Evaluating the Cross-Contamination Risks of *Salmonella* and Generic *Escherichia coli* on Agricultural Ground Covers in Produce Pre-Harvest Production

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Scientific Abstract

The US Food and Drug Administration Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) prohibits the harvest of dropped fruits and vegetables due to potential microbial contamination. Under the FSMA PSR dropped produce includes (i) produce that has detached from the parent plant and unintentionally contacts the ground and (ii) produce that is attached to the parent plant and unintentionally contacts the ground. Due to the benefits of plant growth and pest management, agricultural ground covers are a common horticultural practice implemented in the fresh produce production environment and produce may come into contact with these ground covers. Thus, this thesis aims to (i) quantify the survival of bacteria on different agricultural ground cover types and in different production environments and (ii) evaluate the cross-contamination risk of mulch to fresh produce from different drop heights and contact times. A seven-strain *Salmonella* cocktail was spot inoculated on coupons of biodegradable mulch, landscape fabric, and plastic mulch, and held in a growth chamber (23°C, 55% RH). At 0, 0.06, 0.17, 1, 2, 3, 5, 7, 30, 60, 90, and 140 days post-inoculation (dpi), coupons were enumerated for *Salmonella*. Coupons of plastic mulch were also spot inoculated with a green-fluorescent protein-tagged generic *Escherichia coli* and held in a growth chamber, greenhouse, and field environment for enumeration at 0, 0.06, 0.17, 0.41, 1, 2, 3, 5, and 7 dpi. Fresh cucumber, jalapeño, and tomato were dropped from 0, 1, 2, 4, and 6 ft using height-modified PVC (polyvinyl chloride) pipes onto generic *E. coli* inoculated plastic mulch, as well as tomato onto inoculated biodegradable mulch. Produce samples were enumerated after 3 s of

mulch contact. Fresh cucumber, jalapeño, and tomato were also grown in contact with generic *E. coli* inoculated plastic mulch for 0, 1, 3, 5, and 7 days post-placement in the field. *Salmonella* survived on all ground covers for up to 140 dpi in the growth chamber. From 0 to 30 dpi, biodegradable mulch had the lowest *Salmonella* reduction, followed by landscape fabric and then plastic mulch ($P < 0.05$). No significant differences in ground cover type and *Salmonella* reduction were observed at 90 dpi ($P > 0.05$). Plastic mulch had the highest reduction of generic *E. coli* in the field followed by the greenhouse and growth chamber over 7 dpi ($P < 0.05$) with field and greenhouse coupons achieving approximately a 6-log reduction by 0.17 and 7 dpi, respectively. Ground cover type and environment impacted bacterial survival and highlighted the importance of growing environment in risk management. Cucumber and tomato samples dropped from 4 (33%; 17%) and 6 (100%; 43%) ft were damaged, respectively. In general, generic *E. coli* transferred to the tested commodities regardless of drop height or contact time. These findings support that dropped produce should not be harvested due to potential damage and when surfaces were contaminated, transfer was likely to occur. Similarly, if surfaces were contaminated, regardless of contact time (0, 1, 3, 5, and 7 d), transfer was likely to occur indicating cross-contamination poses a food safety risk despite unintentional or intentional ground contact. Food safety efforts should focus on minimizing visible contamination, as outlined in the FSMA PSR, that may contaminate fresh produce in the production environment. Growing produce in contact with the ground alone may not be the sole factor in the contamination of fresh produce, as a contamination event is needed.

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General Audience Abstract

The US Food and Drug Administration Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) is a federal regulation that sets science-based standards for the safe production, harvest, and handling of fresh fruits and vegetables. Within the harvesting requirements of the PSR, produce that drops to the ground cannot be harvested because of potential microbial contamination. Dropped produce includes (i) detached from the plant and touching the ground and (ii) attached to the plant and touching the ground. The ground can include different surfaces including agricultural ground covers, a common horticultural practice that increases plant growth and decreases pests. This thesis aims to evaluate the food safety risk of (i) bacteria on different ground covers and in different growing environments and (ii) fresh produce contacting the ground while detached (i.e., drop) and attached to the plant. Three ground cover types, biodegradable mulch, landscape fabric, and plastic mulch, were inoculated with *Salmonella* and held in a growth chamber with moderate conditions (23°C, 55% RH). At 0, 0.06, 0.17, 1, 2, 3, 5, 7, 30, 60, 90, and 140 days, sample coupons were evaluated for *Salmonella* counts. Plastic mulch was inoculated with green-fluorescent protein-tagged generic *Escherichia coli (E. coli)* and placed in a growth chamber, greenhouse, and field for 0, 0.06, 0.17, 0.41, 1, 2, 3, 5, and 7 days to investigate survival. At the selected time-points, generic *E. coli* counts were evaluated on plastic mulch. Fresh cucumber, jalapeño, and tomato were either dropped through height-modified PVC (polyvinyl chloride) pipes at 0, 1, 2, 4, and 6 ft onto generic *E. coli* inoculated plastic mulch and only tomato onto generic *E. coli* inoculated biodegradable mulch

(detached produce), or grown in contact with generic *E. coli* inoculated plastic mulch in the field (attached produce) for 0, 1, 3, 5, and 7 days. *Salmonella* was present on all ground covers for up to 140 d in the growth chamber. From 0 to 30 d, biodegradable mulch had the highest concentration of *Salmonella*, followed by landscape fabric and then plastic mulch; however, no differences in material were observed at 90 d. Plastic mulch had the lowest concentration of generic *E. coli* in the field followed by the greenhouse and growth chamber over 7 d. Ground cover types (biodegradable mulch, landscape fabric, and plastic mulch) and growing environment (field, greenhouse, and growth chamber) influenced bacterial survival and should be considered in food safety management and assessment of preharvest contamination risks. Cucumber and tomato samples dropped from 4 and 6 ft were often damaged, compared to lower drop heights. Bacteria transferred to cucumber, jalapeño, and tomato regardless of drop height and contact time. Dropped produce should not be harvested due to damage and likelihood of cross-contamination. Contamination was not influenced by contact time confirming fresh produce in contact with a contaminated source is likely to become contaminated despite unintentional or intentional ground contact. Thus, food safety efforts should focus on minimizing visible contamination on surfaces that may contact fresh produce in the growing environment.

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Dedication

I dedicate this work to my family for their unconditional and unwavering love, support, and encouragement. Thank you for continually setting high standards of success to show me the limits are endless.

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Chapter 1: Introduction

In 2015, the final rule of the US FDA Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) set the first science-based mandatory standards for fruits and vegetables produced for human consumption (16). Fresh fruits and vegetables that are not on the rarely consumed raw list (34 commodities) are covered by the rule (14). Subpart K of the FSMA PSR prohibits the harvesting of dropped fresh produce (14). The term dropped is defined as produce that unintentionally drops to the ground before harvest (14). The FDA has clarified that fresh produce (i) detached from the parent plant and unintentionally contacting the ground and (ii) attached to the parent plant and unintentionally contacting the ground is classified as dropped produce and thus unacceptable to harvest (13, 15). There are examples of produce not considered a drop described in the PSR including root crops that grow underground (such as carrots), crops that grow on the ground (such as cantaloupe), or produce that is intentionally dropped to the ground as part of harvesting (14). For the purposes of this research thesis, produce still attached to the parent plant contacting the ground is classified as a droop. However, drooped produce is not used in the PSR and additional PSR guidance.

Dropped produce is prohibited from harvest because of the potential increased risk of microbial contamination from damage that may occur from the drop (physical action of produce falling to the ground) (13). The exclusion of drooped produce (produce still attached to the parent plant with no physical action of produce falling to the ground) indicates that ground contact alone, regardless of damage, poses a potential food safety risk. Previous studies have suggested that bacteria can transfer to produce from ground contact and may contain higher amounts of bacteria, compared to non-dropped, undamaged, or non-ground contacted produce, but are limited by ground type, commodity, and applicability (1, 3, 4, 7, 9, 10, 12). “Ground” has

not been defined by the FSMA PSR and could include different ground surfaces depending on the horticultural practices implemented by produce growers. A common horticultural practice is mulching with synthetic agricultural ground covers. Ground covers have beneficial effects on plant growth and management, including increased soil temperature, moisture conservation, earlier production, greater yields, and increased crop quality (5, 6, 8, 11) and are typically laid over fruit and vegetable planting beds with plants staggered in intentional openings throughout the planting row. Since ground covers are close to the plant, dropped or drooped produce may contact these surfaces; however, research on the cross-contamination risk of ground covers is limited.

In 2020, the Northeast Center to Advance Food Safety unpublished survey found that handling dropped produce was a commonly misunderstood practice (2). There is a lack of research on the microbiological risks of harvesting dropped and drooped fresh produce that encompasses different commercial ground cover types and fresh produce commodities. Understanding the microbiological risks of such produce may better inform on-farm risks regarding harvesting practices and clarify the harvesting requirements of the PSR. Due to limited research, the risk of contamination between dropped and drooped produce or assessment of the risk posed by ground covers cannot yet be concluded. Risks associated with dropped produce need to further be elucidated to determine appropriate handling practices.

Therefore, the objective of this thesis is to evaluate (i) the survival of *Salmonella* on different ground covers (biodegradable mulch, landscape fabric, and plastic mulch) over 140 days in a growth chamber, (ii) the survival of generic *Escherichia coli* on plastic mulch in different growing environments (growth chamber, greenhouse, and field) over 7 days, (iii) the impact of drop height (0, 1, 2, 4, and 6 ft) on transfer of generic *Escherichia coli* to fresh

cucumbers, jalapeños, and tomatoes, and (iv) the impact of contact time (0, 1, 3, 5, and 7 d) on the transfer of generic *Escherichia coli* to fresh cucumbers, jalapeños, and tomatoes.

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Chapter 2: Review of Literature

Pre-Harvest Microbiological Hazards

Contaminated fresh produce has been linked to foodborne illness outbreaks. From 1998 to 2016, 30 to 60 single and multistate outbreaks occurred sickening 900 to 3,000 people annually with 46 percent of foodborne illnesses associated with fresh produce (37, 55). Foodborne illness outbreaks due to produce have resulted in more illnesses than any other food category (37). These produce-associated outbreaks have been attributed to pathogenic *Escherichia coli*, *Salmonella*, Norovirus, and *Listeria monocytogenes*, as well as, *Vibrio, Shigella, Cryptosporidium, Giardia, Cyclospora, Toxoplasma gondii*, and Hepatitis A virus (37). More recently, from 2010 to 2017, 85 multistate fresh produce outbreaks occurred with Hepatitis A and *Cyclospora cayetanensis* accounting for only 2 of these outbreaks, while the remaining were attributed to pathogenic bacteria: *S. enterica* (67.5%), *E. coli* (27.4%), and *L. monocytogenes* (4.8%) (11).

While contamination can occur at any point throughout the various steps of the farm-to-fork chain, potential sources across all commodities include growing, harvesting, packing, and holding (60). At the farm level, common routes of fresh produce contamination include water, soil amendments, animals, worker health and hygiene, and equipment and buildings (60). Food safety prevention practices need to be applied at all points of fresh produce production; however, the importance of focusing on pre-harvest hazards is emphasized, as produce contaminated in the first step of the chain is unlikely to be inactivated at a later point (1). Although there are several routes for pre-harvest contamination, contaminated water and soil are the most predominant sources of pathogenic contamination (2). Pre-harvest risk may fluctuate depending on the production environment and practices (e.g., greenhouse, open field, aquaponics, hydroponics).

Pinpointing one produce commodity to blame for the foodborne illness outbreaks is challenging, as poor agricultural and manufacturing practices can cause potential contamination and illness in any commodity (60). Low and high-risk fresh produce share many of the same characteristics, therefore, factors contributing to foodborne illness contamination include agricultural practices and handling during growing, harvesting, and postharvest, physical characteristics of the crop, consumer and retail handling practices, and rates of consumption (60). Fresh produce poses a substantial public health risk, as it is normally consumed in its raw state with no processing steps to reduce the microbial load and eliminate microbial hazards. The lack of treatment or kill step in fresh produce is attributed to the high rate of foodborne illness outbreaks and illnesses.

Due to the lack of processing treatments, pathogenic bacterial contamination (e.g., *Salmonella*, *E. coli*, *L. monocytogenes)* is one of the risks associated with fresh produce (36). Specifically, *Salmonella* is naturally found in the gastrointestinal tracts of animals, birds, reptiles, and humans, and can contaminate the pre-harvest environment by animal or human feces (58). *Salmonella* is a gram-negative, non-spore-forming, facultative anaerobe belonging to the bacterium family *Enterobacteriaceae* (58). When ingested, even in low doses, the infected person may experience salmonellosis including diarrhea, fever, and stomach cramps with onset between six hours to six days and symptoms lasting up to seven days, on average (20, 58). While *Salmonella* prefers conditions of 37°C and a pH of 6.5 to 7.5, select strains may grow between temperatures of 2°C and 54°C and pH of 4.0 to 9.5 with survival even shown in frozen conditions for greater than a year (40, 42). Additionally, *Salmonella* requires 0.93 water activity to grow, however, it has been shown to survive in as little as 0.18 (38). The frequency of outbreaks is a result of *Salmonella's* hardiness (40).

However, detection of pathogenic bacterial contamination may be difficult due to low population levels, prevalence, and non-uniform distribution of pathogens in the environment (35). Instead, nonpathogenic indicators and or index organisms are often utilized to evaluate the presence of potential pathogen contamination (9). The presence of indicator or index organisms does not confirm pathogen contamination has occurred but indicates the conditions may be favorable for growth or survival in the environment. Generic *E. coli* is used as an indicator of fecal contamination or unsanitary conditions (63). Therefore, generic *E. coli* is often used in research as a surrogate for foodborne pathogens, as it is unsafe to introduce pathogens into the environment and requires strict protocols to do so properly.

In an effort to reduce potential microbial hazards, numerous produce food safety programs have been developed. Good Agricultural Practices (GAP) is a voluntary program that uses third-party audits to verify food safety practices during the production, packaging, handling, and storing of fruits and vegetables. Additionally, other programs focus on the safety of specific commodities, such as the Tomato Good Agricultural Practices (T-GAP) and California and Arizona Leafy Greens Marketing Agreement (LGMA). While all these programs are voluntary, the US FDA Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) is the first fresh produce regulation in which a set of minimum best practices are required (67).

US FDA Food Safety Modernization Act Produce Safety Rule

Signed into law in 2011, FSMA aimed to shift food safety from a reactive to a proactive approach to prevent food contamination (65). The goal of FSMA was to strengthen the food system through seven foundational rules that focus on the food system as a whole; as well as specific standards and related programs that focus on specific sectors of the industry, such as the FDA FSMA PSR. In 2016, the final rule of the PSR, Title 21 Code of Federal Regulations (CFR)

Part 112 Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption, went into effect providing the first science-based mandatory standards for fruits and vegetables grown and produced for human consumption (67).

