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Food Safety Risk in an Indoor Microgreen Cultivation System

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Food Safety Risk in an Indoor Microgreen Cultivation System

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

by

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Bachelor of Science in Nutrition, 2005

May 2020
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Abstract

Microgreens are immature sprouts of edible plants, sharing some similarities with sprouted seeds and petite leafy greens. Since they are most often grown in containers in buildings or greenhouses, they present a new area for food safety research at the intersection of the built environment and produce farming. Contamination by human pathogens has been extensively studied in other types of produce typically eaten raw, including sprouted seeds, which have been implicated in numerous outbreaks of salmonellosis over the last several decades. There is a paucity of knowledge about the microgreen sector of the fresh-cut industry; thus, it was determined that a survey of operational details, microgreen varieties grown, and food safety practices would be needed to determine research directions. Following a nationwide survey of US-based microgreen farmers, two laboratory experiments were conducted using the most common production system type and microgreen varieties. Soil-free growing media (SFGM) was inoculated with *Listeria monocytogenes* FSL R2-574 and *Salmonella enterica* Javiana in a plant-free bench scale experiment as well as during cultivation of sunflower microgreens in a fully indoor, artificially lit, stacked track system similar to that of the microgreen farmers surveyed. It was found that the type of SFGM influenced survival of these two pathogens, which are commonly associated with sprouted seed outbreaks as well as several recent microgreen product recalls. Furthermore, it was found that survival of these pathogens was enhanced in the presence of the microgreen root environment. These results are important for informing system design decisions by microgreen farmers.

Acknowledgements

Dedication

It is with genuine gratitude that I dedicate this work to my husband and partner in all things, Jacob Haqq Misra. Without his tireless emotional and intellectual support, this work may not have been completed. This thesis is only the beginning of all the great science we will do together in the decades to come.

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Chapter 2. Submitted to Food Protection Trends on 2/13/2020.

Chapter 3. Submitted to the Journal of Applied Microbiology on 2/1/2020.

Introduction: Why study indoor microgreen cultivation systems?

I.   **What is controlled environment agriculture?**

Controlled environment agriculture (CEA) encompasses a variety of non-traditional farming

methods that take place inside climate controlled buildings. Examples of CEA may include

greenhouses or high tunnels, which have transparent or translucent walls that let in natural

sunlight, or spaces with opaque walls that rely on artificial lighting. Greenhouses and fully

indoor spaces may require varying degrees of climate modulation such as heating, cooling, and

humidity control. Indoor farmers often use soil-free horticulture techniques that include

hydroponics, aquaponics, aeroponics, or growing on mats and soil alternatives.

The term "zero acreage farming" or z-farming has been coined to describe methods of indoor

farming that do not burden arable land (Specht et al. 2014, Thomaeir et al. 2015). CEA is also

referred to as protected agriculture because its climatic conditions are tightly controlled

(McCartney et al. 2018). The most commonly used term appears to be "vertical farming"

(Despommier 2011, Martin et al. 2016, McCartney et al. 2018, Mok et al. 2014, Shamshiri et al

2018, Specht et al. 2014). Vertical farming may refer to either vertically stacked artificially lit

shelves, or vertically inclined surfaces, such as outdoor "green walls" (Specht et al. 2014).

II.   **Common CEA crops and techniques**

Indoor farming systems may include hydroponics, aquaponics, aeroponics, trays, gutters, or pots

with soil or soil-free media (FAO 2014). Hydroponics is a soil-free growing technique that

involves submerging plant roots into soil-free media such as gravel, vermiculite, perlite, or

pumice and flooding with a precisely mixed nutrient solution. In addition, some systems use only

nutrient solution with no rooting medium. Methods may include flood-and-drain, nutrient film technique (NFT), or deep water raft culture (DWC) (Sharma et al. 2019). Aquaponics is a type of hydroponic system that uses nitrogen-rich aquaculture wastewater as the nutrient solution instead of more precise chemical nutrient mixtures (Forchino et al., 2017). Aeroponics involves suspending plants above ground so that their roots are exposed to air and then sprayed with a nutrient solution, a technique that is used mainly for growing root crops for the herbal supplement industry (Hayden et al., 2015). Non-hydroponic soil-free techniques include growing in coco coir or on mats made of either synthetic or natural fibers (Verhagen and Boon, 2008, Carlile et al, 2015, Sarkar et al., 2018). Crops most commonly grown indoors include leafy greens, herbs, and microgreens. (Agrilyst, 2016). On hydroponic and aquaponic farms in particular, lettuce, tomatoes, peppers, and strawberries are among the primary crops grown (Agrilyst, 2016).

III.   **The Appeal of Indoor Farming**

The CEA concept is intended as a more sustainable alternative to traditional field cultivation. Proponents claim that it allows resource-efficient, intensive, year-round fruit and vegetable production in a variety of climates, on land that is not suitable for farming (Despommier 2011 and 2013, McCartney et al., 2018). Claims have been made that CEA will potentially solve problems such as feeding a growing population by intensifying food production (Touliatos et al. 2016), adapting agriculture to climate change (Tirado et al. 2010), reducing food miles (Specht et al., 2014, Eigenbrod et al., 2015) and saving water (Kozai et al., 2016, Martin and Molin, 2018).

Critics of CEA point to the high start-up capital and energy needed to recreate the outdoors, such as artificial lighting, plumbing, heating, and cooling (Banerjee et al., 2014, Kalantari et al., 2015). Others say its promise of feeding people in urban centers is overstated in terms of meeting nutritional needs (Van Iersel 2013) and acceptance by target consumers (Guthman 2008). Additionally, above-ground farming requires either soil or soil-free media to be purchased and often used only once—a point which weakens the case for indoor farming as resource-efficient and economical (Banerjee et al. 2014). Research has also pointed to risk of chemical contamination of produce from polluted city air (Mok et al. 2014, Specht et al. 2014, Thomaeir et al. 2015). Notably, the risk of pathogen contamination was not adequately addressed (Mok et al. 2014, Specht et al. 2014, Thomaeir et al. 2015).

IV. Food Safety in CEA

The risk of contamination of produce by human pathogens in controlled-environment farming has only been minimally investigated. A systematic review of CEA literature (Thomaier et al. 2015) did not reveal any food safety studies on fresh produce grown in controlled, indoor environments. There has been discussion of food contamination by industrial pollutants such as heavy metals, pesticides/herbicides, asbestos, petroleum products, and solvents, suggesting that CEA may protect crops better than outdoor urban agriculture (Mok et al. 2014, Specht et al. 2014). For example, crops grown in outdoor urban gardens may have reduced yields, lower quality, and may be more susceptible to pests and plant diseases (Bell et al. 2011); thus, these issues may be mitigated by bringing plants into controlled settings.

Food safety is also important for sustainability because food production systems susceptible to contamination by pathogens counteract the food security and human health aspects of sustainable development. A 2016 survey of 198 indoor farms by the company Agrilyst reported that small ($<$ 1,500 ft.^{2} or 140 m^2) CEA farms appear more likely to be fully indoors rather than in greenhouses (Agrilyst 2016). Scaling up indoor operations for large-scale production may increase the number of food safety failure points. Previous research on small to medium sized farms and farmers' market vendors' food safety practices demonstrates that these groups typically struggle to maintain consistent food safety practices (Harrison et al. 2013, Behnke et al. 2012). This is discussed in further detail in Chapter 2.

In addition, pathogens may recirculate easily in air handling systems and water supplies of closed environments such as buildings. Microbiome studies of the built environment suggest that humans are a main driver of microbial diversity in these settings, and a wide variety of microorganisms occupy unique niches in buildings (Kelley and Gilbert 2013, Mahnert et al. 2015, Stamper et al., 2016). The built environment may have overall lower biological diversity compared to outdoor environments (Hanski et al. 2012, Berg et al. 2014), which may limit competitive inhibition among microbial species, particularly between human pathogens and environmental microorganisms (Meadow et al. 2013).

Human handling contributes significantly to contamination of fresh produce. Human pathogens commonly associated with contamination of fresh produce include bacteria *Listeria monocytogenes*, *Salmonella* serovars, as well as human noroviruses (Ahmed et al 2014, Sivapalasingam et al. 2004, Herman et al. 2015, and Bennett et al. 2018). Research on *L.*

monocytogenes (Carpentier et al. 2011) as well as extensive study of *Salmonella* biofilm formation on abiotic surfaces (Fatica et al., 2011, Iibuchi et al. 2010, Kusumaningrum et al., 2003) demonstrates that these pathogens have characteristics which allow survival in the built environment, particularly that of food production, for weeks to months or even years. The microbiome of soil-free culture may be different from soil based growing environments (Koohakan et al. 2004), suggesting that this may be a source of the variation between indoor and outdoor farming. Thus, the aspects of indoor vegetable and leafy green production where human handling is a significant factor, such as during planting or packaging, are appropriate research targets, as well as studying the interaction between human pathogens and the various types of soil-free growing media available for indoor farming applications.

V. **Microgreens**

Microgreens may serve as a model crop for indoor farming research. These immature shoots of crops such as sunflower, peas, chard, beets, spinach, kale, and cilantro are a popular choice for indoor farmers according to our US-based microgreen grower survey (see Chapter 2), and another survey showing that 63 of the 198 farms interviewed produced microgreens (Agrilyst 2016). They are often grown indoors on stacked, artificially lit shelves, or in greenhouses, and considered to be nutrient-dense (Weber 2017, Treadwell et al., 2016). Their seed to harvest time is approximately 7 to 21 days (Kyriacou et al. 2016). Their relatively short life cycle combined with their premium price and year-round production makes this crop profitable for small farmers (gross sales < 250,000 USD/year) and attractive to entrepreneurs (Charlebois et al. 2018). They have a short shelf life of approximately one week even under refrigeration and are used in small quantities as garnishes, toppings, or seasonings (Xiao et al. 2012, Mir et al. 2017).

Microgreens have been chosen as the focus for this research because of the similarities they share with high risk crops, specifically leafy greens and sprouts. There have been numerous product recalls of microgreens related to *Salmonella* and *L. monocytogenes* since 2016 (CFIA 2018a-f, 2019a, and 2019b; US FDA 2016, 2018, and 2019), but no reported illnesses. Although no recalls have yet been associated with viruses, this does not exclude them from future risk assessment. Microgreen production has multiple steps where human hands are involved, which is a principle route of contamination for pathogens where humans are the main reservoir (Escudero et al. 2012, Rönnqvist et al. 2014).

This thesis first discusses the literature that has directly addressed food safety issues in microgreen cultivation systems as well as the rationale for further research into this emerging raw salad crop (Chapter 1). Then, a survey was conducted to understand operational details and food safety practices of microgreen producers in the United States (Chapter 2). Finally, several experiments were conducted to determine the survival of common produce-associated pathogens *L. monocytogenes* and *S. enterica* on four types of soil-free growing media (SFGM) used in an indoor, artificially lit shelf system (Chapter 3). Survival of each pathogen was then tested with and without the presence of sunflower microgreens, and transfer to the final product was also assessed (Chapter 4).

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Chapter 1: Microgreens—A review of food safety considerations along the farm to fork continuum

I.   **Abstract**

The food safety implications of microgreens, an emerging salad crop, have been studied only minimally. The farm to fork continuum of microgreens and sprouts has some overlap in terms of production, physical characteristics, and consumption. This review describes the food safety risk of microgreens as compared to sprouts, potential control points for microgreen production, what is known to date about pathogen transfer in the microgreen production environment, and where microgreens differ from sprouts and their mature vegetable counterparts. The synthesis of published research to date may help to inform Good Agricultural Practices (GAPs) and Good Handling Practices (GHPs) for the emerging microgreen industry.

II.   **Introduction**

One in ten people worldwide contract illnesses from food contaminated with infectious agents, and 420,000 of those cases result in death (Alegbeleye et al., 2018; Hoffmann et al., 2017). The World Health Organization reported in 2015 that Africa, Southeast Asia, and the Eastern Mediterranean bear the greatest burden, while the Americas and Europe bear the least (World Health Organization, 2015). Nevertheless, the most recent report of confirmed cases of foodborne illness from the Centers for Disease Control and Prevention (CDC) in the United States concluded that in 2015 alone there were 902 food-borne disease outbreaks resulting in 15,202 illnesses, 950 hospitalizations, 15 deaths, and 20 food product recalls (Center for Emerging Diseases, 2015). The true figures could be greater as these events are from confirmed outbreaks. Scallan et al. (2011) reported that an estimated 47.8 million cases of domestically

acquired food-borne illness may occur annually in the United States. A 2013 CDC report on the attribution of illnesses to food commodities showed that 46% of the foods involved in outbreaks are produce, causing 23% of the fatalities (Painter et al., 2013). Further, the CDC's Food-borne Disease Outbreak Surveillance System reported that out of 120 multi-state outbreaks between 2010 and 2014, 17 were from fruits, 15 were from vegetable row crops, 10 were from sprouts, and 9 were from seeded vegetables (e.g. cucumbers, mini peppers) (Crowe et al., 2015). A myriad of pathogens can contaminate produce, including spore-forming bacteria, non-spore forming bacteria, viruses, parasites, and prions. The multi-state outbreak report by Crowe et al. (2015) demonstrates that the most common produce-associated bacterial pathogens are *Salmonella enterica*, *Listeria monocytogenes*, and shiga toxin-producing *Escherichia coli*. Human norovirus, the leading cause of food-associated acute gastroenteritis, is responsible for 5% of all food-borne illnesses of known etiology in the United States (Scallan et al., 2011) and 65% of those in Canada (Thomas et al., 2013). A search on September 7, 2018 for 'norovirus' and 'food' in the CDC's National Outbreak Reporting System (NORS) Database revealed that norovirus is the major cause of outbreaks associated with leafy greens. After multiple ingredient foods and foods considered 'unclassifiable,' 'vegetable row crops,' 'other,' 'mollusks,' and 'fruits' are the most common food categories implicated in norovirus outbreaks.

A 2013 report by the European Food Safety Authority (EFSA) attributed an increase in cases of foodborne illness (from 18% to 26%), hospitalizations (from 8% to 35%) and deaths (5% to 46%) between 2007 and 2011 to one large verocytotoxigenic *Escherichia coli* (VTEC) outbreak in Germany in 2011. Fenugreek sprouts were identified as the infected food and over 3800 people were affected (European Food Safety Authority, 2013). The EFSA later reported that

active surveillance of eight European Union (EU) member states revealed one sample of 344

collected was positive in 2016 compared to zero positive samples out of 444 collected from six

member states in 2013 (European Food Safety Authority, 2017). Produce-associated outbreaks in

the United States have also increased in the last two decades, from 8% of foodborne illness

outbreaks between 1998 and 2001 to 16% between 2010 and 2013 (Bennett et al., 2018).

Alegbeleye et al. (2018) postulated that increases in produce-related outbreaks are at least

partially due to improved surveillance and reporting. However, they suggest a true increase in

produce-associated illness may simply be a result of increased consumption of fruits and

vegetables. Data collected by the United States Department of Agriculture's Economic Research

Service (ERS/USDA) from 1990 to 2016 show that while head lettuce availability per capita and

domestic production has gone down, there has been an increase in availability and production of

romaine lettuce and a slight increase in spinach availability. There has also been an increase in

imported fresh vegetables that is suggested to correspond with an increase in imported Romaine

and head lettuce (Fig. 1).

An increase in importing supports the assertion by Alegbeleye et al. (2018) that agriculture has

become more globalized. Globalization adds challenges in regulating food safety since practices

differ between countries, such as water quality management and waste water treatment.

According to a report by the International Food Policy Research Institute (IFPRI), developing

countries often have difficulty meeting the strict food safety requirements of developed nations

(Käferstein, 2003). Lastly, agriculture has become more intensive due to increased demand for

fresh fruits and vegetables, so produce may be more likely to be in close proximity to potential

sources of contamination such as livestock. In these settings, fresh produce may become contaminated via the soil, irrigation water, wildlife, insects, livestock, pets, or soil amendments such as manure (Alegbeleye et al., 2018).

As the consumption of fresh produce is changing, so are the types of fresh produce available. Microgreens, which are the immature shoots of products such as sunflower, peas, chard, beets, spinach, kale, and cilantro, are an emerging salad crop. They are often grown in trays indoors or in greenhouses and are touted for their reported high nutrient content. Microgreens have recently grown in popularity in developed countries due to increased interest in gourmet cooking, healthy eating, and indoor gardening. They have a relatively short shelf life even in refrigeration and are used in small quantities as garnishes, toppings, or seasonings (Delian et al., 2015; Kyriacou et al., 2016; Mir et al., 2017; Treadwell et al., 2010; Xiao et al., 2012).

Microgreens may be easily confused with sprouted seeds, which have been frequently implicated in food-borne illness (Gensheimer and Gubernot, 2016). However, while microgreens share some characteristics with sprouts, they share others with fresh herbs and petite greens. Examples of fresh herbs include basil, thyme, and cilantro and examples of petite greens include baby spinach and spring mix. While there is a growing body of literature on both microgreen nutrition and physiology, only eight studies since 2009 have specifically examined the food safety risk of microgreens. However, leafy green and sprout safety has been studied extensively. The purpose of this review is to compare microgreens to other raw salad crops previously shown to be linked to food-borne illness and identify potential control points given what is currently known about how raw produce is colonized by disease-causing microorganisms.

III. Traits of high-risk crops: how microgreens compare

Produce can become contaminated at any point along the farm to fork continuum. Common control points for growers include irrigation water, soil amendments such as manure or compost, livestock and wild animal fecal contamination, worker health and hygiene, field and harvest sanitation, sanitation of packing facilities, post-harvest water and handling, value-added processing, storage, transportation, and distribution (Olaimat and Holley, 2012; Suslow, 2003). The crops with the greatest risk of becoming contaminated with human pathogens include lettuce, spinach, parsley, basil, berries, green onions, melons, sprouts, and tomatoes (Alegbeleye et al., 2018). Each of these crops have earned their high-risk status because of growing conditions that facilitate the growth or transfer of microorganisms, production methods that expose the product to contaminants from animals or humans, and physiological characteristics of the plant that facilitate contact and binding with microorganisms. Microgreens share some traits with these high-risk crops.

i. Tissue damage increases susceptibility

Harvesting by cutting may increase susceptibility to contamination. For example, tomato stem scars result from picking or cutting a tomato from its stem during harvest, and research in this area demonstrates that tissue damage can expose produce to contaminants. Lin and Wei (1997) demonstrated that *Salmonella* Montevideo clusters around tomato stem scars at 10^3 colony forming units (CFU). At greater inoculum doses of 10^4 and 10^5 CFU, *Salmonella* Montevideo spread to the interior of the tomato. Lettuce and spinach are often vehicles of produce-associated foodborne illness (Gao et al., 2016; Waitt et al., 2014; Wang et al., 2017). Damage to leaves, stems, and roots sustained during post-harvest processing may facilitate pathogen contamination.

Like tomatoes, lettuce is harvested by cutting, and the cut site may be a route of entry for

pathogens. Aruscavage et al. (2008) demonstrated that *Escherichia coli* O157:H7 survived better

on lettuce split along the central vein compared to healthy, undamaged leaves. Microgreen

harvesting also involves cutting by hand above the root, but to our knowledge there is no

research indicating whether the cut end of a microgreen is susceptible to contamination as

observed in lettuce and tomatoes. Sprouted seed production, however, has no cutting step

(United States and Food Drug Administration, 2017a). Therefore, contamination at the cut edge

is one contamination susceptibility of microgreens not shared by sprouted seeds.

Surface characteristics combined with tissue damage of lettuce leaves and other leafy greens may

create opportunities for contamination. For example, Wang et al. (2017) and Gao et al. (2016)

have demonstrated that lettuce leaf surfaces express glycoproteins that are biochemically similar

to histo blood group antigens (HBGA) in mammals and serve as attachment sites for norovirus

capsid proteins. Human noroviruses are the primary cause of foodborne illness associated with

leafy greens (Sivapalasingam et al., 2004; Herman et al., 2015; Bennett et al., 2018). Gao et al.

(2016) demonstrated that enzymatic degradation of red leaf lettuce, Romaine lettuce, and celery

tissue by cellulase R10 increases binding of human norovirus capsid proteins, likely due to

exposing additional binding sites. However, binding of norovirus capsid protein to HBGAs did

not occur with basil, indicating that pathogen attachment may depend at least partially on plant

variety.

Lectins and adhesins on leaf surfaces also act as binding sites for bacteria such as *Salmonella* and

E. coli O157:H7. These pathogens are implicated in many of the outbreaks traced to spinach and

lettuce (Deng and Gibson, 2017). A review by Berger et al. (2010) concluded that plant variety

and bacterial species both play a role in the ability of contaminants to attach to plant surfaces.

Even among *Salmonella enterica* serovars, they found that there is considerable variation in

attachment ability and mechanism. Major cell components involved in attachment include the

pilus curli, the O antigen capsule, and cellulose synthesis necessary for biofilm formation. *E. coli*

variants also use curli when attaching to tomatoes, spinach, and alfalfa roots. *E. coli* attachment

to leafy vegetables is also aided by its filamentous type III secretion system and its flagellum

(Berger et al., 2010; Olaimat and Holley, 2012). Such a phenomenon demonstrated on the leaves

of full sized vegetables suggest that it is likely to occur on microgreen leaves as well, though

more studies are needed to determine the susceptibility of individual microgreen varieties to

particular pathogens.

ii. **Hand harvesting and farm worker hygiene**

Because microgreens are typically harvested by hand, it is worth considering the risks that

producers themselves contribute through inadequate hygiene. *Salmonella* is the most common

cause of produce-associated infections, so an extensive body of research has been focused on

understanding how this animal fecal organism finds its way to fresh fruits and vegetables

(Olaimat and Holley, 2012; Waitt et al., 2014). Inadequate worker hygiene is a major

contributing factor to contamination of produce by human pathogens, especially for hand-

harvested crops like strawberries (Moore et al., 2015). Of the pathogens identified in a review by

Todd et al. (2009) of outbreaks involving food workers between 1927 and 2006, *Salmonella*

species and norovirus were the most prevalent for the bacterial and viral categories, respectively,

for all food vehicles studied. Specifically, in produce, however, *Salmonella* was only implicated

in 4.6% of outbreaks and *Shigella* was the most commonly implicated pathogen, representing 21.2% of outbreaks involving food handlers. Todd et al. (2009) focused primarily on the service end of the food continuum, particularly restaurant workers, which made up the majority the studies reviewed.

Inadequate hygiene practices by farm workers also pose a risk at the production end of the food continuum. Bartz et al. (2017) conducted a matched-pair epidemiological study of 11 farms and calculated the odds ratios of the presence of indicator organisms on worker hands to the presence of indicator organisms on produce. The indicator organisms chosen were total coliforms, *E. coli*, Enterococcus, and coliphage and the target produce included cantaloupe, jalapeno peppers, and tomatoes. When *E. coli* was found on hands, the handled produce was nine times more likely to contain *E. coli*. When coliphage was present on worker hands, the handled produce was eight times more likely to contain coliphage. Surprisingly, there was no significant relationship between bacteria or phage in either soil or irrigation water. These data suggest that transfer from worker hands was the main contributor of contaminants.

When the production environment and harvesting techniques are combined with specific physiological interactions between produce and pathogens, the risk is compounded. Sprouts, the agricultural product most closely resembling microgreens, will be described shortly as a perfect storm of these three factors. Microgreens are similar to high-risk crops such as lettuce, berries, green onions, melons, sprouts, and tomatoes because they, too, are frequently consumed raw. Good Agricultural Practices (GAP) and Good Handling Practices (GHP) with respect to personal

hygiene and glove use are therefore even more crucial to prevent microgreens from suffering the same fate as other uncooked produce.

iii. Sprouts: an ideal disease vector

Sprouted seeds are an agricultural product most closely resembling microgreens. These young germinated seeds are often eaten raw (U.S. Department of Health and Human Services, 2015) and exemplify the intersection of production, growth, and handling conditions that allow pathogens to thrive. A search for "sprouts" in the CDC's Food Outbreak Online Database (FOOD) showed that products such as alfalfa, clover, and bean sprouts have been implicated in 53 outbreaks, 1876 illnesses, 209 hospitalizations, and numerous product recalls between 1998 and 2016 (Table 1). *Salmonella enterica*, shiga-toxin producing *E. coli*, *L. monocytogenes*, and human norovirus genogroup I were implicated in the 1876 food-borne illnesses from sprouts between 1998 and 2016, with *Salmonella enterica* alone responsible for 1675 illnesses (Table 2). The illnesses associated with norovirus genogroup I were from a single outbreak. In early 2018, the sandwich franchise Jimmy John's recalled alfalfa sprouts from its 2727 locations due to patrons in Wisconsin, Minnesota, and Illinois becoming ill with *Salmonella* serovar Montevideo that could be traced back to two seed lots from two Minnesota growers (Flynn, 2018).

Interestingly, *Salmonella enterica* appears to be the cause of more than three quarters of the reported illnesses resulting from contaminated sprouts (Table 1), and organic soil amendments may be a contributing factor (Jung et al., 2014). In particular, alfalfa sprouts appear to have been the most common variety among reported sprout-linked illnesses between 1998 and 2016, followed by mung bean and clover sprouts. One outbreak (32 illnesses) was traced specifically to

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alfalfa seeds (Table 3). Alfalfa and clover seeds are produced in large fields primarily for animal forage, and may be fertilized with manure. A subset of these seeds are sold to sprout producers. If proper sterilization or heat-pelleting of manure is not performed prior to application, seeds used for sprouts may be contaminated (Taormina et al., 1999).

Sprouts are produced by soaking seeds and then germinating them in a moist environment for approximately 5–7 days. Therefore, they may be exposed to temperatures and moisture levels optimal for the growth of mesophilic bacteria, including many human pathogens. Germination conditions provide ample time for pathogen proliferation and internalization (Warriner et al., 2005). Multiple studies have shown that pathogenic bacteria are capable of proliferating in the sprout germination environment, including enterohemorrhagic *E. coli* on radish sprouts (Itoh et al., 1998) and Vibrio cholerae O1, *Salmonella* Typhi, and *Escherichia coli* O157:H7 in alfalfa sprouts (Castro-Rosas and Escartin, 2000). Furthermore, there is evidence that growth of *Salmonella* during the sprouting process is capable of leading to outbreaks (Erdozain et al., 2013; Stewart et al., 2001).

By contrast, microgreens are immature seedlings of edible plants wherein their seeds are soaked only briefly, if at all, and harvested above the growth media after 10 to 21 days, between the opening of the cotyledon and the showing of the first set of true leaves (Fig. 2). Both microgreens and sprouts are often grown in greenhouses, high tunnels, and climate-controlled buildings. Since sprouted seeds have been implicated in a large number of high profile food-borne illness outbreaks as well as recalls over the past two decades (Gensheimer and Gubernot, 2016), this has led to the suspicion that microgreens may be similarly susceptible. Indeed, there

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are enough similarities between microgreens and sprouts to warrant thorough investigation into this emerging product. So far, there are no reported outbreaks or illnesses associated with microgreens. However, there have been 7 microgreen product recalls since 2016 due to contamination by either *Salmonella* or *L. monocytogenes* in the finished product as reported by the FDA Food Recalls, Withdrawals, and Safety Alerts Database (US Food and Drug Administration, 2016, 2018, 2019) and by the Canadian equivalent (Canadian Food Inspection Agency, 2018a, 2018b, 2018c). No consumer illnesses were reported; in all cases the contamination was discovered during routine quality control procedures.

IV. **The Produce Safety Rule and Guidance for the Sprout Industry**

The Food Safety Modernization Act (FSMA) was signed into law on 2011 as a sweeping measure to prevent food contamination. The Produce Safety Rule (81 FR 57784) is the section of the FSMA finalized in November 2015 (U.S. Department of Health and Human Services, 2015) that focuses on the prevention of contamination before, during, and after the production of fresh fruits and vegetables typically eaten raw. The Produce Safety Rule contains specific guidelines for sprouts, but not for microgreens. Requirements for sprouts include routine testing of the growing environment and agricultural water for the presence of *Listeria* species, testing each batch of spent sprout irrigation water or sprouts for *E. coli* O157:H7, *Salmonella* species, and other pathogens when necessary. The rule also requires that proper corrective actions are taken if contamination is found.

