however it had a significantly higher fresh and dry shoot mass than the inoculated treatment with a saponin addition of 100 $\mu g \cdot mL^{-1}$.

Lettuce head diameter after 840 h (5 weeks) was numerically greatest among the non-inoculated treatment without saponins and the inoculated treatment without saponins (Table 12). However, they were statistically similar to the two inoculated treatments with the lower levels of saponins (12.5 and 25 $\mu g \cdot mL^{-1}$). The inoculated treatment with a saponin addition of 50 $\mu g \cdot mL^{-1}$ had a significantly smaller head diameter than the treatments with no saponins and lower level saponins. The inoculated treatment with a saponin addition of 100 $\mu g \cdot mL^{-1}$ had a head diameter that was statistically smaller than all other treatments evaluated.

Discussion

Part 1:

Effects of saponins on growth of E. coli

Treatment effects were found to be the most influential on growth of *E. coli.* Over time, all treatments exhibited growth and decline (Fig. 1). The rate of growth and decline were decidedly different amongst the treatment levels. The experimental hypothesis was that saponins would have an antibacterial effect on *E. coli.* This would suggest that more saponins would equate to less *E. coli.* The resulting outcome of the experiment was the opposite. The greatest population of *E. coli* was consistently found in the inoculated treatment containing the highest concentration of saponins. At its highest population (336 h), this treatment produced 3 log increases over treatment 2, which contained no saponin addition (Table 6). This result was reliably seen at every time point beyond the initial first hours of the experiment.

The results found here were consistent with those found in the work of Arabski et al. (2011) on triterpenoid saponins. This experiment was conducted using steroidal saponins extracted from *Yucca shidigera* found in the southwest US and northwest Mexico. As discussed previously in this manuscript, the aglycone structure of each saponin compound determines its biological properties. Using the results from this study and those found by Arabski et al., one could surmise that both steroid and triterpenoid saponins react similarly to stimulate the growth of *E. coli.* The leading postulate to the reasoning of increased bacterial growth is that saponins increase cell permeability. However, instead of opening intercellular space to potentially harmful extracellular abiotic conditions, the newly formed pores in the bacterial membranes allowed the passage of nutrients to flow into the cell, letting the *E. coli* prosper.

The results seen in this experiment contradict those found in Chapter 1, in which saponins inhibited the growth and survival of *E. coli.* The primary difference between these two experiments is the addition of plants into the system ecology. A plant's rhizosphere can contain up to 100 times the amount of microorganisms found in soil without plants (Haas, Keel, and Reimmann, 2002). This rich biodiversity of microbes is home to a group known as rhizobacteria, which produce beneficial secondary metabolites that enhance plant growth through a variety of mechanisms (Sturz and Christie, 2003). A few notable rhizobacteria are found within the genera *Pseudomonas*, *Streptomyces,* and *Bacillus* (Emmert and Handelsman, 1999; Haas, Keel, and Reimmann, 2002). Brown et al. (1976) were able to isolate naturally occurring sulphur-containing carboxylic acids from strains of *Streptomyces* which are very potent inhibitors of *E. coli.* Soetan et al. (2006) reported that saponins only produced inhibitory effects on Gram-positive bacteria. *Streptomyces* is a Gram-positive bacterium, leading the investigator in the current study to postulate that the greater saponin concentrations inhibited beneficial rhizobacteria like *Streptomyces,* which allowed *E. coli* to survive in a less competitive environment.

It is important to note that early time points in this experiment had very low populations of *E. coli* to report. A study by Cooper, Bennett, and Lenski (2001) involving *E. coli* thermal dependence also indicated that most bacterial loss was seen in early stages of the experiment, when adaptation is the most rapid. Bacterial injury was observed on a great deal of the TSA plates. Typical colony morphology appeared circular, convex, and smooth. *E. coli* that was recovered and cultured at early time points were irregular in shape and size. Initially this experiment used MacConkey agar without the TSA overlay. Recovery became less and less as water temperatures dropped in the nutrient reservoirs due to changing seasons. *E. coli* will grow

over the temperature range of 10° C to 49° C, but it will grow at a progressively slower rate when temperature is raised above 40° C or below 20° C (Jones, VanBogelen, and Neidhardt, 1987; Cooper, Bennett, and Lenski, 2001). As shown in Table 1, temperature readings were below 20° C for the early stages of the first two replications. *E. coli* was present (indicated by subsequent aliquots), but in low numbers and in some cases undetectable. A pre-enrichment step was deemed necessary to facilitate bacterial recovery. In this case it was the addition of TSA to the MacConkey plates in the form of an overlay. This gave injured bacteria an opportunity to repair themselves in the nutrient rich environment and increased laboratory success in proper enumeration of *E. coli.*