While the PSR covers produce, only select commodities that are classified as raw agricultural commodities are regulated under this rule (59). A raw agricultural commodity is "any food in its raw or natural state, including all fruits that are washed, colored, or otherwise treated in their unpeeled natural form prior to marketing" (e.g., berries, herbs, leafy greens, legumes, melons, tree nuts, root vegetables) (64). Therefore, "produce that is rarely consumed raw [including but not limited to asparagus, beets, corn, eggplants, potatoes, and pumpkins], produce that is produced by an individual for personal consumption or produced for consumption on the farm or another farm under the same management, and any produce that is not a raw agricultural commodity" is exempt (61). Additionally, only select farms are covered by this rule depending on the average monetary value of produce, the average monetary value of the food, the percentage of sale distribution, and the miles the food travels, further explained in Title 21 CFR Part 112 Subpart A (61).

Specifically, Title 21 CFR Part 112 Subpart K, Growing, Harvesting, Packaging, and Holding Activities is of interest. Part 112.114 states that dropped covered produce cannot be distributed (61). Dropped covered produce is defined as produce that is regulated by the PSR that drops to the ground before harvest (61). Dropped produce does not include root crops that grow underground (such as carrots), crops that grow on the ground (such as cantaloupe), or produce intentionally dropped for harvest (such as almonds) (61). However, "ground" is never strictly defined causing ambiguity about what surfaces this includes (e.g., soil, plastic mulch, straw). In the FDA response to public comments regarding the reasoning for excluding dropped produce

from harvest; the FDA cited concern that produce may experience damage to the outer layer, even if this layer is not consumed, allowing access to the inner portion of the produce causing contamination, and resulting in a potential food safety risk (59).

Additionally clarified in the FDA public comment response is that "even if articles of produce are still attached to the plant when they contact the ground," it is still considered dropped produce and should not be harvested, which is reiterated in an FDA fact sheet (59, 62). This type of produce has been classified as drooped produce, although this term is never noted in the PSR or additional guidance. For this research scope, dropped produce is fully detached from the parent plant and unintentionally contacts the ground while drooped produce is still attached to the parent plant and unintentionally contacts the ground. However, both are considered dropped produce by the PSR and consequently, unacceptable produce to harvest, regardless of when the fresh produce contacts the ground (pre-harvest or during harvest). The FDA excludes dropped produce from harvest due to potential damage, but damage to drooped produce is unlikely due to continual attachment to the parent plant causing the produce to remain intact; thus, the FDA is implying ground contact alone poses a food safety risk. The contact differences between dropped and drooped produce should be further evaluated to quantify the risk associated with dropped produce.

Additional Produce Food Safety Programs and Dropped Produce

While the PSR is binding to all covered farms in the US, additional programs may be voluntary, or market driven. Several of these programs regarding fresh produce note a similar stance on contact with the ground. Specifically, USDA GAP states in their policies and procedures "microbial contamination or cross-contamination of fresh produce during pre-harvest and harvest activities may result from contact with soils... [and] may be a means where food

contamination may occur" (52). USDA Harmonized GAP and USDA Harmonized GAP Plus+ state in their standards, "product that contacts the ground shall not be harvested unless the product normally grows in contact with the ground" and written procedures should be in place for harvesting "produce that comes in contact with the soil (e.g., drops)" (53, 54). These GAP programs align with PSR as they infer that unintentional contact with the ground is considered microbial contamination and should be excluded from harvest. However, USDA GAP only states contact with soil but USDA Harmonized GAP and USDA Harmonized GAP Plus+ state contact with the soil or ground, which adds to the ambiguity of what is defined as ground contact depending on the standards abided by.

T-GAP also specifically mentions "drops" in their guidelines and audit forms stating, "tomatoes that have fallen from the plant to the ground (i.e., "drops") shall not be harvested" and "workers are trained and follow the policy that product that has dropped on the ground is discarded" (29, 30). Although drops are mentioned, "fallen from the plant to the ground" and "dropped on the ground" also create ambiguity about what classifies as a drop (30). "Fallen" may be interpreted as detached, potentially not including droop produce in harvesting exclusions.

As for the California and Arizona LGMA, the language is similar for both agreements stating, "prohibit ground or soil contact to avoid cross-contamination and minimize the potential introduction of contamination during and after harvest operations" and "discard and do not pack any lettuce/leafy greens dropped on the ground or soil during harvest" (5, 10). Both states' LGMA audits focus on minimizing potential contamination due to ground or soil contact during harvest, specifically by worker handling practices, rather than natural dropping or drooping, which is unlikely in lettuce production due to the plant-growing nature.

Dropped and Drooped Fresh Produce Food Safety Risks

As previously mentioned, the FDA excludes dropped produce from harvest due to the increased risk of microbial contamination. In response to public comments on the FSMA PSR Final Rule, the FDA cited two studies that indicated dropped and damaged produce (apples and pears) contained more bacteria than non-dropped produce, implicating damage and ground contact caused increased rates of contamination (26, 45, 59). However, the FDA acknowledged these commodities include edible outer layers; therefore, referenced commodities with outer layers not typically consumed that were implicated in previous outbreaks (i.e., mangoes, papayas, cantaloupes), providing reasoning for the inclusion of select commodities despite outer layer typical consumption (59). Additionally, two more studies cited in the FDA draft guidance for industry provided evidence that structural damage to produce (e.g., lettuce) may introduce or increase pathogen contamination (6, 7, 21). However, the studies cited by the FDA are limited in fresh produce commodity scope.

Visible or non-visible damage to fresh produce from dropping potentially introduces surface and/or internal contamination that is unlikely to be removed at any further production steps. Prior research has suggested pathogens exhibit increased persistence on injured tissues compared to non-injured (27, 32, 33, 49). It is important to note that damage and/or contamination of fresh produce in the early stages of production may cause downstream risks due to a lack of processing treatments. For example, previously contaminated produce (e.g., dropped) may contaminate water with no or ineffective sanitizer control and may cross-contaminate other produce. Additionally, fresh produce that is improperly pre-cooled, rinsed, or washed, may allow infiltration of bacteria from contaminated water into the produce through

damaged portions (possibly caused by dropping), stem scars, cracks, and crevices due to temperature differentials between water and produce (51).

In addition to contamination via damage (dropped produce), the FDA indicates ground contact may be a contamination point due to prohibiting the harvest of produce unintentionally growing in contact with the ground (drooped produce). Previous studies have suggested that tomatoes, cucumbers, and cantaloupe in contact with contaminated ground may increase the chance of fresh produce contamination due to higher microbial counts on ground contacted produce (24, 31, 41, 44). While the FDA noted cantaloupes as implicated in previous outbreaks and research may suggest increased microbial counts due to growing in contact with the ground, this commodity is excluded from the dropped produce requirement (exclusions include crops that grow on the ground); therefore, may not provide the most appropriate reasoning for excluding ground contacted produce. Research quantifying the microbial risk due to injury and ground contact of in-field dropped, drooped, and non-dropped or non-drooped produce is minimal.

Doren et al. (25) conducted a literature review on the food safety risk of harvesting dropped and drooped produce. This review highlighted several factors that previous studies suggested influence the contamination risk of ground contacted produce, including ground moisture, ground surfaces, crop features, and contact time (25). Specifically, higher moisture may create more conducive conditions for bacterial transfer and survival (50, 69). A study revealed that production systems, such as trellising, could decrease bacterial transfer levels to cucumbers, but when other factors that favored bacterial proliferation were present (e.g., increased moisture via rainfall), these benefits were eliminated (41). However, crop features (e.g., crevices, stem scars, surface characteristics) cause the extent of transfer to differ among produce commodities (25). Bare soil has been shown to have a higher reduction in pathogens

compared to mulch (e.g., straw, plastic, biodegradable, paper) with speculation that the moisture loss over a growing season in bare ground contributes to this reduction (69). Similarly, soil-contacted tomatoes have shown a similar or lower risk than plastic mulch contacted tomatoes (50). Mulch may promote the persistence of pathogens in the soil due to the creation of an ideal growth environment but may also protect produce by limiting contact with the bare soil (25).

While ground contact time may not be a large risk factor in dropped produce during harvest, the inclusion of drooped produce indicates timing may be important. Contact time effect has been minimally researched, regarding this research scope; however, various factors, such as moisture, ground type, the direction of transfer, and region may play a role (50). Bhullar et al. (8) observed no difference between percent transfer and tested contact times (5 s, 1 min, 10 min, 1 h, and 4 h) when dropping produce onto tile and carpet. Surface transfer occurred when the produce contacted contaminated flooring no matter the time of contact (8). However, this study may have limited in-field applicability for drooped produce due to differing environmental conditions and coupon surfaces and damage potentially incurred from dropping.

Produce Commodities of Interest

Except for the exclusions noted in the FSMA PSR (root crops, crops that grow on the ground, and crops intentionally dropped for harvest), most produce commodities have the potential to drop and/or droop, or unintentionally contact the ground in varying capacities. For this research thesis purpose, fresh cucumbers, jalapeño peppers, and cherry tomatoes were selected as the commodities of interest to research and evaluate the food safety risk of harvesting dropped and drooped produce. Since these produce commodities are covered by the FSMA PSR, they are commonly consumed in their raw state (61). Specifically, tomatoes are one of the most consumed vegetables, and cucumber consumption has steadily increased since the 1970s (56,

57). Additionally, small hot pepper varieties (e.g., jalapeños) provide a similar shape with a different surface texture and weight compared to cucumbers. The similarities and differences between the two commodities may help determine if other factors, such as surface texture and weight, play a role in the microbial risk of dropped and drooped fresh produce.

In the 85 multistate fresh produce outbreaks previously mentioned, cucumbers, tomatoes, and peppers were three of the ten commodities implicated, which may be due to the common raw state consumption and frequency of consumption (11). Previously, the CDC reported vine-stalk vegetables (e.g., tomatoes and cucumbers) and *Salmonella* as the third most common pathogen-commodity pair responsible for outbreak-related illnesses (22). The Eastern Shore of Virginia was implicated in four *Salmonella* outbreaks associated with tomatoes from 2000 to 2010 (31). While implicated less, cucumber outbreaks have been associated with *Salmonella* in the Delmarva Peninsula (Maryland, Delaware, and Virginia) (4). Hot peppers are the primary cause of pepper outbreaks and have been implicated in several cases from 2008 to 2016 (11, 34).

Another FDA FSMA rule, the Food Traceability Rule, will be enacted in 2026 (66). This rule features a list of foods that require additional traceability records due to an evaluation and ranking of the foods' potential hazards (66). Currently, fresh cucumbers, peppers, and tomatoes are included in the Food Traceability Rule, as they have exceeded the risk score threshold (66). Table 1 specifically highlights CDC outbreak investigations of the fresh produce commodities of interest implicated in *Salmonella* foodborne illness outbreaks (2006-2023). Overall, being covered by the PSR, the likelihood of dropping and drooping, frequency of consumption, and previous outbreak implications have caused fresh cucumbers, peppers, and tomatoes to be commodities of interest.

Table 1. Multistate outbreak investigations led by the CDC from 2006 to 2023 associated with fresh cucumbers, peppers, and tomatoes due to *Salmonella* contamination.

Year	Commodity	Microbiological Agent	Contamination Source	Reference
2006	Tomato	*Salmonella* Newport	Unknown	(13)
	Tomato	*Salmonella* Typhimurium	Unknown	(12)
2008	Hot Peppers	*Salmonella* Saint Paul	Contaminated water	(14)
2013	Cucumber	*Salmonella* Saint Paul	Unknown	(15)
2014	Cucumber	*Salmonella* Newport	Unknown	(16)
2015	Cucumber	*Salmonella* Poona	Unknown	(18)
2016	Cucumber	*Salmonella* Oslo	Unknown	(17)
	Hot Peppers	*Salmonella* Anatum	Unknown	(19)

Relevant Horticultural Practices

Peppers, cucumbers, and tomatoes can grow without support; however, trellising is often desired for vertical growth due to the plants' growing nature. Although this horticultural practice is not required for the cultivation of these commodities, trellising can support fruit reducing disease pressure and space requirements, increase photosynthetic rates due to increased light exposure, increase yield and fruit quality, and protect from ground contact (41). While trellising and natural vertical growth of select varieties protect against ground contact by lifting the plant off the ground, drooped produce can still occur for all selected commodities, as the commodities grow and increase in weight or due to naturally low-growing fruit. However, trellising creates additional confusion regarding the PSR dropped produce. For example, cucumbers grown in the field are often not trellised but instead left to trail and grow on the ground, excluding this commodity from the dropped produce harvesting requirement according to subpart K of PSR (exclusions include crops that grow on the ground) (43). Once the cucumber plant is elevated off

the ground via trellising, fruit that unintentionally contacts the mulch would be classified as a droop and unacceptable to harvest.

Ground surfaces in fresh produce production may include ground covers, which is another horticultural practice not required but often accompanied by trellising. Plastic mulch is a common ground cover option because its benefits include higher and earlier yields, increased soil temperature, and reduced evaporation, weeds, fertilizer leaching, and soil compaction (47). Cucumbers, tomatoes, and peppers have shown significant early harvest, yield, and quality increases when grown in conjunction with plastic mulch (39). Specifically, cucumbers trellised with plastic mulch increased yields by almost one and a half times more than cucumbers not trellised with plastic mulch (43). In addition to plastic mulch, there are other commercial synthetic ground covers available that are implemented generally to achieve the same benefits as plastic mulch, but the characteristics and efficacy of benefits may differ slightly.

Landscape fabric is more durable than other mulch options, such as plastic mulch, and thus may be reused over multiple growing seasons, which decreases plastic waste from year to year (28). In addition to durability, landscape fabric is known for its permeability which allows water and gas to penetrate through the fabric (23, 28). Biodegradable mulch has become another ground cover of interest due to concerns about microplastic contamination. Unlike plastic mulch and landscape fabric, biodegradable mulch can be tilled into the soil after the growing season and broken down by the soil microbial community into carbon dioxide, water, and microbial biomass (48). If disposed of improperly, plastic mulch and landscape fabric can contaminate the environment with microplastics; however, these materials are hard to recycle due to the cost and labor required (3, 68). The lack of mechanical properties of biodegradable mulch has caused it to be utilized less than the other synthetic ground cover options (46). Because of the benefits of

ground cover use, it is a frequent agricultural practice implemented in fresh produce production, and thus dropped and drooped fruit have a high likelihood of contacting these materials.