Responses to comments on the Produce Safety Rule (Comments, Sub-part A, pg. 74497) clarify that microgreens, fresh herbs, and edible flowers are all covered under the Produce Safety Rule

Part 112 "Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption" that governs all other produce eaten raw. This is because, despite microgreens' similarities to sprouts, the FDA maintains that microgreens are not sprouts due to their age at harvest and differences in harvesting practices and are therefore not covered under the sprout requirements in Part 112 Sub-part M of the rule. However, the FDA encourages producers of microgreens to voluntarily comply with the sprout guidelines. For microgreen operations that utilize hydroponics and aquaponics, the FDA recommends that producers comply with the agricultural water and soil amendment provisions addressed in Part 112, sub-part E and F, respectively.

i. **Good Agricultural Practices**

Good Agricultural Practices (GAPs) and Good Handling Practices (GHPs) are voluntary audits of on-farm food safety practices that produce growers may undergo in order to demonstrate compliance with the standards set forth by produce industry guidance documents. Commodity specific guidelines include the 1998 "Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables (FDA, 1998)," the updated 2011 "Produce GAPs Harmonized Food Safety Standard (United States Department of Agriculture, 2018)," and "Compliance with and Recommendations for Implementation of the Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption for Sprout Operations: Guidance for Industry," (United States Food and Drug Administration, 2017a, 2017b). These are non-binding recommendations that assist growers in complying with the Produce Safety Rule. The Produce Safety Alliance (PSA) and the Sprout Safety Alliance (SSA) exist to help growers comply with

the requirements of the Produce Safety Rule by offering training, educational programming, and assistance with GAP self-audits (Calvin, 2013).

ii. **Are commodity-specific guidelines for microgreens needed?**

There are presently no commodity specific guidelines for microgreens. It may not be necessary to establish a separate sub-part to the Produce Safety Rule specifically for microgreens, as many of the general guidelines are sufficient to address any potential issues related to microgreens. However, because microgreens share some traits in common with full-sized fresh produce and other traits in common with sprouts, it may be necessary to develop a guidance for industry to help microgreens growers navigate and comply with the various sub-parts of Part 112 of the Produce Safety Rule that apply to them.

V. **Potential Control Points for Microgreens**

Microgreens have the potential to become contaminated by pathogens from seed to harvest. Possible control points on the production continuum are outlined here. Some of these control points are common to all raw produce, while some are unique to microgreens.

i. **Irrigation Water and Irrigation Methods**

Microgreens are often grown in greenhouses, high tunnels, and climate-controlled buildings where contact with livestock, insects, and wildlife is minimal. Additionally, indoor and greenhouse operations tend not to use fertilizers, manure or otherwise, because the product is harvested after only one to three weeks (Treadwell et al., 2010; Xiao, 2013; Xiao et al., 2014b). Irrigation water, however, is of particular concern when it comes to sprouts and microgreens,

especially those grown hydroponically. Studies conducted in the field indicated that norovirus, for example, can directly contact and attach to vegetables and fruits from experimentally contaminated irrigation water (Alum et al., 2011; Stine et al., 2005).

The type of irrigation technique affects the risk of contamination. Produce irrigation water acquires pathogens during transportation through either canals, ditches, or pipes. Outdoor transportation exposes water to soil bacteria and parasites while pipes expose the water supply to biofilms. Some types of "sustainable" irrigation systems may compound the risk of microbial contamination, such as gray-water recycling and rainwater collection tanks. Drip irrigation reduces the risk of produce contamination compared to overhead spray irrigation due to limiting exposure of the edible portion of the plants to the water (Painter et al., 2013; Solomon et al., 2002).

Surface water sources such as nearby rivers, lakes, and streams have been to blame for many large outbreaks of food-borne illness. In 2011, 390 elementary schools and child care facilities contracted norovirus from contaminated frozen strawberries imported from China. The investigators hypothesized that, due to the size of the outbreak, the source may have been norovirus-contaminated irrigation water (Bernard et al., 2014). A 2012 outbreak of *Salmonella* Litchfield in Australia affecting 26 people was traced back to contaminated river water that was being used to wash papayas. In the United States, an *E. coli* O157:H7 outbreak in 2006 in prepackaged spinach affecting 205 people was traced back to contaminated surface water; the clinical isolate was detected in nearby river water and in cow and pig feces from a nearby farm (Gelting, 2007). Four outbreaks of acute gastroenteritis associated with norovirus isolates from

cabbage kimchi occurred in South Korea between 2008 and 2012 and were traced back to contaminated irrigation water (Cho et al., 2014).

Since microgreens are grown in trays in greenhouses or on artificially lit shelves indoors, producers may be more likely to water from municipal sources, groundwater, gray water, or collected rainwater. A review by Uyttendaele et al. (2015) concluded that municipal water is of the best microbial quality, followed by groundwater, gray water, and collected rainwater. Groundwater quality can be compromised, however, if the reservoir is too shallow, if heavy rainfall floods reservoirs with feces and microorganisms on land, or a nearby septic system or sewage line leaks. Roof-top collected rainwater may become contaminated by bird droppings and insects found on rooftops.

ii. **Decontamination of the seed**

Seed contamination is a well-known problem in the sprout industry. If seeds are contaminated, pathogens can become internalized from the beginning of the growing process and once incorporated are very difficult to remove (Wang and Kniel, 2016). Because of this, a significant body of literature has grown out of efforts to determine effective seed disinfection procedures. The FDA cites 20,000 ppm calcium hypochlorite as the standard method of chemical disinfection (US Food and Drug Administration, 1999), though adoption of this practice by growers may vary widely. Harrison (2017) reported, for example, that many growers selling at farmers' markets had limited food safety knowledge related to fresh produce, leading to the assumption that disinfection practices are not standard. Additionally, sprout producers who are seeking organic certification may not be permitted to use chlorine compounds on their products at levels

exceeding the Environmental Protection Agency's standards for drinking water, which are 0.8 ppm (Organic Standards, EPA Water Standards)

A review of sprout seed disinfection techniques by Ding et al. (2013) found that across 44 published articles, 18 of which tested the FDA recommendation of 20,000 ppm calcium hypochlorite, the standard 10 to 15-minute soak enabled a mean reduction in bacterial load of 3.08 log CFU/g with a standard deviation of 2.03 log CFU/g. The concentrated hypochlorite treatment had roughly twice the variability of the non-chemical methods such as heat treatment and irradiation compared in Ding et al. (2013), likely due to slightly differing protocols used by growers and the physical characteristics of the seed. For example, rough textured or scarified seeds were more difficult to disinfect than smooth seeds. It was hypothesized that bacteria and viruses are able to hide in the crevices of the seed surface and evade contact with disinfectants. Microgreen varieties such as pea shoots and sunflower are smooth in texture, but other varieties such as chard and beet have a rough, irregular surface (Fig. 3). Therefore, investigations into seed disinfection strategies for different microgreen varieties may be necessary.

With sprouts and microgreens, germination rate is a critical factor in production. Ding et al. (2013) demonstrated that physical methods such as heat treatment also boast high log CFU/g reductions, but it is a balancing act to achieve adequate reduction without compromising germination rate. High pressure treatment, out of all of the methods surveyed by Ding et al. (2013), demonstrated the lowest variability (standard deviation=0.94 log CFU/g) and the highest mean reduction of 5.09 log CFU/g with insignificant effects on seed germination rate. High-pressure treatment also has the advantage of being amenable to organic certification, though

potentially more expensive for small operations than chemical treatments because it requires special equipment (Wuytack et al., 2003).

Biological control is a relatively new attempt at dealing with seed contamination, though it is difficult to assess effectiveness of the methods because of the very specific environmental conditions of each approach. Studies have involved competition by communities of normal flora (Matos and Garland, 2005) and bacteriophage (Kocharunchitt et al., 2009) to control levels of unwanted bacteria with some success. There are potential health risks associated with these methods due to the many unknowns involved, and may be difficult to scale beyond the bench.

iii. **The relationship between post-harvest washing, spoilage, and contamination**

Since microgreens have a relatively short shelf life of 3-5 days even in refrigeration and are used in small quantities (Kou et al., 2014), it is important to determine if there is any connection between produce spoilage and contamination by human pathogens. As stated in a previous section, plant tissue damage creates opportunities for pathogen attachment or entry. In addition to damage by human handlers and harvesting tools (Lin and Wei, 1997; Moore et al., 2015; Bartz et al., 2017), enzymatic digestion by spoilage microorganisms may facilitate contamination. Gao et al. (2016) demonstrated this possibility in their study on virus attachment to lettuce leaves. Virus attachment to the leaf surface increased significantly after enzymatic digestion by cellulase. They also found that virus attachment increased when the leaf cuticle was peeled back, suggesting that the cuticle offers some protection.

Damage may also occur during post-harvest washing. In an effort to determine if post-harvest calcium chloride wash would have a measurable effect on shelf life of broccoli microgreens, Kou et al. (2015) found that the washing procedure itself decreased shelf life from 21 days to 14 days due to mechanical damage during rinsing, spinning, and drying. They also found that chlorine washes at 50 and 100 ppm were not effective at altering shelf life.

Refrigeration temperatures may also play a role. Kou et al. (2013) found that buckwheat microgreens stored at 1 °C suffered tissue damage, whereas buckwheat microgreens stored at 5 °C and 10 °C did not. The tissue damage corresponded to a greater increase in aerobic plate counts (APC) toward the end of the storage period. However, Xiao et al. (2014b) found that radish microgreens retained their quality best at 1 °C compared to 5 °C and 10°C. It is possible that there is a differential tolerance to temperature among microgreen varieties. They also found that a 100 ppm chlorine wash did not extend shelf life as aerobic mesophilic bacteria (AMB) increased by almost 4 log CFU by the seventh day of storage.

It appears to be important to prevent the growth of both pathogenic and spoilage related microorganisms earlier in the production chain, especially since post-harvest washing may cause tissue damage. Kou et al. (2014) tested the effects of a pre-harvest spray of calcium chloride, rather than a post-harvest wash. The spray seemed to have a beneficial effect on the post-harvest quality and shelf life of broccoli microgreens based on reduced tissue electrolyte leakage and lower microbial growth during storage.

VI. **Microgreen safety**

While there is a growing body of work on the health benefits of microgreens, there are very few reports on microgreen safety. Only eight reports of specific investigations into food safety risk of microgreens have been published to date, the first of which was Lee et al. (2009). After washing Chinese cabbage (*Brassica campestris* var. *narinosa*) microgreens in distilled water and two different concentrations of chlorine (50 ppm and 100 ppm) at two different water temperatures (5 °C and 25 °C), post-storage quality measurements and APC were compared. The data suggest that both concentrations of chlorinated water reduced APC more effectively than non-chlorinated water. Warmer wash water appeared to have a slightly stronger effect on reducing APC compared to cooler wash water. However, by the sixth day of storage, APC had increased from 7 log CFU to greater than 9 log CFU for test groups and controls. Additionally, the authors stated that as other measures of microgreen quality decreased, APC increased.

Chandra et al. (2012) studied Chinese cabbage microgreens and compared quality measurements, total coliforms, and APC after washing in four disinfectant mixtures and holding at 5 °C for 9 days. The disinfectant mixtures used were tap water (control), 100 mL/L chlorine, a citric acid/ascorbic acid mixture (0.25 percent w/v of each), and a 0.50 percent w/v citric acid solution followed by a 50 percent ethanol spray. The effect of packaging material was also considered. Two sets of microgreens were treated by the aforementioned methods and then were stored in either polypropylene or polyethylene containers. In both container types, APC was lower in microgreens treated with 100 ppm chlorine and the citric acid/ethanol treatment. Similar to Lee et al. (2009), counts rebounded around the sixth day to a log CFU level exceeding pre-wash levels.

Total coliform counts demonstrated by Chandra et al. (2012) sharply increased over three days in storage, and then began to slightly decrease after the 9th day. They failed to return to baseline levels. This pattern was observed regardless of treatment method or storage container, although the 100 ppm chlorine and citric acid/ethanol spray treatments resulted in overall lower log CFU/g of coliform bacteria compared to the other treatments for both types of packaging. These results were reported to be statistically significant at a p-value less than 0.05. The researchers stated that the reason for this decrease in proliferation is unclear and may be a result of multiple confounding variables in the storage environment including water content, pH, storage temperature, and relative humidity. Nevertheless, it can be surmised by these results that none of the sanitizing treatments tested were able to effectively reduce the log CFU/g of coliform bacteria on cabbage microgreens sufficiently enough to prevent regrowth.

Xiao et al. (2014) performed several experiments exploring the proliferation of two strains of *E. coli* on experimentally contaminated radish seeds. The starting inoculation levels were compared to the harvest levels of these *E. coli* strains at both the sprout stage and the microgreen stage. The microgreen stage had consistently lower counts at harvest relative to the inoculation level, even though the microgreens and sprouts came from the same batch of contaminated seeds. Watering overhead or from below made no significant difference in the proliferation of *E. coli* on the edible parts of the microgreen; however, the inedible parts showed greater growth that appeared to correspond with greater levels in the soil.

Xiao et al. (2015) compared the type of growth media on the proliferation of *E. coli* O157:H7 from seed to harvest of radish microgreens. Radish seeds were inoculated at low and high levels

of *E. coli* and radish microgreens were grown in a peat moss based soil substitute and in a hydroponic system. Compared to soil-grown microgreens, there was a large, statistically significant increase in proliferation of *E. coli* on the hydroponically grown plants. This occurred on both the edible and inedible plant parts as well as the hydroponic water. The researchers suggested that there could be competitive microbiota in the germination mix that inhibits the growth of *E. coli* compared to the hydroponic media.

These findings suggest that exposure to moisture is a significant contributing factor to the spread of *E. coli* in microgreen growing systems. In addition to *E. coli* cell counts, the researchers also assessed the spatial distribution of *E. coli* cells on various parts of the microgreen using a green fluorescent protein (GFP) labeled *E. coli* strain viewed with laser confocal scanning microscopy. Spatial analysis showed that the seed coat was the most densely populated part of the microgreen, whereas the hypocotyl and cotyledon were much less densely populated.

A comparison of the native microbial populations on different types of growth media was performed by Di Gioia et al. (2017). They measured AMB, yeast and molds (YM), Enterobacteriaceae, and *E. coli*. Their data showed that food-grade plastic mats had the lowest overall AMB and YM levels, whereas peat had the highest levels. Peat and jute-kenaf grown microgreens had the highest levels of AMB and YM, and peat had the highest levels of Enterobacteriaceae. The microgreens grown on textile fibers and food-grade plastic mats had no detectable levels of Enterobacteriaceae or *E. coli* in the edible portion of the plant, indicating that they were not as easily transferred to the edible part of the plant from those media. Conversely,

the jute-kenaf fiber growing media did not have detectable levels of Enterobacteriaceae but this group of bacteria was strongly detected on the microgreens.

Researchers have also investigated the role of contaminated hydroponic nutrient water on the persistence and transmission of viruses, using murine norovirus (MNV) as a surrogate for human norovirus, the primary cause of food-borne disease outbreaks in the US. Wang and Kniel (2016) grew kale and mustard microgreens in a hydroponic system that was artificially contaminated by 3.5 log PFU/mL of MNV on the 8th day of growth. Water and microgreen tissue samples were collected at 2, 4, 8, and 12 h immediately following inoculation. After day 8, water and microgreen tissue samples were collected daily until the 12th day. This design enabled monitoring of detectable levels of virus taken up by the plants in addition to the rate of die-off toward the end of harvesting.

Virus survival immediately following inoculation remained relatively consistent at ~2 log plaque-forming units per milliliter of water (PFU/mL) for up to 12 h of sampling. By day 12, MNV only decreased by around 1 log PFU/sample (statistically significant) in both varieties of microgreens. This decrease was similar for internalized virus as well as its concentration in the hydroponic nutrient water. The virus was also detected at around 1–2 log PFU/mL in the hydroponic water for up to 16 days post-inoculation and contaminated the next crop of microgreens at detectable levels in both root and shoot tissue. These findings demonstrate that MNV can persist at detectable levels in hydroponic systems for at least several weeks from an initial inoculation of 3.5 log PFU/mL. There were no statistically significant differences overall between kale and mustard.

Wright and Holden (2018) studied the colonization of nine varieties of microgreens by shiga-toxin producing *E. coli* serovar Sakai (STEC). Experiments were conducted on seeds contaminated directly at 3 log CFU/g and on seeds grown with contaminated irrigation water at 7 log CFU/g. Varieties tested were amaranth, broccoli, kale, mustard, coriander, rocket, basil, parsley, and radish. Colonization for eight of the nine microgreen varieties exceeded 8 log CFU/g of fresh weight. Basil was the only variety to show a final STEC level of less than 8 log CFU/g with 7.21 log CFU/g of fresh weight. Previous research by Gao et al. (2016) has shown that basil is also less likely to be colonized by a norovirus surrogate, again pointing to possible plant variety differences.

Reed et al. (2018) was able to demonstrate differences in colonization between *Salmonella enterica* serovars Hartford and Cubana on alfalfa sprouts and Swiss chard microgreens. External factors tested were growth media, storage time, contamination of either seed or water, and inoculation level. For sprouts and microgreens grown from contaminated seeds, increasing the inoculation level from 10 to 100 CFU/g of seed had the most influence on colonization of both microgreens and sprouts, regardless of serovar. However, for sprouts, increasing storage time from 7 to 28 days allowed *S. enterica* levels to decrease by half. For microgreens, Cubana was less prolific at 10 CFU/g of seed, but was equivalent to Hartford once inoculation was increased by one order of magnitude. A community analysis demonstrated that the sprout rhizosphere was more species-rich compared to microgreens. Hydroponic media showed overall greater colonization by both serovars compared to either soil mixture, which is consistent with previous research by Xiao et al. (2015) and Wang and Kniel (2016).

VII. **Future Research**

Given what is currently known about bacterial and viral contamination of microgreens, many questions remain. Sunflower microgreens and pea shoots have not yet been the subject of any microbiological or viral studies, yet they are popular for producers due to the low cost of seeds, consistent germination rate, and high average fresh weight (Personal communication with beginning growers). They are also popular for beginners who may be even less attentive than established commercial operations to food safety protocols. Reed et al. (2018) and Wang and Kniel (2016) are so far the only investigators that compared multiple microgreen varieties. These as well as Gao et al. (2016) suggest that there is a species effect for both contaminant and product, though the sample sizes were small. Furthermore, most of the research into microgreen safety has been focused on bacteria, particularly *Salmonella* spp. and *E. coli*, likely due to regulatory requirements and the prevalence of these microbes in food-borne illness outbreaks. Viral contamination of microgreens should be explored further, in particular the attachment of norovirus to microgreen leaves, internalization of the virus during the growing process, and possible prevention measures. Further research on the contributions of hand harvesting versus cutting are recommended. Only Di Gioia et al. (2017) compared different types of growth media on contamination risk; these experiments need to be replicated and expanded. Additionally, earlier papers that measured AMB and coliform levels along with spoilage indicators suggested that these two factors may have an inverse relationship, though no formal correlation has been shown. Due to the short shelf life of microgreens and their tendency to be used only in small quantities, understanding the relationship between spoilage and contamination by pathogens is important.

VIII. **Conclusion**

The limited amount of data available suggests that microgreens may very well be of lower risk than sprouts in terms of food-borne illness, but the background level of bacteria is greater than that of conventional vegetables (Chandra et al., 2012; Lee et al., 2009) and is more similar to sprouts. Hydroponically grown microgreens appear to be much more susceptible to bacterial colonization compared to any solid media tested (Wang and Kniel, 2016; Xiao et al., 2015). Spoilage and shelf life may be linked to contamination by pathogens (Gao et al., 2016; Kou et al., 2013; Kou et al., 2015; Xiao et al., 2014a, 2014b). The variety of microgreen and the serovar of the contaminant may influence risk. Postharvest washes appear so far to be ineffective and may actually increase contamination risk due to tissue damage that invites pathogens among other microorganisms (Kou et al., 2015). Pre-harvest spraying with disinfectants may provide a valid alternative the post-harvest wash for ameliorating surface contamination. Seed decontamination appears to be a critical ongoing discussion (Kou et al., 2014).

IX. References

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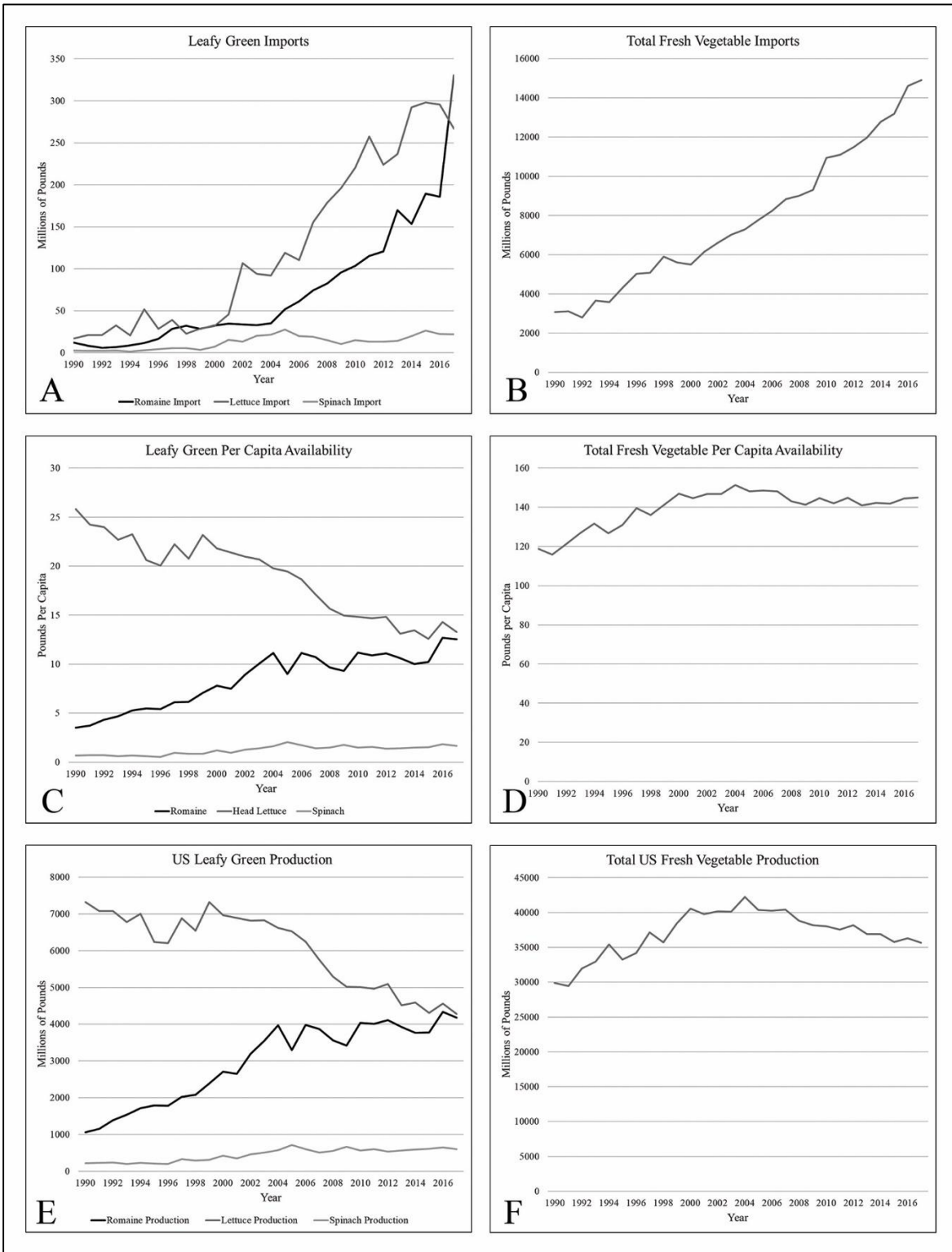
Figure 1. Leafy green consumption and availability. Lettuce, leafy green, and total fresh vegetable imports (A and B), per capita availability (C and D), and production (E and F) in the United States from 1990 to 2016. Source: ERS/USDA, Accessed June 4, 2018.
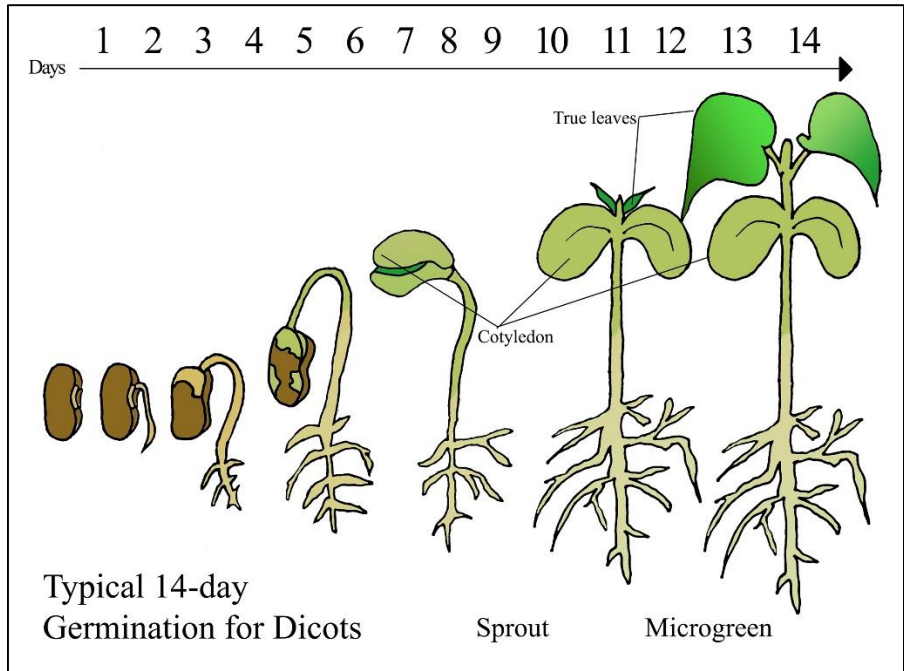
Figure 2. Microgreens and sprouts differ by age at harvest. A typical 14-day germination period for a dicot, using the common garden bean as an example. Germination period for microgreens and sprouts varies by plant variety.
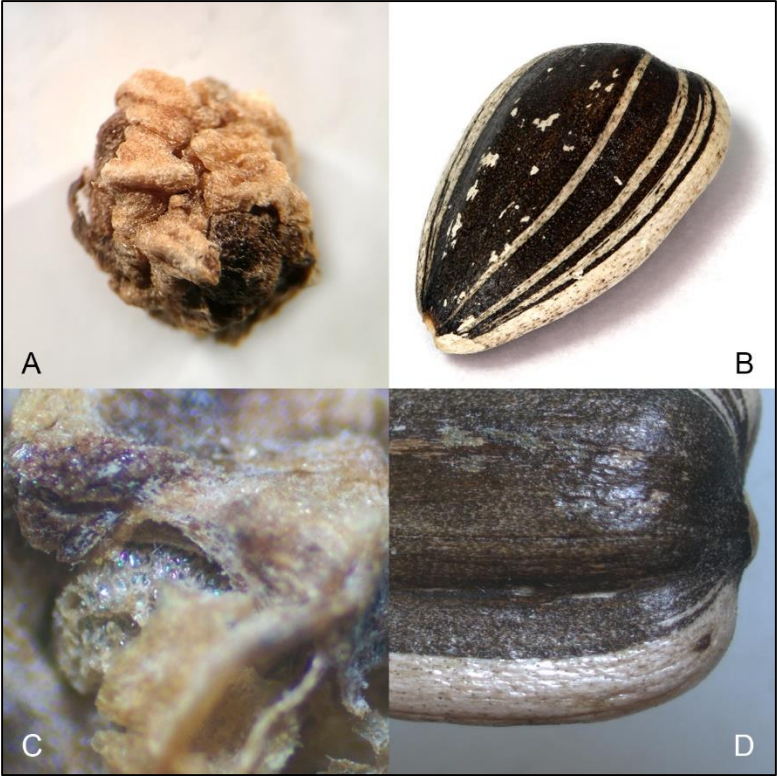
Figure 3. Differences in seed topography. A) Swiss chard seed 17.5×, Olympus SZ60; B) sunflower seed, public domain; C) Swiss chard seed 150×, AccuScope 3072/Excelis SMZ143; D) sunflower seed 150×, AccuScope 3072/Excelis SMZ143.

XI. **Tables**

Table 1: Sprout Outbreaks by Etiology

Etiology	Illnesses	Hospitalizations	Deaths
L. monocytogenes	27	21	2
Norovirus Genogroup I	32	0	0
S. enterica	1675	160	2
Shiga-toxin producing *E. coli*	133	28	1
Total	1867	209	5

Sprout outbreaks by etiology from 1998 to 2016. Source: Centers for Disease Control and Prevention National Outbreak Reporting System (NORS). Accessed June 4, 2018.

Table 2: Sprout Outbreaks by Year

Year	Outbreaks	Illnesses	Hospitalizations	Deaths
1998-2001	12	711	56	0
2002-2005	10	166	16	1
2006-2009	11	425	31	0
2010-2013	11	293	49	1
2014-2017	9	272	57	3
Total	53	1867	209	5

Morbidity and mortality related to foodborne disease outbreaks linked to consumption of sprouts in the U.S. from 1998 to 2016. Source: Centers for Disease Control and Prevention National Outbreak Reporting System (NORS), Accessed June 4, 2018

Table 3: Sprout Illnesses by Food Vehicle

Product	Total Illnesses
alfalfa seeds	32
alfalfa sprouts	1059
bean sprouts	68
clover sprouts	212
mung bean sprouts	394
sprouts, unspecified	55

Sprout Illnesses by Food Vehicle from 1998 to 2016. Source: Centers for Disease Control and Prevention National Outbreak Reporting System (NORS). Accessed June 4, 2018.