Yet another, more controversial, explanation is provided for the lack of bacterial recovery at early time points. Although *E. coli* is documented as a non-sporulating microbe (Ray and Bhunia, 2014), some believe that it can enter a dormant-like stage known as viable but nonculturable (VBNC) state (Winfield and Groisman, 2003). These microbiologists have hypothesized that bacteria enter the VBNC state in response to sub-optimal environmental conditions, such as low temperature, and therefore are unable to detect using known laboratory methods for recovery (Ravel et al., 1995; Winfield and Groisman, 2003). Although the bacteria are metabolically active, they remain undetected until favorable conditions allow for resuscitation to an active form. This could explain the reason this experiment encountered low *E. coli* population recovery early on, and without any additional inoculum, recovered relatively high populations at subsequent time points. However, it should be noted the VBNC hypothesis is not widely accepted in the scientific community.

Internalization of E. coli through the root system

This experiment also included an assay for the presence of *E. coli* on or within the edible portions of the lettuce grown. The results were omitted from the statistical analysis due to the simplicity of the findings. An *E. coli* presence or absence screening was conducted on a total of 180 heads of lettuce throughout the duration of the experiment. A total of eight plants were found to have *E. coli* on or within their leaf tissue. The most likely source of contamination of these plants was accidently administered by the researcher. Plants were harvested in a manner similar to that of a hydroponic grower using an NFT system and packaging product with the root system intact. In doing so, wet root systems were lifted over adjacent plant sites causing contamination through dripping water. Great care was taken to avoid this contamination source, nonetheless some plants were contaminated. The low number of contaminated plants suggests that *E. coli* cannot be internalized through the root system. This evidence is contrary to that found by Solomon, Yaron, and Matthews (2002). The discrepancy of the before mentioned study and this study could be the result of differing identification techniques. Solomon et al. used sophisticated microscopy for detection of bacterial internalization. However, the present study is supported by Hora et al. (2005) who did not find internalization in aerial plant portions of spinach when roots had been inoculated.

Part 2:

Effects of saponins on plant growth and development

Although individual ANOVA's run at each time point revealed mean separations, the most important time point to address is 840 h (week 5). This time point reflects the most accurate time of maturation for 'Rex' lettuce and therefore conveys the most fundamental information to a grower considering the use of saponins in a recirculating hydroponic NFT system. Under every growth measurement, the non-inoculated treatment without saponin addition and the inoculated treatment without saponin addition numerically produced the highest yields on average. The fact that these treatments were the only treatments tested that did not include the saponin intervention indicates the economic practicality of this treatment as a mitigation tool for *E. coli* or any other microbe when growing lettuce in an NFT system.

Stymied growth of lettuce was clearly related to an increase in saponin solution. It is difficult to say whether this reduced growth pattern was due to the active ingredient (steroid saponins) or other ingredients within the solution or a combination of these factors. The provided saponin solution used in this experiment is not currently on the market, however there are similar products available to consumers from the manufacturer. These similar products are used as supplements for livestock feed to control ammonia and other noxious gasses in the immediate environment conveying air quality improvements. A complete list of ingredients is listed in Table 1 (Ch. 1). The formulation of the tested saponin extract product is not necessarily engineered for plant growth in a hydroponic NFT system.

The most likely cause of limited plant growth at higher concentrations of saponins is an increase in damaged plant cell membranes. Saponins are essentially nonionic surfactants, which

have phytotoxic effects on plant membranes by increasing permeability (Riechers et al., 1994). The damage caused to the root zone inhibited nutrient uptake and retarded the growth cycle.