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Chapter 3: Survival of *Salmonella* on Biodegradable Mulch, Landscape Fabric, and Plastic Mulch

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Abstract

Ground covers are an agricultural practice utilized in fresh produce production to improve plant growth and manage diseases and pests. There are several factors to consider when selecting an appropriate commercial ground cover; however, food safety, specifically the survival of foodborne pathogens is not currently included in these factors. Fresh produce may contact ground covers in the production environment, but limited research exists on the fate of foodborne pathogens on these surfaces over time. This research was conducted to evaluate the survival of *Salmonella* on biodegradable mulch, landscape fabric, and plastic mulch ground covers. New rolls of each ground cover were obtained and constructed to fit a 100x15 mm Petri dish. Ground cover coupons were spot inoculated with 100-µl of a diluted seven-strain *Salmonella* cocktail to achieve ca. 6 log CFU/cm^2. Coupons were held in a climate-controlled growth chamber (23°C, 55% RH) and enumerated for *Salmonella* at 0, 0.06, 0.17, 1, 2, 3, 5, 7, 30, 60, 90, and 140 days post-inoculation (dpi). If counts fell below the limit of detection (< 0.12 log CFU/cm^2), enrichments were performed using the FDA BAM *Salmonella* protocol. *Salmonella* survived up to 140 dpi on all tested ground covers, with a >5 log reduction. *Salmonella* was detected highest on landscape fabric (83% of coupons), compared to biodegradable mulch (13%) and plastic mulch (50%) ($P < 0.05$). From 0 to 30 dpi, biodegradable mulch (2.47 ± 0.26 log CFU/cm^2) had the lowest reduction, followed by landscape fabric (3.07 ± 0.30 log CFU/cm^2) and plastic mulch (3.86 ± 0.72 log CFU/cm^2). At 60 dpi, reduction stabilized across all materials (ca. 4 log CFU/cm^2 reduction), and at 90 dpi, no significant difference in *Salmonella* reduction was observed between each ground cover type ($P > 0.05$). No *Salmonella* growth was observed on any of the ground covers tested. While findings

showed *Salmonella* survival differed on ground covers in the short term (0-30 dpi), >5 log *Salmonella* reductions were observed long term.

Highlights

- *Salmonella* survival was significantly different on tested ground cover types.
- Biodegradable mulch had the lowest reduction of *Salmonella* from 0 to 30 dpi.
- Landscape fabric had the lowest reduction of *Salmonella* at 140 dpi.
- *Salmonella* survived on all ground cover types for up to 140 dpi.
- By 140 dpi, *Salmonella* reduction was >5 log on all ground cover types.

Mulching is an agricultural practice used as a soil protective covering to control pests, increase soil temperature, conserve moisture, contribute to earlier production, greater yields, and increase crop quality (11, 21). While the level of these benefits may vary based on the ground cover, synthetic mulches are utilized for the same purpose. Biodegradable mulch, plastic mulch, and landscape fabric are some synthetic mulch options used in fruit and vegetable production. Commonly, these mulches are purchased in a continuous sheet and laid over planting beds secured with soil or stakes. Plants are staggered in punctured holes throughout the ground cover. As the plant grows, it may be intentionally (e.g., strawberry) or unintentionally (e.g., staked tomato) grown on the ground covers.

While ground covers generally provide similar agricultural benefits, there are practical advantages and disadvantages (e.g., availability, durability, price) of the various available types (10). Historically, non-degradable plastic mulches (e.g., landscape fabric, plastic mulch) were primarily used due to their high stability and lower cost; however, these mulches are difficult to recycle, making them costly and labor-intensive to dispose of (2, 27). Further, if disposed of improperly, non-degradable plastic mulches can contaminate the environment with microplastics (2, 27). Concerns of microplastic contamination have resulted in a shift in interest toward alternative biodegradable mulch options (12). Biodegradable mulch can be broken down by soil microorganisms into carbon dioxide, water, and microbial biomass, but these mulches are used less frequently due to a lack of mechanical properties (e.g., more delicate) (20, 22).

Food safety is often not a consideration when selecting a mulch partially due to limited research on the fate of foodborne pathogens on ground covers. Depending on the crop, the plant may be intentionally grown in contact with the ground cover (e.g., cantaloupe, strawberry) or may unintentionally contact the ground cover as weight increases or due to low-growing fruit

(e.g., staked tomato, pepper). Previous research has indicated that contaminated plastic mulch can transfer bacteria to fresh cucumber and tomato fruit (14, 16, 25). Additional research has been conducted on the microbial activity of soil under mulch but not on the mulch surface (4, 13, 18). Specifically, foodborne pathogens (i.e., *Escherichia coli, Salmonella, Listeria Monocytogenes*) have exhibited increased persistence in soil under mulch compared to bare soil indicating ground covers may alter produce production food safety risk (13). Therefore, this study sought to evaluate the survival of *Salmonella* on three ground covers (biodegradable mulch, landscape fabric, and plastic mulch) over 140 d at 12 time-points under growth chamber conditions of 23°C and 55% RH.

Materials and Methods

Materials

Three different industry-relevant ground cover types used in the field were selected: embossed white on black plastic mulch (1.25 mil, Ginegar Plastics, Inc., Santa Maria, CA, USA), biodegradable mulch (6 mil, Bio360 Mulch, Berry Hill Irrigation, Inc., Buffalo Junction, VA, USA) and landscape fabric (5 oz, Pro 5 Weed Barrier, Johnny's Selected Seeds, Winslow, ME, USA). Ground cover types were purchased new and stored until use. All ground covers were cut with sterile scissors to fit a 100x15 mm Petri dish (Corning, Inc., Corning, NY, USA). A total of 30 coupons of each ground cover type were used for each time-point of 0, 0.06, 0.17, 1, 2, 3, 5, 7, 30, 60, 90, and 140 days post-inoculation (dpi; n=1,080). All coupons beyond 0.06-dpi were held in a growth chamber (Percival E41L2, Percival Scientific Inc., Perry, IA, USA) with environmental conditions set to 23°C and 55% relative humidity. Data loggers (WatchDog B-Series Button Loggers, Spectrum Technologies, Inc., Aurora, IL, USA) were used to confirm the stability of environmental conditions for the duration of the time-points.

Selection of Salmonella Strains

A seven-strain cocktail of *Salmonella* spp. was used in this study. The serotypes studied and their sources are as follows: *Salmonella enterica* serotypes Agona (Clinical, Alfalfa Sprouts Outbreak), Enteritidis (Clinical, Peach Outbreak), Montevideo (Clinical, Tomato Outbreak), Newport (Clinical, Onion Outbreak), Newport (Environmental, Creek Sediment), Poona (Clinical, Cucumber Outbreak), and St. Paul (Clinical, Pepper Outbreak). Strains were previously adapted to 80 ppm rifampicin (TCI America, Inc., Portland, OR, USA).

Bacterial Inoculum Preparation

Frozen stock cultures (-80°C) of each *Salmonella* strain were streaked for isolation onto tryptic soy agar (TSA; Bacto, BD, Franklin Lakes, NJ, USA) containing 80 ppm rifampicin (TSAR) and incubated at 37 ± 2°C for 24 h. An isolated colony was transferred to 10 mL tryptic soy broth (TSB; Bacto, BD, Franklin Lakes, NJ, USA) containing 80 ppm rifampicin (TSBR) and incubated at 37 ± 2°C for 24 h. After incubation, a 10-μL loop of culture was transferred to fresh 10 mL TSBR and incubated at 37 ± 2°C for 24 h. A 0.1-mL aliquot of the second overnight culture was spread across TSAR (100x15 mm) and incubated at 37 ± 2°C for 24 h. Each bacterial lawn was harvested by adding 5 mL 0.1% peptone water (Fisher BioReagents, Thermo Fisher Scientific, Inc., Waltham, MA, USA), loosening the lawn with a sterile spreader, and pipetting the suspension into a sterile centrifuge tube. A bacterial cocktail slurry was produced by combining 2 mL of each *Salmonella* strain. The cocktail slurry was diluted in 0.1% peptone water to achieve an approximate concentration of 10^8 log CFU/mL. Final concentrations were verified by enumeration on TSAR and xylose lysine deoxycholate agar (XLD; Criterion, Hardy Diagnostics, Santa Maria, USA) containing 80 ppm rifampicin (XLDR) by ten-fold serial dilutions.

Inoculation and Enumeration Procedures

Prior to spot inoculation, coupons were surface sterilized with 70% ethanol (VWR International, LLC, Radnor, PA, USA) and air-dried. Twenty 5-µL aliquots (100-µL total) were spot inoculated on sterilized coupons. The 0-dpi time-point treatment was enumerated immediately after spot inoculation to determine the starting coupon inoculum level. All remaining time-points were dried for 90 minutes at ambient temperature in a biosafety cabinet and then placed in the growth chamber, except the 0.06-dpi (90-minute) time-point which was enumerated at this time to quantify the effect of air drying on the coupons. The remaining coupons were randomly chosen for enumeration at the selected time-points. All coupons were enumerated by placing each in a 7 in x 12 in non-filtered sterile sampling bag (Fisherbrand, Thermo Fisher Scientific, Inc., Waltham, MA) and washing with 20 mL of 0.1% peptone water. Each coupon was hand-massaged for 30 s, shaken for 30 s, and hand-massaged for 30 s. Samples were enumerated by spread plating via serial dilutions with 0.1-mL aliquots plated on TSAR and XLDR in duplicate. To lower the limit of detection, 0.1-mL aliquots were plated directly in duplicate from the sampling bag. When *Salmonella* populations appeared to reach the limit of detection, 0.25-mL aliquots were plated to four plates each in duplicate. Colonies were counted manually and log10 transformed based on coupon size (log CFU/cm^2).

Enrichment of Cells

Coupons below the limit of detection (< 0.12 log CFU/cm^2) were enriched according to a modified FDA BAM method (26). Briefly, 1 mL of coupon rinsate was diluted in 10 mL of 1.0% buffered peptone water (Difco, BD, Franklin Lakes, NJ, USA) and incubated at 37 ± 2°C for 24 h. Post-incubation, 0.1 mL was transferred to 9.9 mL of Rappaport Vassiliadis (RV) Broth (Difco, BD, Franklin Lakes, NJ, USA) and incubated at 42°C ± 2°C for 48 h. After 48 h, a 10-µL

loop of RV broth was streaked for isolation onto TSAR and XLDR agar and incubated at 37 ± 2°C for 24 h.

Data Analysis

Enumeration was reported based on coupon size (100x15 mm; 15 cm^2). Log10 transformation (log CFU/cm^2 = log (CFU_{mulch} x 20 mL wash water / 15 cm^2)) and *Salmonella* reduction (reduction = (mean (0 dpi log CFU/cm^2) – log CFU/cm^2) were calculated using Microsoft Excel. When counts fell below the lower limit of detection (< 0.12 log CFU/cm^2), -0.18 log CFU/cm^2 was assumed. All additional statistical analyses were performed in RStudio (V4.2.3). Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) Test were used to determine significant differences in time and ground cover type ($P \leq 0.05$).

Results

No growth of *Salmonella* was observed at any time-point during the study duration (140 dpi). Significant differences were observed between counts on TSAR and XLDR agar plates ($P < 0.5$) with TSAR counts approximately 0.63 log CFU/cm^2 higher; thus, data were analyzed separately, with the results of TSAR included below and with XLDR included in the supplemental materials (Supplemental Materials, Fig. 2-3, Table 1-2).

Survival of Salmonella on Biodegradable Mulch

The starting concentrations of biodegradable mulch at 0 dpi ranged from 5.67 to 6.53 log CFU/cm^2, with a mean concentration of 5.95 log CFU/cm^2 (± 0.20) (Fig. 1, Table 1). Over the course of the study period, the concentration of biodegradable mulch decreased, reaching a concentration of -0.01 log CFU/cm^2 (± 0.66) by 140 dpi. By 30 dpi, there was a significant reduction in concentration to 3.48 log CFU/cm^2 (± 0.24) ($P < 0.05$). This trend continued, and by 90 dpi, the concentration further decreased to 1.04 log CFU/cm^2 (± 0.64) ($P < 0.05$). A >5 log

reduction of *Salmonella* was observed by 140 dpi (5.96 ± 0.65 log CFU/cm^2) (Table 2, Supplemental Materials, Fig. 1). By 140 dpi, *Salmonella* was detected on 4/30 (13%) coupons with the remaining coupons (26/30) below the limit of detection.

Survival of Salmonella on Landscape Fabric

On 0 dpi, the landscape fabric had initial concentrations ranging from 5.41 to 5.98 log CFU/cm^2, with a mean concentration of 5.71 log CFU/cm^2 (± 0.15). Over the initial 30 dpi, a significant reduction in *Salmonella* concentration was observed, with the concentration decreasing to 2.64 log CFU/cm^2 (± 0.29) ($P < 0.05$). From 30 dpi onwards, the concentration of *Salmonella* steadily decreased, with significant changes observed up to 140 dpi ($P < 0.05$). By 140 dpi, the concentration of *Salmonella* was 0.20 log CFU/cm^2 (ca. 5.50 log CFU/cm^2 reduction from 0 dpi). On 90 and 140 dpi, S*almonella* was detected on 29/30 (97%) coupons and 25/30 (83%), respectively.

Survival of Salmonella on Plastic Mulch

The starting concentrations of plastic mulch on 0 dpi ranged from 5.82 to 6.76 log CFU/cm^2, with a mean concentration of 6.20 log CFU/cm^2 (± 0.21). By 30 dpi, there was a significant decrease in *Salmonella* concentration to 2.34 log CFU/cm^2 (± 0.66) ($P < 0.05$). This trend continued, and by 90 dpi, the concentration further decreased to 1.43 log CFU/cm^2 (± 0.43) ($P < 0.05$). At 140 dpi, the concentration of *Salmonella* was 0.06 ± 0.39 log CFU/cm^2 with a >6 log reduction observed (6.14 ± 0.37 log CFU/cm^2). *Salmonella* was detected on 15/30 (50%) coupons by 140 dpi.

Comparison of Salmonella Reduction Across Ground Cover Types

Significant differences were observed between ground cover type and *Salmonella* reduction at 0 to 30 dpi time-points *($P < 0.05$)*, except 0.17 dpi (4 h). From 0 to 30 dpi,

biodegradable mulch had the lowest reduction of *Salmonella* (2.47 ± 0.2 log CFU/cm^2), followed by landscape fabric (3.07 ± 0.30 log CFU/cm^2), and plastic mulch (3.86 ± 0.72 log CFU/cm^2) ($P < 0.05$). Both plastic mulch and landscape fabric achieved a >1 and >3 log reduction by 1 and 30 dpi, respectively, while biodegradable mulch did not exceed those reductions (>1 and >3 log CFU/cm^2) until 5 and 60 dpi, respectively. By 60 dpi, a >4 log CFU/cm^2 reduction was achieved across all ground cover types. No significant differences were observed between biodegradable mulch and landscape fabric *Salmonella* reduction at 60 dpi ($P > 0.05$). A <1 log difference was observed between the tested ground cover types at 60 and 90 dpi. By 90 dpi, no significant differences were observed between *Salmonella* reduction and ground cover type *($P > 0.05$)*. While biodegradable mulch (5.96 ± 0.65 log CFU/cm^2) and plastic mulch (6.14 ± 0.37 log CFU/cm^2) reductions were not significantly different at 140 dpi ($P > 0.05$), they were significantly greater than landscape fabric (5.50 ± 0.36 log CFU/cm^2) ($P < 0.05$). Plastic mulch had equal or higher reduction than other tested ground covers at almost all sampling points. *Salmonella* reduction on landscape fabric was higher than biodegradable mulch and lower than plastic mulch until longer time-points in which reduction was lowest at 140 dpi. However, all ground cover types achieved a >5 log CFU/cm^2 *Salmonella* reduction by 140 dpi. By 140 dpi, 13% (4/30), 83% (25/30), and 50% (15/30) of biodegradable mulch, landscape fabric, and plastic mulch coupons were positive for *Salmonella* by enrichment or concentrations above the limit of detection.