Chapter 2: Characterization of Microgreen Businesses in the United States with Emphasis on Food Safety

I.   **Abstract**

Microgreens are an emerging industry about which little is known. This study represents the first national survey of microgreen growers in the United States. A total of 176 respondents completed an online survey including questions about farm demographics, growing techniques, microgreen varieties grown, and relevant farm food safety practices. Microgreen operations earning less than 10,000 USD/year in microgreen revenue (62%) that produce microgreens in trays on stacked, artificially lit shelves (40.3%) dominated the response pool. Most farms who responded to the survey opened after 2010 (75%). These farms primarily grew microgreens using peat, coco coir, or soil. Sunflower, peas, and radish were the most popular microgreen varieties produced. It was found that common deficits among microgreen growers include poor routine documentation, limited growing media and agricultural water testing, and widely variable post-harvest storage practices. Strengths of the industry include self-reported routine hand-washing and equipment sanitation, greater average education level, and awareness of food safety training resources. In addition to supporting training and outreach efforts, this study aims to inform the research community of growing systems, microgreen varieties, and production practices that would be relevant for future microgreen food safety studies.

II.   **Introduction**

Farming systems that present alternatives to traditional field production of fresh produce are on the rise. The most reliable and recent estimates are between 5-15% of total agricultural production in developing nations (Zessa et al., 2010). In developed countries such as the United

States, the number of farmers markets and community-supported agriculture (CSA) organizations supplied by small urban producers has grown by more than 50% since the mid-2000s (Mok et al., 2014). By 2014, consumers in the United States purchased almost 800 million USD in indoor-grown crops (Lensing, 2018). This increase in popularity is often attributed to concurrent interests in preventing climate change impacts on farm productivity (McCartney et al. 2018, Gruda et al. 2019), access to fresh food for an increasingly urbanized population (Benke and Tomkins, 2017; Shamshiri et al. 2018), and for space travel research (Kyriacou et al. 2017, Zabel et al. 2016). Modern indoor farming was popularized as vertical farming by Despommier (2013) and has since evolved into a myriad of system types under the umbrella term of "controlled environment agriculture" (CEA).

While there is a growing body of literature investigating the profitability and productivity of CEA (Eaves and Eaves, 2018; O'Sullivan et al. 2018, Thomaier et al. 2015, Specht et al. 2014, Touliatos et al. 2016), less is known about food safety risks related to these production systems or the crops typically grown within them. For example, microgreens—an emerging raw salad product produced using CEA—are immature shoots of common vegetables harvested above the root at 10-20 days old (Kyriacou et al. 2016). Similar to leafy greens, microgreens can be produced outdoors, fully indoors, or in greenhouses, as well as in hydroponic systems or in soil or soil-alternative based systems (Kyriacou et al. 2016). And similar to sprouts, they are harvested at a young age after germinating in a warm, moist environment (Kyriacou et al. 2016). These characteristics of microgreens make it a target crop for studying the food safety of CEA-grown produce. Therefore, since microgreen production shares some similarities with sprouts and leafy greens, they may have similar food safety risks.

Sprouted seeds (referred to as 'sprouts') are a raw salad crop frequently compared to microgreens. Sprouts have been implicated in more than 1,800 cases of foodborne illness since 1996, many of which were linked to *Salmonella* spp. (CDC NORS). Leafy greens are also frequently associated with foodborne illness, making up approximately 38% of all produce-associated outbreaks (Bennett et al. 2018). Romaine lettuce grown in the Yuma, AZ region has been implicated in several high-profile outbreaks of Shiga toxin-producing *Escherichia coli* (STEC) since 2018 (Bottichio et al., 2018). While the majority of traceback investigations have not revealed a causal link, in 8 of 32 outbreak investigations conducted since 1995, improper post-harvest washing procedures were identified, as well as STEC contamination of irrigation water and animal excrement found in the growing fields (Kintz et al., 2018). However, contamination can occur at any point along the production continuum (Olamait et al., 2012). While there have been no known outbreaks associated with microgreens, there have been multiple product recalls of microgreens related to *Salmonella enterica* and *Listeria monocytogenes* since 2016 in the United States (US FDA 2016, 2018, and 2019) and Canada (CFIA 2018a-f, 2019a, and 2019b). This history underscores an urgent need to elucidate potential risk factors within microgreen production that may render these products susceptible to contamination and possible foodborne outbreaks as the industry grows.

Regulatory oversight for the safety of produce in the United States falls under the Food and Drug Administration's (FDA) Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption, 21 CFR Part 112, commonly referred to as the Produce Safety Rule (PSR). The rule was adopted by the FDA in response to the Food Safety Modernization Act (FSMA) of 2011. The rule establishes best practices for the prevention of foodborne pathogen

contamination of "covered produce," defined as produce that is typically eaten raw. The PSR requires that growers meet certain standards for the use of biological soil amendments of animal origin, worker health and hygiene practices, irrigation water quality, equipment and surface sanitation practices, and the handling of wild and domesticated animals in the farm environment (U.S. Department of Health & Human Services, 2015). However, growers who earn less than 25,000 USD in annual produce sales are exempt from the rule, as well as any produce grower who earns less than 500,000 USD but half or more of all sales of covered produce are direct to consumers or food retail businesses (U.S. Department of Health & Human Services, 2015). Understanding the size and other characteristics of microgreen businesses will determine if they tend to be exempt from the PSR and if common industry practices exist which might be risk factors for contamination of microgreens with human pathogens.

Furthermore, improved understanding of the farm food safety practices among practitioners of these unique farming styles, generally categorized as CEA, will assist training and outreach efforts targeting compliance challenges faced by these businesses. While certain standards put forth by the PSR invariably apply to all fresh produce growers, such as hygiene and irrigation water quality, CEA growers may face challenges more similar to packing plants than that of conventional field growers. There are no established guidelines for the production of microgreens at a commercial scale, with the exception of a recommendation within the PSR that microgreen growers voluntarily comply with the sprout recommendations (U.S. Department of Health & Human Services, 2015). Lastly, laboratory research directly examining food safety risks of common microgreen production systems should be informed by current industry trends and practices, which are largely unknown.

While multiple surveys have been conducted to assess food safety practices on farms growing produce typically eaten raw (Parker et al. 2016, Adalja et al. 2018, Cannon et al. 2013, Astill et al. 2019), little is known about these practices within the emerging microgreen market. Two previous surveys of aquaponics facilities—a farming style resembling certain types of microgreen production—assessed only general production methods and demographics with the primary objective of determining profitability and sustainability of this subset of the indoor farming industry (Love et al., 2014 and 2015). Unfortunately, farm food safety practices were not examined. Agrylist, a greenhouse management software company, has conducted one of the only annual, comprehensive surveys of the indoor farming industry for which data is freely available (Agrylist 2016 and 2017). However, the survey is conducted for the purposes of market research, and as such it does not focus on understanding grower compliance with food safety regulations. It also focuses on all types of produce grown in CEA farms, rather than just microgreen farms. Given these knowledge deficits, an online survey was designed and implemented for the purpose of understanding the demographics, farm characteristics, and food safety practices of microgreen farmers in the United States.

III. Materials and Methods

i. Ethics statement

The study was reviewed by The University of Arkansas Institutional Review Board (IRB No: 1809144516) which determined it to be exempt and not human subjects research. The survey contained a cover page with a description of the research objectives as well as a consent question that had to be answered before the participant could begin the survey. The survey did not collect personally identifying information such as farm name, participant name, street address, phone

number, or email address. However, the survey did collect the US zip code for each farm in order to assess geographic distribution of farms surveyed and any regional differences in responses.

ii. **Survey development and implementation**

We collected 142 complete responses with an additional 34 incomplete responses (total = 176) between October 1, 2018 and March 30, 2019. Unless otherwise specified, all percentages reported are calculated with 176 as the denominator. Unanswered questions represent the response "No response" and are considered in the dataset. The survey was designed and distributed using the Qualtrics platform (Provo, UT, USA). Participant inclusion criteria required that respondents sold microgreens to United States customers. Recruitment was conducted within online communities on social media sites Facebook and Reddit dedicated to microgreen growing and sales, hydroponic crop production, sustainability, and gardening. Additional respondents were recruited through email broadcasts on customer lists of a few popular seed and indoor farming supply companies. Lastly, approximately 80 emails were sent, with follow-up messages a week later, using the database LocalHarvest.org to search for all farms and CSAs in the United States that list "microgreens" as one of their available products. To incentivize completion, a discount coupon was offered from the seed and supply businesses who distributed the survey link.

iii. **Survey Questions**

The survey question styles included 44 multiple-choice, 18 multiple-answer, 8 fill-in-the-blank, 1 ranking, 1 short answer, and 1 multiple-choice matrix. Not all questions were asked to all respondents; the questions viewed were generated based on answers given to previous questions.

Questions were grouped by the following topics: farm demographics, product information, growing system, growing media, irrigation water, seed storage and handling, sanitation and worker hygiene, post-harvest washing, post-harvest storage, tracking and documentation, food safety training, and grower education. Following acceptance of the informed consent statement, growers were asked their country of origin and if they sold microgreens to United States customers. If the respondent selected "no" to that question, they would be routed to an ending page telling them that the study being conducted is on microgreen businesses with US customers only, regardless of farm location.

Validation of the survey instrument was performed by academic as well as industry professionals. Question wording, appropriateness of questions, survey flow, and coverage of food safety topics were adjusted based on feedback from an expert in food safety education and outreach. Significant attention was paid to minimizing the total number of questions, limiting matrix, fill-in-the-blank, and multiple-response questions, as well as the overall time required to complete the survey. Following expert evaluation, three graduate students performed a pilot test of the survey and were provided with pre-determined survey responses designed to guide them through specific pathways to test reliability. Finally, adjustments were made based on feedback on the overall survey experience from two microgreen farmers who acted as non-scientific reviewers. Completion time was estimated by the Qualtrics platform to be 15 minutes or less.

iv. **Estimating Total Production**

Total microgreen production was standardized to pounds per month, even when respondents reported their total monthly production in trays, kilograms, or ounces. The conversion factor for

the weight of microgreens produced per tray (0.45 pounds per 10"x20" tray) was determined by using the average of typical yields per 10"x20" tray for seven microgreen varieties (sunflower, pea shoots, radish, kale, cabbage, amaranth, and basil) as suggested by one of the responding farms (Personal communication).

v. **Data Analysis**

Data from Qualtrics were exported and analyzed in Excel (Microsoft, Redmond, WA, USA) and the R statistical platform (version 3.6.0) including the packages *descr* (Aquino, 2018), *maps* (Deckmyn, 2018), *ggplot2* (Wickham, 2016), and *data.table* (Dowle and Srinivasan, 2019). Chi-square tests for independence were performed between categorical variables to determine if statistically significant relationships exist between key food safety practices and farm characteristics where the answer type was multiple choice. For comparing numerical to categorical responses, Kruskal-Wallis tests were used. Kruskal-Wallis is a non-parametric analysis of variance that is more robust than ANOVA for non-normally distributed datasets (Kruskal and Wallis, 1952). Because the data were skewed strongly toward smaller, beginning farms growing microgreens in trays on stacked shelves and the sample sizes of the other groups were much smaller, improved accuracy of Chi-square tests was attempted by adding a Monte Carlo simulated p-value to reduce risk of a Type 1 error (Rai et al. 2001).

For multiple-response questions, the large number of possible answer choices ($p = 122$), and thus a large number of predictors relative to samples ($n = 143$), as well as non-normally distributed data, necessitated the use of the R package *glmnet* (Friedman et al. 2010). This generalized linear modeling approach with Lasso was used to determine if linear relationships exist between key

food safety practices and selected farm characteristics where multiple responses were given. A key benefit of Lasso is preventing over-fitting of the data and selecting only the most relevant predictors for such high-dimensional data.

IV. Results

i. Demographics

a. *Geographic Distribution*

The survey captured growers across the United States (Figure 1). Fewer farms reported Western US zip codes; however, this regional response rate difference is consistent with a previous nationwide survey of produce farmers, also showing lower farm density in that region (Adalja et al. 2018).

b. *Farm Size*

Farm size was calculated by yearly revenue from microgreens, monthly microgreen production output, and by number of employees. Farm size by number of employees is reported in Table 1. For revenue, respondents were asked "What is your yearly revenue from microgreens?" and were given the option to choose from five revenue categories or "Prefer not to respond." There were 71 farms earning less than 5,000 USD/year, 28 farms earning between 5,000-9,999 USD/year, 10 farms earning between 10,000-24,999 USD/year, 9 farms earning between 25,000-49,999 USD/year, and 6 farms earning greater than 50,000 USD/year. 18 farms preferred not to answer, and 34 farms did not choose a response.

Monthly production level was reported in 10"×10" trays, 10"×20" trays, pounds, ounces, kilograms, or "other." The values reported by respondents were then standardized to pounds per month for comparison using the method described previously (Section III,iv). Farms earning less than 5,000 USD/year in revenue (n = 71) averaged 14.7 ± 18.9 pounds per month, farms earning between 5,000-9,999 USD/year in revenue (n = 28) averaged 45.20 ± 59.4 pounds per month, farms earning between 10,000-24,999 USD/year in revenue (n = 10) averaged 97.47 ± 144.4 pounds per month, farms earning between 25,000-49,999 USD/year in revenue (n = 9) averaged 420.39 ± 1,043.4 pounds per month, and farms earning greater than 50,000 USD/year in revenue (n = 6) averaged 7,629 ± 8,635 pounds per month. The high standard deviations associated with these production estimates are likely due in some part to the error prone method of standardizing pounds per tray described previously, and to a lesser extent, due to respondents entering their total farm production instead of just microgreen production and the differing sample sizes of each revenue category.

c. *Education and Farming Experience*

Growers' education level was primarily at the bachelor's level (23.9%) or "some college" (18.2%). "Some college" does not distinguish between participants who are still in college or who never completed college. The third most common education level is an associate's degree, representing 9.7% of respondents. This rate is similar to the national average, where 33.4% of US citizens hold a bachelor's degree (US Census Bureau, 2016).

Most microgreen growers (48.3%) reported having learned to grow microgreens using websites and online videos. The second most popular method of learning to grow microgreens included

"informally from other growers," (12.5%) "books and magazines," (9.1%) and "social media groups" (8%). However, high representation from Internet-learners is possibly due to the Internet-intensive survey participant recruitment procedures.

The microgreen growers surveyed appeared to be mostly produce farmers, either growing only microgreens or microgreens along with other plant products. Livestock production on microgreen farms was less common. Among microgreen growers, 31% of farms produced other vegetable crops, 2% of farms produced livestock and animal products, 10% of farms produced both, and 24% of farms produced only microgreens. Thirty-two percent of respondents declined to answer the question. The most common vegetable crops included produce typically eaten raw (36.3%) and produce rarely eaten raw (25.5%). The most common animal products include poultry (8%) and eggs (7.4%).

Most farms who responded are newly opened, with 74% of the farms in the survey opening after 2010, most of which fell into the "Less than $5000/year" revenue category, suggesting that most of these very small farms are beginners. Interestingly, farms opening after 2010 were more likely to be raising livestock or animal products (3.1%), or both animal and plant products (13.8%) compared to those farms that opened before 2010. Of the older farms, 61.5% produced other plants or crops, and 15% grew only microgreens. This suggests that in addition to beginning growers, more experienced fresh produce farmers are adopting microgreen production.

d. *Produce Safety Rule (PSR) Exemption*

The PSR exempts farms earning less than 25,000 USD/year in revenue, as well as farms earning less than 500,000 USD/year where at least half of sales are direct to customers or food retail outlets (U.S. Department of Health & Human Services, 2015). However, respondents were only asked what their yearly revenue was for microgreens. For farms who produced other vegetable crops, their total produce revenue may exceed the exemption threshold and thus some of these farms may not be exempt. Furthermore, even farms earning greater than 50,000 USD/year in microgreen revenue, whether or not they sell other covered produce, may still primarily sell direct to customers, grocery stores, and restaurants rather than wholesalers and would be exempt. Therefore, it is possible that nearly all respondents in this survey are exempt from the PSR.

ii. **Growing Techniques**

a. *Growing Systems*

The survey inquired about the system type and location where half or more of the respondent's microgreens are produced. System type is defined as the production system design, whether that is aquaponics, hydroponics, in ground, containers, raised beds, or trays on shelves. System location refers to the setting where the production takes place, whether that is fully indoors in a room with opaque walls, such as a storefront, warehouse or residential building; a greenhouse or hoop house with translucent or transparent walls; or completely outdoors. The most common combinations were an indoor residential space with trays on stacked shelves (26.7%), a container farm inside a climate-controlled greenhouse (8.5%), and an indoor commercial space with trays on stacked shelves (6.8%). All combinations of system type and location are shown in Table 2.

Farms earning greater than 50,000 USD/year in microgreen revenue did not use trays on stacked shelves, whereas at least half of all other revenue categories did. The predominant production methods in the highest revenue category were unstacked container farms (50%) and hydroponic systems (16.7%). Hydroponic systems were less common among farms earning less than 25,000 USD/year. Of those growers who preferred not to disclose their yearly microgreen revenue, 17% used hydroponics and 39% used trays on stacked shelves, possibly suggesting a mixture of high and low earning farms unwilling to give income information.

b. *Growing Media*

Most growers who responded to the survey utilized trays on stacked, artificially lit shelves, while cultivating in a soil blend or soil substitute, particularly organic soil or peat blended with an aerator such as perlite and occasionally, a biological soil amendment. The most common types of media used include peat moss (17.6%), organic soil (15.3%), and coco coir (14.2%). The most common additives included perlite (31%) and vermiculite (19.3%). Many growers did not report using any soil amendments (37%). However, the most common were worm castings (8.5%), green compost (6.2%), food compost (4.5%), and manure (2.3%). One grower used a unique fertilization mixture containing ingredients such as kelp meal, fossilized bat guano, and aged forest products.

Participants were also asked how they disposed of their used growing media, and it was found that a single-use approach with growing media is uncommon. 43.8% of growers reported that they compost spent media after harvesting microgreens; 5.1% of growers selected "We use it to grow other plants"; and 1.1% (2 growers) reported that they reuse the media to grow more

microgreens. It is unknown what the end use of the composted growing media is for the 43.8%

of growers who produce it, and thus future investigations into this practice may be warranted.

c. *Irrigation Method*

Microgreens can be watered by either overhead spray irrigation or by sub-irrigation. Bottom-

watering or drip irrigation, where the water does not touch the microgreens, was reported by

33% of respondents. Overhead watering, where the water does touch the edible portion of the

microgreens, was reported by 23.9% of respondents. This question was left blank by the other

42.6% respondents. Previous microgreen food safety studies comparing the risks of overhead vs.

sub-irrigation are limited, though it has been studied in other leafy greens (Rock et al. 2019).

Neither Işık et al. (2020) nor Xiao et al. (2015) found statistically significant differences in the

transfer of *E. coli* O157:H7 to microgreens between the two watering methods, while Solomon et

al. (2002) did detect a difference in *E. coli* O157:H7 transfer to lettuce.

d. *Production Environment*

Approximately half of all farms (51.1%) reported monitoring environmental conditions of their

growing space. The average ambient temperature, water temperature, and relative humidity for

each production environment type are shown in Table 3. Non-responses were excluded from this

analysis (69/176). The 33 respondents who reported all three variables were used for this

comparison. By contrast, relative humidity in sprouted seed production environments tend to be

closer to 70% (Xiao et al. 2014). This may indicate a possible difference in food safety risk

between microgreens and sprouted seeds. Studies in other types of covered produce (Stine et al.

2005, Tian et al. 2013) indicate the possibility that low relative humidity is generally linked to pathogen inactivation, though it may ultimately depend on pathogen and produce type.

iii. **Agricultural Water**

The most common sources of irrigation water include municipal water (32.4%) and well water (29.5%). Rainwater collection (2.8%), surface water (1.1%) and greywater (0.6%, only one farm) were also used. The majority of farms did not impose any end-user water treatment beyond what may be performed at the source, such as at a municipal water treatment plant. Activated charcoal, reverse osmosis, and sediment filtration were the most commonly used methods among the few respondents who treated their water. Discussion of water testing and treatment, which are key food safety practices, can be found in Section V, part ii.

iv. **Microgreen Varieties**

Sunflower, pea shoots, and radish were the top three most commonly grown microgreens (Table 4). Possible reasons for this preference include ease of cultivation and short seed-to-harvest period; the low cost of seeds relative to other varieties; and the high fresh weight yield per unit of tray area, leading these varieties to be the most profitable. Thus, it is critical that microgreen food safety research focuses on these varieties. So far, no research has been published that investigates the food safety risk of sunflower and pea shoots. Radish microgreens have appeared in three studies (Xiao et al. 2014 and 2015, Wright et al. 2018).

v. **Key Food Safety Practices**

Chi-square tests of association were performed to identify any statistically significant relationships between farm characteristics and food safety practices that are relevant to the PSR. Farm characteristics tested included farm size by revenue, farm size by number of employees, number of employees directly handling microgreens, whether or not the farm has passed a Good Agricultural Practices (GAP) audit, number of previous food safety trainings taken, last completed education level, type of production system, and monthly microgreen production in pounds. These characteristics were tested against the following practices: documentation, water testing, seed disinfection, hand washing, post-harvest washing, grow media testing, and sanitation. Table 5 summarizes these relationships. The values for n varied across each comparison because the statistical tests required exclusion of "NA" values. Sample sizes for each comparison are cited within the text.

a. *Growing Media Testing*

When participants (n = 104) were asked "Do you test your soil or growing media for bacteria?", responses included "Yes" (11.5%), "No" (87.5%), and "I don't know." (1%). Testing frequency was reported as follows: 2% of growers tested twice a year, 4% of growers tested 4 times per year, 2% of growers tested more than 4 times per year, 87.5% of growers did not test growing media, 4% of growers tested their growing media but did not know how often, and one grower (1%) did not know if their farm's growing media was tested for bacteria. Statistically significant relationships were found between grow media testing at least once per year and both the total number of employees (p = 0.015) and the total number of employees who directly handle the microgreens (p = 0.001). This may indicate that larger microgreen operations are better equipped

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to engage in routine quality assurance procedures such as microbiological testing of media. As most operations used peat or soil and did not engage in any media testing, it is difficult to determine, statistically, if type of growing medium influences testing frequency.

b. *Water Testing and Treatment*

The only farm characteristic that had a statistically significant relationship with irrigation water testing at least once per year was the type of production system ($p = 0.01$). The source of irrigation water (e.g. municipal, groundwater, surface water, rainwater, etc.) was hypothesized to be an influencing factor, but was not significant in our data ($p = 0.49$) as most operations used either groundwater or a municipal water source. However, linear regression showed that "collected rainwater" was a negative predictor of water testing (See Section V and Table 2-S1 in the Appendix), though only 2 growers used it. A summary table of the number of farms in each testing frequency group by system type are presented in Table 6.

For water treatment, 46.6% of respondents did not treat their water and 35% did not answer the question ($n = 176$). The most popular type of water treatment method among those who did treat their water included activated charcoal filtration (6.2%), a sediment filter (6.2%), and reverse osmosis (5.1%). Respondents were allowed to choose more than one response for this question, so percentages will not add up to 100. There were many unique combinations of water treatment reported by respondents, but the most common combination of water treatment methods was a sediment filter along with an activated charcoal filter, used by 5 growers. Water treatment by water source is reported in Table 7.

c. *Seed Disinfection*

Statistically significant relationships existed between pre-germination seed disinfection and two farm characteristics: production system type (p = 0.001) and total number of employees (p = 0.011). Interestingly, those farms who did not disinfect their seeds prior to germination had an average of 29 total employees while farms who did disinfect their seeds averaged 4 total employees. An in depth survey of 19 food safety experts and 32 produce growers (Parker et al., 2016) also challenges the assumption that larger farms are more likely to engage in more food safety practices than smaller farms. The authors found that if a recommended food safety practice is more challenging to implement on a larger scale, large farms are less likely to do it. Seed disinfection may be one of those practices.

For growing system type, 40 stacked-tray growers (n = 71) disinfected their seeds, 28 did not, and 2 did not respond. For all other growing system types combined (n = 47), a greater proportion of growers did not disinfect their seeds compared to those who did. In particular, 17 out of 22 container farms reported not disinfecting seeds. Among growers of all system types who reported having a seed disinfection step (n = 49), 42 (85%) used a hydrogen peroxide soak. Sodium hypochlorite (3 respondents) and vinegar (1 respondent) were also reported.

d. *Harvest, Post-Harvest Washing, and Storage*

Post-harvest washing was performed by 34 farms (19.3%) and not performed by 77 farms (43.8%), while 65 farms did not respond to the question. The most common varieties washed after harvest were "all varieties" (20 farms, 11.4%), "sunflower" (10 farms, 5.7%), and "radish" (4 farms, 2%). While these were the most commonly grown varieties, thus most commonly

washed, a few respondents noted in the free response "other" field that they only soaked the larger seeds or those with thick seed coats. There were no significant relationships found between post-harvest washing of microgreens and any of the farm characteristics tested.

The most common microgreen harvest method is to hand cut with scissors or a knife, a technique used by 56% of respondents. An additional 21% sold their microgreens as a "living tray." A living tray refers to the sale of the microgreens live and unharvested, in their original growing container. Hand picking and other methods of harvesting were uncommon, and 37% of participants did not answer the harvest technique question. The most common post-harvest storage method was in a refrigerator or cooler (52%), while 3% of growers stored their microgreens at room temperature, and the remaining growers did not respond to this question. The average refrigerated storage time from harvest to sale for cut microgreens was 14.6 ± 14.1 h (n = 92), and the average room temperature storage time was 36.8 ± 37 hours (n = 5). For living tray storage, the average cooler time was 20.7 ± 17.4 hours (n = 7) and room temperature storage was 18 ± 25 hours (n = 25). Thus, room temperature storage is more common among growers who sell living trays. Nevertheless, it is concerning that growers who store cut microgreens at room temperature do so for a longer period of time on average than those who use a cooler and that storage times among growers suffer from high variability.

e. *Sanitation and Hygiene*

Respondents were asked how often they cleaned various food contact surfaces such as tools, growing trays, preparation tables, and floors (n = 143). Daily cleaning of at least one of these surfaces was common among respondents (64%). Equipment sanitation is broken down by

surface type and frequency in Table 8. There were no statistically significant relationships

between daily sanitation of at least one surface and any of the farm characteristics tested.

Respondents were asked about worker handwashing during production (n = 112). The practice is

common, with 95.5% of respondents reporting "yes" to the question "Do workers routinely wash

their hands during microgreen production?". When asked which specific production steps

workers routinely washed their hands, 32% reported washing before handling seeds, before

harvesting, and before packaging. Another 20% of farms reported washing at those steps as well

as before watering microgreens. An additional 17% of farms reported washing at all steps as well

as at random times throughout the day. There was a statistically significant relationship between

handwashing and disposable glove use (p = 0.025), where farmers who washed hands routinely

were more likely to also use disposable gloves. However, no other farm characteristics tested

were found to be related to handwashing. Disposable glove use among farms was 32.4%, and the

steps where disposable gloves were most commonly used included during harvest (27.4%) and

packaging (26.7%). An additional 16% of respondents reported using gloves while handling

seeds.

f. *Documentation and Tracking*

Respondents were asked to report which farm processes they routinely documented and were

allowed to give more than one answer. Using this input, the number of farm processes

documented was counted, and the assumption was made that a greater number of farm processes

documented implies a greater degree of documentation compliance. "No routine documentation"

was assigned a score of "0". Statistically significant relationships were found between number of

farm processes documented (0-8 processes) and annual microgreen revenue (p = 0.003), passing

a GAP audit (p = 0.001), and number of previous food safety trainings attended (p = 0.001).

A greater proportion of farms earning over 25,000 USD/year had high documentation numbers

compared to farms earning less than the Produce Safety Rule exemption cut-off (Table 9). The

observed relationship between annual microgreen revenue and documentation is consistent with

findings from a previous produce grower survey (Adalja et al. 2018) showing that written

documentation was more prevalent among commercial sized farms.

Additionally, it appears that passing a GAP audit or attending food safety training influences

number of processes documented. A greater proportion of farms with high documentation

numbers (5-8 processes) had previously passed a GAP audit, whereas only one farm who passed

a GAP audit had a documentation number of "1." Conversely, the majority of farms that had not

pursued or passed a GAP audit documented 4 processes or fewer. Overall, the most common

processes documented (n = 176) include Standard Operating Procedures (26%), Water Testing

(24.4%), Cleaning (23.3%), Employee Food Safety Training (22.7%), Shipping and Receiving

(20.4%), Growth Media Testing (11.4%), and Recalls (8.5%). Further, 22.7% of respondents

reported "No Routine Documentation."

vi. **Multiple Linear Regression of Food Safety Practices**

The same seven key food safety practices (documentation, water testing frequency, seed

disinfection, routine hand washing, post-harvest washing of microgreens, growing media testing

frequency, and daily surface sanitation) analyzed by Chi-square tests were also tested by linear

regression, using *glmnet* with Lasso ($\alpha = 1$, using cross validation to obtain λ_{min}), against

predictors collected from MA questions (certification type, food safety training type, method of learning to grow microgreens, production of other farm products aside from microgreens, growing media type, microgreen variety grown, irrigation water source, and water treatment method). See Data Analysis section for rationale for not testing these responses with Chi-square tests.