Another postulate that is worth considering encompasses dissolved oxygen (DO) in the nutrient water. Unfortunately, due to equipment failures, DO was not measured across all replications of the experiment and therefore not included in the statistical analysis. Lettuce grows sufficiently at DO levels of at least 4 ppm (=4 $mg \bullet L^{-1}$) (Brechner and Both, 1996). Using the limited measurements recorded in this study, DO levels drop drastically as more saponins are added to the NFT system (Fig. 2). Levels do not fall below 4 $mg \bullet L^{-1}$ until saponins are added at a concentration of 50 $\mu g \bullet mL^{-1}$ and above (Table 13). Saponins are well known for their ability to foam in aqueous solutions (Francis et al., 2002) as detergent like compounds. Increased amounts of foam were observed at increasing saponin levels in this experiment. The amounts of foam were large enough to obstruct gas exchange between the nutrient reservoir of the NFT system and the atmosphere (Fig. 3). A correlation cannot be stated, but appears to be consistent with DO levels, saponin treatment, and plant growth.

Conclusions

The primary objectives of this research study was to identify whether saponins could be used as a natural bactericide for *E. coli* and what, if any, effects that would have on plant growth and development of 'Rex' lettuce grown in a hydroponic NFT system. The fact of the matter is that increasing saponin levels not only failed to elicit a bactericidal effect, but promoted the growth of *E. coli*. All the while plant health and vigor suffered in the presence of increasing amounts of saponin levels. Based on these merits, this product would not be recommended for the intended use of bacterial mitigation in hydroponic irrigation water.

Furthermore, the saponin solution was rather unpleasant to work with. Not only did the saponins create a foam barrier to the nutrient tanks, but it also had a foul odor. Another negative side effect of the saponin solution was the occlusions it would tend to manifest in the hydroponic equipment. Small water passages clogged with solids and had to be intermittently cleared of obstruction. On a large scale operation, clogged dripper emitters can easily lead to lost product. Pumps also needed extensive cleaning between replications. The saponin solution increased nutrient water viscosity and created more strain on pumps to deliver water to the root zone.

E. coli does not appear to travel through root systems into the edible portions of lettuce plants. Only cultural practices relocated *E. coli* from the nutrient water to edible surfaces of plant material in this study.

In this study, the negative control was juxtaposed to the positive control and found no statistical differences in fresh shoot weight (p-value = 0.74). What this means is that *E. coli* living in the irrigation water and interacting with the vast community of microorganisms

surrounding the root zone does not negatively impact the growth and development of ‘Rex’ lettuce in a hydroponic NFT system.

E. coli recovery was inadequate when using MacConkey agar growth media. Due to bacterial injury, a pre-enrichment step should be implemented in future research to ensure proper recovery and enumeration of bacteria. A tryptic soy agar overlay on MacConkey agar was used in this experiment and is recommended for future study.

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Tables

Table 1. Weekly average water temperature for each block.

Block	Week	P-value[z]	Avg. temperature (°C)[y]
1	1	0.4874	18.46
	2	0.6590	18.06
	3	0.3518	18.63
	4	0.9460	18.14
	5	0.9942	19.06
2	1	0.0958	19.37
	2	0.9999	20.00
	3	0.7181	19.43
	4	0.3297	19.37
	5	0.3221	19.20
3	1	0.4671	21.11
	2	0.9933	22.37
	3	0.9841	23.34
	4	0.9870	23.50
	5	0.1837	26.57

[z]P-value based on Student's T-test comparing weekly averages for selected treatments.
[y]Average temperature is based on seven daily readings prior to time point.

Table 2. Effect tests for treatment (TRT), time in weeks (Time), and water temperature (°C) (WT).

Source	P-value
TRT	0.0073*
Time	0.0927
WT	0.7562
Time*TRT	0.7425
Time*WT	0.7467
TRT*WT	0.3270
Time*TRT*WT	0.5691

*Indicates significant value at $\alpha = 0.05$

Table 3. Comparison of colony forming units (CFU) means by saponin treatment 1 h after inoculation of *E. coli* (time point = 0 h).