Discussion

Salmonella survival differed on ground covers in the short term (0-30 dpi)

The reduction of *Salmonella* was lowest on biodegradable mulch from 0 to 30 dpi, compared to landscape fabric and plastic mulch. For this study, the selected biodegradable mulch

was made of Mater-Bi®, a plant-based bioplastic that is biodegradable and compostable due to the primary composition of corn starch. Starch-based mulches are broken down by the plant and soil microbial community when tilled into the soil due to the secretion of enzymes by soil microorganisms that are capable of degrading naturally occurring polymers, such as starch (5). As the starch is broken down into carbon dioxide, water, and microbial biomass, the soil microbes involved can utilize its components for metabolic purposes (5, 20). In general, biodegradable mulches alter the soil microbiome indicating these types of ground covers impact microbes (4, 13, 18). Because *Salmonella* is capable of digesting starch, like soil microorganisms involved in the degradation process, biodegradable mulch may impact the survival of *Salmonella* due to the composition of corn starch as opposed to landscape fabric (polypropylene) and plastic mulch (embossed plastic), where starch and other naturally occurring polymers are not included in the ground cover composition (17).

Bandyopadhyay et al. (3) concluded that starch-based mulches were more favorably degraded by bacteria in comparison to other commercial biodegradable mulches without starch compositions; however, foodborne pathogens were not the bacterial communities investigated in the previous study. Additionally, Strantz and Zottola (24) found under select conditions the growth of *Salmonella* and other targeted bacteria was enhanced in media containing polyethylene film with cornstarch in comparison to polyethylene film without. Previous literature indicates starch from biodegradable mulches does impact various bacterial communities, and *Salmonella*'s capability to digest starch may play a role in its survival. This work establishes biodegradable mulch impacted the survival of *Salmonella* in the short term (0-30 dpi), compared to other tested ground cover types; however, starch's role in the survival of *Salmonella* on biodegradable mulch

cannot yet be concluded. The mechanisms by which starch-based biodegradable mulches affect the survival of foodborne pathogens should be further researched.

Landscape fabric had the lowest reduction of Salmonella at 140 dpi

By 140 dpi, *Salmonella* reduction was lowest on landscape fabric, compared to biodegradable and plastic mulches. Approximately 83% of landscape fabric coupons were positive for *Salmonella*, as opposed to 13% and 50% for biodegradable mulch and plastic mulch coupons, respectively, at 140 dpi. The landscape fabric selected for this study was made of woven polypropylene fabric and marketed for its ability to allow air and water transfer. In general, landscape fabric is characterized as porous, compared to other commercial ground cover options (6, 9, 19, 23). Porous materials and the survival of foodborne pathogens in the pre-harvest environment have been minimally studied; however, the porous nature of landscape fabric may allow for *Salmonella* to be absorbed and protected within the niches of the fabric resulting in higher survival compared to other materials tested. This is only speculation as further research is required to better understand the impact of the composition on ground covers and foodborne pathogen survival.

Salmonella survival was significantly different on tested ground cover types, but each achieved a >5 log reduction by 140 dpi.

While survival varied by time-point, a >5 log reduction of *Salmonella* was achieved for all tested ground cover types. The present study was conducted solely in a climate-controlled growth chamber with little to no conditions conducive to reducing *Salmonella* levels, such as UV light, temperature, or relative humidity, which are commonly uncontrolled in the production environment. Conditions included moderate temperature (23°C) and relative humidity (55%) with minimal monitored fluctuations over 140 days and the exclusion of artificial light, except in

the removal of samples for enumeration. In practice, ground covers would be utilized in an environment with much less climate control, such as in open-field production. Environmental factors (e.g., bacterial antagonists, rainfall, sunlight, temperature, relative humidity) do impact the survival of *Salmonella*, thus it is expected that different results would be observed in a realistic ground cover environment (1, 7, 15). The varying *Salmonella* survival supports that not all ground covers pose the same food safety risk and should be considered in a fresh produce operation's risk management.

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Figures

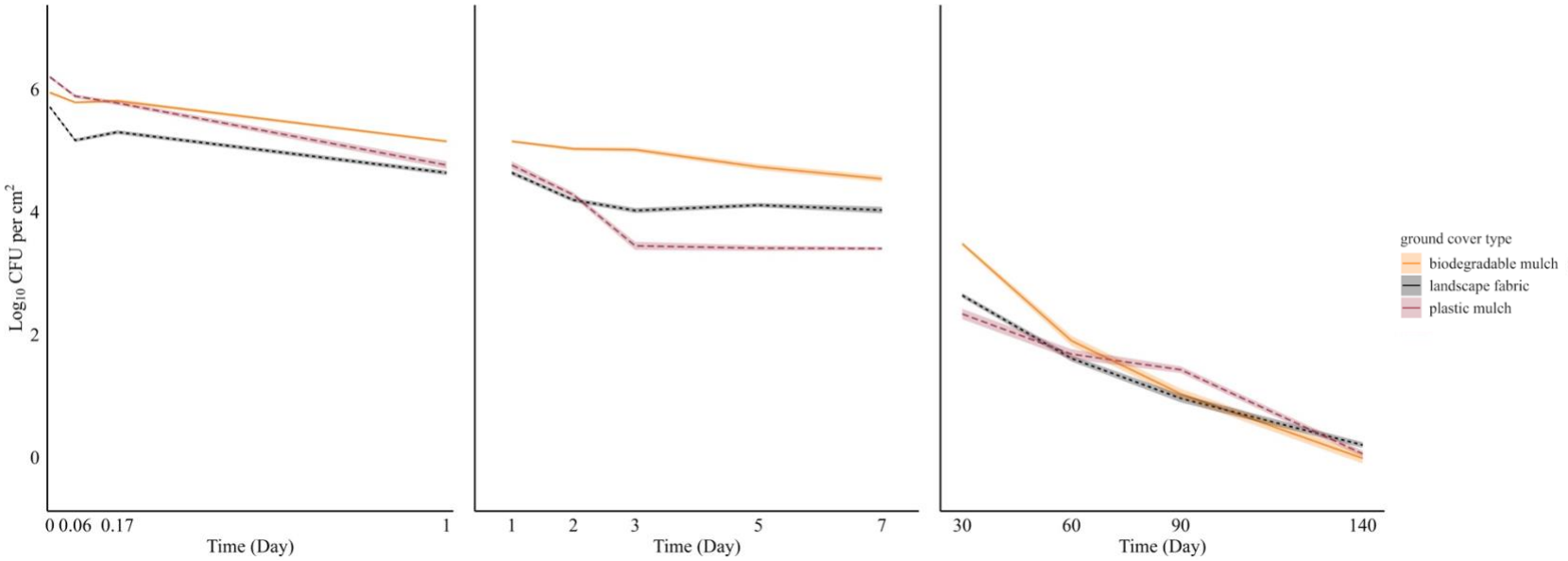

Figure 1. Biodegradable mulch, landscape fabric, and plastic mulch log CFU/cm^2 of *Salmonella* over 0 to 140 days enumerated on tryptic soy agar. Shading represents standard error.

Tables

Table 1. Log CFU/cm^2 of *Salmonella* over 0 to 140 days for biodegradable mulch, landscape fabric, and plastic mulch enumerated on tryptic soy agar.

Time (days)	Ground Cover Type								
	Biodegradable Mulch			Landscape Fabric			Plastic Mulch		
0	5.95±0.20[a]		Aa[b]	5.71±0.15		Ba	6.20±0.21		Ca
0.06	5.79±0.13		Aa	5.17±0.22		Bb	5.89±0.24		Cb
0.17	5.81±0.23		Aa	5.30±0.25		Bb	5.78±0.26		Ab
1	5.15±0.17		Ab	4.64±0.29		Bc	4.76±0.48		Bc
2	5.03±0.23		Ab	4.20±0.27		Bd	4.28±0.32		Bd
3	5.01±0.30		Ab	4.02±0.33		Bd	3.45±0.51		Ce
5	4.73±0.39		Ac	4.11±0.27		Bd	3.41±0.34		Ce
7	4.54±0.41		Ac	4.03±0.41		Bd	3.40±0.20		Ce
30	3.48±0.24		Ad	2.64±0.29		Be	2.34±0.66		Cf
60	1.90±0.63		Ae	1.61±0.39		Bf	1.68±0.62		ABg
90	1.04±0.64	(4/4)[c]	Af	0.96±0.51	(2/3)	Ag	1.43±0.43		Bh
140	-0.01±0.66	(2/28)	Ag	0.20±0.36	(11/16)	Bh	0.06±0.39	(8/23)	ABi

[a] Mean ± standard deviation

[b] Capital letters indicate significant differences ($P < 0.05$) across each ground cover type and time. Lowercase letters indicate a significant difference ($P < 0.05$) within each ground cover type.

[c] Positive coupons post-enrichment

Table 2. Log CFU/cm^2 reductions[a] of *Salmonella* over 0 to 140 days for biodegradable mulch, landscape fabric, and plastic mulch enumerated on tryptic soy agar.

Time (days)	Ground Cover Type								
	Biodegradable Mulch			Landscape Fabric			Plastic Mulch		
0	0.00±0.00[b]		Aa[c]	0.00±0.00		Aa	0.00±0.00		Aa
0.06	0.16±0.13		Aa	0.54±0.22		Bb	0.31±0.14		Cb
0.17	0.14±0.23		Aa	0.41±0.25		Bb	0.43±0.19		Bb
1	0.80±0.17		Ab	1.07±0.29		Bc	1.44±0.48		Cc
2	0.92±0.25		Ab	1.51±0.27		Bd	1.92±0.28		Cd
3	0.93±0.26		Ab	1.69±0.33		Bd	2.75±0.62		Ce
5	1.21±0.38		Ac	1.60±0.27		Bd	2.79±0.32		Ce
7	1.41±0.43		Ac	1.68±0.41		Bd	2.80±0.26		Ce
30	2.47±0.26		Ad	3.07±0.30		Be	3.86±0.72		Cf
60	4.05±0.63		Ae	4.10±0.39		Af	4.52±0.65		Bg
90	4.91±0.65	(4/4)[d]	Af	4.75±0.51	(2/3)	Ag	4.77±0.39		Ag
140	5.96±0.65	(2/28)	Ag	5.50±0.36	(11/16)	Bh	6.14±0.37	(8/23)	Ah

[a] Reductions were compared to log CFU/cm^2 0 dpi values.
[b] Mean ± standard deviation
[c] Capital letters indicate significant differences ($P < 0.05$) in reduction across each ground cover type and time. Lowercase letters indicate a significant difference ($P < 0.05$) in reduction within each ground cover type.
[d] Positive coupons post-enrichment.

Supplemental Materials

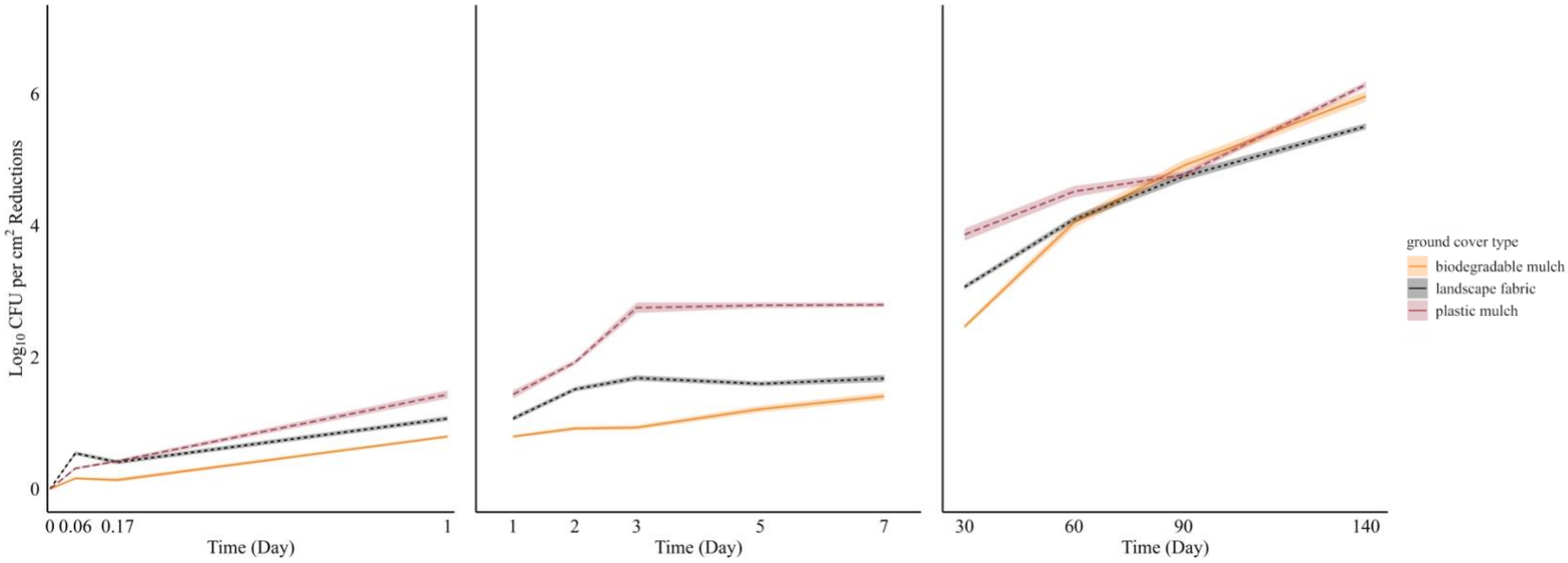

Figure 1. Biodegradable mulch, landscape fabric, and plastic mulch log CFU/cm^2 reductions of *Salmonella* over 0 to 140 days enumerated on tryptic soy agar. Log CFU/cm^2 reductions were calculated from the 0-day time-point. Shading represents standard error.

Injured cells are unable to form colonies on selective media (XLDR) causing less recovery than that of non-selective media (TSAR) explaining the significant differences in this present study (7).