Variation in documentation level (Adjusted R-squared = 0.55) could be negatively predicted by not having any certifications (such as GAP, third-party sustainability, or certified organic) and by irrigating with untreated water (regardless of source). Positive predictors of variation in documentation include passing a GAP audit, a food safety lecture at work, GFSI training, and having a county health card (Table 10).

Variation in water testing frequency could be predicted (Adjusted R-squared = 0.62) by multiple categories each for food safety training type, method of learning to grow microgreens, other farm products produced, growing media type, microgreen variety grown, irrigation water source, and water treatment method. See Table 2-S1 in the Appendix for individual categories and their coefficients and p-values. Some variables were unexpected to be predictors of water testing frequency and may be an artifact of the associations and correlations between predictors.

vii. **Survey Limitations**

The survey respondents were predominantly very small farms, earning less than 10,000 USD in annual microgreen revenue. This is likely due to the utilization of online microgreen growing communities as the primary recruitment strategy, which may be biased toward small-scale and beginning growers. However, when commercial-scale farms were successfully reached using

direct emails, they were often reluctant to answer the majority of the survey questions. Two

farms directly expressed concern about the sharing of trade secrets with potential competitors.

Furthermore, as it is an emerging industry, these data may be reflective of a true greater

proportion of beginning growers to large scale commercial operations. Nevertheless, confidence

in the statistical relationships demonstrated, particularly with the linear regression, is low. This is

because categories did not have equal values of n; data were not normally distributed; and

overall sample sizes in each category were low except for those favoring small, beginning farms

growing microgreens in trays on stacked shelves. Therefore, future surveys should aim for a

larger sample size and targeted recruitment of commercial scale, non-exempt microgreen farms.

V.   **Discussion**

It may be assumed from these survey results that the microgreen industry is dominated by very

small operations, earning less than 10,000 USD/year in microgreen revenue, though it is possible

a greater number of commercial growers exist who were not interested in responding to the

survey or otherwise not reached by recruitment efforts. Most growers in the < 10,000 USD/year

category produce radish, sunflower, and pea microgreens in peat or soil, using trays on stacked

shelves in artificially lit residential or commercial facilities. Hydroponic microgreen production

and unstacked container farms in greenhouses are also approaches taken, but these systems may

be more common among greater revenue farms.

In terms of key food safety practices, the industry has some strengths and weaknesses.

Microgreen farmers appear generally aware of food safety training opportunities, many of whom

have attended more than one. Routine worker handwashing and equipment sanitation are both

relatively common practices, which appears consistent with previous work (Adalja et al. 2018, Lichtenberg et al. 2016). In terms of the production environment, overall % relative humidity in the microgreen growing environment may be lower than that of sprouted seeds. Also, the most common sources of agricultural water used by microgreen growers, municipal water and well water, are considered as low risk compared to surface water (Alegbeleye et al., 2018). A recent survey also found that produce growers in general have adopted safer agricultural water sources (Astill et al. 2019).

Most of the survey respondents do not perform microbiological testing on their growing medium. However, even though the PSR does not explicitly require microbiological soil testing, the importance of environmental monitoring of food contact surfaces (Jones et al. 2018) and preliminary data on differential survival of common foodborne pathogens on soil-free growing media types (Di Gioia et al. 2017, Wright et al. 2018, and Chapter 3) indicates that the growing media is not without risk. Testing of growing media is not only uncommon among microgreen growers, but it appears not to be influenced by any farm characteristics tested. The importance of soil testing may not be included in requirements for passing a GAP audit nor included in farm food safety trainings since it is not explicitly required by the PSR. Therefore, if this relationship is reflective of reality, it is not surprising. Furthermore, the only discussion of growing media in the PSR is related to the proper use of biological soil amendments of animal origin (US FDA, 2015). Biological soil amendments are used infrequently among microgreen producers, and of the small number who do, worm castings were mentioned most often. By contrast, two surveys of field-grown produce farmers (Astill et al. 2019, Adalja et al. 2018) indicated that manure use is quite common.

Routine documentation of farm procedures is also not common, with most farms documenting one practice or none at all. If genuine, the moderate statistical relationship between documentation and greater farm revenue, greater numbers of food safety trainings attended, and passing a food safety audit may suggest that increasing the rate of food safety training of very small microgreen operations may increase documentation practices. A previous survey found a similar relationship between revenue on documentation (Adalja et al. 2018) as the present study. It may be that larger farms have a greater need for documentation, or they have more resources to implement it. It is worth considering, however, that many of microgreen farms may not prioritize routine documentation due to being exempt from the PSR.

Microbiological testing of irrigation water is required under the Produce Safety Rule (U.S. Department of Health & Human Services, 2015). Among microgreen growers surveyed, water testing appears more common than growing media testing but still uncommon overall. Hydroponic growers appear to be more likely to test their water four or more times per year (5/12) compared to tray growers, who test around once per year (18/71). Many respondents did not answer this question, making it difficult to rely on these percentages. Nevertheless, regression analysis showed water testing frequency can be positively predicted to some degree by attending a greater number of food safety trainings. This could be explained as growers who are more conscientious about food safety issues in general both engage in regular water testing and attend food safety trainings, or that food safety trainings are at least somewhat effective in encouraging farmers to test their irrigation water. Water testing becomes an even more important educational objective when taking into account that the majority of microgreen growers surveyed

do not implement any water treatment (or did not respond to the question), such as reverse osmosis, ultraviolet light, or other filtration method.

Recommendations for training and outreach efforts include greater consideration for the impact of soil-free growing media on food safety risk; the importance of routine documentation of farm procedures; irrigation water testing; and proper storage of microgreens prior to sale. Recommendations for future research include greater consideration for the most commonly grown varieties of microgreens, differential risk among soil-free growing media and production system types, and the utility of applying similar seed disinfection practices to microgreen production presently used for sprouted seeds. Environmental monitoring best practices for microgreen growers may also be needed if the commercial popularity of CEA-farmed produce continues to increase.

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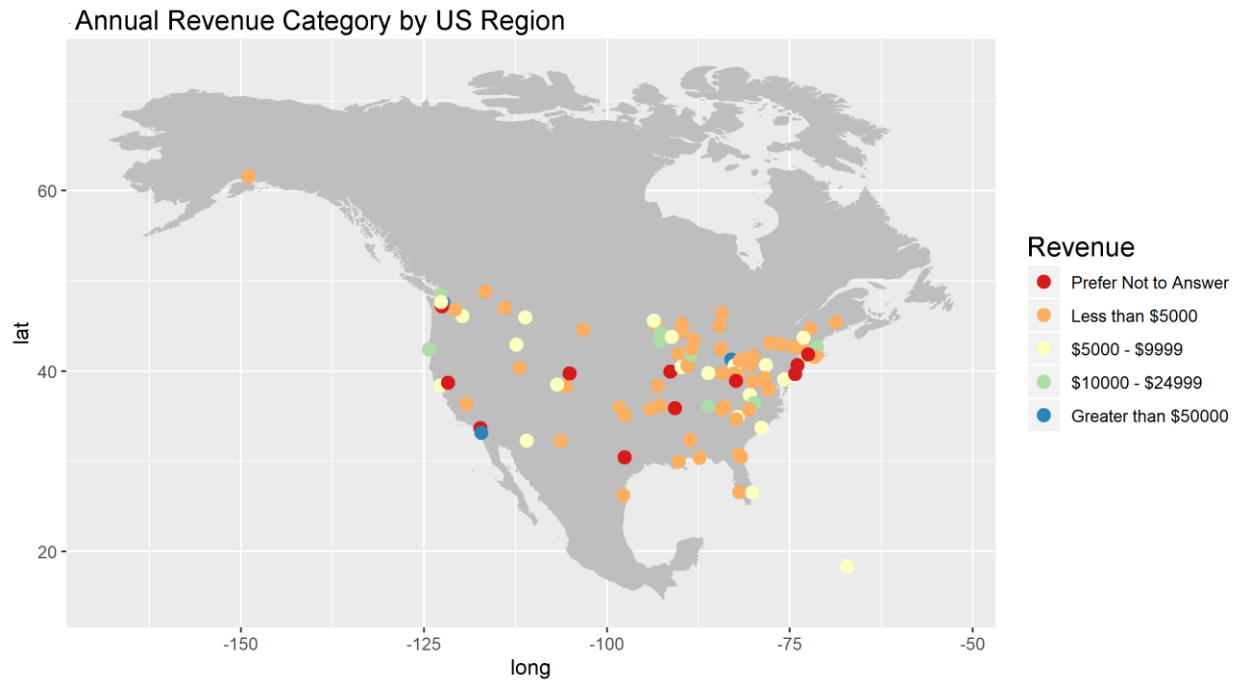
Figure 1. Farm geographic distribution by revenue category. The nine farms in the 25,000-49,999 USD/year category are not shown because none of those farms provided a zip code.

VIII. **Tables**

Table 1. Number of farm employees by revenue category

Revenue	Avg. # Employees	SD	n
Less than $5000	7.3	9.1	27
$5000 - $9999	3.7	1.9	19
$10000 - $24999	5.6	6.4	7
$25000 - $49999	3.3	1.5	3
Greater than $50000	127.8	170.7	5
Prefer Not to Answer	141.3	316.0	6

SD = standard deviation, n = total number of respondents that answered the question (NA responses were omitted)

Table 2. Combinations of System Type and Location

System Location	Aquaponics	Container farm	Hydroponics	In ground	Raised beds	Trays on stacked shelves
Climate controlled greenhouse	2	15	2	0	1	4
Indoors- commercial	1	1	5	1	0	12
Indoors- residential	0	2	5	1	0	47
Non-climate controlled greenhouse	0	3	0	1	0	7
Outdoors	0	1	0	6	0	1

n = 176, "NA" responses omitted. Shading allows easy visibility of most (darkest) to least (lightest) common combinations.

Table 3. Environmental Conditions

System Location	Water (°C)	Air (°C)	RH (%)	n
Climate controlled greenhouse	18.1 ± 14	20.7 ± 16	65.8 ± 9.7	6
Indoors - commercial	18.9 ± 6.5	20.7 ± 4	60.0 ± 0	3
Indoors - residential	18.5 ± 9.5	22.3 ± 4.5	51.3 ± 12	24

These conditions were reported by microgreen growers who answered all three questions (n = 33).

Table 4: Frequency of Microgreen Varieties Produced

Variety	%	#	Variety	%	#	Variety	%	#
Radish	29%	42	Pea Tendrils	7%	10	Bean	3%	4
Sunflower	28%	40	Cabbage	7%	10	Tatsoi	2%	3
Pea Shoots	27%	39	Mizuna	5%	7	Cress	2%	3
Arugula	18%	26	Beet	5%	7	Chard	1%	2
Broccoli	16%	23	Amaranth	5%	7	Bok Choy	1%	2
Kale	15%	21	Cilantro	4%	6	Wasabi	1%	1
Mustard	11%	16	Nasturtium	3%	5	Rapini	1%	1
Basil	9%	13	Kohlrabi	3%	5	Lemongrass	1%	1
Other	8%	12	Popcorn	3%	4	Chives	1%	1
Daikon	8%	12	Pak Choy	3%	4	Celery	1%	1

Respondents (n = 143) were allowed to choose up to five varieties from a list of thirty varieties, with a free response "Other" category for writing in varieties not listed in the choices.

Table 5: Summary of significant relationships found using Chi-Square tests

Correlate	Documentation	Water testing	Seed disinfection	Hand washing	Post-harvest Washing	Grow media testing	Daily Sanitation of Surfaces
Farm size by Revenue Category	0.003	0.073	0.745	0.341	0.971	0.291	0.942
# of Total Employees	0.503	0.631	0.011	0.158	0.873	0.015	0.688
# of Empl. handling microgreens	0.149	0.454	0.106	0.100	0.409	0.001	0.126
Passed a GAP Audit	0.001	0.211	0.470	1.000	0.430	0.634	0.209
# of previous food safety trainings	0.001	0.201	0.823	0.613	0.662	0.123	0.790
Last completed Education level	0.809	0.374	0.710	0.138	0.396	0.925	0.346
Growing System Type	0.065	0.010	0.001	0.151	0.630	0.321	0.499
Production (lbs/month)	0.321	0.598	0.646	0.245	0.539	0.334	0.182

The relationships that are significant at $p < 0.05$ are shaded gray. Shaded boxes are the significant relationships before Bonferroni correction. Darker shaded regions remained significant after correction.

Table 6. Water testing frequency by system type

System Type	Once a year	More than once a year	No testing
Aquaponics	0	2	0
Unstacked Containers	6	3	3
In Ground	3	1	0
Hydroponics	0	5	0
Raised beds	0	0	1
Trays on shelves	18	8	5

Total responses, n = 118. Non-response values (58/176) have been excluded.

Table 7. Water treatment by water source

Water Treatment Method	Municipal Water	Well Water	Collected Rainwater	Other
Activated charcoal filter	1 (0.7%)	6 (4.2%)	4 (2.8%)	0
Chlorine filter	5 (3.5%)	1 (0.7%)	0	0
Lemon juice	1 (0.7%)	0	0	0
Reverse osmosis	5 (3.5%)	3 (2.1%)	1 (0.7%)	1 (0.7%)
Sediment filter	4 (2.8%)	5 (3.5%)	2 (1.4%)	0
Ultraviolet light	4 (2.8%)	3 (2.1%)	1 (0.7%)	0
Water softener	2 (1.4%)	1 (0.7%)	0	0
Untreated	36 (25.1%)	44 (30.7%)	2 (1.4%)	1 (0.7%)

For the irrigation water source question, respondents (n = 143) were allowed to choose more than one answer so columns and rows totals will not add up to 143. Percentages are calculated out of 143 responses.

Table 8. Frequency of Sanitation of Production Surfaces and Equipment

Frequency	Floors	Prep Tables	Tools	Trays
Daily or more	41 (28.6%)	74 (51.7%)	83 (58.0%)	43 (30.0%)
2-4 times a week	34 (23.7%)	22 (15.3%)	15 (10.4%)	18 (12.6%)
Once a week	19 (13.2%)	8 (5.6%)	8 (5.6%)	32 (22.3%)
Once a month	10 (6.9%)	4 (2.8%)	3 (2.1%)	12 (8.4%)
Never	6 (4.2%)	3 (2.1%)	1 (0.7%)	4 (2.8%)

Percentages are calculated out of 143 responses.

Table 9. Number of Processes Documented by Revenue

Number of Processes Documented	Less than $5000	$5000 - $9999	$10000- $24999	$25000- $49999	Greater than $50000	Prefer Not to Answer	No response	Total
0	16 (22%)	5 (18%)	1 (10%)	2 (22%)	2 (33%)	8 (44%)	34 (50%)	68
1	31 (44%)	10 (36%)	3 (30%)	2 (22%)	1 (17%)	6 (33%)	0	53
2	3 (4%)	4 (14%)	1 (10%)	2 (22%)	0	1 (6%)	0	11
3	6 (9%)	2 (7%)	1 (10%)	1 (11%)	0	0	0	10
4	7 (10%)	2 (7%)	2 (20%)	1 (11%)	0	1 (6%)	0	13
5	4 (6%)	1 (4%)	1 (10%)	0	1 (17%)	0	0	7
6	3 (4%)	2 (7%)	1 (10%)	0	0	1 (6%)	0	7
7	1 (1.5%)	2 (7%)	0	1 (11%)	0	1 (6%)	0	5
8	0	0	0	0	2 (33%)	0	0	2
Total	**71**	**28**	**10**	**9**	**6**	**18**	**34**	**176**

Percentages are based on column totals for each revenue level.

Table 10. Negative and Positive Predictors of Documentation

β-hat	Predictor	p-value
Positive		
0.2316	GAP audit passed	0.0819
0.2565	Food safety lecture training at work	0.0077*
0.0601	GFSI Training	0.0080*
0.0963	County health card	0.1484
Negative		
-0.2925	Irrigation with untreated water	0.0015*
-0.2817	No certifications	0.1225
y-Intercept		
1.1339		

The coefficients (β-hat) in this table represent those of a single linear equation with an adjusted R squared value of 0.55. Values marked with an asterisk are statistically significant at $p < 0.05$. Untreated water refers to water that is not treated by the grower. This may include municipal water that is treated at the plant.

Chapter 3: Survival of *Salmonella enterica* and *Listeria monocytogenes* on different types of soil-free microgreen growing media

I. Abstract

The production of microgreens in controlled–environment agricultural (CEA) settings is increasing. These systems utilize soil alternatives such as fibrous or synthetic mats, peat, perlite, or coco coir. It is not well understood how the risk of foodborne pathogen transmission may be affected by the type of soil–free growing medium (SFGM). This study aims to measure survival of *Listeria monocytogenes* and *Salmonella enterica* subsp. Javiana over a typical 10–day microgreen growing period on four different SFGM types in the absence of microgreens and fertilizers. Samples of coco coir, a *Sphagnum* peat/vermiculite mix, Biostrate® mats, and hemp mats were inoculated with a bacterial cocktail of approximately 3×10^6 CFU/mL per SFGM sample along with a positive control of bacteria in PBS. Samples were allowed to incubate at room temperature for up to 10 days with sample collection on day 0, 1, 3, 6, and 10. Statistically significant differences in pathogen survival were observed across multiple time points for hemp mats and Biostrate® mats compared to coco coir, peat, and bacteria in PBS ($p < 0.05$). *Salmonella* showed greater overall survival compared to *Listeria* ($p < 0.0002$). For hemp and Biostrate®, there was an initial increase in growth (~1 log) for both *Listeria* and *Salmonella* after 1 day while both pathogens began to decline on coco coir, peat, and in PBS. By day 10, *Salmonella* persisted at the initial inoculum concentration for hemp and Biostrate® while declining by 1–2 log CFU/mL on coco coir, peat, and in PBS. *Listeria* also persisted at the original inoculum level of 10^6 CFU/mL in hemp and Biostrate®. Conversely, *Listeria* decreased to 1 log CFU/mL for peat and below the detection limit for coco coir and bacteria in PBS. Overall, it was concluded that there are survival differences between bacterial pathogens in soil–

free microgreen systems, and these survival differences may be further impacted by the specific SFGM material used.

II.   **Introduction**

An estimated 9.4 million foodborne illnesses from 31 identified pathogens occur per year in the United States (Scallan et al., 2011). Human bacterial pathogens, including non–typhoidal *Salmonella* and *Listeria monocytogenes*, are significant contributors to this annual burden of disease. Approximately 3.6 million (39%) of the estimated 9.4 million illnesses are caused by bacteria. Non–typhoidal *Salmonella* is the leading bacterial pathogen, attributed to an estimated 11% of illnesses and 27% of hospitalizations. Furthermore, of the approximately 800 deaths estimated to occur each year in the US from foodborne bacterial pathogens, non–typhoidal *Salmonella* and *L. monocytogenes* are the top two etiologic agents—with the latter characterized by a 16% mortality rate (Scallan et al., 2011).

In recent decades, fresh produce has been increasingly implicated in foodborne illness outbreaks related to *Salmonella* and *L. monocytogenes* (Warriner et al., 2009). Data from the US Centers for Disease Control and Prevention's (CDC) National Outbreak Reporting System (NORS) revealed that fruits and vegetables have been implicated in 185 outbreaks (~12,000 illnesses) caused by *Salmonella* and *Listeria* from 1998 to 2017. Produce—typically eaten raw—is frequently associated with these outbreaks due to a variety of factors including poor worker hygiene during harvest and packing, cross-contamination from soil amendments (e.g., manure and compost), and contaminated seeds, irrigation water, or soil (Alegbelye et al., 2018, Gil et al., 2016, Olamait et al., 2012). Additionally, post–harvest washing of raw produce may have a

limited effect due to the tendency of contaminants to become internalized within the plant tissue during cultivation (Hirneisen et al., 2012).

Produce of particular interest include sprouted seeds (referred to as 'sprouts') and leafy greens as these are frequently implicated in outbreaks. For example, a search for "sprouted seeds" in the CDC NORS database from 1998–2017, there were 42 *Salmonella* outbreaks involving sprouts— a popular raw salad crop that is produced in an environment of high water activity and temperatures favorable to bacterial growth (US FDA, 2019). Leafy greens have also been extensively studied due to their frequent involvement in outbreaks (Herman et al., 2015, Self et al., 2019, Sharapov et al., 2016, Turner et al., 2019). Microgreens, an emerging raw salad crop, share some traits with both leafy greens and sprouts (Riggio et al., 2018). These immature shoots of common vegetables are gaining attention as a potential vector for foodborne pathogens (Riggio et al., 2018). While sprouts germinate for up to 5 days and are consumed whole (i.e. including the root system), microgreens are grown in soil, soil alternatives, or hydroponic systems in ways that are similar to controlled environment leafy green production and are harvested above the root system after 10 to 21 days (Mir et al., 2017). At this time, there have been no reported outbreaks related to microgreens. However, there have been an increasing number of microgreen recalls associated with possible *L. monocytogenes* or *Salmonella* contamination. In Canada, 6 of the 7 recalls were due to *L. monocytogenes* and were classified as "high risk," while one was determined to be "moderate risk" and involved *Salmonella* (CFIA, 2018a-f, CFIA, 2019a and 2019b). In the United States, two recalls also involved *L. monocytogenes* (US FDA, 2016 and 2019) and one involved *Salmonella* (US FDA, 2018).

Despite these differences, the microgreen growing environment could also enable the proliferation of pathogens. At present, there are 9 published studies that have specifically addressed food safety-related microbiological characteristics of microgreen production (Bergspica et al. 2020, Chandra et al. 2012, Di Gioia et al., 2017, Isik et al. 2020, Lee et al., 2009, Reed et al., 2018, Wright and Holden, 2019, Xiao et al., 2014 and 2015). For example, the behavior of Shiga toxin–producing *Escherichia coli* (STEC) under microgreen production conditions revealed that contaminated seeds and growing media could successfully transfer STEC to the edible product (Xiao et al., 2015). Furthermore, the authors implied that hydroponic microgreen production might confer a greater transfer risk than the soil–grown counterpart. Indoor production using potting soil and soil alternatives are popular among microgreen producers. Examples of soil alternatives include coco coir, peat mixed with perlite or vermiculite, gravel, sand, and fibrous mats made from textiles, biodegradable felt, hemp, coco coir, cellulosic materials (Kennedy 2018), wood fiber, and synthetics (Di Gioia et al., 2017, Sarkar and Majumdar 2018, Wright and Holden, 2018). Microgreens can also be produced hydroponically, with or without a rooting medium (Weber 2017, 2018). Since soil is an important source of contamination for field-grown leafy greens (Alegbelye et al., 2018), it is important to determine if the growing media used in indoor horticulture is a similarly important contamination route.

Only two previous studies have addressed differential survival of microorganisms on soil–free growing media (SFGM). Between jute/kenaf, polypropylene, textile fiber mats, and fertilized peat, it was demonstrated that peat and jute-kenaf mats were associated with the highest numbers of colony forming units per gram (CFU/g) of background aerobic mesophilic bacteria (AMB),

yeasts, and mold compared to textile and polypropylene mats. Furthermore, transfer of background levels of Enterobacteriaceae and *E. coli* to microgreens was greater for peat and jute-kenaf mats compared to the textile and polypropylene mats (Di Gioia et al., 2017). Similarly, a comparison of three types of felt growing pads (20% rayon/80% polyester, 100% polyester, and a wool/burlap blend), perlite, and plastic mesh contaminated with STEC in the absence of plants revealed differences between substrates. However, the only statistically significant difference in STEC levels was between the polyester pads (8 log CFU/g) and the plastic mesh (5 log CFU/g) (Wright and Holden, 2018). Thus far, no studies have directly assessed the survival of *Salmonella* and *L. monocytogenes* on SFGM in the absence of confounding influences from plants or fertilizers.

Therefore, in order to understand how different SFGM materials may influence pathogen transfer to microgreens, it is necessary first to assess differences in bacterial survival on each SFGM material. If the bacterial concentration changes over the growing period, this persistence, growth, or decline may convey an increased or decreased risk of pathogen uptake by the microgreens, complicating the effect of initial contaminant concentration. The present study was conducted to determine if four types of SFGM (coco coir, peat/vermiculite, Biostrate® mats, and hemp mats) showed differential growth support of *L. monocytogenes* and *Salmonella* Javiana. It was hypothesized that the SFGM with the highest carbon and micronutrient content would be most supportive of bacterial persistence and/or growth.

III. Materials and Methods

i. Selection and Preparation of SFGM

Due to the wide variety of soil alternatives available, the material choice for this study was based

on our recent survey of microgreen growers (n = 176) who sell in the US (Chapter 2). In our

survey, the most popular growing media for microgreen producers included peat with perlite or

vermiculite, potting soil, coco coir, and various organic fiber pads such as hemp, burlap, and

Biostrate®. Fibrous mats were used in both hydroponic and non–hydroponic production systems.

Thus, a *Sphagnum* peat and vermiculite mix (hereafter referred to as 'peat'), coco coir, hemp

fiber mats, and Biostrate® biodegradable fiber mats were chosen for the present study.

SFGM samples included a 3.5-cm square of Biostrate® (Grow-Tech, South Portland, ME, USA)

with an average weight of 0.29 ± 0.06 g; a 2.5-cm square of hemp mat (BioComposites Group,

Alberta, Canada) with an average weight of 1.01 ± 0.16 g; a 5–cm^3 sample of coco coir

(UBICON, Woodridge, IL, USA) with an average weight of 1.13 ± 0.11 g; and a 5–cm^3 sample

of Jiffy–Mix® Soilless Starter Peat/Vermiculite mix (Harris Seeds, Rochester, NY, USA) with an

average weight of 0.92 ± 0.13g. The weight and volume of each SFGM material was chosen

based on its water retention capacity, which is discussed further in Section Vb.

The water retention capacity of each material was approximated by placing pre-weighed, dry

SFGM in a 10" × 20" germination tray (Harris Seeds, Rochester, NY) with a single hole in the

bottom, resting above a collection beaker. Volumes of distilled water were subsequently added to

each material until excess water began to drain into the beaker. At the completion of drainage,

the water that drained into the beaker was poured into a graduated cylinder to measure the

volume of the excess. The excess distilled water was subtracted from the initial volume added to get an approximate water retention capacity. Results of the water retention determination are in Table 3-S2 of the Appendix.

ii. **Chemical Analysis of SFGM**

Each type of SFGM was analyzed at the Fayetteville Agricultural Diagnostic Laboratory at the University of Arkansas (Fayetteville, AR) for total carbon, total nitrogen, nitrate-nitrogen, minerals, pH, and electrical conductivity (EC). Peat and coco coir were submitted for analysis in their original state while Biostrate® and hemp mats were pre-processed using sterile scissors to shred and homogenize prior to analysis. Biostrate® and hemp were also analyzed for acid detergent fiber (ADF) including lignin and cellulose, neutral detergent fiber (NDF) including lignin, cellulose, and hemicellulose, and acid detergent lignin (ADL). Peat and coco coir could not be analyzed for ADF and NDF due to technical limitations of the methods.

The diagnostic laboratory performed a saturation extract on all four media samples in preparation for mineral analysis, nitrate nitrogen, and EC. The mineral analysis was performed using the Melich-3 method by inductively coupled plasma mass spectrometry (ICP) as described in Zhang et al., (2014). Total nitrogen and carbon were determined by combustion as described in Campbell et al., (1992), and nitrate nitrogen was determined by UV–Vis spectroscopy as described in Peters et al., (2003). EC and pH were determined by electrode using the soil EC and pH methods described in Sikora et al., (2014) and Wang et al., (2014), respectively. The %ADF, %ADL, and %NDF were determined by the AOAC filter bag method for A200 (AOAC 1990, Van Soest et al., 1991).

iii. **Preparation of Bacterial Cultures**

Bacteria used in this study include *L. monocytogenes* (FSL R2–574) isolated from a soft cheese outbreak and *Salmonella enterica* subsp. Javiana (ATCC BAA1593) isolated from a tomato outbreak. *L. monocytogenes* was streaked for isolation from a glycerol stock on Modified Oxford Medium (MOX) agar (HiMedia Laboratories, Mumbai, India) with antimicrobial supplement (BD Difco, Franklin Lakes, NJ, USA) containing colistin sulfate (10 mg/L) and moxalactam (20 mg/L). Similarly, a glycerol stock of *S.* Javiana streaked on Xylose Lysine Tergitol–4 (XLT4) agar (Criterion, Philadelphia, PA, USA) with a 4.6 mL/L Tergitol 4 agar supplement (BD Difco). The inoculated XLT4 and MOX plates were incubated at 37°C for 18 h and 35°C for 24 h, respectively. A single colony from each plate was transferred to separate 50 mL conical tubes of 10 mL of Brain–Heart Agar Infusion (BHI) broth (BD Difco) for *L. monocytogenes* and Tryptic Soy Broth (TSB, BD Difco) for *S.* Javiana and incubated overnight at 35°C at 120 rpm in a shaking incubator (Thermo Scientific MaxQ 4000).