Treatment[z]			
E. coli[y]	Active Ingredient	Mean (Ln[CFU/mL+1])	Mean (CFU/mL)[x]
-	0	0	0 a
+	0	< 0.01	$< 1 \times 10^{-2}$ a
+	12.5 μg•mL^{-1}	0.3662	4.42×10^{-1} a
+	25 μg•mL^{-1}	0.73241	1.08×10^{0} a
+	50 μg•mL^{-1}	1.9154	5.79×10^{0} a
+	100 μg•mL^{-1}	1.66348	4.28×10^{0} a

[z]Treatment intervention not yet applied, displayed for treatment relevance.
[y]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^3 CFU/mL, negative (-) indicates *E. coli* not present.
[x]Means with different letter(s) are significantly different using a student's t test ($\alpha = 0.05$).

Table 4. Comparison of colony forming units (CFU) means by saponin treatment 1 h after treatment.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 a
+	0	< 0.01	$< 1 \times 10^{-2}$ a
+	12.5 μg•mL^{-1}	0.96346	1.62×10^{0} a
+	25 μg•mL^{-1}	1.82409	5.19×10^{0} a
+	50 μg•mL^{-1}	2.03344	6.64×10^{0} a
+	100 μg•mL^{-1}	2.55321	1.18×10^{1} a

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^3 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a student's t test ($\alpha = 0.05$).

Table 5. Comparison of colony forming units (CFU) means by saponin treatment 168 h (1 week) after treatment.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 a
+	0	5.72774	3.06×10^{2} b
+	12.5 μg•mL^{-1}	8.21378	3.69×10^{3} bc
+	25 μg•mL^{-1}	10.26297	2.87×10^{4} cd
+	50 μg•mL^{-1}	11.69847	1.20×10^{5} d
+	100 μg•mL^{-1}	12.14945	1.89×10^{5} d

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^3 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a student's t test ($\alpha = 0.05$).

Table 6. Comparison of colony forming units (CFU) means by saponin treatment 336 h (2 weeks) after treatment.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 a
+	0	5.00292	1.47×10^{2} bc
+	12.5 µg•mL^{-1}	7.59516	1.99×10^{3} cd
+	25 µg•mL^{-1}	9.8575	1.91×10^{4} de
+	50 µg•mL^{-1}	11.56304	1.05×10^{5} e
+	100 µg•mL^{-1}	12.91615	4.07×10^{5} e

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^{3} CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a student's t test ($\alpha = 0.05$).

Table 7. Comparison of colony forming units (CFU) means by saponin treatment 504 h (3 weeks) after treatment.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 a
+	0	3.51353	3.26×10^{1} b
+	12.5 µg•mL^{-1}	8.01023	3.01×10^{3} c
+	25 µg•mL^{-1}	9.22139	1.01×10^{4} cd
+	50 µg•mL^{-1}	10.82477	5.02×10^{4} cd
+	100 µg•mL^{-1}	12.049	1.71×10^{5} d

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^{3} CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a student's t test ($\alpha = 0.05$).

Table 8. Comparison of colony forming units (CFU) means by saponin treatment 672 h (4 weeks) after treatment.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 a
+	0	2.04083	6.69×10^{0} a
+	12.5 µg•mL^{-1}	6.54505	6.94×10^{2} b
+	25 µg•mL^{-1}	7.56964	1.94×10^{3} bc
+	50 µg•mL^{-1}	10.08137	2.39×10^{4} cd
+	100 µg•mL^{-1}	10.97707	5.85×10^{4} d

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^{3} CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a student's t test ($\alpha = 0.05$).

Table 9. Comparison of colony forming units (CFU) means by saponin treatment 840 h (5 weeks) after treatment.

Treatment			
E. coli[z]	Active Ingredient	Mean (Ln[CFU•mL^{-1}+1])	Mean (CFU•mL^{-1})[y]
-	0	0	0 a
+	0	2.04949	6.76 x 10^0 a
+	12.5 µg•mL^{-1}	5.34673	2.09 x 10^2 b
+	25 µg•mL^{-1}	5.83143	3.39 x 10^2 b
+	50 µg•mL^{-1}	7.39178	1.62 x 10^3 b
+	100 µg•mL^{-1}	10.62053	4.09 x 10^4 c

[z]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^3 CFU/mL, negative (-) indicates *E. coli* not present.
[y]Means with different letter(s) are significantly different using a student's t test ($\alpha = 0.05$).