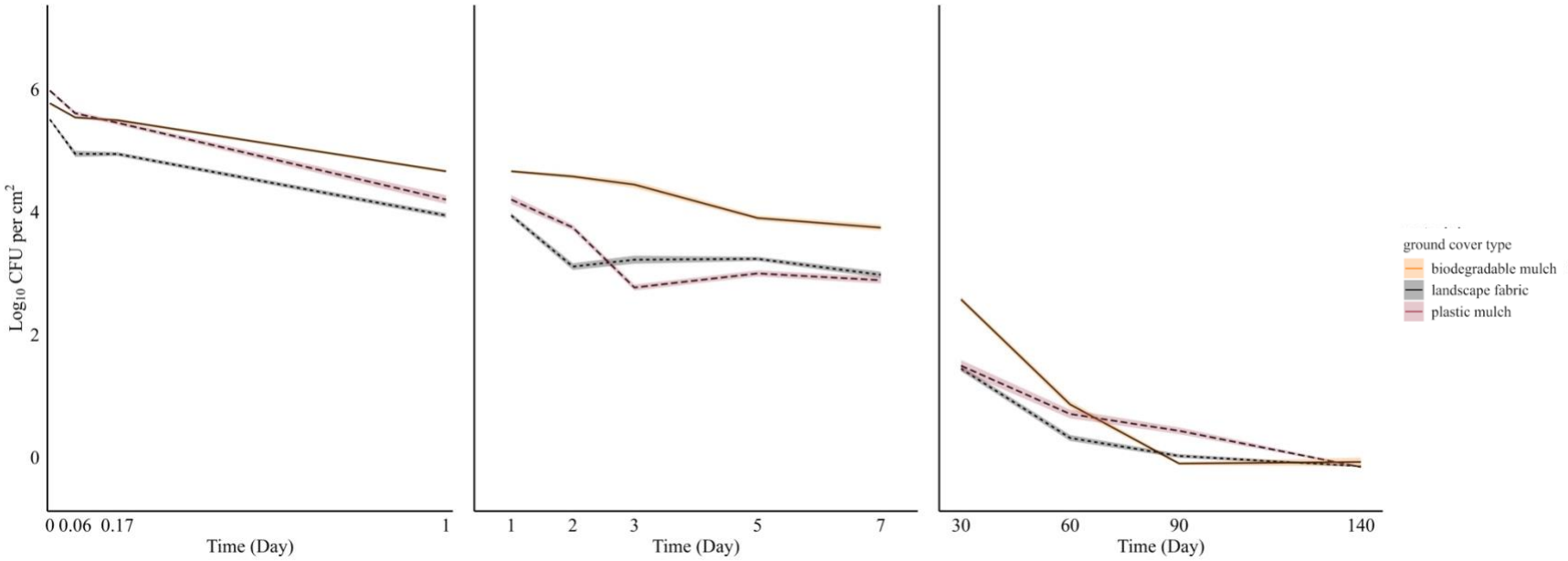

Figure 2. Biodegradable mulch, landscape fabric, and plastic mulch log CFU/cm^2 of *Salmonella* over 0 to 140 days enumerated on xylose lysine deoxycholate agar. Shading represents standard error.

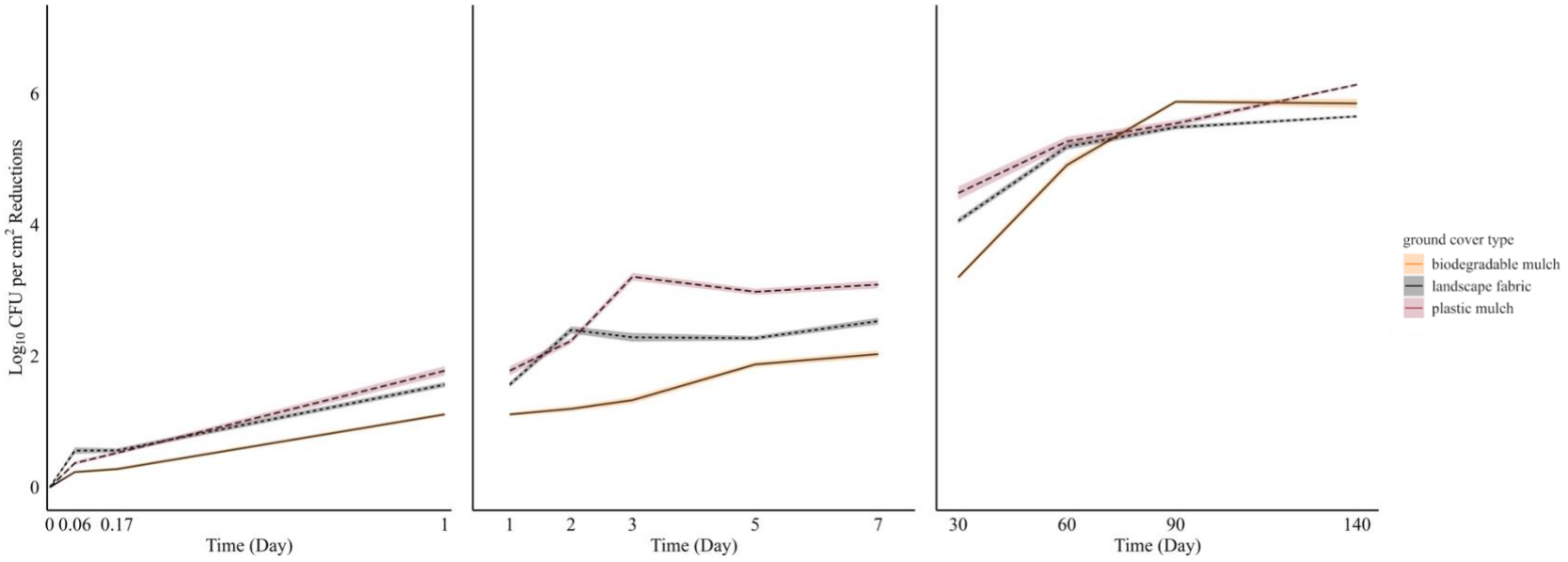

Figure 3. Biodegradable mulch, landscape fabric, and plastic mulch log CFU/cm^2 reductions of *Salmonella* over 0 to 140 days enumerated on xylose lysine deoxycholate agar. Log CFU/cm^2 reductions were calculated from the 0-day time-point. Shading represents standard error.

Table 1. Log CFU/cm^2 reductions[a] of *Salmonella* over 0 to 140 days for biodegradable mulch, landscape fabric, and plastic mulch enumerated on xylose lysine deoxycholate agar.

Time (days)	Ground Cover Type								
	Biodegradable Mulch			Landscape Fabric			Plastic Mulch		
0	0.00±0.00[b]		Aa[c]	0.00±0.00		Aa	0.00±0.00		Aa
0.06	0.23±0.16		Ab	0.56±0.39		Bb	0.37±0.21		Cb
0.17	0.28±0.20		Ab	0.56±0.25		Bb	0.52±0.21		Bb
1	1.11±0.22		Ac	1.56±0.30		Bc	1.78±0.55		Cc
2	1.20±0.30		Acd	2.40±0.43		Bde	2.23±0.34		Cd
3	1.33±0.40		Ad	2.29±0.51		Bd	3.21±0.46		Ce
5	1.87±0.33		Ae	2.27±0.27		Bd	2.98±0.36		Ce
7	2.03±0.40		Ae	2.53±0.40		Be	3.09±0.46		Ce
30	3.20±0.34		Af	4.06±0.34		Bf	4.48±0.77		Cf
60	4.91±0.45	(3/3)[d]	Ag	5.19±0.38	(10/10)	Bg	5.27±0.56	(6/6)	Bg
90	5.88±0.22	(26/26)	Ah	5.49±0.25	(19/20)	Bh	5.54±0.39	(8/8)	Bh
140	5.85±0.55	(3/29)	Ah	5.65±0.12	(25/30)	Bh	6.14±0.11	(15/30)	Ci

[a] Reductions were compared to log CFU/cm^2 0 dpi values.
[b] Mean ± standard deviation
[c] Capital letters indicate significant differences ($P < 0.05$) in reduction across each ground cover type and time. Lowercase letters indicate a significant difference ($P < 0.05$) in reduction within each ground cover type.
[d] Positive coupons post-enrichment.

Table 2. Log CFU/cm^2 of *Salmonella* over 0 to 140 days for biodegradable mulch, landscape fabric, and plastic mulch enumerated on xylose lysine deoxycholate agar.

Time (days)	Ground Cover Type								
	Biodegradable Mulch			Landscape Fabric			Plastic Mulch		
0	5.77±0.19[a]		Aa[b]	5.51±0.14		Ba	5.98±0.20		Ca
0.06	5.54±0.15		Ab	4.95±0.39		Bb	5.61±0.29		Ab
0.17	5.50±0.21		Ab	4.95±0.25		Bb	5.46±0.26		Ab
1	4.66±0.20		Ac	3.95±0.30		Bc	4.20±0.55		Cc
2	4.58±0.28		Acd	3.11±0.43		Bde	3.75±0.37		Cd
3	4.45±0.44		Ad	3.22±0.51		Bd	2.77±0.38		Ce
5	3.90±0.33		Ae	3.24±0.27		Bd	3.00±0.38		Ce
7	3.75±0.40		Ae	2.98±0.40		Be	2.89±0.42		Be
30	2.58±0.33		Af	1.45±0.34		Bf	1.50±0.71		Bf
60	0.87±0.46	(3/3)[c]	Ag	0.32±0.38	(10/10)	Bg	0.71±0.55	(6/6)	Ag
90	-0.10±0.21	(26/26)	Ah	0.02±0.25	(19/20)	Ah	0.44±0.43	(8/8)	Bh
140	-0.07±0.56	(3/29)	Ah	-0.14±0.12	(25/30)	Ah	-0.16±0.08	(15/30)	Ai

[a] Mean ± standard deviation

[b] Capital letters indicate significant differences ($P < 0.05$) across each ground cover type and time. Lowercase letters indicate a significant difference ($P < 0.05$) within each ground cover type.

[c] Positive coupons post-enrichment.

Chapter 4: Survival of Generic *Escherichia coli* on Plastic Mulch Held in Different Growing Environments

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Abstract

Plasticulture including the use of plastic mulch to decrease pests and increase plant growth is used in fresh produce production. Fresh produce is grown in various environments (e.g., open-field, greenhouse) with the continual use of plastic mulch. Harvestable portions of fruit and vegetable plants may grow directly or indirectly on plastic mulch, depending on crop and horticultural practices utilized. Limited data exists on the potential food safety risk if contaminated. This study aims to evaluate the survival of generic *Escherichia coli* on plastic mulch held in three environments: field, greenhouse, and growth chamber. New plastic mulch was cut into 100x15 mm Petri dishes and spot inoculated with a 100-µl of green fluorescent protein-tagged generic *Escherichia coli* strain (TVS 353) at ca. 6 log CFU/cm^2. Post-drying (90 min), coupons were held in a growth chamber (23°C, 55% RH), greenhouse (29°C, 50% RH), and field (24°C, 80% RH). Coupon samples were randomly selected for enumeration at 0, 0.06, 0.17, 0.41, 1, 2, 3, 5, and 7 days post-inoculation (dpi). Enrichments were performed if counts fell below 0.12 log CFU/cm^2. All counts and enrichments were confirmed using UV light. Generic *E. coli* reduction on plastic mulch was significantly different between environments ($P < 0.05$). Generic *E. coli* on plastic mulch survived the worst in the field with a reduction of >6 log CFU/cm^2 by 0.17 dpi (4 h), and negative for generic *E. coli* post-enrichment by 5 dpi. Generic *E. coli* survived on plastic mulch in a greenhouse and growth chamber for at least 7 dpi. Generic *E. coli* concentrations began to fall below the limit of detection (< 0.12 log CFU/cm^2) by 5 dpi in the greenhouse, while generic *E. coli* concentrations remained countable in growth chamber held samples, at approximately 4.01 log CFU/cm^2 at 7 dpi. Generic *E. coli* survival was impacted by the growing environment highlighting surfaces like plastic mulch have different food safety risks, and may require different management (e.g., sanitation protocols).

Highlights

- Generic *E. coli* survival was significantly different between environments.
- Generic *E. coli* survived up to 7 d in the growth chamber and greenhouse.
- By day 7, 4/30 mulch coupons were countable for generic *E. coli* in the greenhouse.
- Generic *E. coli* was negative post-enrichment by 5 d on mulch coupons held in the field.

Plasticulture is the practice of using plastic materials in agriculture for a broad range of applications, including irrigation, fumigation, and pest management in the pre-harvest environment (12). Specifically, plastic mulch is utilized to suppress weeds, control insect pests, conserve water, increase soil temperature, and minimize erosion resulting in a shorter time to harvest and increased yields (14). Because of these benefits, plastic mulch is a common horticultural practice used in fresh produce production. Depending on the commodity and production style, edible portions of the crop may intentionally or unintentionally contact plastic mulch during the growing cycle, posing a potential cross-contamination risk of foodborne pathogens. Bacteria have been shown to transfer from plastic mulch to fresh produce upon contact (9, 13, 15).

The environmental conditions may vary, but horticultural practices, such as the use of plastic mulch, are still utilized across open-field and greenhouse production. From 1998 to 2019, the production of fresh fruits and vegetables in controlled environmental agriculture (CEA) operations almost tripled to optimize yields, improve crop quality, reduce risks posed to open-field production, and extend the growing season (6). CEA encapsulates a wide range of growing environments and practices other than conventional field production, such as aquaculture, hydroponics, vertical farming, and greenhouses. CEA provides a protected growing environment; however, the level of control can range from full to minimal depending on the production system (6). While more protected from the environment than open-field production, greenhouses may still be exposed to a variety of fluctuating or uncontrolled conditions depending on the type.

It is unsafe for public health to introduce pathogenic bacteria into the environment, thus indicator organisms, such as generic *Escherichia coli*, are used in research to quantify the risks

of a targeted hazard (e.g., plastic mulch, growing environment). Generic *E. coli* is found naturally in the intestinal tract of humans and animals and serves as an indicator of fecal contamination (17). While foodborne pathogens may behave differently, indicator organisms can indicate if conditions are conducive for pathogen survival (3). For example, the Food Safety Modernization Act Produce Safety Rule uses generic *E. coli* as an indicator of post-harvest water quality (16). Since plastic mulch is implemented across open-field and greenhouse production, evaluating the survival of generic *E. coli* can better quantify food safety risks depending on the production environment to focus food safety efforts. The objective of this study was to assess the survival of Generic *E. coli* on plastic mulch as affected by the growing environment (field, greenhouse, and growth chamber) across 7 days.

Materials and Methods

Experimental Design

Three environments, a field, greenhouse, and growth chamber, in Blacksburg, Virginia (USA) were selected for this study. The field was nearby at the Virginia Tech Urban Horticulture Center and, the greenhouse research facility was on Virginia Tech's campus. The growth chamber (Percival E41L2, Percival Scientific Inc., Perry, IA, USA) was held in a Biosafety Level 2 laboratory with maintained conditions of 23°C and 55% relative humidity. All environmental conditions were monitored with data loggers (WatchDog B-Series Button Loggers, Spectrum Technologies, Inc., Aurora, IL, USA) placed in each environment and compared to nearby Virginia Tech WeatherSTEM stations when applicable (i.e., greenhouse, field).