Overnight cultures were centrifuged at $4000 \times g$ for 10 min at 10°C to pellet the bacteria. The pellet was washed twice in 10 mL of sterile phosphate buffered saline (PBS, pH = 7.4) using the same centrifugation speed and time and then re-suspended in 10 mL of sterile PBS. The bacterial cocktail was prepared by adding 1 mL each of the prepared cultures into a sterile 15 mL tube and vortexed briefly at maximum speed. A 10–fold dilution series was prepared, and the cocktail was enumerated by spread plate on XLT4 and MOX agar and incubated as described previously. The bacterial cocktail contained approximately 10^9 CFU/mL each of *S.* Javiana and *L. monocytogenes* and was diluted to a final concentration of 10^6 CFU/mL in sterile PBS prior to inoculation of SFGM.

iv. **Inoculation Method**

Two replicates for each SFGM material were inoculated with 3 mL of the bacterial cocktail while a third was used as an un-inoculated control, spotted with 3 mL of sterile PBS. Additional tubes were set up as 1) a positive control containing 3 mL of the bacterial cocktail in PBS (10^6 CFU/mL) but no SFGM, and 2) an un–inoculated control tube containing only 3 mL of sterile PBS and no SFGM, for a total of 14 tubes. An identical set of 14 tubes was prepared for each collection day – day 0, day 1, day 3, day 6, and day 10 (see Figure 3-S1 in Appendix). All five sets of tubes were simultaneously inoculated on day 0. The tubes were incubated at room temperature on the lab bench with the caps loose to retain moisture but allow for aeration until sampling at the designated time point.

v. **Recovery of Bacteria from SFGM**

Immediately after inoculating all tubes, the day 0 set of tubes was processed for recovery of bacteria. To elute, each tube was filled with 12 mL of PBS (total = 15 mL) and pulse–vortexed at maximum speed every 15 s for 1 min. One milliliter of eluent was removed and diluted in a 1:10 dilution series. Peat and coco coir were allowed to settle for 30 s before pipetting the liquid to avoid particulates clogging the pipette tip. For each dilution level, 100 μL was plated onto selective agar as described in Section III,, subpart iii. The elution and enumeration processes were repeated at day 1, 3, 6, and 10 post-inoculation (p.i.).

a. *Recovery Efficiency and Assay Detection Limit*

Prior to beginning the experiment, the recovery efficiency of the elution method was determined by inoculating SFGM samples prepared as described in Section III, i. with a cocktail of *S.*

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Javiana and *L. monocytogenes* at a concentration of 10^6 CFU/mL inside of sterile 50 mL

centrifuge tubes. The bacterial cocktail was prepared as described previously in Section III, iii.

Bacteria were allowed to acclimate for 1 h at room temperature and then eluted as described in

Section III, v. For the assay detection limit, it was assumed that no fewer than 1 CFU could

theoretically be detected in each 100 µL of eluent plated. Thus, the concentration of the 15-mL

eluent from each SFGM sample must be at least 10 CFU/mL, or 150 CFU per sample, to be

above the limit of detection. For peat and coco coir samples, there was some suspicion that

bacterial attachment to media particles might impact recovery as *Salmonella* in particular can

interact with soil particles (Turpin et al., 1993). However, recovery of *Salmonella* from peat was

only somewhat lower than the other SFGM types, and coco coir recovery was the same as for

Biostrate® (Table 3-S3 in the Appendix). Therefore, recovery was not likely to be significantly

impacted by these particulate interactions.

vi. **Sanger Sequencing of Isolates**

As background microorganisms appeared on SFGM blanks, colonies were picked, and glycerol

stocks were prepared. Molecular biology grade glycerol (MP Biomedicals, Irvine, CA) was

diluted to 50% concentration with sterile Millipore water, and the final 50% glycerol mixture

was filter sterilized through a 0.45–micron syringe filter membrane (Corning, City, State) and

stored at 4°C until use. One colony each from the XLT4 and MOX plates were selected with a

sterile inoculating loop and inoculated into 5 mL of TSB in glass culture tubes and incubated at

37°C for 24 h. Following incubation, 500 µL of the TSB culture was mixed with 500 µL of

sterile 50% glycerol in 2 mL cryogenic tubes and stored at –80°C until further analysis.

a. *Amplification of the 16S rRNA Gene by PCR*

A single colony from each un-inoculated control plate was chosen and grown to an approximate

concentration of 10^9 CFU/mL overnight in either BHI if the isolate was found on MOX plates or

TSB if the isolate was found on XLT4 plates. DNA was extracted from liquid cultures using the

Qiagen UltraClean Microbial DNA Kit (Cat. #12224, Qiagen, Valencia, CA) by following the

manufacturer's instructions and quantified on a NanoDrop 1000 Spectrophotometer (Thermo

Fisher Scientific, Wilmington, Delaware, USA).

Amplicon sequencing of the V4 region of the 16S rRNA gene for each isolate was performed

with the primer set 515F/806R designed by Caporaso et al. (2011) and by following the protocol

by the Earth Microbiome Project (Thompson 2018).

b. *Sequencing and Identification*

The resulting PCR products were sequenced using the Sanger method (Sanger et al. 1977) at the

Arizona State Genomics Center (Tempe, AZ, USA). NCBI BLAST was used to determine the

identity of each isolate. Results of BLAST queries are shown in Table 2, and the raw sequence

data are included in Table 3-S7 in the Appendix.

vii. **Statistical Analysis**

The first and second experiments, which were conducted approximately one month apart but

using an identical protocol, demonstrated unequal variances but equal means both overall and for

each sampling day. Therefore, it was determined that the experiments could be pooled into a

single dataset. Day 10 was not included in this comparison because no sampling was performed on day 10 for Experiment 1. Results of this analysis are available in the Appendix, Table 3-S1.

The R software platform (R Core Development Team, version 3.6.0) was used to perform statistical analysis along with the library "lsmeans" (Lenth, 2016). To compare mean survival among SFGM materials, a separate One–Way ANOVA was performed for each sampling day and pathogen at a 0.05 significance level followed by post-hoc pairwise comparisons using Tukey's HSD test (Tukey 1949). Overall, pathogen survival differences were determined by a student's t-test at a 0.05 significance level.

IV. **Results**

i. **Overview**

Mean log CFU/mL among SFGM materials for each pathogen at each incubation time were compared. Relationships and p-values for all SFGM comparisons are reported in Table 3 as well as Table 3-S3, 3-S4, 3-S5 in the Appendix. Using a separate one–way ANOVA and Tukey HSD post-hoc comparisons for each sampling day, statistically significant differences were observed from day 1 through day 10 for *L. monocytogenes*. Growth dynamics for both pathogens followed a general pattern of statistically significant increases at 24 h p.i. for Biostrate® and hemp mats, followed by a decline for all SFGM except for *S.* Javiana on Biostrate®, which remained at approximately 10^6 CFU/mL for the duration of the experiment. No statistically significant growth occurred on peat, coco coir, and in bacteria in PBS, and instead followed a steady decline across the 10–day incubation period.

ii. **Survival of *Salmonella* Javiana on SFGM**

Survival of *S.* Javiana is shown in Figure 1. At 24 h p.i., the survival of *S.* Javiana was greater by 0.8 log CFU/mL on hemp than on all other SFGM types, but gradually dropped off over the study period. In general, peat, coco coir, and PBS showed no significant differences between one another and remained as such for the duration of the experiment, while Biostrate® and hemp demonstrated either growth or persistence. At 10 days p.i.—a typical microgreen harvest time point—survival on Biostrate® was 1.5 log CFU/mL greater than bacteria in PBS, 1.8 log CFU/mL greater than peat, and 2.2 log CFU/mL greater than coco coir; all differences were statistically significant. Hemp was significantly greater than peat by 1.1 log CFU/mL and coco coir by 1.5 log CFU/mL, but was no different from bacteria in PBS. Overall, statistically significant decreases between day 0 and day 10 occurred on coco coir, peat, and bacteria in PBS, but initial inoculum levels were maintained for Biostrate® mats and hemp mats (Table 3).

iii. **Survival of Listeria monocytogenes on SFGM**

Survival of *L. monocytogenes* is shown in Figure 2. At 24 h p.i., the *L. monocytogenes* population increased by 2 log CFU/mL and by 1 log CFU/mL on Biostrate® whereas peat, coco coir, and the bacteria in the PBS control maintained the original inoculum concentration. On day 3 p.i., survival on Biostrate® and hemp still supported 2 log CFU/mL of bacterial cells compared to peat and coco coir. *L. monocytogenes* did not survive well in PBS only, as evidenced by significant die-off. This decline in numbers continued for peat and coco coir through the sixth day. On day 10 p.i., survival on both Biostrate® and hemp was approximately 6 log CFU/mL, similar to the original inoculum. Survival of *L. monocytogenes* on these media were both more than 5 log CFU/mL greater than on peat, which was only 1.3 log CFU/mL by the end of the

experiment. Bacteria concentrations on coco coir and PBS fell below the assay detection limit (Table 3).

iv. **Pathogen Differences**

A student's *t*-test indicated that *S.* Javiana persisted at a greater overall average concentration (6.35 ± 0.75 log CFU/mL) than *L. monocytogenes* (5.33 ± 2.28 log CFU/mL) at p = 0.0002. The increase was 1.02 log CFU/mL with a 95% confidence interval of 0.49 to 1.55 log CFU/mL. Overall survival differences and the greater variation in *L. monocytogenes* survival can be observed in Figures 1 and 2.

v. **Compositional Analysis of SFGM**

Biostrate® and hemp contain a greater percentage of organic matter as evidenced by 73.2% lignin in Biostrate® and 70.9% cellulose in hemp. Biostrate® and hemp have greater quantities of total carbon, 48.94% and 42.99%, respectively, compared to coco coir and peat, which are both approximately 12% total carbon. The electrical conductivity of coco coir (1036 µΩ/cm) and peat (780 µΩ/cm) were greater than Biostrate® (32 µS/cm) and hemp (96 µΩ/cm). Sodium, potassium, and iron levels were also greater in peat and coco coir compared to Biostrate® and hemp. The complete compositional analysis of SFGM is shown in Table 1.

vi. **Differences in Recovery Efficiency Between Pathogens**

Recovery efficiency differed between pathogen species. *L. monocytogenes* was recovered at a rate of 75%, and *S.* Javiana was recovered at a rate of 40% compared to the original inoculum concentration. Among SFGM, recovery of Gram-negative *S.* Javiana from peat and hemp were

less than for Biostrate® and coco coir. For Gram-positive *L. monocytogenes*, recovery from peat was the poorest, but there was less variation in general between SFGM types (Table 3-S3 in the Appendix).

vii. **Background Microorganisms Isolated from SFGM**

Seven unknown organisms appeared on the un-inoculated blank SFGM samples, primarily on hemp, but with one representative each from peat, Biostrate®, and coco coir. The sequences of the 16S rRNA gene amplicons and the identities of these organisms are shown in Table 2. None of these background organisms appeared on the inoculated samples or on negative control plates for the PBS used to prepare suspensions.

V. **Discussion**

i. **Differences Between SFGM Materials**

The present study investigated differences in survival between two common produce–associated pathogens (*S.* Javiana and *L. monocytogenes*) on four types of SFGM (coco coir, peat, Biostrate® mats, and hemp mats) to determine if SFGM material influenced pathogen survival independently of plant roots and fertilizers. The hypothesis was that organic carbon-rich SFGM would be more supportive of bacterial growth than inorganic or synthetic substrates. Biostrate® and hemp grow mats, which were greater in total carbon than coco coir and peat, supported the growth of *S.* Javiana and *L. monocytogenes* R2–574, while coco coir and peat did not. Generally, these results are consistent with previous work indicating that choice of growing medium can impact microbiological characteristics of soil-free systems (Di Gioia et al., 2017, Grunert et al., 2016, Koohakan et al., 2004, Macarisin et al., 2013, Reed et al., 2018, Xiao et al., 2015).

Di Gioia et al., (2017) demonstrated that peat and polyethylene terephthalate (PET) mats were positive for Enterobacteriaceae, the family to which *Salmonella* spp. belongs and an important hygiene indicator. On peat, 2 log CFU/g was detected at planting, and 5.5 log CFU/g was recovered from harvested Rapini microgreens. For PET mats, 1 log CFU/g was detected at planting, but recovery was below the detection limit in harvested microgreens. However, another type of SFGM tested in this study, jute–kenaf mats, tested negative for Enterobacteriaceae at planting but revealed nearly 4 log CFU/g in the harvested microgreens. These results suggest that material type may be more predictive of pathogen transfer than initial contaminant levels.

It is worth noting that while the peat used in Di Gioia et al., (2017) showed growth of Enterobacteriaceae, the peat in the present study only supported persistence of *S.* Javiana but not growth. However, the present study investigated only SFGM, without cultivation of microgreens. Thus, it is possible that the presence of plant roots contributes to microbial survival in a growing medium that would otherwise not support microbial growth. Furthermore, Di Gioia and others (2017) measured background Enterobacteriaceae, a community likely comprised of multiple genera, which may or may not include any *Salmonella* subspecies, let alone *S.* Javiana. Reed et al. (2018) demonstrated that *Salmonella* survival may even differ between types of peat as well as serovar.

Other work, however, is consistent with evidence provided by the present study. Xiao et al., (2015) grew radish microgreens in a peat mix and on PET hydroponic growing mats and observed a ~2 log CFU/g decline in *E. coli* O157:H7 on peat between planting and harvest from both 3.7 log CFU/g and 5.7 log CFU/g initial inocula. Furthermore, while Di Gioia et al., (2017) observed minimal background contamination of PET mats and the lowest bacterial transfer to

plants, Xiao et al., (2015) observed a ~2 log CFU/g increase in the surrounding hydroponic nutrient solution and greater transfer to plants. However, it is difficult to tell if it is the PET mats or the hydroponic nutrient solution that facilitates pathogen growth in this type of microgreen cultivation system.

Wright and Holden (2018) reported a plant-free comparison of SFGM using polyester, polyester/rayon, wool/burlap, perlite, and plastic mesh. These authors demonstrated a 2 to 3 log CFU/mL increase in STEC after harvest at as many as 19 days (dependent upon microgreen variety) in all three mat types as well as perlite, while plastic mesh appeared to show no change in population. These results suggest a difference between organic (fibrous mats) and synthetic media (plastic), but no difference between inorganic (perlite) and organic media. The present study did not make any comparisons to synthetic media due to low reported use by our survey respondents (Chapter 2). However, in light of previous findings demonstrating greater pathogen transfer risk related to hydroponic nutrient water where synthetic media was used (Xiao et al., 2015), such an investigation would be useful. In conclusion, peat demonstrates potentially contradictory results, showing either a 1.5 – 3 log increase in Enterobacteriaceae (Di Gioia et al., 2017) or a 2 log CFU/g decrease in *E. coli* O157:H7 as in Xiao et al., (2015) and a 2 log CFU/mL decrease in *S.* Javiana in the present study. Perlite appears to support STEC growth (Wright and Holden, 2018). Importantly, fibrous mats high in organic carbon, such as polyester or jute-kenaf, appear to support growth of STEC (Wright and Holden, 2018) as well as generic *E. coli* and Enterobacteriaceae (Di Gioia et al., 2017). The present study supports these data, as the fibrous, organic carbon-rich Biostrate® and hemp mats were supportive of the growth of *L. monocytogenes* and *S.* Javiana.

ii. **Hemp and Biostrate Support Growth of *L. monocytogenes***

L. monocytogenes survival followed a similar pattern on both hemp and Biostrate®, characterized by a logarithmic increase during the first day followed by stabilization. The fact that there were few significant differences among peat, coco coir, and bacteria in PBS indicates that coco coir and peat do not, on their own, provide nutritional support for the growth of *L. monocytogenes.* Analysis of chemical constituents of peat and coco coir, particularly related to total carbon content, provides some evidence for this assumption.

Most previous work examining *Listeria* survival in growing media has involved agricultural and forest-sourced soil (Dowe et al., 1997, Jiang et al., 2004, Locatelli et al., 2013, McLaughlin et al., 2011, Vivant 2013a & b). It is well known that *Listeria* species are able to survive in soil due to their tolerance for a wide range of temperatures and ability to grow under sub-optimal conditions (Welshimer 1960). It has been demonstrated that *L. monocytogenes* prefers fertile soil over clay soils (Welshimer 1960, Locatelli et al., 2013). *Listeria* is saprophytic (Ivanek et al., 2006), suggesting that its preference for decaying organic matter indicates potential to survive better in media of high organic carbon content, such as manure-amended soils (Jiang et al., 2004). The peat and coco coir used in the present study both had a total carbon content of 12% compared to the 42% and 49% of Biostrate® and hemp mats, respectively. Total carbon was measured as carbon dioxide resulting from the combustion of the material, so this does not necessarily represent total organic carbon, though the high percentages of cellulose and lignin suggest a high percentage of total organic carbon.

In media with low total carbon content (peat and coco coir), there was no discernable growth observed for *L. monocytogenes*, but instead an approximate 4 log CFU/mL reduction over 10 days (Figure 2). This reduction is consistent with previous *L. monocytogenes* survival studies in soil (McLaughlin et al., 2011) showing that three strains of *L. monocytogenes* incubated at both 25°C and 30°C in soil samples collected from a forested region in Ireland declined by approximately 4 log CFU/g over 6 days. Therefore, *L. monocytogenes* survival in peat and coco coir may be similar to that of forest soil, though without a complete characterization of soil and peat using the same analytes, and without directly comparing survival experimentally, the data are difficult to compare.

Competitive inhibition by diverse communities of native soil microorganisms may contribute to the suppression of growth for *L. monocytogenes* (Vivant et al., 2013a,b). McLaughlin et al., (2011) observed the growth of 1 log CFU/g after a one–day incubation in sterilized forest soil, compared to a decline in fresh forest soil. They also showed that a competitive *in vitro* assay between aerobic soil isolates and *L. monocytogenes* resulted in a moderate decline in *L. monocytogenes*. A comparison of 100 soil samples across France also demonstrated that soil microbial communities influenced *L. monocytogenes* survival in soils (Locatelli et al., 2013). In the present study, background microorganisms *Bacillus cereus*, *Klebsiella*, and *Curtobacterium* were detected on hemp mats, where survival was high. *Klebsiella* was also found on coco coir, where survival was poor. *Enterobacter* was found on Biostrate®, where survival was high. Therefore, these organisms do not appear to affect survival of *L. monocytogenes* on these SFGM types. In cases such as coco coir, poor survival may be attributable to other organisms not recovered or biochemical effects of the media. Survival was also poor on peat, and peat was the

only SFGM type where *Pseudomonas* was recovered. There is some prior evidence that *Pseudomonas* may suppress the growth of *L. monocytogenes* in co-culture (Buchanan and Bagi, 1999).

Moisture level in growing media likely influences *L. monocytogenes* survival (Dowe et al., 1997). After one week of exposure to air, moist soil samples inoculated with *L. monocytogenes* began to decline in numbers compared to capped samples, indicating that *L. monocytogenes* will survive longer in moist environments (McLaughlin et al., 2011). Water retention capacity differences between SFGM types may contribute to survival differences due to varying susceptibility to desiccation over time. Biostrate® and hemp mats both had greater water retention capacities (8.8 mL/g and 10 mL/g, respectively) compared to coco coir and peat (3 mL/g for both), which may have contributed to improved survival of *L. monocytogenes* on those media (Table 3-S3 in the Appendix). However, sensitivity to desiccation may be strain specific. For example, across 8 strains of fish slaughterhouse–associated *L. monocytogenes* that were cultured in high and low salt concentrations and allowed to desiccate on stainless steel surfaces, *L. monocytogenes* strain EDG was more sensitive to the other seven tested. Interestingly, survival for all strains was improved when grown in fish juices with high organic matter content as well as greater NaCl. (Vogel et al., 2010). In the present study, *L. monocytogenes* survival was best on SFGM with the highest apparent organic matter as indicated by total carbon and % NDF (Table 1). Despite peat and coco coir having greater EC (Table 1), and thus greater salt content, the low organic matter may have been a contributing factor to poorer survival. Drying likely occurred on all SFGM materials over the duration of the experiment, so it is possible that the

greater organic matter present in Biostrate® and hemp mitigated some die–off despite possible drying.

iii. *S*. Javiana survived on all SFGM tested

Survival of *Salmonella* in the soil is a subject of intense study due to its presence in animal manure fertilizers and its link to outbreaks attributed to contaminated produce. It is well known to persist in farm environments among livestock, soil, and plants via feed, water, and equipment (Jacobson et al., 2012). However, studies are lacking that examine factors influencing *Salmonella* survival in soil alternatives.

Our results showed that *S*. Javiana persisted over the 10–day incubation period with values ranging from 4.6 to 8.2 log CFU/mL depending on the type of SFGM, with hemp mats and Biostrate® mats providing the most support. Although peat and coco coir showed an approximate 2 log decline over the study period, these data generally indicate that *S*. Javiana is nutritionally supported by all four types of SFGM tested in the present study, but was also able to survive similarly well in PBS alone. Sterile PBS was selected as both the suspension and elution buffer to ensure that all nutrients potentially supporting bacterial growth would be from the SFGM. This is consistent with the abundance of data showing robust survival abilities of *Salmonella* spp. (Jacobson et al., 2012, Kenyon et al., 2011, Kumar et al., 2018, Rychlik and Barrow 2005, Semenov et al., 2011, Spector et al., 2012, Stocker and Makela 1986). In general, factors affecting *Salmonella* survival in soil include temperature, moisture, soil type, presence of plants, exposure to ultraviolet light, inoculation level, method of application of bacteria (in experimental conditions), and protozoan predation (Jacobson et al., 2012). Characteristics that aid in

Salmonella survival include biofilm production (Kumar et al., 2018) and the ability to tolerate both aerobic and anaerobic environments (Semenov et al., 2011).

While it appeared that *S.* Javiana was able to survive in the presence of all SFGM, only hemp mats and Biostrate® mats demonstrated statistically significant growth at any time point. Thus, it may be surmised that hemp and Biostrate® are more nutritive than coco coir and peat. However, while *L. monocytogenes* did not survive well in coco coir and peat, with levels of 1 log CFU/mL or less by day 10, *S.* Javiana persisted at 4.67 log CFU/mL or greater by day 10, even in bacteria in PBS. *S.* Javiana may have been relying upon a survival mechanism adapted for nutrient poor conditions that is not expressed by *L. monocytogenes* R–574. For example, *S.* Typhimurium may respond to unfavorable conditions by initiating a starvation stress response (SSR) that is specific to carbon–poor environments, allowing it to become more efficient at using nutrient sources and initiating other cellular protection mechanisms (Spector & Kenyon 2012). It is not known if *S.* Javiana is also capable of SSR.

iv. **Study Limitations**

a. *Absence of Plant Roots*

The present study only observed survival on SFGM alone. Because previous work (DiGioia et al., 2017, Wright and Holden, 2018, Xiao et al., 2015) performed growing media comparisons in microgreen production systems, it will be necessary to demonstrate if differences in pathogen survival on SFGM exist when microgreen roots are present in the medium. The nutrient-rich microenvironment surrounding plant roots may be taken advantage of by pathogens as well as native microorganisms associated with the growing medium (Reed et al., 2018).

b. *Differing Water Retention Capacities Among SFGM*

Peat, coco coir, Biostrate®, and hemp each have different water retention capacities (Table 3-S2 in Appendix) and densities, which complicates determining the appropriate inoculation volume and sample mass. The water retention capacity was used to determine the appropriate volume of bacterial cocktail to add to each SFGM sample so that all of the bacteria added would be in contact with the SFGM material without excess liquid pooling in the bottom of the tube. Sample mass had to be adjusted so that each sample mass was close to saturation at the same volume, requiring different sample masses. Because of the differing masses of the samples, a 0.29-g piece of Biostrate at a 42% total carbon would have provided the inoculum with same total carbon (0.12 g) as a 1-g sample of peat that was 12% total carbon. Lastly, re-wetting of the growing media to simulate daily watering during microgreen production was not performed. Thus, the overall decline observed in both pathogens across all growing media may have been due to gradual water loss over the 10-day experimental period, and in general, water retention capacity of SFGM may be a more important survival factor than organic carbon availability. This may be elucidated by an experiment where one side of the microgreen growing tray is inoculated but left unplanted during production as watering is routine.

VI. **Conclusion**

Soil–free growing media used in microgreen production is differentially supportive of both *S.* Javiana and *L. monocytogenes*. Biostrate® and hemp supported growth and persistence, while a *Sphagnum* peat/vermiculite mix and coco coir were less supportive. *L. monocytogenes*, in particular, showed a significant decline over a 10–day period (~5 log CFU/mL), while *S.* Javiana experienced only a small decline (~2 log CFU/mL). However, previous work comparing SFGM

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susceptibility indicates that the presence of plant roots in the medium may complicate these differences. To our knowledge, this is the first comparison of survival among SFGM involving a *S. enterica* serovar and *L. monocytogenes,* and the first study comparing coco coir, Biostrate®, and hemp. Further research to elucidate the role of plant roots on pathogen survival is warranted. Growers entering the microgreen industry should be aware of potential risks associated with their choice of horticultural media when designing production systems, as carbon-rich and high water-retaining grow mats such as those examined in this study may confer increased risk for key foodborne pathogens.

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Figure 1. 10-Day Survival of *Salmonella* Javiana on SFGM. The lines trace the mean log CFU/mL of *S.* Javiana recovered from each type of growing medium (Biostrate®, coco coir, hemp, peat, and the control) for each collection time. The additional points represent the individual measurements that make up each mean. Un-inoculated controls did not contain detectable concentrations of *Salmonella* and therefore are not shown in the plot.
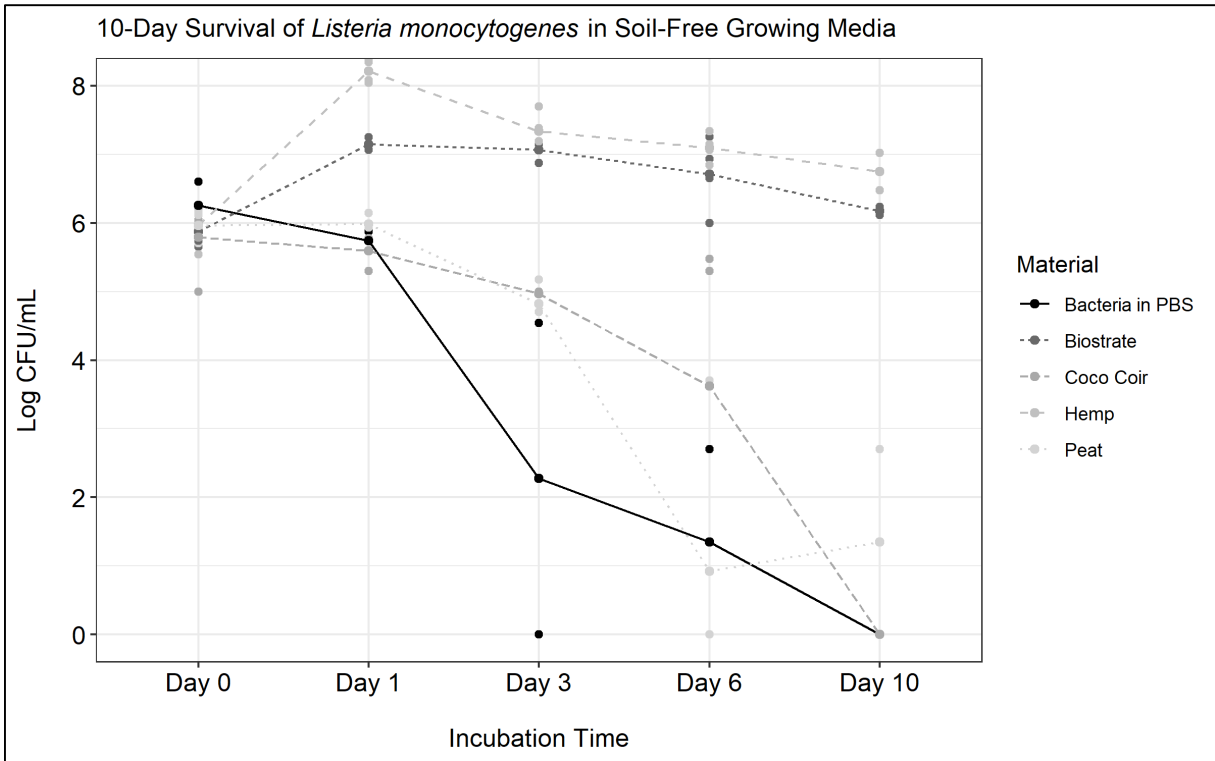
Figure 2. 10-Day Survival of *Listeria monocytogenes* on SFGM. The lines trace the mean log CFU/mL of *L. monocytogenes* recovered from each type of growing medium (Biostrate®, coco coir, hemp, peat, and the no-media control "Bacteria in PBS") for each collection time. The additional points represent the individual measurements that make up each mean. Un-inoculated controls did not contain detectable concentrations of *L. monocytogenes* and therefore are not shown in the plot.