Table 10. Mean fresh shoot relative weight by week[zy].

Treatment		Time (Weeks)				
E.coli[x]	Saponin	1	2	3	4	5
-	0	100 ab	100 a	100 a	100 a	100 a
+	0	108 a	80 ab	100 a	86 ab	101 a
+	12.5 µg•mL^{-1}	97 ab	65 ab	79 a	73 b	75 b
+	25 µg•mL^{-1}	103 a	56 bc	76 a	72 b	80 ab
+	50 µg•mL^{-1}	69 bc	25 cd	30 b	31 c	44 c
+	100 µg•mL^{-1}	59 c	15 d	8 b	7 c	14 d

[z]Mean responses displayed as percent of negative control (without *E. coli*, without saponin addition).
[y]Means with different letter(s) are significantly different using a Tukey's honest significance difference test ($\alpha = 0.05$).
[x]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^3 CFU/mL, negative (-) indicates *E. coli* not present.

Table 11. Mean dry shoot relative weight by week[zy].

Treatment		Time (Weeks)				
E.coli[x]	Saponin	1	2	3	4	5
-	0	100 ab	100 a	100 a	100 a	100 a
+	0	105 ab	79 ab	99 a	90 ab	95 a
+	12.5 µg•mL^{-1}	102 ab	67 abc	79 a	72 b	70 b
+	25 µg•mL^{-1}	109 a	66 bc	79 a	78 ab	87 ab
+	50 µg•mL^{-1}	77 b	40 c	38 b	38 c	46 c
+	100 µg•mL^{-1}	70 c	33 c	18 b	12 c	16 d

[z]Mean responses displayed as percent of negative control (without *E. coli*, without saponin addition).
[y]Means with different letter(s) are significantly different using a Tukey's honest significance difference test ($\alpha = 0.05$).
[x]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^3 CFU/mL, negative (-) indicates *E. coli* not present.

Table 12. Mean head diameter by week[zy].

Treatment		Time (Weeks)				
E.coli[x]	Saponin	1	2	3	4	5
-	0	100 a	100 a	100 a	100 a	100 a
+	0	101 a	95 a	96 ab	95 a	98 a
+	12.5 µg•mL^{-1}	100 a	89 a	85 bc	86 b	89 a
+	25 µg•mL^{-1}	104 a	87 ab	82 c	82 b	88 a
+	50 µg•mL^{-1}	96 ab	73 b	68 d	65 c	74 b
+	100 µg•mL^{-1}	88 b	73 b	52 e	42 d	48 c

[z]Mean responses displayed as percent of negative control (without *E. coli*, without saponin addition).
[y]Means with different letter(s) are significantly different using a Tukey's honest significance difference test ($\alpha = 0.05$).
[x]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^3 CFU/mL, negative (-) indicates *E. coli* not present.

Table 13. Dissolved Oxygen (mg•L^{-1}) average measurements by treatment[z].

Treatment		
E.coli[y]	Saponin	DO(mg•L^{-1})
-	0	8.03
+	0	7.96
+	12.5 µg•mL^{-1}	7.28
+	25 µg•mL^{-1}	5.35
+	50 µg•mL^{-1}	3.91
+	100 µg•mL^{-1}	1.00

[z]Table does not have data for all replicates and therefore is merely anecdotal.
[y]Positive (+) indicates presence of *E. coli* inoculated at an initial population of 10^3 CFU/mL, negative (-) indicates *E. coli* not present.