This study consisted of two independent trials with 15 replications each for all time-points of 0, 0.06, 0.17, 0.41, 1, 2, 3, 5, and 7 days post-inoculation (dpi; n=810) for each

environment. Embossed white-on-black plastic mulch (1.25 mil, Ginegar Plastics, Inc., Santa Maria, CA, USA) was purchased new for use. Plastic mulch coupons were constructed to fit a 100x15 mm Petri dish (Corning, Inc., Corning, NY, USA) and surface sterilized with 70% ethanol (VWR International, LLC, Radnor, PA, USA). Coupons were affixed with double-sided mounting tape (Scotch, 3M, St. Paul, MN, USA) to the Petri dish before placement in the field and greenhouse environments. Additionally, Petri dishes were affixed to a 41-qt plastic storage bin (Sterlite Corporation, Townsend, MA, USA) with drainage holes in the field to eliminate bin flooding due to potential rainfall. The field used was in production, and coupon bins were placed parallel to planting rows on a declining slope of bare soil secured with stakes to hold bins in place. Greenhouse coupon bins were placed directly on the cement floor of the greenhouse.

Inoculum Preparation

Rifampicin-resistant (80 ppm), green-fluorescent protein-tagged generic *E. coli* TVS 353 was the model organism. Inoculum preparation was adapted from Blessington et al. (2). Briefly, the frozen stock culture (-80°C) was streaked onto tryptic soy agar (TSA; Bacto, BD, Franklin Lakes, NJ, USA) containing 80 ppm rifampicin (TSAR; TCI America, Inc., Portland, OR, USA) and incubated at 37 ± 2°C for 24 h. A single colony was transferred to 10 mL tryptic soy broth (TSB; Bacto, BD, Franklin Lakes, NJ, USA) containing 80 ppm rifampicin (TSBR) and incubated, with a successive 10-µL loop transfer of the overnight culture to fresh 10 mL of TSBR with incubation conditions at 37 ± 2°C for 24 h for both TSBR cultures. A 1-mL aliquot of the second overnight culture was spread across a large TSAR petri plate (150x15 mm; Corning, Inc., Corning, NY, USA) and incubated at 37 ± 2°C for 24 h. The resulting bacterial lawn was flooded with 9 mL 0.1% peptone water (Fisher BioReagents, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and dislodged with a sterile cell spreader. The suspension was

pipetted into a sterile centrifuge tube. To reach an approximate concentration of 10^8 log CFU/mL, the culture slurry was two-fold diluted in 0.1% peptone water. In duplicate, final concentrations were confirmed by enumeration on TSAR by 0.1 mL serial dilutions.

Coupon Inoculation

Seventy percent ethanol was used to surface sterilize plastic mulch coupons (VWR International, LLC, Radnor, PA, US). Twenty 5-µL spots (100-µL) of the diluted inoculum were spot inoculated to plastic mulch coupons. Immediately after spot inoculation, the 0-dpi time-point was enumerated. The remaining treatments were dried in a biosafety cabinet at ambient temperature for 90 minutes, and the 0.06-dpi (90-minute) time-point was enumerated post-drying to quantify the loss of generic *E. coli* due to the air drying process. All remaining coupons were moved to their corresponding sample site and enumerated for environmental impact on generic *E. coli* survival. Coupons were randomly selected at each time-point for enumeration.

Coupon Enumeration and Enrichment

Each coupon was placed in a 7 in x 12 in non-filtered sterile sampling bag (Nasco Whirl-Pak, Fisherbrand, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 20 mL of 0.1% peptone water and hand-massaged for 30 s, shaken for 30 s, and hand-massaged for 30 s. Samples were serially diluted, and 0.1-mL aliquots were surface plated on TSAR in duplicate. In cases of low bacterial levels, 0.1-mL aliquots were directly surface plated from the non-filtered sterile sampling bag to TSAR, in duplicate. When counts were near the limit of detection, 1 mL (0.25-mL aliquots each) of the coupon rinsate was directly surface plated from the non-filtered sterile sampling over four TSAR plates, in duplicate. All generic *E. coli* counts were confirmed under UV light for the presence of green-fluorescent protein. Colonies were counted manually by placing TSAR plates in a UV light box and only fluorescing colonies were included in counts.

Following a modified *E. coli* FDA BAM method, coupons below the limit of detection (< 0.12 log CFU/cm^2) were enriched (17). Briefly, 1 mL of the rinsate was pipetted into 10 mL of 1.0% buffered peptone water (Difco, BD, Franklin Lakes, NJ, US) and incubated at 37 ± 2°C for 24 h. Subsequently, 1 mL was transferred to 10 mL of *E. coli* Broth (HiMedia Laboratories, Maharashtra, India) and incubated at 37 ± 2°C for 24 h. Post-incubation, a 10-µL loop of broth was streaked in duplicate onto TSAR and incubated at 37 ± 2°C for 24 h. Samples with growth were confirmed using UV light for the presence of green-fluorescent protein. If the growth fluoresced under a UV light box, growth was recorded as positive.

Statistical Analysis

All counts were log-transformed based on coupon size (log CFU/cm^2 = log (CFU_{mulch} x 20 mL wash water / 15 cm^2)). For counts that fell below the lower limit of detection of 0.12 log CFU/cm^2, a value of -0.18 log CFU/cm^2 was designated. To calculate reduction, the average log CFU/cm^2 of the 0-dpi time-point was calculated for each trial, and all time-points beyond the 0-dpi of the corresponding trial were subtracted from this value (reduction = (mean (0 dpi log CFU/cm^2) – log CFU/cm^2). Log transformation and reduction calculations were computed in Microsoft Excel. All remaining statistical analyses were conducted using RStudio version 4.2.3. Significant differences ($P \leq 0.05$) in time and environment were determined using Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) Test.

Results and Discussion

Weather Data Across Tested Environments

Data loggers and WeatherSTEM data for each corresponding environment (i.e., greenhouse, field) minimally differed in temperature and relative humidity results (Supplemental Materials, Table 1-2). Greenhouse data loggers were placed directly in the greenhouse with

coupon bins, and the corresponding WhetherSTEM station monitored outdoor conditions, accounting for the larger differences seen in this environment's weather data across monitoring systems. Greenhouse trials were conducted in August and field trials in September. For the greenhouse, data loggers reported an average day and night temperature (°C) of 31.6 and 24.2 and relative humidity (%) of 41.8 and 64.1, respectively. For the field, data loggers reported an average day and night temperature (°C) of 28.5 and 17.6 and relative humidity (%) of 68.0 and 95.5, respectively. An average of 0.06 in of rainfall and 1.55 UV index was reported during the duration of field trials. On average, greenhouse temperature was reported higher than field temperature, and field relative humidity was reported higher than that of the greenhouse.

Survival of Generic Escherichia coli on Plastic Mulch in Growth Chamber

At 0 dpi, the starting concentrations of generic *E. coli* on plastic mulch in the growth chamber ranged from 5.56 to 6.13 log CFU/cm^2, with a mean concentration of 5.88 log CFU/cm^2 (± 0.14) (Table 1, Fig. 1). Over the course of the sampling period, generic *E. coli* declined to 4.01 log CFU/cm^2 (± 0.37) by 7 dpi. At 2 dpi, ca. 1 log reduction (1.00 ± 0.29 log CFU/cm^2) was observed with a final reduction (0-7 dpi) of 1.86 log CFU/cm^2 (± 0.40) by 7 dpi (Table 2, Supplemental Materials, Fig. 1).

Survival of Generic Escherichia coli on Plastic Mulch in Greenhouse

Initial concentrations of generic *E. coli* on plastic mulch in the greenhouse ranged between 5.85 and 6.39 log CFU/cm^2, with a mean concentration of 6.14 log CFU/cm^2 (± 0.14). From 0.17 to 7 dpi, generic *E. coli* concentrations significantly declined at all 7 sampled time-points to -0.04 log CFU/cm^2 (± 0.35) at 7 dpi. Starting at 5 dpi and into 7 dpi, coupons began to fall below the limit of detection with generic *E. coli* detected on 14/30 (47%) and 4/30 (13%) mulch coupons, respectively. A >6 log reduction was observed by 7 dpi.

Survival of Generic Escherichia coli on Plastic Mulch in Field

At 0 dpi, the starting concentrations of generic *E. coli* on plastic mulch in the field ranged from 6.07 to 6.46 log CFU/cm^2, with a mean concentration of 6.29 log CFU/cm^2 (± 0.08). At 0.17 dpi, generic *E. coli* concentrations significantly declined to 0.05 log CFU/cm^2 (± 0.41) with a >6 log reduction (6.24 ± 0.43 log CFU/cm^2). By 1 dpi, generic *E. coli* counts fell below the limit of detection (-0.18 ± 0.00 log CFU/cm^2) with a reduction of 6.47 log CFU/cm^2 (± 0.04). Coupons were negative post-enrichment (0/30; 100%) by 5 dpi.

Comparison of Generic Escherichia coli Reduction Across Tested Growing Environments

From 0.41 to 7 dpi, significant differences were observed between generic *E. coli* reduction and environment for all 7 sampled time-points (0.41, 1, 2, 3, 5, and 7 dpi; $P < 0.05$). As predicted, the most controlled environment (growth chamber) resulted in the lowest reduction of generic *E. coli*. Plastic mulch coupons held in the growth chamber had <2 log reduction with relatively high generic *E. coli* presence (4.01 ± 0.37 log CFU/cm^2) remaining by 7 dpi in comparison to other environmental treatments. *Salmonella* was detected on 30/30 (100%) of growth chamber coupons with 4/30 (13%) and 0/30 (0%) detected on greenhouse and field plastic mulch, respectively, by 7 dpi. Greenhouse and field coupons exceeded the 7 dpi growth chamber reduction (1.86 ± 0.40 log CFU/cm^2) by 1 dpi (1.99 ± 0.40 log CFU/cm^2) and 0.17 dpi (6.24 ± 0.43 log CFU/cm^2), respectively. By 7 dpi, generic *E. coli* reduction on greenhouse plastic mulch (6.18 ± 0.37 log CFU/cm^2) was more closely aligned with the field (6.47 ± 0.04 log CFU/cm^2). Greater variability in log reductions was observed on coupons held in the greenhouse environment compared to the field or growth chamber environments. Over the 7 dpi sampling period, the generic *E. coli* reduction of 1.86 log CFU/cm^2 (± 0.40) was lowest in the growth

chamber followed by 6.18 log CFU/cm^2 (± 0.37) in the greenhouse and 6.47 log CFU/cm^2 (± 0.04) in the field.

Plastic mulch held in the growth chamber had <2 log reduction of generic *E. coli* by day 7. In general, growth chambers strive to isolate samples from the outside environment and can maintain uniform conditions. Generic *E. coli* was subjected to moderate growth chamber conditions of 23°C and 55% relative humidity with the exclusion of artificial light, except when retrieving plastic mulch samples for enumeration. In this study, external factors, such as UV light, temperature, relative humidity, were not present to reduce generic *E. coli* levels on the plastic mulch held in the growth chamber thus a minimal reduction was observed. While fresh produce is unlikely to grow in conditions as controlled as the growth chamber tested, it establishes the survival of bacteria on plastic mulch is possible and is a potential food safety hazard. The >6 log generic *E. coli* reduction on plastic mulch held in the field and greenhouse environments suggests the food safety risk of plastic mulch alone is minimal.

Field plastic mulch achieved a >6 log reduction by 0.17 days (4 h) compared to 7 days for greenhouse mulch, showing an immediate reduction in the field environment and a slower reduction in the greenhouse. In open-field production, fresh produce is exposed to several routes of contamination, including water, soil, soil amendments, workers, insects, and animals (1). The closed nature of greenhouses protects the growing environment from select potential hazards (e.g., soil, soil amendments, animals, insects), depending on the production system. In addition to minimizing contaminates, natural external factors present in the field, such as precipitation, wind, and UV light, are limited or better controlled in a greenhouse environment (4). The lower susceptibility of greenhouse-grown fresh produce to contaminants or environmental factors does not imply lower food safety risks as the controlled conditions still create an environment

conducive to pathogens (7, 8). This is supported by this present study, as the reduction of generic *E. coli* observed was slower in the greenhouse environment. Immediate and continual reduction of generic *E. coli* on field plastic mulch indicates external factors (e.g., sunlight, relative humidity, temperature, rainfall) that, while more difficult to control in-field, may also reduce the present bacterial loads (5, 10, 11). These findings suggest that food safety risks may be different in field and greenhouse environments and, thus, require different risk mitigation strategies.

The differences in generic *E. coli* reduction over time dependent on the environment indicate management strategies and assessment of risk will be dependent on the growing environment. These findings suggest if contamination is left unmanaged, infrequent microbial contamination in a greenhouse may be reduced slowly and frequent contamination in the field may be reduced quickly. However, small microbial contamination events can cause large contamination issues downstream and should not be left unmanaged, regardless of the environment. The prevention and elimination of foodborne pathogens by implementing more frequent food-safe mitigation practices may be key to reducing food safety risks in more protected environments, such as greenhouses, due to the slower reduction observed. Effective prevention and elimination tactics of controlled environments should be evaluated to provide growers with effective measures to reduce such food safety risks.