IX. **Tables**

Table 1. Chemical composition of SFGM materials

Analyte, unit	Biostrate®	Hemp	Coco Coir	Peat
% ADL_{OM} (lignin)	73.2	4.93	*NA*	*NA*
% ADF (lignin + cellulose)	91.97	75.91	*NA*	*NA*
% NDF (lignin + cellulose + hemicellulose)	94.82	86.12	*NA*	*NA*
% Cellulose (%ADF – %ADL)	18.77	70.98	*NA*	*NA*
% Hemicellulose (%NDF – %ADF)	2.85	10.21	*NA*	*NA*
% Nitrogen	0.09	0.31	0.13	0.2
% Carbon	48.94	42.99	12.9	11.91
pH	6.3	5.85	6.3	5.15
EC (µmhos/cm)	32	96	1036	780
NO_3-N, mg/L	0.3	0.1	0.8	46
P, mg/L	1.04	1.68	8.88	2
K, mg/L	2.07	3.74	216	46.5
Ca, mg/L	0.5	4.2	2.48	28.8
Mg, mg/L	0.26	1.41	1.65	52.1
Na, mg/L	2.04	7.21	53.7	19.9
S, mg/L	0.34	5.65	10.1	37.1
Fe, mg/L	0.11	0.31	2	2.16
Mn, mg/L	0.001	0.07	0.02	0.2
Zn, mg/L	0.002	0.16	0.04	0.05
Cu, mg/L	0.02	0.02	0.02	0.05
B, mg/L	0.02	0.02	0.2	0.11

Units for each analyte are on the left hand column. Percent refers to the % dry weight of the sample. ADF = acid detergent fiber, NDF = neutral detergent fiber, ADL = acid detergent lignin, EC = electrical conductivity. The unit "µmhos/cm" is a measure of conductance, also known as a "Siemen" or the reciprocal of an ohm (resistance). ADL, ADF, and NDF for coco coir and peat was not performed, shown as "NA", due to limitations of the forage analysis method.

Table 2. Background Isolates: Colony Morphology, Source, and Identity

#	Description	SFGM, Plate media	Day	Genus
1	Large colony, translucent	Hemp, XLT4	6	*Klebsiella*
2	Very small colony, clear	Peat, XLT4	6	*Pseudomonas*
3	Large colony, waxy, white	Hemp, XLT4	6	*Klebsiella*
4	Large colony, white	Biostrate®, XLT4	6	*Enterobacter*
5	Large colony, pale yellow	Coir, XLT4	6	*Klebsiella*
6	Medium-sized colony, translucent, esculin reaction	Hemp, MOX	6	*Bacillus cereus*
7	Small colony, weak esculin reaction, slow growing (~36 h)	Hemp, MOX	1	*Curtobacterium*

Source includes the SFGM material where the isolate was found and on which type of selective media, and Day includes the first day where the contaminant appeared. 16S rRNA gene sequences are provided in the Appendix in Table 3-S7.

Table 3. Survival of *S.* Javiana and *L. monocytogenes* at day 10

A. *S.* Javiana

Material	Mean (log CFU/mL)	SE	Lower CI	Upper CI	Significance
Coco Coir	4.67	0.175	4.18	5.16	a
Peat	4.99	0.175	4.51	5.48	a
Buffer Only	5.35	0.248	4.67	6.04	ab
Hemp	6.15	0.175	5.66	6.64	bc
Biostrate®	6.86	0.175	6.38	7.35	c

B. *L. monocytogenes* R2-754

Material	Mean (log CFU/mL)	SE	Lower CI	Upper CI	Significance
Buffer Only	0.00	0.975	-2.71	2.71	a
Coco Coir	0.00	0.689	-1.91	1.91	a
Peat	1.35	0.689	-0.56	3.26	a
Biostrate®	6.17	0.689	4.26	8.09	b
Hemp	6.75	0.689	4.84	8.66	b

These values represent the mean survival by day 10, a typical microgreen harvest time point. Values with different letters are significantly different at $p < 0.05$. The overall significance of the ANOVA is shown at the bottom. Individual p-values for pairwise comparisons for all sampling days are available in the Appendix. The significance of the ANOVA for *S.* Javiana is $p = 0.0041$ (A), and the significance of the ANOVA for *L. monocytogenes* is $p = 0.0059$ (B).

Chapter 4: Transfer and survival of *Salmonella enterica* and *Listeria monocytogenes* from soil-free growing media to sunflower microgreens

I.   **Abstract**

Microgreens are immature shoots of edible plants often eaten as a raw salad green and are susceptible to contamination by bacterial pathogens commonly associated with produce-borne illness outbreaks. This study aims to measure survival of *Listeria monocytogenes* and *Salmonella enterica* subsp. Javiana on two types of soil-free growing medium (SFGM) during sunflower microgreen cultivation, as well as the degree of pathogen transfer to the edible product. *S.* Javiana and *L. monocytogenes* FSL R2-584 were inoculated onto two types of SFGM -- sphagnum peat with vermiculite and Biostrate® biodegradable mats. Following, sunflower microgreens (*Helianthus annuus* cultivar Black Oil) were cultivated on half of the inoculated tray for 10 days, with the other side left unplanted. At harvest, concentrations of the two pathogens in the growing medium at the start and completion of the growing cycle, as well as in the harvested microgreens, were determined. Overall, pathogen levels on SFGM declined more on peat than on Biostrate®, declined more without the presence of microgreen roots than when the tray was planted, and declined more for *L. monocytogenes* compared to *Salmonella* Javiana. Statistically significant differences were found on Biostrate, where *S.* Javiana growth was greater on the planted side of the tray compared to the unplanted side ($p = 0.02$). There were also survival differences between the two pathogens. On the unplanted side of Biostrate, there was a statistically significant difference between *L. monocytogenes*, which experienced a decline, and *S. Javiana*, which experienced growth. These findings indicate that pathogen survival in microgreen cultivation systems may partially depend on the growing medium chosen. The data

also show that the sunflower microgreen root environment may be a source of nutritional support for these two human pathogens.

II.   Introduction

Microgreens are an emerging raw salad product similar to sprouted seeds and lettuce. However, while there are similarities, some aspects of microgreen production differ from that of sprouted seeds (referred to as 'sprouts') and lettuce. While sprouts are germinated for 5 days in a warm, mostly closed, moist environment (US FDA 2017 and 2019), microgreens are germinated for up to 72 hours in either hydroponic nutrient solution, soil, or a soil-substitute (Muchjajib et al. 2015, Treadwell et al. 2016, Weber 2017, Di Gioia et al. 2017) and then allowed to grow for 10 to 20 days – approximately during the opening of the cotyledon or the formation of the first set of true leaves. Lettuce, by contrast, is typically grown in a field or hydroponically and reaches the mature rosette stage after 90 days (Smith et al. 2011). Produced as a "baby" variety, lettuce may also be cultivated in container farm greenhouses and harvested at 38 – 43 days (Grahn et al. 2015).

The production environment and conditions under which leafy greens are grown may influence the plant's uptake of bacteria, including human pathogens that contribute to produce-associated foodborne illness (Olaimat et al 2012, Alegbeleye et al. 2018). Approximately 16% of foodborne illness outbreaks are linked to produce according to the most recent data from 2013, and 37% of those outbreaks were linked to leafy greens (Bennett et al. 2018). A search for "sprouted seeds" and "food" in the Centers for Disease Control and Prevention's National Outbreak Reporting System (CDC NORS) showed that sprouted seeds alone have been involved in over 1,800

foodborne illnesses since the mid-1990s. Microgreens have not yet been responsible for any

known illnesses or outbreaks, but have been implicated in multiple product recalls in the US (US

FDA 2016, 2018, and 2019) and Canada (CFIA 2018a-f, 2019a, and 2019b) due to possible

contamination with *Salmonella enterica* and *Listeria monocytogenes*.

Human and plant pathogens alike are known to utilize the plant root system to gain access to

internal plant tissues thus rendering post-harvest washing ineffective (Olaimat et al. 2012,

Bernstein et al. 2016). Therefore, studying aspects of leafy green production that may increase

the risk of contamination via the growing medium is a necessary preventive strategy. Pathogen

uptake into leafy greens from soil has been extensively studied (Warriner et al. 2003, Deering et

al. 2012, Hirneisen et al. 2012, Erickson et al. 2012, 2016, and 2019, Zheng et al. 2013, Zhang et

al. 2015, DiCaprio et al. 2015, Bernstein et al. 2016, Gao et al. 2017, Karanja et al. 2018). In

addition to soil, several studies have explored pathogen uptake by hydroponic crops (DiCaprio et

al. 2012, Hull et al. 2016, Moriarty et al. 2018 and 2019). Review articles on internalization risk

(Hirneisen et al. 2012, Macarisin et al. 2014, Carducci et al. 2015, Riggio et al. 2019) suggest

that pathogen uptake may be affected by a number of factors including pathogen species,

growing medium, and plant variety. Previous work investigating the uptake of foodborne

pathogens in microgreen growing systems have examined soil (Xiao et al. 2014), peat (Xiao et

al. 2015, Di Gioia et al. 2017, Reed et al., 2018) and fibrous mats (Di Gioia et al. 2017, Wright et

al. 2018), and hydroponic nutrient solution (Xiao et al. 2015). Research in this area has been

focused on *Escherichia coli*, another important foodborne pathogen frequently associated with

outbreaks in leafy greens (Turner et al. 2019). Furthermore, microgreen varieties studied in a

food safety context have included radish (Xiao et al. 2014 and 2015), cabbage (Chandra et al.

2012), kale (Wang et al. 2016, Photchanachai et al. 2018), mustard (Wang et al. 2016), Rapini (Di Gioia et al. 2017), and herb varieties (Wright et al. 2018), but not sunflower microgreens or pea shoots, which are two of the most commonly grown microgreens (see Chapter 2). To our knowledge, this is the first microgreen pathogen transfer study involving *Salmonella* and *L. monocytogenes* in sunflower microgreens cultivated in soil-free growing media (SFGM).

III. **Materials and Methods**

i. **Preparation of Bacterial Cultures**

Bacteria used in this study include *L. monocytogenes* (FSL R2–574) isolated from a soft cheese outbreak and *Salmonella enterica* subsp. Javiana (ATCC BAA1593) isolated from a tomato outbreak. *L. monocytogenes* was streaked for isolation from a glycerol stock on Modified Oxford Medium (MOX) agar (HiMedia Laboratories, Mumbai, India) with antimicrobial supplement (BD Difco, Franklin Lakes, NJ, USA) containing colistin sulfate (10 mg/L) and moxalactam (20 mg/L). Similarly, a glycerol stock of *S.* Javiana streaked on Xylose Lysine Tergitol–4 (XLT4) agar (Criterion, Philadelphia, PA, USA) with a 4.6 mL/L Tergitol 4 agar supplement (BD Difco). The inoculated XLT4 and MOX plates were incubated at 37°C for 18 h and 35°C for 24 h, respectively. A single colony from each plate was transferred to separate 50 mL conical tubes of 10 mL of Brain–Heart Agar Infusion (BHI) broth (BD Difco) for *L. monocytogenes* and Tryptic Soy Broth (TSB, BD Difco) for *S.* Javiana and incubated overnight at 35°C at 120 rpm in a shaking incubator (Thermo Scientific MaxQ 4000).

Overnight cultures were centrifuged at $4000 \times g$ for 10 min at 10°C to pellet the bacteria. The pellet was washed twice in 10 mL of sterile phosphate buffered saline (PBS, pH = 7.4) using the

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same centrifugation speed and time and then re-suspended in 10 mL of sterile PBS. A bacterial cocktail of both pathogens was prepared by adding 1 mL each of the prepared cultures into a sterile 15 mL tube and vortexing briefly at maximum speed. A 10–fold dilution series was prepared by placing 100 µL of culture into 900 µL of PBS. The cocktail was enumerated by spreading 100 µL of each dilution on either XLT4 and MOX agar plates and incubated as described previously. The bacterial cocktail contained approximately 10^9 CFU/mL each of *S. Javiana* and *L. monocytogenes* and was diluted to a final concentration of 10^6 CFU/mL in sterile PBS prior to inoculation of SFGM.

ii. **Preparing the Microgreen Trays**

Two types of SFGM, Biostrate® 185 Felt Sheets (Harris Seeds, Catalog #41461-00-00-833, Rochester, NY) and a peat/vermiculite blend (Soilless Jiffy-Mix, Harris Seeds, Catalog #04035-00-00-900, Rochester, NY), were chosen for this study. Biostrate® is used in hydroponic microgreen production, while peat is a common choice for stacked shelf systems. Prior to adding grow media, each empty tray was disinfected with 70% ethanol and allowed to air dry in a UV-light treated biosafety cabinet. Following, the appropriate amount of SFGM was weighed into each tray using sterile containers. Each peat tray contained 600 g peat that was moistened with 1000 mL of sterile distilled water. Each Biostrate® tray contained one ~20g Biostrate® mat that was moistened with 400 mL of sterile distilled water. Two trays of each SFGM were set aside for inoculation while two trays of each type of SFGM were covered and used as un-inoculated controls.

iii. **Inoculation of Growing Media**

Inside the biosafety cabinet, each non-blank microgreen tray was inoculated with 50 mL of a 10^6 CFU/mL cocktail of the two target organisms. To inoculate, a 50 mL serological pipette was filled with the cocktail and dripped across the peat or Biostrate® in a zig-zag motion from the top of the tray to the bottom. This resulted in approximately 5 mL of cocktail spread across the tray every 2 in (5 cm) as measured on the long edge of the tray. The un-inoculated trays were treated with 50 mL of only PBS using the same method. After inoculation, but before planting, a sampling procedure to obtain initial bacterial counts was carried out as described in Section v.

iv. **Microgreen Cultivation**

Approximately 70 grams of organic sunflower seeds (*Helianthus annuus* cultivar Black Oil, Cat# 2160SG, Johnny's Seeds, Maine, USA) for each tray were soaked in 500 mL of sterile distilled water in a foil-covered beaker for 6 hours prior to planting. At the time of planting, each beaker of soaked seeds was strained through an autoclaved metal strainer to remove excess water. Following, seeds were poured from the strainer and, with a gloved hand disinfected with 70% ethanol, spread evenly over half of the tray, attempting to avoid clumping of seeds. Gloves were changed between trays. The other half of the tray was left unplanted to compare survival of microorganisms on the SFGM with and without the presence of microgreen roots. Un-inoculated trays were planted and sampled first to avoid accidental cross-contamination.

Microgreen germination and growth were carried out in a climate controlled room with an ambient temperature held between 68°F - 72°F (20 - 22°C) and a relative humidity level of approximately 70%. After planting, each tray was covered with a second germination tray,

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disinfected with 70% ethanol, and overturned to form a lid so that germination would take place in near darkness. Twice per day, the lids were lifted, and seeds were misted with sterile distilled water to keep them moist through the germination process. After 72 hours, the covers were removed, and the lights were turned on. The photoperiod was 18 hours on and 6 hours off, using three GrowBright 4-foot T5 6400K (5000 lumens) Compact Fluorescent Lamps (HTG Supply, Pennsylvania, USA) per shelf. The microgreen trays were positioned approximately 10" (25 cm) from the lights. The blank trays were grown on a separate but identically constructed shelf above the inoculated trays in order to prevent accidental cross-contamination during watering. Watering was performed using an overhead pouring method for all four trays. From day 3 until day 10, every 24 hours, the Biostrate® trays were watered with 200 mL of sterile distilled water and the peat trays were watered with 400 mL of sterile distilled water. To evenly distribute the water across the trays, each tray was carefully tilted back and forth four times after watering.

v. **Harvesting and Sampling**

a. *SFGM*

Before spreading the seeds over the growing media, initial SFGM samples were collected to verify the initial concentration that could be recovered using our elution method. From each inoculated tray, six total samples were collected. Three were collected from the unplanted side and three from the planted side (outer edge, middle, and inner edge for each). On the un-inoculated trays, only two samples were taken from each side (middle and outer edge). A sampling diagram is available in the Appendix (Figure 4-S1). Sampling of Biostrate® was conducted by lifting the mat with sterile forceps, removing a 2.5 cm^2 piece of media with sterile scissors, and placing it inside a sterile 50-mL centrifuge tube. Sampling of peat trays was

conducted by removing approximately 5-mL samples of peat using a sterile metal scoop and placing these samples inside a 50-mL centrifuge tube. At harvest, this sampling method was repeated. When sampling the planted side of each tray, attempts were made to remove as much root tissue from the mat as possible, but some was inevitably left behind as the root system is often deeply embedded in the media. Since the grow media samples were wet as a result of bacterial elution, a dry weight was obtained after the plate assay by pouring off excess liquid and allowing the media samples to dehydrate at 80°C for 16 hours. Then, each dehydrated media sample was weighed, and weights were recorded for later CFU/g calculations.

b. *Microgreens*

In locations near where SFGM was sampled, 5 – 7 microgreens (approximately 2-3 grams of microgreens per sample) were held with sterile forceps and then cut 1 cm above the root system using sterile scissors and placed into stomacher bags. After sampling, each bag of microgreens was weighed, and the actual sample weight determined by subtracting the weight of an empty bag. These sample fresh weights were recorded for later CFU/g calculations.

vi. **Elution and Recovery**

a. *SFGM*

To elute, each tube was filled with 10 mL of 1X PBS (pH = 7.5) and pulse–vortexed at maximum speed every 15 s for 1 min. One milliliter of eluent was removed using a 1-mL serological pipette and diluted in a 1:10 dilution series. Peat was allowed to settle briefly (~10 s) before pipetting the liquid to avoid particulates clogging the pipette tip (further discussed in Section V, part iii, subpart b). The serological pipette allowed improved aspiration of the peat

eluent because of the larger opening compared to a 1-mL micropipette tip, minimizing loss of microorganisms that may have attached to the peat particulates. The dilutions for each sample were plated and enumerated as described previously.

b. *Microgreens*

To elute, each stomacher bag was filled with 10 mL of PBS and stomached for 3 minutes on 240 rpm (Stomacher 400 Circulator; Seward, Worthing, United Kingdom). To further assist homogenization of the microgreen tissue (~ 2.5 g per bag), manual crushing was performed for another 5 minutes after stomaching, until the buffer became green and turbid and all stem and leaf material was broken into very small pieces. The resulting liquid (~7-9 mL) was pipetted into a 15 mL conical tube and the stomacher bag was discarded. The eluent was then diluted in a 10-fold dilution series and plated as described previously (Section III, i.)

vii. **Statistical Analysis**

The R software platform (R Core Development Team, version 3.6.0) was used to perform statistical analysis along with the library "emmeans" (Lenth 2019) and "ggplot2" (Wickham 2016). Pathogen concentrations expressed as log CFU/g of *S.* Javiana and *L. monocytogenes* were considered to be separate responses. After determining the existence of slight but statistically significant differences in starting inoculum levels, the growth of *S.* Javiana and *L. monocytogenes* were calculated instead as the difference between harvest and initial. Positive values represent growth, and negative values represent decline. A separate two-way ANOVA was conducted for each pathogen, and then pathogen differences were assessed by a separate three-factor ANOVA. Microgreens were assessed independently from SFGM, in their own set of

ANOVA tests, since pathogen levels were obtained using different extraction methods that demonstrated different recovery efficiencies.

IV. **Results**

i. *Salmonella* **Javiana survival and transfer to sunflower microgreens**

Absolute measurements of mean log CFU/g of *Salmonella* Javiana recovered from SFGM and microgreens at planting ("Initial") and harvest are shown in Figure 1. A two-way ANOVA revealed no statistically significant differences in *Salmonella* Javiana levels recovered from Biostrate®-grown microgreens and peat-grown microgreens. The mean concentration in Biostrate®-grown microgreens was 5.45 ± 0.83, while that of peat-grown microgreens was 3.58 ± 1.11 (Table 1) ($p = 0.076$). Within each set of SFGM conditions, there were no statistically significant differences in pathogen growth/decline from planting to harvest when comparing planted (-0.77 ± 0.72 log CFU/g) and unplanted (-1.33 ± 0.43 log CFU/g) sides of the peat tray ($p = 0.44$) (Table 1). However, statistically significant differences were found on Biostrate®. Over the 10-day growing period, the planted side showed a 2.69 ± 0.07 log CFU/g increase and the unplanted side showed an increase of 1.30 ± 0.22 log CFU/g ($p = 0.019$).

ii. *L. monocytogenes* **survival and transfer to sunflower microgreens**

Absolute measurements of mean log CFU/g of *L. monocytogenes* recovered from SFGM and microgreens at planting and harvest are shown in Figure 2. A two-way ANOVA revealed no statistically significant differences between relevant variables (Table 2). Biostrate®-grown microgreens incurred a 4.21 ± 1.84 log CFU/g uptake while peat-grown microgreens incurred a 2.27 ± 0.67 log CFU/g uptake ($p = 0.16$). The planted side of Biostrate® experienced only a 0.23

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± 0.66 log CFU/g increase in growth over the 10-day period, while the unplanted side of

Biostrate® incurred a decline of 2.26 ± 0.73 log CFU/g (p = 0.064). The planted side of peat

showed a decline of 1.89 ± 1.56 log CFU/g, while the unplanted side showed a decline of 3.27 ±

0.78 log CFU/g (p = 0.39).

iii. **Between-pathogen differences**

Results of a three-factor ANOVA (Table 3, Table S3) show that the microgreen pathogen levels

recovered were neither significantly different for *L. monocytogenes* and *S.* Javiana overall (p =

0.11), nor for microgreens grown in Biostrate® (p = 0.60) or peat (p = 0.56). Despite the lack of

statistical significance, a possible interaction effect was observed where *S.* Javiana levels in both

types of microgreens were greater than those in *L. monocytogenes* by approximately 1 log

CFU/g. There was a statistically significant difference between *L. monocytogenes* and *S.* Javiana

on the unplanted side of the Biostrate® trays (p = 0.0008), where a 2.26 ± 0.73 log CFU/g decline

in *L. monocytogenes* was observed, and *S.* Javiana experienced a 1.3 ± 0.23 log CFU/g increase.

The planted side of Biostrate® also showed statistically significant differences in a pattern similar

to the unplanted side (p = 0.021). For peat, there was no difference between the planted and

unplanted sides for either bacterium, and all SFGM conditions experienced a decline. However,

it is worth noting that the greater declines were observed with *L. monocytogenes* in general, as

well as on peat over Biostrate, and on unplanted trays over planted trays.

V. Discussion

i. Pathogen Transfer to Microgreens

Biostrate® mats appear to support overall survival for both *Salmonella* Javiana and *L. monocytogenes* compared to peat. Both pathogens appeared to increase in concentration over the 10-day microgreen production cycle on Biostrate® and showed an overall decline on peat. These results are consistent with previous work in our lab (refer to Chapter 3) and are generally supported by findings of Di Gioia et al. (2017), Reed et al. (2018), Wright et al. (2018), and Xiao et al. (2015) that demonstrate differential pathogen survival across multiple types of microgreen growing media, with and without roots present.

While the overall concentration of *L. monocytogenes* and *S.* Javiana recovered from the Biostrate®-grown microgreens generally appeared greater than peat-grown microgreens by 2 log CFU/g, the difference was not statistically significant. The initial inoculum level on Biostrate® was 1 log CFU/g greater than on peat due to limitations of the experimental methods (Section V, iii, c) and is likely a major contributor to the 2 log CFU/g difference. Therefore, if pathogens experienced a decline on peat and growth on Biostrate®, but resulted in similarly contaminated microgreens, this may indicate that 1) pathogens on peat did not decline to low enough numbers to prevent a detectable level in microgreens and 2) perhaps uptake occurred early, during or just after germination, when differences in starting inocula on the media were more similar than they were at harvest. However, research on *E. coli* O157:H7 and *Salmonella enterica* (Bernstein et al. 2007, Brandl et al. 2008, Pu et al. 2009, Kroupitski et al. 2019) has shown that pathogen colonization of leafy greens is not always found to be related to plant age, and when a relationship is found, colonization favors developmental stages beyond leaf emergence. For the

first assumption, repeating this study with both low and high inocula may elucidate the minimum

level of SFGM contamination required for a detectable transfer.

ii. **The Presence of Roots on SFGM**

a. *Salmonella Javiana*

Salmonella Javiana experienced an overall increase between planting and harvest on Biostrate®

and an overall decline in concentration on peat, indicating that peat may be less nutritionally

supportive or that organisms endemic to peat may suppress the growth of *Salmonella* Javiana

(Table 1). In general, the presence of plant roots appeared to aid in the survival of *Salmonella*

Javiana, with the planted side of each media type showing an overall greater level at harvest

regardless of whether there was an overall decline or an overall increase in bacterial titer across

the growing period (Figure 1). Ongeng et al. (2011) found similar results with *S.* Typhimurium in

field-grown cabbage, where the levels of bacteria in manure-amended bulk soil were lower than

the levels in the cabbage plant rhizosphere after being irrigated with contaminated water.

Similarly, *S.* Typhimurium declined in soil but persisted for up to 4 weeks in the rhizosphere of

parsley spray-irrigated with water inoculated at 8.5 log CFU/mL, 7.5 log CFU/ml, and 6.5 log

CFU/mL. (Kisluk and Yaron, 2012).

b. *Listeria monocytogenes R2-574*

In general, the presence of plant roots aided in the survival of *L. monocytogenes* on both SFGM

types. The declines in growth observed seemed only to be abated on the planted side of

Biostrate®. Under that condition, *L. monocytogenes* levels maintained their initial concentration,

but did not increase (Figure 2). Though it appears that the beneficial effect of microgreen roots is

more pronounced on Biostrate® than it is on peat, once corrected for initial inoculum concentration, that difference was not statistically significant (Table 2). These results may be somewhat supported by Jablasone et al. (2004), which found that *L. monocytogenes* persistence in the rhizosphere was different only in lettuce, where co-inoculating with root-associated isolate *Enterobacter cloacae* reduced L. monocytogenes levels by approximately 1 log. While more research is needed, *L. monocytogenes* survival may be impacted by differences in plant rhizosphere more than *Salmonella*, possibly due to suppression by endemic root bacteria.

c.   *Between-Pathogens*

The most important questions to address in the between-pathogen comparisons are 1) whether *Salmonella* Javiana and *L. monocytogenes* are impacted differently by the presence of roots on different types of SFGM and 2) if the microgreens produced are differentially contaminated. For the first question, we ask if there is a larger difference in one pathogen over another between initial and harvest on the planted side compared to the unplanted side. On the planted side of Biostrate®, the change in *Salmonella* Javiana levels was 2.47 log greater than for *L. monocytogenes* and 3.56 log greater on the unplanted side of Biostrate®. It appears that the survival of *Salmonella* is aided by microgreen roots to a greater degree than for *L. monocytogenes* on this media type. For the second question, since there were no statistically significant differences in recovery from microgreens by SFGM type, it is unsurprising that there were also no between-pathogen differences in transfer to microgreens.

From a practical standpoint, it may seem irrelevant to compare survival of pathogens on SFGM with and without microgreens, as the transfer of pathogens to the edible product is of greatest

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concern for industry. However, any differences in survival on SFGM with and without plant

roots suggest the possibility that the rhizosphere composition plays a role in the survival of

foodborne pathogens in indoor microgreen cultivation systems. Root exudates specific to plant

varieties, as well as the organisms belonging to the root microbiome, may enhance or suppress

the growth of *Salmonella* spp., *L. monocytogenes,* and other major foodborne pathogens. For

example, in a comparison of *L. monocytogenes* (NCTC 13372) and *E. coli* O157:H7

internalization between lettuce, cultivated rocket, wild rocket, corn salad, and basil,

internalization of both pathogens occurred in the salad products but not in basil or in the basil

growing medium. The authors suggest that basil may produce root exudates that limit the growth

of these organisms (Chitarra et al. 2014). Therefore, it may be possible to identify greater risk

microgreen varieties and the SFGM types that, upon interaction with those microgreens, worsen

or mitigate pathogen transfer.

iii. **Study Limitations**

a. *Differing recovery efficiency of microgreens and SFGM*

Preliminary tests of cut microgreens and SFGM samples that were surface-inoculated with a

known quantity of bacteria revealed that pathogen recovery from microgreens (10^{-2}) is an order

of magnitude less than recovery from SFGM (10^{-1}). Due to these differences, separate statistical

analyses were used for SFGM levels and microgreen levels.

b. *Bacterial interaction with peat particles*

For peat samples, there was some suspicion that bacterial attachment to media particles might

impact recovery as *Salmonella* in particular is known to interact with soil particles (Turpin et al.,

1993). However, recovery of *Salmonella* from peat was only somewhat lower than for Biostrate[®] (Chapter 3 Appendix), but still within the same order of magnitude. Therefore, recovery was not likely to be significantly impacted by these interactions.

c. *Inoculation Technique*

Attempting to inoculate SFGM so that the concentration is the same regardless of thickness and density is error-prone. The method chosen for this study was chosen for its simplicity, as it ensures an equal number of cells per tray. Upon initial sampling, it was found that peat log CFU/g measurements were about 1 log lower than for Biostrate[®]. It may be assumed that this means fewer bacterial cells are accessing the microgreen roots in peat than in Biostrate[®]. However, this is not known. When laid in its tray at the appropriate depth, peat is several times thicker than the Biostrate[®] mats. The precise degree of this difference is difficult to measure due to variation in how densely packed the peat is, so attempting to add different concentrations of bacteria to each media type in order to achieve the same per-gram concentration may not be successful. Any unseen impact of adding a different number of cells per tray for each media type may be worsened by the effect of watering, which may unevenly redistribute cells around the tray during the growing process. Furthermore, the assumption that peat microgreen roots are accessing fewer cells does not account for cell motility that may allow the bacteria to migrate toward the plant roots in search of nutrients. Therefore, it is recommended that a future investigation compare inoculation strategies to determine the least biased method of testing pathogen uptake between different types of soil-free growing media of varying volumes and densities.