Figures

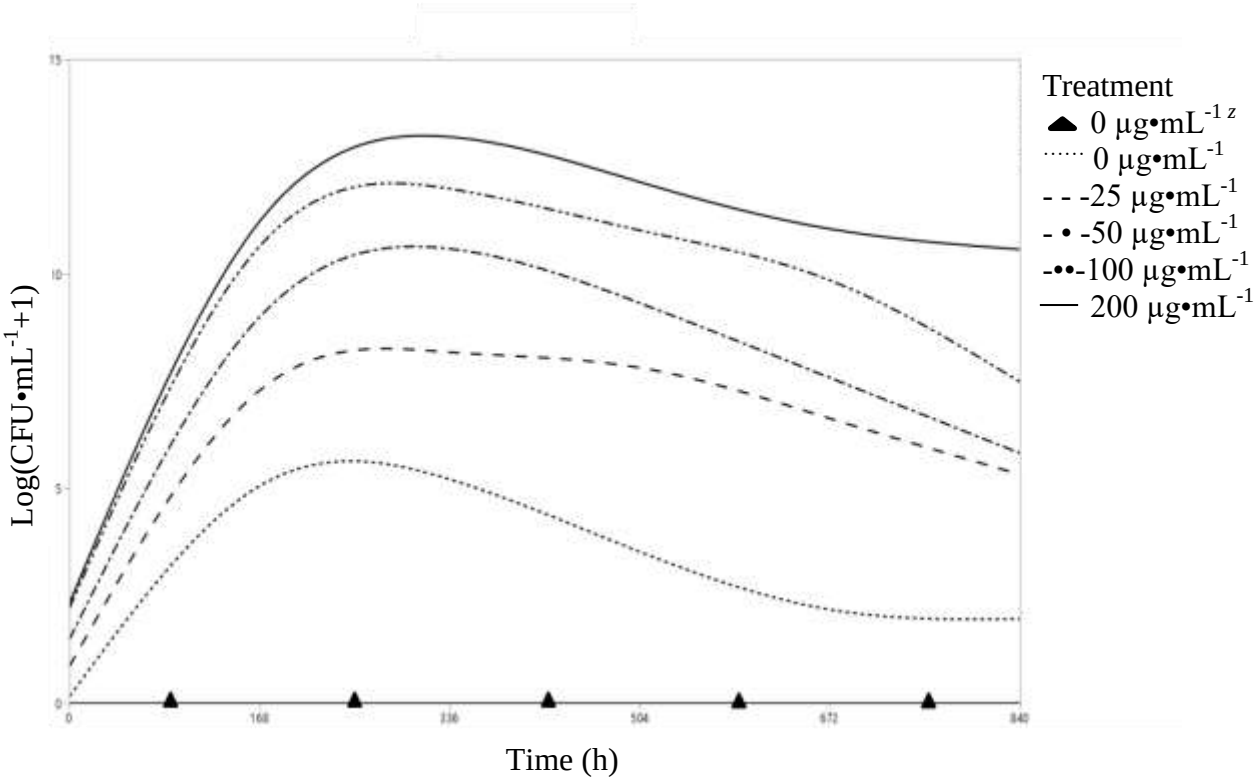

Fig. 1. Growth curves of *E. coli* population by treatment over time.
[z]Indicates the non-inocultaed treatment.