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Figures

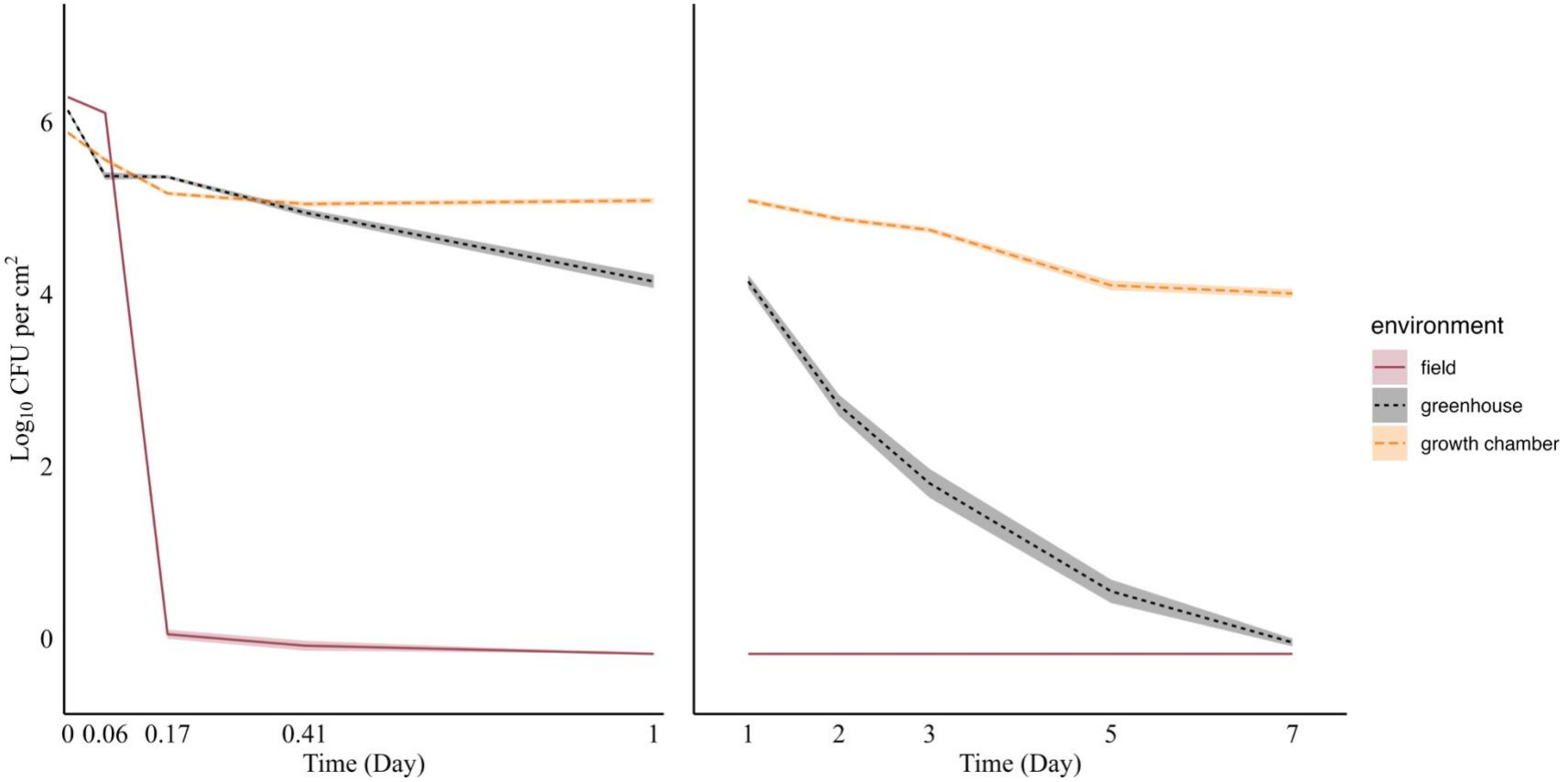

Figure 1. Generic *Escherichia coli* log CFU/cm^2 across 0 to 7 days on plastic mulch held in the growth chamber, greenhouse, and field environments. Standard error is represented by shading.

Tables

Table 1. Mean ± standard deviation of log CFU/cm^2 of generic *Escherichia coli* across 0 to 7 days on plastic mulch held in the growth chamber, greenhouse, and field environments.

Time (days)	Environment							
	Growth Chamber		Greenhouse			Field		
0	5.88±0.14	Aa[a]	6.14±0.14	Ba		6.29±0.08	Ca	
0.06	5.56±0.22	Ab	5.38±0.34	Bb		6.11±0.13	Cb	
0.17	5.17±0.18	Ac	5.36±0.15	Bb		0.05±0.41	Cc	(3/22)
0.41	5.05±0.23	Ac	4.95±0.34	Ac		-0.08±0.45	Bd	(3/29)
1	5.09±0.25	Ac	4.15±0.59	Bd		-0.18±0.00	Cd	(2/30)
2	4.88±0.24	Ad	2.71±0.95	Be		-0.18±0.00	Cd	(1/30)
3	4.75±0.25	Ad	1.80±1.30	Bf		-0.18±0.00	Cd	(1/30)
5	4.10±0.44	Ae	0.55±1.06	Bg	(3/19)[b]	-0.18±0.00	Cd	(0/30)
7	4.01±0.37	Ae	-0.04±0.35	Bh	(0/26)	-0.18±0.00	Cd	(0/30)

[a] Capital letters indicate significant differences ($P < 0.05$) across each environment and time. Lowercase letters indicate a significant difference ($P < 0.05$) within each environment.
[b] Coupons positive post-enrichment.

Table 2. Mean ± standard deviation of log CFU/cm^2 reductions[a] of generic *Escherichia coli* across 0 to 7 days on plastic mulch held in the growth chamber, greenhouse, and field environments.

Time (days)	Environment							
	Growth Chamber		Greenhouse			Field		
0	0.00±0.00	Aa[b]	0.00±0.00	Aa		0.00±0.00	Aa	
0.06	0.31±0.26	Ab	0.76±0.29	Bb		0.19±0.11	Cb	
0.17	0.70±0.22	Ac	0.78±0.17	Abc		6.24±0.43	Bc	(3/22)
0.41	0.82±0.28	Ac	1.19±0.40	Bc		6.37±0.46	Cd	(3/29)
1	0.79±0.23	Ac	1.99±0.66	Bd		6.47±0.04	Cd	(2/30)
2	1.00±0.29	Ad	3.43±0.97	Be		6.47±0.04	Cd	(1/30)
3	1.13±0.26	Ad	4.34±1.33	Bf		6.47±0.04	Cd	(1/30)
5	1.77±0.48	Ae	5.59±1.10	Bg	(3/19)[c]	6.47±0.04	Cd	(0/30)
7	1.86±0.40	Ae	6.18±0.37	Bh	(0/26)	6.47±0.04	Cd	(0/30)

[a] Reductions were compared to log CFU/cm^2 0 dpi values.
[b] Capital letters indicate significant differences ($P < 0.05$) in reduction across each environment and time. Lowercase letters indicate a significant difference ($P < 0.05$) in reduction within each environment.
[c] Coupons positive post-enrichment.

Supplemental Materials

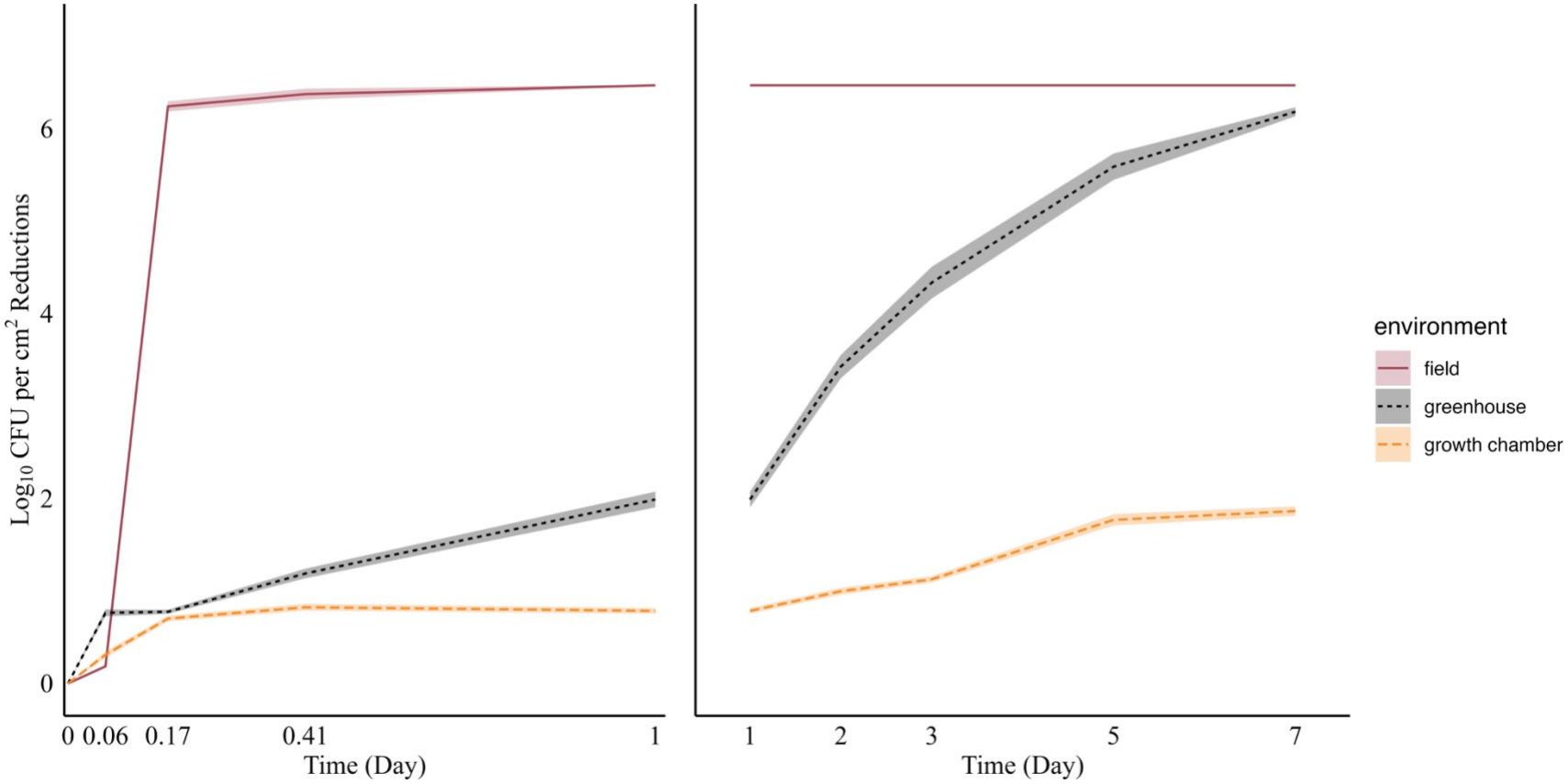

Figure 1. Generic *Escherichia coli* log CFU/cm^2 reductions across 0 to 7 days on plastic mulch held in the growth chamber, greenhouse, and field environments. Reductions were compared to the mean log CFU/cm^2 of the 0-day time-point. Shading represents standard error.

Table 1. The weather data for the greenhouse.

Source	Environment	Date Range		Average Temperature (°C)		Temperature Range (°C)		Average Relative Humidity (%)		Relative Humidity Range (%)	
				day	night	min	max	day	night	min	max
WatchDog Data Logger	Greenhouse	08/16/23,	08/25/23	31.6	24.2	22.2	39.4	41.8	64.1	26.3	69.7
WeatherSTEM	VT CALS Turf Grass	08/16/23,	08/25/23	23.4	17.9	14.6	28.9	66.3	83.3	44.5	93.7

Table 2. The weather data for the field.

Source	Environment	Date Range		Average Temperature (°C)		Temperature Range (°C)		Average Relative Humidity (%)		Relative Humidity Range (%)		Average Rainfall (in)	Average UV Index
				day	night	min	max	day	night	min	max		
WatchDog Data Logger	Field	09/04/23,	09/12/23	28.5	17.6	15.7	40.4	68.0	95.5	36.4	100.0	-[a]	
WeatherSTEM	Urban Horticulture	09/04/23,	09/12/23	23.2	18.0	16.2	28.9	81.3	97.9	59.3	100.0	0.06	1.55

[a] Rainfall and UV index were not measured by data loggers.

Chapter 5: Effect of Drop Height on Transfer of Generic *Escherichia coli* to Fresh Cucumber, Jalapeño, and Tomato

Formatted for submission to the Journal of Food Protection (Research Paper)

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Keywords: Biodegradable Mulch, Dropped Produce, *Escherichia coli*, Plastic Mulch, Pre-Harvest, Produce Safety

Abstract

The US FDA Food Safety Modernization Act Produce Safety Rule (PSR) prohibits harvesting dropped produce. Dropped produce is produce that drops to the ground before harvest and is excluded from harvest due to potential microbial contamination incurred from damage. This study aimed to evaluate the microbial risk of dropped produce on simulated contaminated mulch from various drop heights (0, 1, 2, 4, and 6 ft). Fresh cucumber, jalapeño, and tomato were dropped onto green-fluorescent protein-tagged generic *E. coli* spot inoculated dried plastic mulch coupons of ca. 5 log CFU/cm^2 through height-modified PVC (polyvinyl chloride) pipes of 1, 2, 4, and 6 ft. All commodities were also touched to inoculated dried plastic mulch coupons without dropping (0 ft). After 3 s of contact with the mulch coupons, the fruit and inoculated coupon were enumerated to determine generic *E. coli* concentrations. When generic *E. coli* counts of fruit fell below the limit of detection (< 1.30 log CFU/produce), produce was enriched. This protocol was repeated for additional tomato samples dropped on biodegradable mulch. Cucumber and tomato fruit were visibly damaged from 4 and 6 ft. Plastic mulch drop height did not impact generic *E. coli* log percent transfer within the commodity, as generic *E. coli* transferred regardless of drop height. Generic *E. coli* transferred to non-dropped (0 ft) cucumber and tomato. Plastic mulch transferred generic *E. coli* to more cucumber samples than other tested commodities. Significantly higher transfer of generic *E. coli* was observed at all treatment heights for tomato fruit dropped onto biodegradable mulch to tomato fruit dropped onto plastic mulch ($P < 0.05$). Findings indicate when a production surface is contaminated, it is likely to cross-contaminate fresh produce if contacted. To avoid potential downstream contamination risks, dropped produce should not be harvested due to potential damage and contamination, aligning with the FSMA PSR harvesting requirements.

Highlights

- Dropped cucumber and tomato fruits from 4 and 6 ft were visibly damaged.
- Generic *E. coli* transferred to tested commodities regardless of drop height.
- Cucumber and tomato fruits were contaminated even when not dropped (0 ft).
- Biodegradable mulch transferred higher concentrations to tomatoes than plastic mulch.

The US FDA Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) provided the fresh produce industry with the first science-based mandatory standards for growing, harvesting, packing, and holding fresh fruits and vegetables produced for human consumption (34). Regarding harvesting activities, subpart K of the FSMA PSR prohibits the distribution of dropped produce for the fresh market (32). Dropped produce is produce that drops to the ground before harvest when that is not an intended part of the growing or harvesting of that crop (32). The PSR explicitly states root crops that grow underground (such as carrots), crops that grow on the ground (such as cantaloupe), or produce that is intentionally dropped to the ground as a part of harvesting (such as almonds) are excluded from this requirement (32). Dropped produce can only be harvested if commercial processes, a "kill" step, are implemented to adequately reduce the microorganisms present (32).

Dropped produce is excluded from harvest due to potential microbial contamination that may occur from dropping and subsequent damage (31). As cited by the FDA in the FSMA PSR Final Rule, previous studies with apples and pears have indicated that dropped and damaged fresh produce contain higher microbial loads than non-dropped, intact produce (14, 27, 31). Structural damage to leafy greens and apples has been illustrated to enhance bacterial survival and proliferation as opposed to undamaged fruits (2-4, 12, 15, 18, 19, 28, 29). While previously studied commodities and commodities in the present study have outer layers frequently consumed (e.g., apple, tomato, cucumber), produce with outer layers not frequently consumed (e.g., mango, papaya) are also included in this harvesting requirement (31). Damage to the outer layer of fruit can provide access to the inner consumed portions of the produce (31). Microbial contamination of any origin in the initial stages of production is unlikely to become inactivated

at later steps and injured surfaces have been suggested to provide microbial protection from washing and disinfecting treatments (1, 9, 17).