VI.   **Conclusion**

Under the microgreen cultivation conditions used in the present study, there were no statistically significant differences in pathogen-specific and SFGM-specific levels of the target pathogens transferred to indoor, tray-cultivated sunflower microgreens after 10 days of growth. While *Salmonella* Javiana was able to grow to high levels on Biostrate®, it experienced a decline on peat, and *L. monocytogenes* declined on both media types. Despite these differences, it appeared not to impact transfer to microgreens. The decline observed for both pathogens was greater on unplanted media than on planted media, indicating that the root microenvironment may play a role in the survival of human pathogens if the growing medium becomes contaminated. *Salmonella* Javiana appeared to benefit slightly more from the presence of plant roots than *L. monocytogenes*, but only on Biostrate®. These findings raise important questions about the impact of features such as the root microbiome and root exudates that are specific to plant varieties, and the interaction effects of the root and SFGM microbial communities on the suppression of human pathogens in indoor microgreen cultivation.

VII. **References**

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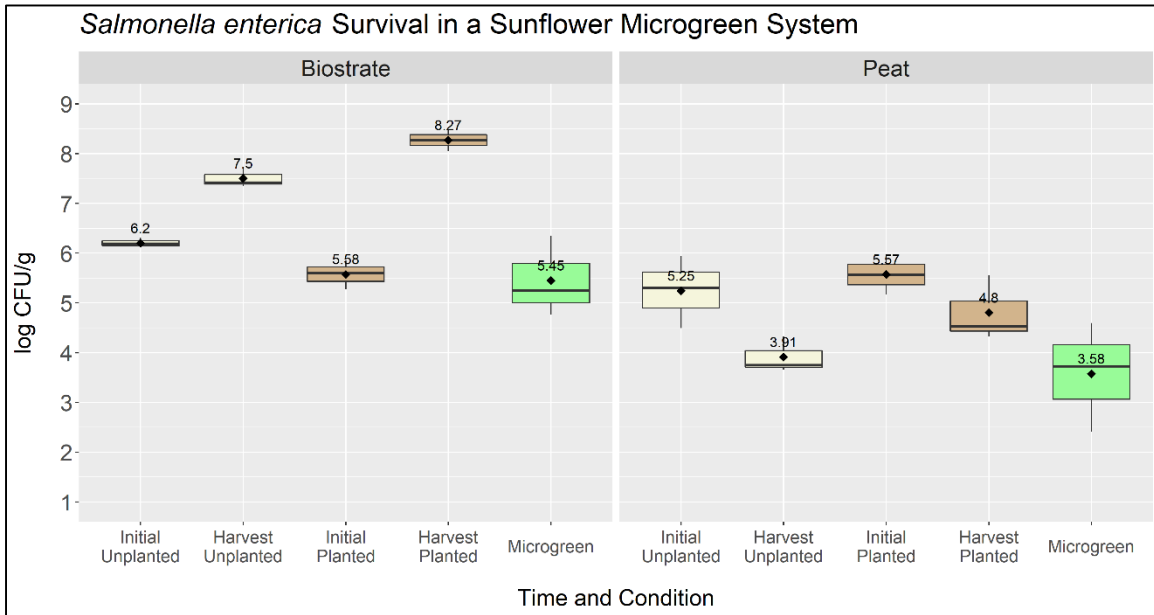
Figure 1. *S. enterica* **transfer to sunflower microgreens grown in Biostrate and peat.** Green boxes represent bacteria recovered from microgreens, tan boxes represent growth on the unplanted side of the tray, and the brown boxes represent bacterial growth on the planted side of the tray. Starred boxes are statistically significant compared to all conditions.
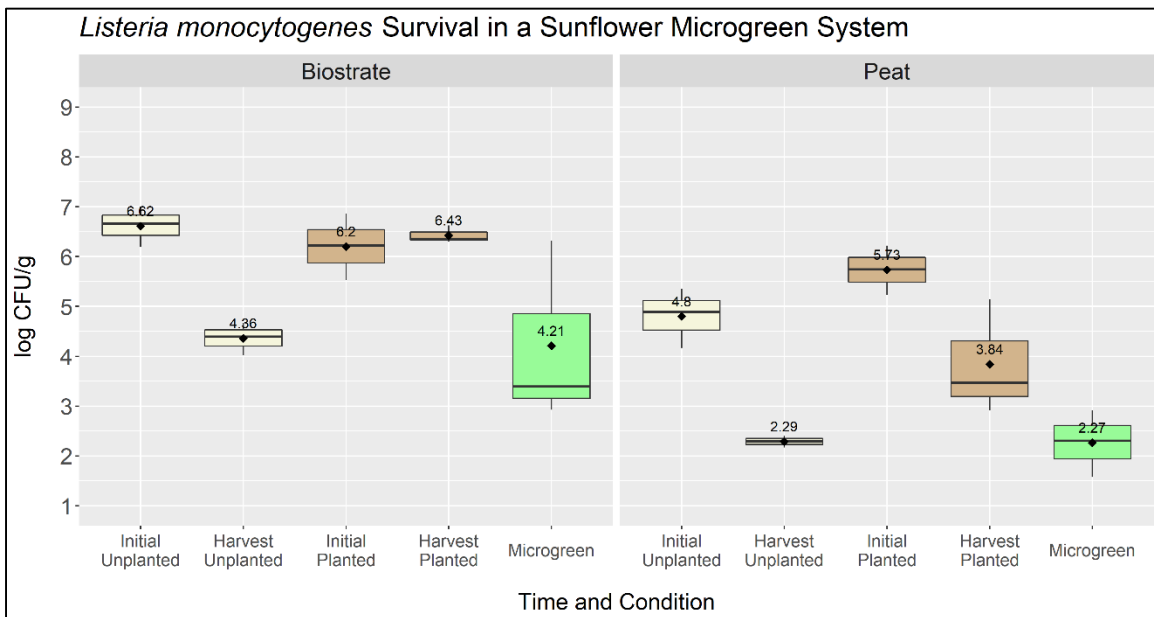
Figure 2. *L. monocytogenes* **transfer to sunflower microgreens grown in Biostrate and peat.** Green boxes represent bacteria recovered from microgreens, tan boxes represent growth on the unplanted side of the tray, and the brown boxes represent bacterial growth on the planted side of the tray. Starred boxes are statistically significant compared to all conditions.

IX. Tables

Table 1. *S. enterica* **mean log CFU/g change between initial and harvest**

Condition	Media	Mean Change	St. Dev.	SE	DF	Low CI	High CI	Group
Microgreen	Biostrate	5.45	±0.81	0.38	12	4.62	6.29	d
	Peat	3.58	±1.10	0.38	12	2.74	4.41	c
Planted	Biostrate	2.70	±0.08	0.38	12	1.86	3.53	bc
	Peat	-0.77	±0.72	0.38	12	-1.60	0.07	a
Unplanted	Biostrate	1.30	±0.23	0.38	12	0.47	2.14	b
	Peat	-1.33	±0.43	0.38	12	-2.17	-0.50	a

Biostrate and peat, and the microgreens grown in each. Differences between groups of the same letter are not statistically significant. Microgreen "differences" are increases only, assuming an initial concentration of 0 log CFU/g because the microgreens had not yet germinated and the seed soak water was negative for the target pathogens. SE = standard error, DF = degrees of freedom, CI = confidence interval, St. Dev. = Standard deviation

Table 2. *L. monocytogenes* **mean log CFU/g change between initial and harvest**

Condition	Media	Mean Change	St. Dev.	SE	DF	Low CI	High CI	Group
Microgreen	Biostrate	4.21	±1.84	0.66	12	2.77	5.65	d
	Peat	2.27	±0.67	0.66	12	0.83	3.70	cd
Planted	Biostrate	0.23	±0.67	0.66	12	-1.21	1.66	bc
	Peat	-1.89	±1.56	0.66	12	-3.33	-0.45	ab
Unplanted	Biostrate	-2.26	±0.73	0.66	12	-3.70	-0.82	ab
	Peat	-3.27	±0.78	0.66	12	-4.71	-1.83	a

Biostrate and peat, and the microgreens grown in each. Differences between groups of the same letter are not statistically significant. Microgreen "differences" are increases only, assuming an initial concentration of 0 log CFU/g because the microgreens had not yet germinated and the seed soak water was negative for the target pathogens. SE = standard error, DF = degrees of freedom, CI = confidence interval, St. Dev. = Standard deviation

Table 3. Between-pathogen differences in mean log CFU/g change between initial and harvest by presence of plant roots and media type

Media	Condition	Pathogen	Mean Change	SD	SE	DF	Low CI	High CI	Group
Biostrate	Microgreen	*Listeria*	4.21	±1.84	0.54	24	3.10	5.33	fg
		Salmonella	5.45	±0.81	0.54	24	4.34	6.57	g
	Planted	*Listeria*	0.23	±0.67	0.54	24	-0.89	1.34	bcd
		Salmonella	2.70	±0.08	0.54	24	1.58	3.81	def
	Unplanted	*Listeria*	-2.26	±0.73	0.54	24	-3.37	-1.14	ab
		Salmonella	1.30	±0.23	0.54	24	0.19	2.42	cde
Peat	Microgreen	*Listeria*	2.27	±0.67	0.54	24	1.15	3.38	def
		Salmonella	3.58	±1.10	0.54	24	2.46	4.69	efg
	Planted	*Listeria*	-1.89	±1.56	0.54	24	-3.01	-0.78	ab
		Salmonella	-0.77	±0.72	0.54	24	-1.88	0.35	abc
	Unplanted	*Listeria*	-3.27	±0.78	0.54	24	-4.39	-2.16	a
		Salmonella	-1.33	±0.43	0.54	24	-2.45	-0.22	abc

Negative values indicate a loss of bacteria, and positive values indicate bacterial growth. Differences between groups of the same letters are not statistically significant. Microgreen "differences" are increases only, assuming an initial concentration of 0 log CFU/g because the microgreens had not yet germinated and the seed soak water was negative for the target pathogens. SE = standard error, DF = degrees of freedom, CI = confidence interval, St. Dev. = Standard deviation

Chapter 5: Conclusion and Future Research Directions

As microgreen food safety, and food safety of indoor agriculture in general, is a relatively new area of research, many open questions remain. The present work has demonstrated that there is a deficit of research studying sunflower microgreens—the most common variety grown—and that trays of some type of particulate growing media such as soil or peat, stacked on artificially lit shelves is the most common microgreen production system for very small microgreen farms. In these systems, we have demonstrated that there is a difference in survival of *L. monocytogenes* R2-574 and *S. enterica* Javiana on four types of soil-free growing media and that survival of both pathogens is enhanced by the microgreen root environment. Further work will investigate differences between sunflower microgreens and pea shoot microgreens.

The conclusions presented in this thesis have allowed the identification of three broad areas of microgreen food safety to address: operational effects, biological effects, and compliance. Operational effects can be described as features of microgreen production over which the operator has some manner of control, and if food safety risks are found, these practices can theoretically be modified or abandoned. Biological effects are risks inherent to the system, such as resistance or susceptibility of certain microgreen varieties, seed varieties, and growing media to pathogen contamination, immutable environmental conditions, and fitness of target pathogens. Compliance refers to farmer ability and desire to adhere to food safety regulatory requirements and any best practices for microgreen production that may be determined.

I.   **Operational Effects**

i.   **Does sub-irrigation decrease contamination risk of the edible product?**

Sub-irrigation has been studied in microgreens twice (Xiao et al., 2015, Işik et al., 2020), with

mixed results. Both studies utilized *E. coli* O157:H7 and radish microgreens. Xiao et al. (2015)

found that sub-irrigation and overhead spray irrigation conferred no statistically significant

difference in transfer to the edible portion of the microgreen for both low (3.7 log CFU/g) and

high (5.6 log CFU/g) inoculation levels, despite greater levels in the growing media and inedible

portion for sub-irrigation under both inoculation levels ($p < 0.05$). Isik et al. (2020) also found no

statistically significant differences in concentration in the edible portion between both watering

methods, but did not differentiate between edible, inedible, and growing media levels.

Furthermore, while Xiao and colleagues (2015) contaminated the irrigation water, Işık and

colleagues contaminated the growing media. Future work comparing multiple microgreen

varieties and contamination routes would provide clarity to this comparison.

ii.   **Is the widely used hydrogen peroxide method effective against seed contaminants?**

Previous work testing hydrogen peroxide as a seed disinfection method has been performed in

the past using sprout production as the model system (Beuchat, 1997, Hong and Kang, 2016).

Beuchat found that a 6% v/v hydrogen peroxide solution was effective at achieving a 3 log

reduction in *Salmonella* populations, and Hong and Kang (2016) found that a 24-h dry heat

treatment followed by 2% v/v hydrogen peroxide soak for 10 minutes reduced *Salmonella*

Typhimurium by 1.66 log CFU/g, compared to 0.26 log CFU/g from dry heat alone. Further,

these treatments improved germination by approximately 10%. The only study in microgreens

examined the utility of a foliar spray to assess possible damage to leaves in systems that use

hydrogen peroxide to disinfect recirculating hydroponic nutrient water (Eicher-Sodo et al., 2019).

iii. **What is the impact of soaking and post-harvest washing on sunflower and peas?**

Non-disinfection soaking of seeds appears uncommon among microgreen producers with the exception of sunflower, peas, and potentially other larger seed types or those with thick seed coats (see Chapter 2). It is unknown if soaking these varieties renders them more susceptible to pathogen contamination throughout the growing cycle or if moisture from routine watering ultimately provides enough moisture for pathogen growth where soaking makes no difference in levels in the edible part of the microgreen. As soaking seeds tends to shorten the germination time, a side by side comparison of soaked and unsoaked microgreens in various contamination scenarios would have to account for the longer growing time of the unsoaked seeds. Longer exposure to pathogens may independently contribute to greater levels at harvest.

iv. **Is there a difference in risk between microgreens sold cut versus "living trays"?**

Previous work on tomato stem scars (Lin and Wei, 2016), cantaloupe rind (Ukuku and Sapers, 2016), and apple wounds (Janisiewicz et al., 1999) lends to the assumption that fresh-cut microgreens bear a greater risk than "living trays" -- microgreens sold in containers with the roots still attached to the growing media. Xiao et al. (2015) showed greater contaminant levels nearer to the cut end of the microgreen. Furthermore, mitigating contamination of cut tissue is not straightforward, and it appears that post-harvest wash water is a key route of contamination. However, depending on the type of growing media used, selling living trays may simply confer different risks than cut microgreens, such as introducing organisms found on growing media to a

food production environment such as a restaurant kitchen. As well, if post-harvest wash water is a key route of contamination in the fresh-cut industry, and many microgreen varieties are not washed at harvest (Chapter 2), then fresh-cut may be less risky than living trays if only for certain types of microgreens. Different production methods and microgreen varieties should be compared to better answer this question.

II.   Biological effects

i.   Are some microgreen varieties at greater risk of contamination than others?

So far, possible differences in susceptibility to pathogen colonization have been found between microgreen varieties. Wright and Holden, (2018) found that basil had statistically significantly less colonization by STEC than other microgreen varieties, the rest of which were not different from one another. Reed et al. (2018) found that the ability of *S. enterica* to grow on sprouting alfalfa seeds was affected by seed storage time, but this was not the case for Swiss chard microgreens. Thus, a variety-associated difference in risk may exist. Future work will involve comparing sunflower microgreens to pea shoot microgreens. These two varieties are popular among beginning growers (Chapter 2), are often soaked to enhance germination, and have the same seed to harvest time. Thus, they are optimal for a first step at elucidating variety-specific effects and have also not previously been studied.

ii.   Do soil-free media types transfer pathogens more or less than soil?

The present work only compared soil-free growing media types, but no comparison was made to soil. The reason for this is that "soil" is not a homogeneous substance, and choosing the type of soil to use as a basis for comparison is difficult. Thus, future work comparing organic,

conventional, potting soil, fertilized potting soil, outdoor collected soil from fields, forests, and peri-urban land may be necessary to determine the optimal reference soil. Previous work studying the survival of *L. monocytogenes* described in Chapter 3 (Dowe et al., 1997, Ivanek et al. 2003, Locatelli et al. 2013, McLaughlin et al., 2011, Vivant et al., 2013a,b) spans a wide variety of soil types, though it is likely the optimal choice will be sourced from a potting soil manufacturer or from a leafy green production field.

iii. **Are container systems more or less risky than hydroponic?**

Two studies have compared microgreens grown in a hydroponic system compared to a non-hydroponic soil-free system (Xiao et al. 2014, Wright and Holden, 2018). Further, a review of different hydroponic system configurations and potential food safety risks concluded that the data is presently insufficient to determine differential risks, as most studies utilized laboratory scale hydroponic systems that cannot be adequately compared to "real life" systems (Riggio et al., 2019).

iv. **Are there different risks between indoor, greenhouse, and outdoor systems?**

The indoor agriculture microbiome has yet to be adequately characterized. There is some evidence that humans are a main driver of the indoor microbiome (Berg et al. 2014), which presents some concern for crop production. As the indoor microbiome has also been found to be less diverse than outdoor environments (Berg et al. 2014, Stamper et al., 2016), this lack of biological diversity may increase the likelihood of pathogen colonization (Vivant et al., 2013). Furthermore, viruses for which humans are the only known reservoir, such as norovirus, a leading cause of foodborne illness linked to leafy greens (Herman et al., 2015), may persist on

indoor surfaces including hydroponic systems (Wang and Kniel, 2016) for extended periods of time. Norovirus has not yet been studied in soil-free microgreen production systems, and since norovirus testing is not routinely performed in environmental monitoring schemes (Rönnqvist et al., 2013), understanding the risk of transfer of this key pathogen to the edible product is warranted.

III. Compliance

i. What factors influence non-compliance with food safety regulatory requirements?

Survey data has demonstrated that small scale and "sustainable" farmers struggle to maintain the food safety practices recommended by the Produce Safety Rule (Adalja et al., 2018, Harrison et al., 2013). Areas of concern include documentation, microbiological testing of water and growing media, employee hygiene, surface and container sanitation, and routine inspections. Chapter 2 outlines some possible factors contributing to non-compliance. However, a larger sample size and more diverse respondents may be necessarily to provide an adequate dataset for regression analyses and other tests of association between behaviors.

ii. Is a microgreen guidance for industry, separate from sprout guidance, necessary?

It seems necessary to develop guidance for industry that is separate from that of sprouted seeds, as microgreens are not similar enough to sprouts for all of the sprout recommendations to apply. While sprouts are submerged in a moist environment for 5 days, microgreen seeds are only soaked and germinated in an environment similar to sprout production for less than 24 hours. Furthermore, the edible portion of the microgreen lives above the soil line, whereas sprouts have no inedible parts. Exposure to a pathogen-friendly environment is thus somewhat different

between these two crops. Further, as microgreens may have a seed to harvest time of up to three times that of sprouts, pathogen growth dynamics will thus be different. Chapter 3 and 4 show that the highest pathogen levels occur within the first 3 days of growth and decreases beyond that window. Microgreens are also produced in a wide variety of system types, whereas sprout production is less diverse. Therefore, guidance for industry should take into account best practices for each method and the relative risk among methods.

IV.   **A Path Forward for Microgreen Producers**

The frequency of microgreen recalls is increasing, most of which are associated with *L. monocytogenes*, and as the industry grows, the risk of an outbreak in microgreens increases. Future investigations into these recalls should include assessments of the production system, particularly with respect to water and growing media, to determine any common traits among the companies implicated. These investigations may be important for guiding future research as well as best practices as new producers enter the industry.

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Appendix

I.   Chapter 2 Supplemental Information

Table 2-S1. Predictors of Water Testing

Beta-hat	Type of Predictor	Description of Predictor	p-value	
-0.0990	Food Safety Training Type	Food safety training at a conference	0.2272	
-0.0535	Food Safety Training Type	Other type of food safety training	0.6353	
-0.0095	Food Safety Training Type	No food safety training	0.8239	
0.0563	Food Safety Training Type	HACCP Training	0.3701	
0.0700	Food Safety Training Type	County health card training	0.0978	
0.2975	Food Safety Training Type	Lecture based training at work	0.1961	
0.7499	Food Safety Training Type	State health card training	1.0000	
0.7560	**Food Safety Training Type**	**Produce Safety Alliance training**	**0.0000**	*
1.1178	**Food Safety Training Type**	**Global Food Safety Initiative (GFSI) Training**	**0.0000**	*
-0.5303	**How Learned to Grow**	**Microgreen growing workshop**	**0.0161**	*
-0.4979	**How Learned to Grow**	**Learned to grow microgreens from a conference**	**0.0030**	*
0.0239	**How Learned to Grow**	**Learned on my own**	**0.0030**	*
0.4316	How Learned to Grow	From a book	0.7172	
0.0483	Livestock Type	Fish	0.7571	
0.2024	Livestock Type	Beef	0.7689	
-0.9117	**Growing Media Type**	**Green compost**	**0.0032**	*
-0.1629	**Growing Media Type**	**Organic soil**	**0.0005**	*
-0.1344	Growing Media Type	Sphagnum peat	0.8013	
-0.0653	Growing Media Type	Conventional soil	0.4062	
-0.0556	Growing Media Type	Worm compost	0.7537	
0.1227	Growing Media Type	Wood fiber	0.6174	
0.2182	Growing Media Type	Did not answer media question	0.4710	
-0.5083	Other Plant Crops	Seedlings	0.4217	
0.3378	Other Plant Crops	Flowers	0.2049	
-0.7458	Microgreen Variety	Pak.Choy	0.3872	
-0.2192	Microgreen Variety	Kohlrabi	0.7911	
-0.2160	Microgreen Variety	Mizuna	0.1049	
-0.0209	Microgreen Variety	Tatsoi	0.7208	
-0.0182	Microgreen Variety	Beet	0.4925	
-0.0075	Microgreen Variety	Radish	0.1392	
0.1193	Microgreen Variety	Nasturtium	0.7135	
0.1584	Microgreen Variety	Celery	1.0000	
0.2969	Microgreen Variety	Popcorn	0.5251	
0.4581	**Microgreen Variety**	**Bok.Choy**	**0.0214**	*
0.7973	**Microgreen Variety**	**Amaranth**	**0.0007**	*
-0.0741	**Water Source**	**Collected rainwater**	**0.0029**	*
0.2175	Water Source	Municipal Water	0.5327	
-0.0283	Water Treatment	No water treatment	0.4015	
0.2270	**Water Treatment**	**Treated with reverse osmosis**	**0.0586**	*
0.9952	**Water Treatment**	**Treated with Ultraviolet Light Filtration**	**0.0000**	*
-0.0904	Y-intercept		0.1812	

Each p-value marked with an asterisk is significant at p < 0.05. Each "Beta-hat" value is the coefficient of each predictor in the overall linear equation with an adjusted R-squared of 0.62.

Chapter 3 Supplemental Information
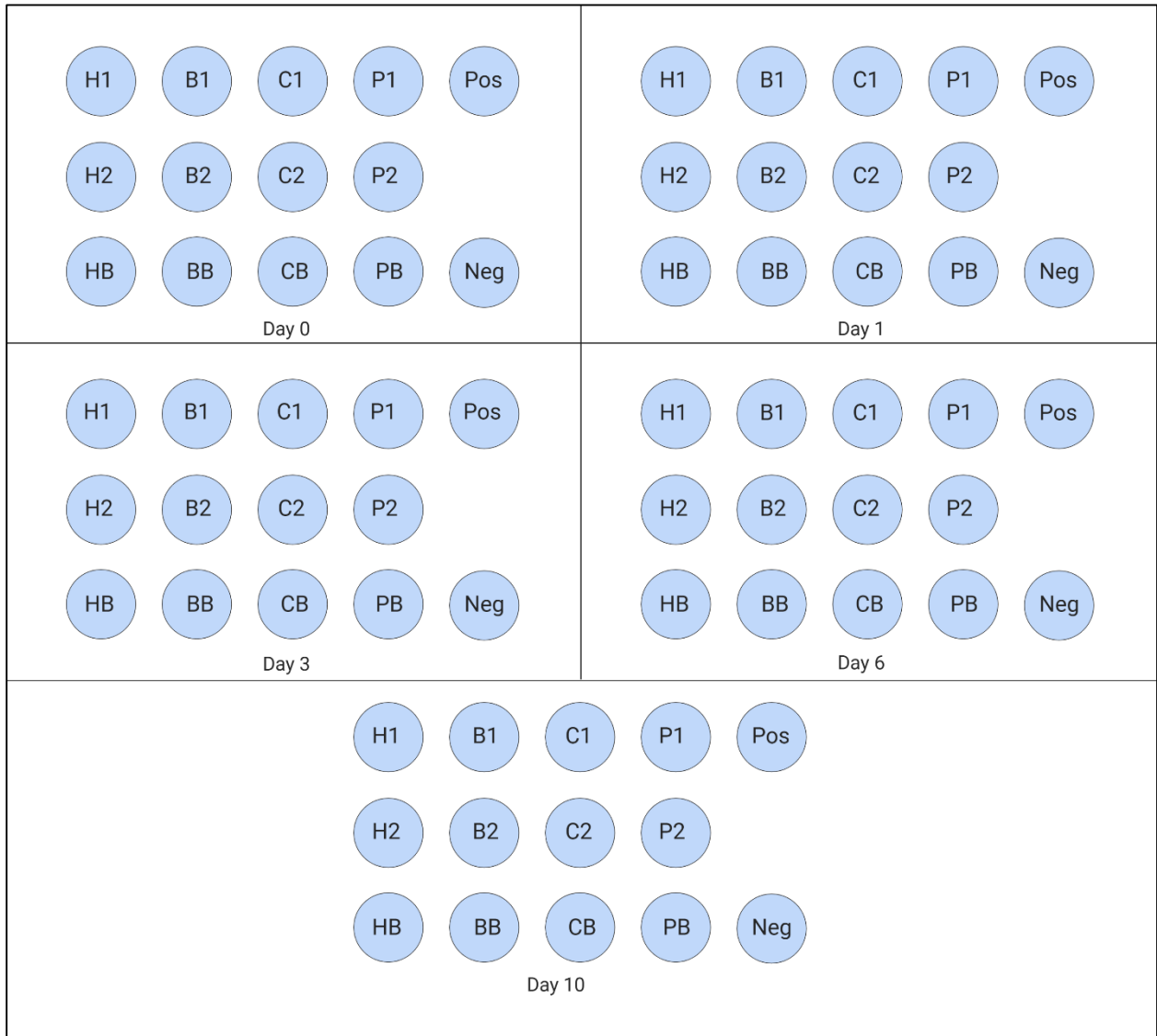
Figure 3-S1. Experimental Set-Up. All tubes were inoculated at the same time from the same cocktail. H1, H2: Hemp replicates. HB: Hemp blank inoculated with sterile PBS only. B1, B2: Biostrate® replicates. BB: Biostrate® blank inoculated with sterile PBS only. C1, C2: Coco Coir replicates. CB: Coco coir blank inoculated with sterile PBS only. P1, P2: Peat replicates. PB: Peat blank inoculated with sterile PBS only. Pos: 10^6 CFU/mL bacterial cocktail in sterile PBS. Neg: the sterile PBS solution used to suspend the cocktail and to inoculate the blanks.

Table 3-S1. Comparison of Experiment 1 and 2 for Pooling Datasets

A. F-Test to Compare Variances

	Listeria monocytogenes		*Salmonella* Javiana	
	Ratio of Variances	p-value	Ratio of Variances	p-value
Overall	0.977	0.952	2.56	0.003
Initial	0.126	0.008	0.227	0.051
One Day	3.803	0.733	6.688	0.013
Three Days	3.724	0.081	1.59	0.525
Six Days	1.39	0.652	0.81	0.768

B. Welch t-Test to compare means (unequal variances)

	Listeria monocytogenes			*Salmonella* Javiana		
	Mean 1	Mean 2	p-value	Mean 1	Mean 2	p-value
Overall	5.420	5.142	0.597	6.334	6.225	0.649
Initial	5.828	6.048	0.196	6.314	6.440	0.132
One Day	6.030	6.543	0.547	6.218	6.616	0.619
Three Days	5.408	5.845	0.631	6.561	6.356	0.573
Six Days	4.348	4.106	0.871	6.259	6.062	0.619

Table 3-S2. Water Retention Capacity of SFGM.

Material	Water Retention Capacity (mL/g)	Avg. sample mass (g)	Water/sample (mL)
Peat/Vermiculite	3.33	0.91	3.03
Hemp	8.88	1.01	8
Biostrate®	10	0.29	2.9
Coco Coir	3.33	1.14	3.79

Water holding capacity for coco coir and peat were determined by 1500 mL of water in 600 g of media, hemp was 45 g/mat and held 400 mL of water, and Biostrate® was 20 g/mat and held 200 mL of water.