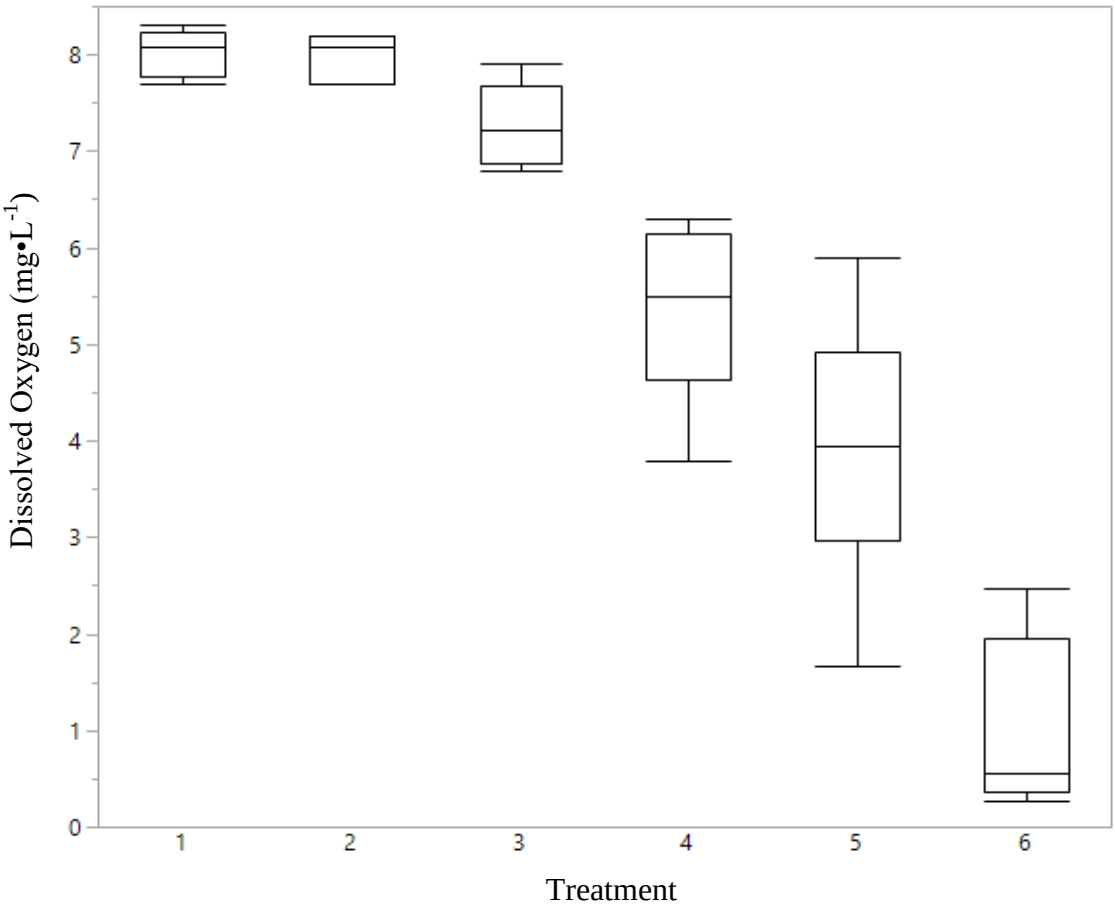

Fig. 2. Box and whisker plots of dissolved oxygen (mg•L^{-1}) for individual saponin treatments in the NFT system (unofficial). Treatment 1=no *E. coli*, no saponin addition; Treatment 2=*E. coli*, no saponin addition; Treatment 3=*E. coli*, 12.5 µg•mL^{-1}; Treatment 4=*E. coli*, 25 µg•mL^{-1}; Treatment 5=*E. coli*, 50 µg•mL^{-1}; Treatment 6=*E. coli*, 100 µg•mL^{-1}.

Fig. 3. Example photo of saponin foaming in NFT system (100 µg•mL-1).

Appendix

Table A.1. List of reported biological activities of saponins (Güçlü-Üstündağ and Mazza, 2007).

Biological activity:
Adaptogenic
Adjuvant
Analgesic activity
Antiallergic
Antiedematous
Antiexudative
Antifeedant
Antifungal
Antigenotoxic
Antihepatotoxic inhibitory effect of ethanol absorption
Anti-inflammatory
Antimicrobial
Antimutagenic
Antiobesity
Anitoxidant
Antiparsitic
Antiphlogistic
Antiprotozoal
Antipsoriatic
Antipyretic
Antispasmodic
Antithrombotic (effect on blood coagulability)
Antitussive (relieving or preventing cough)
Antiulcer
Antiviral
Chemopreventive
Cytotoxic
Diuretic
Effect on absorption of minerals and vitamins
Effect on animal growth (growth impairment), reproduction
Effect on cognitive behavior
Effect on ethanol induced amnesia
Effect on morphine/nicotine induced hyperactivity
Effects on ruminal fermentation
Expectorant
Haemolytic
Hepaprotective
Hypocholesterolemic
Hypoglemic
Immunostimulatory effects

Table A.1 (Cont.).

Biological activity
Increase permeability of intestinal mucosa cells
Inhibit active nutrient transport
Molluscicidal
Neuroprotective
Reduction in fat absorption
Reduction in ruminal ammonia concentrations
Reductions in stillbirths in swine
Ruminant bloat
Sedative