Table 3-S3. Recovery efficiency for each pathogen

Material	Species	Starting CFU/mL	Recovered CFU/mL	Recovery %
Biostrate®	*L. monocytogenes*	2.60×10^6	1.88×10^6	72%
	S. Javiana	3.80×10^6	1.80×10^6	47%
Coir	*L. monocytogenes*	2.60×10^6	1.95×10^6	75%
	S. Javiana	3.80×10^6	1.95×10^6	51%
Hemp	*L. monocytogenes*	2.60×10^6	2.18×10^6	84%
	S. Javiana	3.80×10^6	8.25×10^5	22%
Peat	*L. monocytogenes*	2.60×10^6	1.80×10^6	69%
	S. Javiana	3.80×10^6	1.50×10^6	39%
Average	*L. monocytogenes*			75%
	S. Javiana			40%

Standard deviations are not shown because the CFU/mL recovered was based on a single sample for each material. Averages were determined by adding together recovery rates for all materials for each species and dividing by the total (n = 4)

Table 3-S4. *Salmonella* **Javiana ANOVA**

Day 0					Overall p = 0.29016
Material	Mean	SE	Lower CI	Upper CI	Significance
Hemp	6.261	0.083	6.082	6.441	a
Peat	6.317	0.083	6.137	6.496	a
Biostrate	6.394	0.083	6.215	6.574	a
Coco Coir	6.444	0.083	6.265	6.624	a
Buffer only	6.559	0.118	6.305	6.813	a

Day 1					Overall p = 0.00000
Material	Mean	SE	Lower CI	Upper CI	Significance
Buffer only	5.772	0.202	5.336	6.208	a
Coco Coir	6.090	0.143	5.782	6.399	a
Peat	6.534	0.143	6.226	6.842	ab
Biostrate	7.025	0.143	6.717	7.333	b
Hemp	7.896	0.143	7.588	8.205	c

Day 3					Overall p = 0.00000
Material	Mean	SE	Lower CI	Upper CI	Significance
Coco Coir	5.663	0.134	5.373	5.954	a
Peat	5.920	0.134	5.630	6.210	a
Buffer only	6.175	0.190	5.765	6.586	a
Biostrate	7.041	0.134	6.751	7.331	b
Hemp	7.351	0.134	7.061	7.641	b

Day 6					Overall p = 0.00000
Material	Mean	SE	Lower CI	Upper CI	Significance
Coco Coir	5.264	0.153	4.930	5.599	a
Peat	5.547	0.133	5.257	5.837	a
Buffer only	5.975	0.188	5.566	6.385	ab
Hemp	6.561	0.133	6.271	6.850	b
Biostrate	7.163	0.133	6.873	7.453	c

Day 10					Overall p = 0.00409
Material	Mean	SE	Lower CI	Upper CI	Significance
Coco Coir	4.670	0.175	4.184	5.156	a
Peat	4.991	0.175	4.505	5.477	a
Buffer only	5.352	0.248	4.665	6.040	ab
Hemp	6.151	0.175	5.664	6.637	bc
Biostrate	6.863	0.175	6.376	7.349	c

Each sampling day was analyzed in a separate one-way ANOVA. Differences between materials with the same letter are not statistically significant. Units for Least Squares Mean and upper and lower confidence intervals is in log CFU/mL recovered. Each table is ordered from least to greatest log CFU/mL.

Table 3-S5. *Listeria monocytogenes* **ANOVA**

Day 0					Overall p = 0.68505
Material	Mean	SE	Lower CI	Upper CI	Significance
Coco Coir	5.790	0.182	5.396	6.184	a
Biostrate	5.875	0.182	5.481	6.269	a
Peat	5.962	0.182	5.568	6.357	a
Hemp	5.967	0.182	5.573	6.361	a
Buffer only	6.253	0.258	5.695	6.810	a

Day 1					Overall p = 0.00000
Material	Mean	SE	Lower CI	Upper CI	Significance
Coco Coir	5.589	0.101	5.371	5.808	a
Buffer only	5.739	0.143	5.430	6.047	a
Peat	5.984	0.101	5.765	6.202	a
Biostrate	7.145	0.101	6.927	7.363	b
Hemp	8.209	0.101	7.990	8.427	c

Day 3					Overall p = 0.00013
Material	Mean	SE	Lower CI	Upper CI	Significance
Buffer only	2.272	0.647	0.874	3.671	a
Peat	4.818	0.458	3.829	5.807	b
Coco Coir	4.969	0.458	3.980	5.958	b
Biostrate	7.064	0.458	6.076	8.053	c
Hemp	7.331	0.458	6.343	8.320	c

Day 6					Overall p = 0.00035
Material	Mean	SE	Lower CI	Upper CI	Significance
Peat	0.925	0.812	-0.829	2.679	a
Buffer only	1.350	1.148	-1.130	3.830	a
Coco Coir	3.619	0.812	1.865	5.373	ab
Biostrate	6.709	0.812	4.956	8.463	b
Hemp	7.095	0.812	5.341	8.848	b

Day 10					Overall p = 0.0059
Material	Mean	SE	Lower CI	Upper CI	Significance
Buffer only	0.000	0.975	-2.706	2.706	a
Coco Coir	0.000	0.689	-1.913	1.913	a
Peat	1.350	0.689	-0.563	3.263	a
Biostrate	6.172	0.689	4.259	8.086	b
Hemp	6.749	0.689	4.836	8.663	b

Each sampling day was analyzed in a separate one-way ANOVA. Differences between materials with the same letter are not statistically significant. Units for Least Squares Mean and upper and lower confidence intervals is in log CFU/mL recovered. Each table is ordered from least to greatest log CFU/mL.

Table 3-S6. Pairwise Comparisons from the Tukey HSD post-hoc analysis.

A. *Listeria monocytogenes*

Pair	Day 0	Day 1	Day 3	Day 6	Day 10
Biostrate-Buffer Only	0.754	0.000	0.000	0.015	0.030
Coco Coir- Buffer Only	0.602	0.909	0.032	0.514	1.000
Hemp- Buffer Only	0.890	0.000	0.000	0.009	0.022
Peat- Buffer Only	0.885	0.638	0.045	0.998	0.787
Coco Coir-Biostrate	0.997	0.000	0.043	0.110	0.015
Hemp-Biostrate	0.996	0.000	0.993	0.997	0.969
Peat-Biostrate	0.997	0.000	0.028	0.002	0.035
Hemp-Coco Coir	0.957	0.000	0.020	0.062	0.011
Peat-Coco Coir	0.960	0.098	0.999	0.191	0.666
Peat-Hemp	1.000	0.000	0.013	0.001	0.024

B. *Salmonella* Javiana

Pair	Day 0	Day 1	Day 3	Day 6	Day 10
Biostrate-Buffer Only	0.782	0.002	0.018	0.002	0.034
Coco Coir- Buffer Only	0.927	0.702	0.240	0.077	0.320
Hemp- Buffer Only	0.289	0.000	0.002	0.145	0.225
Peat- Buffer Only	0.475	0.056	0.805	0.387	0.759
Coco Coir-Biostrate	0.992	0.004	0.000	0.000	0.004
Hemp-Biostrate	0.786	0.006	0.505	0.049	0.181
Peat-Biostrate	0.961	0.167	0.000	0.000	0.008
Hemp-Coco Coir	0.545	0.000	0.000	0.000	0.018
Peat-Coco Coir	0.810	0.240	0.667	0.643	0.709
Peat-Hemp	0.989	0.000	0.000	0.001	0.042

Individual p-values for each pair of SFGM at each time point for both pathogens. Bold values are statistically significant at $p < 0.05$.

Table S7. Sequences of the SFGM Background Isolates

#	Genus	Sequence (5' → 3')
1	*Klebsiella*	NNNTCNGNANNCTGGGCGTAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTG AAATCCCCGGNNTCNACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCT TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGA GGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCG AAAGCGTGGGGAGCAAACAGGATTAGAAACCCCNGTAGTCCGGCTGACTGACT ACGTGAGAGAATATCTCGTATGCCGTCTTCTGNNNNNNNNANANNNNNNNGNN NNNNNNTCNTNNNNNNNNNNNNNNGNNNNNNNNNTCATTGANNNNNNCNTGCN NTTNNGNTTGNNTTGGNNNNNGNNNNNNCNANNNNNNNNNNNTGNNNNNNNNN NNNNNGNNGANCNNNNNNNCNNNNNNNNANNNNNNNNNTNNNNNNNNGNNNN NTGNATTNTGNCNTCNGCTCTTCNGTCNGTTNNTCNNTCNTANNNNTNTNNNCN TNNTNNNNNNANANNNNNNNGNNNNNNNNNNNNNTNNNNNNNNGNNNCNA AANNNNNNNATGANNNNNNA
2	*Pseudomonas*	NNNANNCTGGGCGTAAGCGCGCGTANGTGGTTTGGTAAGATGGATGTGAAATC CCCNGNNTCNACCTGGGAACTGCATCCATAACTGCCTGACTAGAGTACGGTAG AGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAAC ACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGC GTGGGGAGCAAACAGGATTANAAACCCNNGTAGTCCGGCTGACTGACTTGGAC ACCGAACATCTCGTATGCCGTCTTCTGCTTGANANAANTTCANGAAAGTCNNTC NTTCCNTTGCCATCNNNNNANNCTTCATTGATNANNTCCTGCAATTCTGCTTCN GTTGGGTTTTGTCCNANCGACCTCATAACGGTGCCNNGTTCCNTTGTGGTGATG GTGCCNTCCCCNTCCNTGTCNAANNNTGAAAANNNNCTTTGAACTCTGCANTC NCCTCTNCTGTCAGTTGATCTGCCNTATGTATATNTNCNTNNNNNNNGTTANANN AAATTANNNNTANAGGGNAATTGTTATCCGCTCACAATTCNNCNNNNTG
3	*Klebsiella*	NNNNNNNNNNNCTGNGCNNNNNCGCACGCAGGCGGTCTGTCAAGTCGGATGT GAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTC TTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGG AGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGC GAAAGCGTGGGGAGCAAACAGGATTAGAAACCCCNGTAGTCCGGCTGACTGAC TGTTACGCATTACATCTCGTATGCCGTCTTCTGCTTGANNNNNTNNCGNANGTC TGTCNNTNNNTTGNNNNNNNNNTNNNNNTCNTTGATCCNGNCCTGCTGTTNNN NTTGNNTTGGNGNCNNNNNNANGCNNNNNNNNTNCCNNTGCNNNNTNNNNNNNT GGTGATNNNGCCNNCCNANTCNNANNNNGANAGTGANNTANNNNNNNNNTGAA NTCTGCNNTCNGGTCTGCNGNNNNNTTGATCTGNNNNTNNGTNTTTCTNCNNTCT AANNNNNNNNNNNNNNNNNNNGNANTNGGNGANNTGNTATCNNGNNNNNNN TTNNNNNNNNGNNNNANNANNNAA
4	*Enterobacter*	NNNCGGANNCTGNGCGTAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAA ATCCCCNGGNTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTG TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGG AATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAA AGCGTGGGGAGCAAACAGGATTAGAAACCCNNGTAGTCCGGCTGACTGACTAG ATAGGACAGGATCTCGTATGCCGTCTTCTGCTTGANTTCAGGAAAGTCAATCGT TCCATTGCCATCANCATCNNNNTCATTGATCATATCCTGCAATTCTGCTTCCGTT GGGTTTTGTCCAAGCGACCTCATAACGGTGCCNAGTTCCTTTGTGGTGATGGTG CCGTCCCCATCCTTGTCNANNGTGAAAAAGCTTCTTTGAACTCTGCAATCGCCT CTTCTGTCAGTTGATCTGCCATATGTATATCTCCTTCTTAAAGTTNNACAAAATT ATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCNCT
5	*Klebsiella*	CGNANNCTGGGCGTAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATC CCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAG AGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAAT ACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGC GTGGGGAGCAAACAGGATTANAAACCCCNGTAGTCCGGCTGACTGACTTGTGA GCACGGTATCTCGTATGCCGTCTTCTGCNNNNNNNNTTNNNANNANGGNANCTC GNATGCCNNCTTNNGNNNNNNNNNNGGANNNNNNNNNNANTANGACNNNNNNNN

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#	Genus	Sequence (5' → 3')
6	*Bacillus cereus*	NNNNNNNTTGGGCGTAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAG CCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAA GAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAA CACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAG CGTGGGGAGCAAACAGGATTANANACCCCGGTAGTCCGGCTGACTGACTAAGC CTACACGTATCTCGTATGCCNCNNTCTGCTTGANNNNNNNCNNNTCTCGNNNG CCNNCTTCTGCNNNNNNNNGNNNNNNNNNNNNNNNNNCNNNNNNNNNNNNTNN
7	*Curtobacterium*	NNNNCGGNNNTTGNGCGTAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGA AATCCCGANGNTCNACCTCGGGCTTGCAGTGGGTACGGGCAGACTAGAGTGCG GTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAG GAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGA AAGCGTGGGGAGCGAACAGGATTANAAACCCNNGTAGTCCGGCTGACTGACTC CATAATCCGTAATCTCGTATGCCGTCTTCTGCTTGANNNNNNNNNNNNNNNNNN NNNNNNNANNCNNNNTTNTNGANNNNNNGNNNNTNTNNNAATTANNNNNNNN NNGNGNTCTNTAANTNNNNNCCNNAAAANNANNGGGNNNNNGNANNNCNNG NNGNNGANANCNACNTCGNTCNNNNNNNNTGNNNNNNNNNCNTNNTNANANNN NNNNNNNNNNNNGNGATCAGATNTNNNNNNNNNTCNCNNNNNATNNANNGN GAGANACNGNNAA

The base call **"N"** represents overlaps in the trace, where more than one base was identified. Values of "N" are automatically excluded in BLAST.

Chapter 4 Supplemental Information
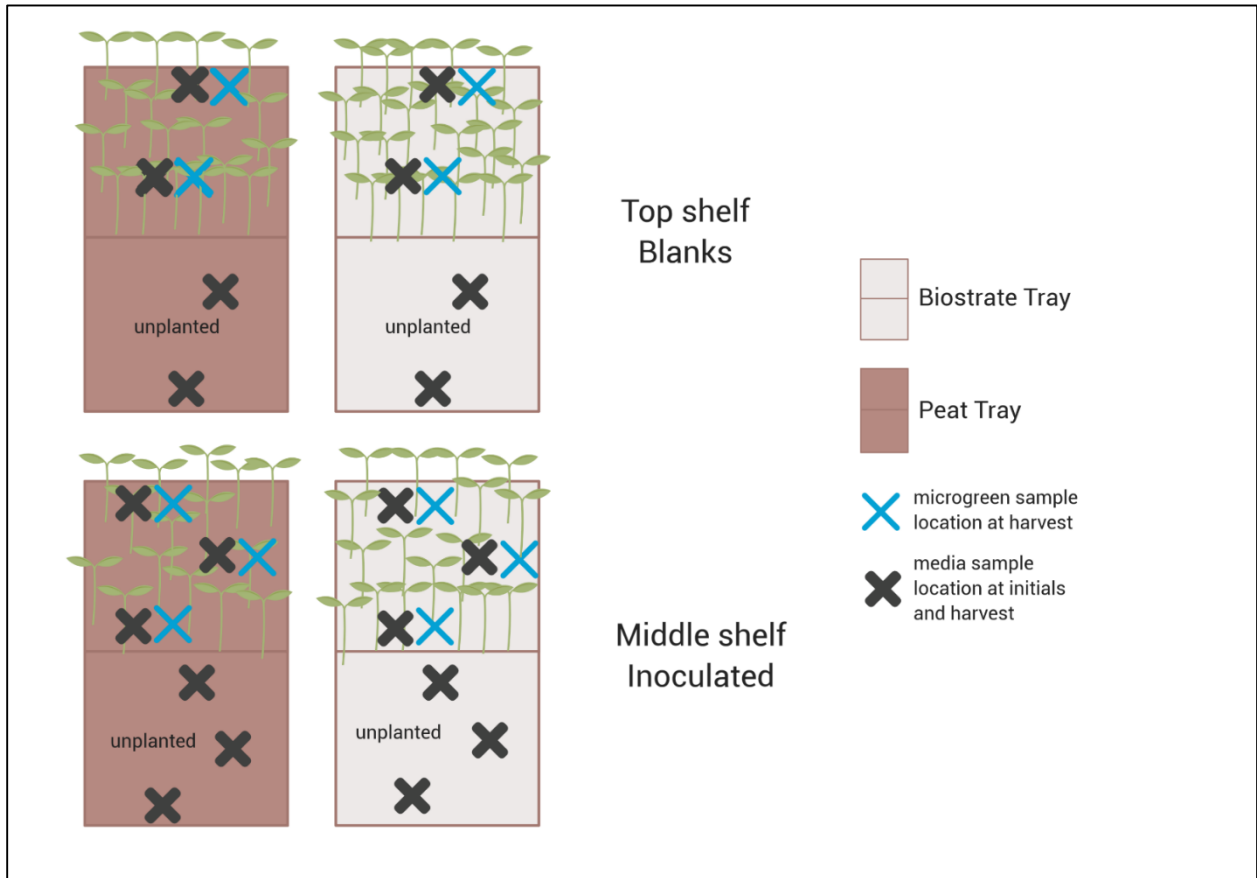
Figure 4-S1. Microgreen Tray Sampling Diagram. For each X, a 2.5 cm^2 sample of Biostrate and a 5-mL sample of peat was taken. Sampling at harvest was performed the same way, but sampled next to the previous sampling location.

Table 4-S1. *Salmonella* **Javiana Microgreen p-values**.

Comparison	Estimate	CI Low	CI High	p-value
Peat vs. Biostrate	-1.87	-4.07	0.32	0.0769

The "Estimate" is the difference between the change in pathogen level between inoculation and harvest (an interaction effect). Negative values indicate that the change in pathogen level of the first variable is smaller than for the second variable. Positive values indicate that the change in pathogen level of the first variable is larger than for the second variable.

Table 4-S2. *Salmonella* **Javiana SFGM p-values**

Comparison		Estimate	Low CI	High CI	p-value
First Variable	*Second Variable*				
Unplanted	Planted	-0.98	-1.56	-0.40	0.00466
Peat	Biostrate	-3.05	-3.63	-2.47	0.00000
Unplanted:Biostrate	**Planted:Biostrate**	**-1.40**	**-2.54**	**-0.25**	**0.01873**
Planted:Peat	**Planted:Biostrate**	**-3.47**	**-4.61**	**-2.32**	**0.00005**
Unplanted:Peat	Planted:Biostrate	-4.03	-5.17	-2.89	0.00002
Planted:Peat	Unplanted:Biostrate	-2.07	-3.21	-0.93	0.00181
Unplanted:Peat	**Unplanted:Biostrate**	**-2.63**	**-3.77**	**-1.49**	**0.00036**
Unplanted:Peat	**Planted:Peat**	**-0.56**	**-1.70**	**0.58**	**0.44130**

The "Estimate" is the difference between the change in pathogen level between inoculation and harvest (an interaction effect). Negative values indicate that the change in pathogen level of the first variable is smaller than for the second variable. Positive values indicate that the change in pathogen level of the first variable is larger than for the second variable.

Table 4-S3. *Salmonella* **means and standard deviations**

Time	Media	Condition	Mean log CFU/g	Standard Dev
Initial	Biostrate	Planted	5.58	0.29
Initial	Peat	Planted	5.57	0.41
Initial	Biostrate	Unplanted	6.20	0.10
Initial	Peat	Unplanted	5.25	0.72
Harvest	Biostrate	Microgreen	5.45	0.81
Harvest	Peat	Microgreen	3.58	1.10
Harvest	Biostrate	Planted	8.27	0.22
Harvest	Peat	Planted	4.80	0.65
Harvest	Biostrate	Unplanted	7.50	0.20
Harvest	Peat	Unplanted	3.91	0.37

Table 4-S4. *Listeria* **Microgreen p-values**

Comparison	Estimate	CI Low	CI High	p-value
Peat vs**.** Biostrate	-1.95	-5.09	1.19	0.1603

The "Estimate" is the difference between the change in pathogen level between inoculation and harvest (an interaction effect). Negative values indicate that the change in pathogen level of the first variable is smaller than for the second variable. Positive values indicate that the change in pathogen level of the first variable is larger than for the second variable.

Table 4-S5. *Listeria* **SFGM p-values**

Comparison		Estimate	CI Low	CI High	p-value
First Variable	*Second Variable*				
Unplanted	Planted	-1.93	-3.27	-0.60	0.010
Peat	Biostrate	-1.57	-2.90	-0.23	0.027
Unplanted:Biostrate	Planted:Biostrate	-2.48	-5.10	0.14	0.064
Planted:Peat	Planted:Biostrate	-2.12	-4.74	0.50	0.119
Unplanted:Peat	Planted:Biostrate	-3.50	-6.12	-0.88	0.012
Planted:Peat	Unplanted:Biostrate	0.36	-2.26	2.99	0.969
Unplanted:Peat	Unplanted:Biostrate	-1.02	-3.64	1.60	0.620
Unplanted:Peat	Planted:Peat	-1.38	-4.00	1.24	0.389

The "Estimate" is the difference between the change in pathogen level between inoculation and harvest (an interaction effect). Negative values indicate that the change in pathogen level of the first variable is smaller than for the second variable. Positive values indicate that the change in pathogen level of the first variable is larger than for the second variable.

Table 4-S6. *Listeria* **means and standard deviations**

Time	Media	Condition	Mean log CFU/g	Standard Dev
Initial	Biostrate	Planted	6.20	0.67
Initial	Peat	Planted	5.73	0.50
Initial	Biostrate	Unplanted	6.62	0.41
Initial	Peat	Unplanted	4.80	0.60
Harvest	Biostrate	Microgreen	4.21	1.84
Harvest	Peat	Microgreen	2.27	0.67
Harvest	Biostrate	Planted	6.43	0.18
Harvest	Peat	Planted	3.84	1.16
Harvest	Biostrate	Unplanted	4.36	0.33
Harvest	Peat	Unplanted	1.53	1.33

Table 4-S7. Between-Pathogen Microgreen p-values

Comparison	Estimate	CI Low	CI High	p-value
Salmonella-Listeria	1.28	-0.32	2.87	0.1017
Peat-Biostrate	-1.91	-3.50	-0.32	0.0244
Salmonella:Biostrate-*Listeria*:Biostrate	1.24	-1.89	4.37	0.6040
Listeria:Peat-*Listeria*:Biostrate	-1.95	-5.07	1.18	0.2660
Salmonella:Peat-*Listeria*:Biostrate	-0.63	-3.76	2.49	0.9125
Listeria:Peat-*Salmonella*:Biostrate	-3.19	-6.31	-0.06	0.0458
Salmonella:Peat-*Salmonella*:Biostrate	-1.87	-5.00	1.25	0.2922
Salmonella:Peat-*Listeria*:Peat	1.31	-1.81	4.44	0.5634

Table 4-S8. Between-Pathogen SFGM p-values

Comparison		Est.	CI Low	CI Hi	p-value
First variable	*Second variable*				
Salmonella	*Listeria*	2.27	1.6	2.94	0.0000
Unplanted	Planted	-1.46	-2.12	-0.79	0.0003
Peat	Biostrate	-2.31	-2.98	-1.64	0.0000
Salmonella:Planted	*Listeria*:Planted	1.8	0.52	3.07	0.0049
Listeria:Unplanted	*Listeria*:Planted	-1.93	-3.21	-0.65	0.0026
Salmonella:Unplanted	*Listeria*:Planted	0.82	-0.46	2.09	0.2955
Listeria:Unplanted	*Salmonella*:Planted	-3.73	-5	-2.45	0.0000
Salmonella:Unplanted	*Salmonella*:Planted	-0.98	-2.26	0.3	0.1673
Salmonella:Unplanted	*Listeria*:Unplanted	2.75	1.47	4.03	0.0001
Salmonella:Biostrate	*Listeria*:Biostrate	3.01	1.74	4.29	0.0000
Listeria:Peat	*Listeria*:Biostrate	-1.57	-2.84	-0.29	0.0139
Salmonella:Peat	*Listeria*:Biostrate	-0.04	-1.31	1.24	0.9998
Listeria:Peat	*Salmonella*:Biostrate	-4.58	-5.86	-3.3	0.0000
Salmonella:Peat	*Salmonella*:Biostrate	-3.05	-4.33	-1.77	0.0000
Salmonella:Peat	*Listeria*:Peat	1.53	0.25	2.81	0.0163
Unplanted:Biostrate	Planted:Biostrate	-1.94	-3.22	-0.66	0.0025
Planted:Peat	Planted:Biostrate	-2.79	-4.07	-1.51	0.0001
Unplanted:Peat	Planted:Biostrate	-3.76	-5.04	-2.49	0.0000
Planted:Peat	Unplanted:Biostrate	-0.85	-2.13	0.42	0.2629
Unplanted:Peat	Unplanted:Biostrate	-1.82	-3.1	-0.55	0.0043
Unplanted:Peat	Planted:Peat	-0.97	-2.25	0.31	0.1719
Salmonella:Planted:Biostrate	*Listeria*:Planted:Biostrate	2.47	0.29	4.66	0.0211
Listeria:Unplanted:Biostrate	*Listeria*:Planted:Biostrate	-2.48	-4.67	-0.3	0.0204
Salmonella:Unplanted:Biostrate	*Listeria*:Planted:Biostrate	1.08	-1.11	3.26	0.6857
Listeria:Planted:Peat	*Listeria*:Planted:Biostrate	-2.12	-4.3	0.07	0.0612
Salmonella:Planted:Peat	*Listeria*:Planted:Biostrate	-0.99	-3.18	1.19	0.7574
Listeria:Unplanted:Peat	*Listeria*:Planted:Biostrate	-3.5	-5.68	-1.31	0.0009
Salmonella:Unplanted:Peat	*Listeria*:Planted:Biostrate	-1.56	-3.74	0.63	0.2758
Listeria:Unplanted:Biostrate	*Salmonella*:Planted:Biostrate	-4.95	-7.14	-2.77	0.0000
Salmonella:Unplanted:Biostrate	*Salmonella*:Planted:Biostrate	-1.4	-3.58	0.79	0.3947
Listeria:Planted:Peat	*Salmonella*:Planted:Biostrate	-4.59	-6.77	-2.4	0.0000
Salmonella:Planted:Peat	*Salmonella*:Planted:Biostrate	-3.47	-5.65	-1.28	0.0010

Comparison		Est.	CI Low	CI Hi	p-value
First variable	*Second variable*				
Listeria:Unplanted:Peat	*Salmonella*:Planted:Biostrate	-5.97	-8.15	-3.78	0.0000
Salmonella:Unplanted:Peat	*Salmonella*:Planted:Biostrate	-4.03	-6.21	-1.84	0.0002
Salmonella:Unplanted:Biostrate	*Listeria*:Unplanted:Biostrate	3.56	1.37	5.74	0.0008
Listeria:Planted:Peat	*Listeria*:Unplanted:Biostrate	0.36	-1.82	2.55	0.9987
Salmonella:Planted:Peat	*Listeria*:Unplanted:Biostrate	1.49	-0.7	3.67	0.3240
Listeria:Unplanted:Peat	*Listeria*:Unplanted:Biostrate	-1.02	-3.2	1.17	0.7387
Salmonella:Unplanted:Peat	*Listeria*:Unplanted:Biostrate	0.92	-1.26	3.11	0.8145
Listeria:Planted:Peat	*Salmonella*:Unplanted:Biostrate	-3.19	-5.38	-1.01	0.0023
Salmonella:Planted:Peat	*Salmonella*:Unplanted:Biostrate	-2.07	-4.26	0.12	0.0702
Listeria:Unplanted:Peat	*Salmonella*:Unplanted:Biostrate	-4.57	-6.76	-2.39	0.0000
Salmonella:Unplanted:Peat	*Salmonella*:Unplanted:Biostrate	-2.63	-4.82	-0.45	0.0129
Salmonella:Planted:Peat	*Listeria*:Planted:Peat	1.12	-1.06	3.31	0.6421
Listeria:Unplanted:Peat	*Listeria*:Planted:Peat	-1.38	-3.57	0.8	0.4066
Salmonella:Unplanted:Peat	*Listeria*:Planted:Peat	0.56	-1.63	2.75	0.9832
Listeria:Unplanted:Peat	*Salmonella*:Planted:Peat	-2.5	-4.69	-0.32	0.0191
Salmonella:Unplanted:Peat	*Salmonella*:Planted:Peat	-0.56	-2.75	1.62	0.9827
Salmonella:Unplanted:Peat	*Listeria*:Unplanted:Peat	1.94	-0.24	4.13	0.1015

The column "Est." is the difference between the change in pathogen level between inoculation and harvest (an interaction effect). Negative values indicate that the change in pathogen level of the first variable is smaller than for the second variable. Positive values indicate that the change in pathogen level of the first variable is larger than for the second variable. CI = confidence interval.

IV.   **R Code and Raw Data Repository Location**

The raw data and R code for plots and statistical analysis can be found at the following public

Github repository: https://github.com/ginamariemisra/mastersthesis