While structural damage has exhibited increased proliferation of bacteria in select commodities, these results may not be applicable across the fresh produce industry due to production and physiological differences of commodities. The extent to which structural damage and bacteria transfer occur from physical dropping in the pre-harvest environment has been limitedly studied. In addition, "ground" is not explicitly defined in the FSMA PSR, and fresh produce may drop onto different ground surfaces depending on the commodity and production system, including soil or mulch. Previously, mulch has been shown to transfer to intact or undamaged fresh produce (22, 26, 30). While damage of any origin poses a potential microbial hazard, the contamination risk of dropped produce has yet to be determined and needs to be further elucidated. This study aimed to evaluate the microbial risk of dropping fresh produce commodities (cucumber, jalapeño, and tomato) from different heights (0, 1, 2, 4, and 6 ft) onto simulated contaminated mulch.

Materials and Methods

Material Selection and Preparation

Fresh produce was grown in a research plot at Virginia Tech's Urban Horticulture Center or greenhouse research facility located on Virginia Tech's campus (Blacksburg, Virginia, USA). Produce was stored post-harvest at 4 ± 2°C and processed within 24 h of harvest. A total of 15 fruits were used for each produce commodity for each drop height: cucumber (*Cucumis sativus* var. *Picolino*; Johnny's Selected Seeds, Winslow, ME, USA), jalapeño pepper (*Capsicum annum* var. *Early Jalapeño*; Johnny's Selected Seeds, Winslow, ME, USA), and cherry tomato (*Solanum lycopersicum* var. *Sakura*; Johnny's Selected Seeds, Winslow, ME, USA). Before

dropping, all produce commodities were weighed (Supplemental Materials, Table 1-2) and washed in 3 ppm bleach solution (Germicidal Bleach Cleaner; Clorox, Oakland, CA, USA) and air-dried in a biosafety cabinet.

Ground cover materials of interest were embossed white-on-black plastic mulch (1.25 mil, Ginegar Plastics, Inc., Santa Maria, CA, USA) and biodegradable mulch (6 mil, Bio360 Mulch, Berry Hill Irrigation, Inc., Buffalo Junction, VA, USA) obtained new from the mulch roll. Cucumber, jalapeño, and tomato were each dropped onto plastic mulch, and only tomato was dropped onto biodegradable mulch, through height-modified PVC (polyvinyl chloride) pipes obtained at and cut by a local hardware store (1, 2, 4, and 6 ft; Lowes, Blacksburg, VA, USA). Coupon and PVC pipe sizes were selected based on commodity size with pepper and tomato ground covers constructed to fit 100x15 mm Petri dish (Corning, Inc., Corning, NY, USA) and dropped through 4 in PVC (IPEX 4-in x 10-ft PVC DWV Foam Core Pipe, IPEX Inc., Oakville, ON**)** and cucumber ground cover to fit 150x15 mm Petri dish (Corning, Inc., Corning, NY, USA) and dropped through 6 in PVC (IPEX 6-in x 10-ft Sch 40 PVC DWV Pipe, IPEX Inc., Oakville, ON). PVC pipes were sanitized according to bleach protocols for food contact surfaces (10).

Bacterial Inoculum Preparation

Inoculum preparation was adapted from Blessington et al. (7) using a rifampicin-resistant (80 ppm), green-fluorescent protein-tagged generic *Escherichia coli* TVS 353. Onto tryptic soy agar (TSA; Bacto, BD, Franklin Lakes, NJ, USA) containing 80 ppm rifampicin (TSAR; TCI America, Inc., Portland, OR, USA), the frozen (-80°C) stock culture was streaked and incubated at 37 ± 2°C for 24 h. After incubation, an isolated colony was transferred to 10 mL of tryptic soy broth (TSB; Bacto, BD, Franklin Lakes, NJ, USA) containing 80 ppm rifampicin (TSBR) and incubated at 37 ± 2°C for 24 h. A 10-µL loop of TSBR culture was transferred to fresh 10 mL

TSBR and incubated at 37 ± 2°C for 24 h. A 1-mL aliquot of the second overnight TSBR was spread across a large TSAR petri plate (150x15 mm) and incubated at 37 ± 2°C for 24 h. The bacterial lawn was harvested by adding 9 mL 0.1% peptone water (Fisher BioReagents, Thermo Fisher Scientific, Inc., Waltham, MA, USA), loosening the lawn with a sterile spreader, and pipetting the suspension into a sterile falcon tube. The culture slurry was diluted in 0.1% peptone water to reach an approximate concentration of 10^8 log CFU/mL with final concentrations verified by enumeration on TSAR by serial dilutions.

Coupon Inoculation and Controlled Drop Treatments

Coupons were surface sterilized with 70% ethanol (VWR International, LLC, Radnor, PA, USA) and air-dried before inoculation. A 100-µL (twenty 5-µL) sample of the diluted inoculum was spot inoculated to sterilized ground cover coupons. Coupons were dried for 90 minutes in the biosafety cabinet and held in a growth chamber (23°C, 55% RH) for 24 h post-inoculation. Immediately after inoculation and post 24 h dry, coupons were enumerated to establish generic *E. coli* concentrations pre-drop. Treatment heights included 0, 1, 2, 4, and 6 ft with 0 ft being no drop and only contact with the inoculated coupon. Produce commodities were dropped blossom end down through sanitized, height-modified PVC pipes (4 in, pepper and tomato; 6 in, cucumber) from the drop heights selected of 1, 2, 4, and 6 ft onto 24 h dried inoculated plastic mulch coupons. Tomatoes were also dropped onto 24 h dried inoculated biodegradable mulch coupons following the same protocol. Dropped and no-drop (0 ft) produce remained in contact with inoculated coupons for 3 s. After 3 s, the produce and inoculated coupon were treated following subsequent enumeration methods. Damaged produce that did not represent marketable produce (e.g., broke into ≥2 pieces, visible cracking) was excluded from further analysis.

Produce and Coupon Enumeration

After 3 s contact, produce was removed from the inoculated coupon and placed in a filtered sterile sampling bag (Nasco Whirl-Pak, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the inoculated coupon was placed in a 7 in x 12 in non-filtered sterile sampling bag (Fisherbrand, Thermo Fisher Scientific, Inc., Waltham, MA, USA), each washed with 20 mL 0.1% peptone water, and hand-massaged for 30 s, shaken for 30 s, and hand-massaged for 30 s. The same enumeration protocol was used for coupons to establish generic *E. coli* concentrations pre-drop. Samples were serially diluted in 0.1% peptone water and 0.1-mL aliquots were surface plated onto TSAR in duplicate. For produce samples with low counts, 0.1-mL aliquots to one plate or 1 mL distributed over four plates (0.25 mL each) of rinsate was directly plated onto TSAR, in duplicate. All plates were incubated at 37 ± 2°C for 24 h and counts were confirmed using UV light. Colonies were counted manually by placing TSAR plates in a UV light box and only fluorescing colonies were included in bacterial counts.

Produce Enrichment

Produce samples below the limit of detection (< 1.30 log CFU/produce) were enriched following FDA BAM method for *E. coli* (33). Briefly, produce samples were stomached (90 s, 4 strokes/s), hand macerated for 90 s, and stomached (90 s, 4 strokes/s). Samples were enriched with 200 mL *E. coli* broth and hand-massaged for 30 s, shaken for 30 s, and hand-massaged for 30 s. Samples were incubated at 37 ± 2°C for 24 h. Post-incubation, samples were streaked onto TSAR and were incubated at 37 ± 2°C for 24 h. After 24 h, plates were examined to determine the presence or absence of generic *E. coli* with the presence confirmed using UV light. If growth fluoresced under a UV light box, *E. coli* presence was marked as positive.

Statistical Analysis

Coupon counts were calculated based on mulch coupon size (cm^2); therefore, 15 cm^2 (100x15 mm) for tomato and pepper and 22 cm^2 (150x15 mm) for cucumber. All counts were log-transformed (mulch samples: log CFU/cm^2 = log (CFU_{mulch} x 20 mL wash water / $cm^2{}_{mulch}$); produce samples: log CFU/produce = log ($CFU_{produce}$ x 20 mL wash water)). Produce samples below the limit of detection (< 1.30 log CFU/produce) were removed from analysis when calculating the mean and standard deviation of log CFU/produce and log percent transfer. Log percent transfer calculations were adapted from Todd-Searle et al. (30) using: Log Percent Transfer (%) = $Logarithm_{10}$ ($CFU_{produce}$ / ($CFU_{produce}$ + CFU_{mulch}) x 100%). Log transformation and log percent transfer calculations were computed in Microsoft Excel. RStudio version 4.2.3 was used for all remaining statistical analyses to evaluate significant differences ($P \leq 0.05$) using Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) Test.

Results

Transfer of Generic Escherichia coli to Cucumbers from Plastic Mulch

Dried coupons for cucumber trials with no produce treatment (post 24 h dry) ranged between 4.47 and 5.77 log CFU/cm^2 with a mean concentration of 5.43 log CFU/cm^2 (± 0.30) (Supplemental Materials, Table 3). Mean concentrations on inoculated coupons after cucumber contact (0-4 ft) ranged between 5.37 (± 0.25) and 5.56 (± 0.14) log CFU/cm^2 (Supplemental Materials, Table 4). Cucumber samples dropped from 4 (5/15) and 6 ft (15/15) were damaged and excluded from further analysis (Table 1). Across all remaining drop heights, the minimum generic *E. coli* transfer was below the limit of detection (< 1.30 log CFU/cucumber) and the maximum was ca. 4.08 log CFU/cucumber (0-4 ft). If generic *E. coli* transfer occurred, cucumber mean counts ranged from 1.87 (± 0.72) to 2.78 (± 0.77) log CFU/cucumber and mean

log percent transfer from 0.68 (± 0.74) to 1.46 (± 0.44) with 9 to 12 cucumbers or 60.0% to 90.0% of cucumbers with transfer (0-4 ft). In cases of 0, 1, and 2 ft, an additional cucumber was positive post-enrichment for generic *E. coli*. Significant differences were observed between drop height 0 to 1, 2, and 4 ft and cucumber generic *E. coli* count or log percent transfer ($P < 0.05$).

Transfer of Generic Escherichia coli to Jalapeños from Plastic Mulch

Dried coupons for jalapeño trials with no produce treatment ranged between 4.61 and 6.47 log CFU/cm^2 with a mean concentration of 5.33 log CFU/cm^2 (± 0.25). Mean concentrations on inoculated coupons after jalapeño contact (0-6 ft) ranged between 5.10 (± 0.28) and 5.29 (± 0.41) log CFU/cm^2. No jalapeño samples were damaged post-drop with a generic *E. coli* minimum count below the limit of detection (< 1.30 log CFU/jalapeño) and a maximum count of ca. 1.90 log CFU/jalapeño across 0 to 6 ft (Table 2). At 0 ft, all generic *E. coli* counts were below the limit of detection with no positive jalapeño fruits post-enrichment. If generic *E. coli* transfer occurred, jalapeño mean counts ranged from 1.30 (± 0.00) to 1.90 log CFU/jalapeño and mean log percent transfer from -0.03 (± 0.01) to 0.45 with 1 to 2 jalapeño fruit or 6.7% to 13.3% of jalapeño fruit with transfer (1-6 ft). Between 1 and 3 more jalapeño samples were positive for generic *E. coli* post-enrichment for drop heights 1, 2, 4, and 6 ft. No significant differences were observed between all drop heights with generic *E. coli* transfer (1-6 ft) and jalapeño log percent transfer ($P > 0.05$).

Transfer of Generic Escherichia coli to Tomatoes from Plastic Mulch

Dried coupons for plastic mulch tomato trials with no produce treatment ranged between 4.84 and 5.93 log CFU/cm^2 with a mean concentration of 5.54 log CFU/cm^2 (± 0.25). Mean concentrations on inoculated coupons after tomato contact (0-6 ft) ranged between 5.50 (± 0.25) and 5.63 (± 0.24) log CFU/cm^2. Tomato samples from 4 (2/15) and 6 ft (6/15) were damaged

post-drop and excluded from further analysis (Table 3). Across all drop heights, the generic *E. coli* minimum count fell below the limit of detection (< 1.30 log CFU/tomato), and the maximum count was ca. 3.16 log CFU/tomato (0-6 ft). If generic *E. coli* transfer occurred, tomato mean counts ranged from 1.40 (± 0.17) to 2.58 (± 0.62) log CFU/tomato and mean log percent transfer from 0.46 (± 0.16) to 1.19 (± 0.43) with 2 to 4 or 13.3% to 44.4% of tomatoes with counts. One to two additional tomato fruits were positive post-enrichment for heights of 0, 1, 2, and 6 ft. No significant differences were observed between all drop heights and generic *E. coli* log percent transfer for tomato ($P > 0.05$).

Comparison of Generic Escherichia coli Transfer Across Commodities from Plastic Mulch

Generic *E. coli* transferred to more cucumber fruit at all tested drop heights compared to jalapeño and tomato (Table 4). At least 9 cucumber fruits showed transfer across all heights with marketable samples compared to at most 2 and 4 fruits for jalapeño and tomato, respectively. Similarly, at minimum 9 cucumber fruits were positive post-enrichment compared to a maximum of 5 for jalapeño and 6 for tomato. In addition, cucumber maximum surface counts were ca. 4.08 log CFU/cucumber versus 1.90 log CFU/jalapeño and 3.16 log CFU/tomato. For all drop heights with generic *E. coli* counts, the log percent transfer was significantly higher for cucumber than jalapeño (1, 2, and 4 ft; $P < 0.05$). However, at drop heights of 0 and 2 ft, no significant differences were observed between the log percent transfer of cucumber and tomato ($P > 0.05$). As for tomato and jalapeño, no significant differences in log percent transfer were observed between both commodities at all drop heights with transfer (1, 2, 4, and 6 ft; $P > 0.05$). While results are not shown in this present study, surface transfer and commodity weight were not correlated.

Transfer of Generic Escherichia coli to Tomatoes from Biodegradable Mulch and Plastic Mulch

Dried coupons for biodegradable mulch tomato trials with no produce treatment ranged between 5.32 and 6.13 log CFU/cm^2 with a mean concentration of 5.69 log CFU/cm^2 (± 0.21). Mean concentrations on inoculated coupons after tomato contact (0-6 ft) ranged between 5.66 (± 0.29) and 5.80 (± 0.25) log CFU/cm^2 (Supplemental Materials, Table 5). Tomato samples dropped onto biodegradable mulch were damaged at 4 (3/15) and 6 ft (7/15) and removed from further analysis (Table 5). Generic *E. coli* counts on biodegradable mulch dropped tomato ranged from below the limit of detection (< 1.30 log CFU/tomato) to ca. 4.68 log CFU/tomato across all drop heights. If generic *E. coli* transfer occurred from biodegradable mulch to tomatoes, counts ranged from 3.08 (± 0.86) to 3.85 (± 0.53) log CFU/tomato and mean log percent transfer from 1.44 (± 0.50) to 1.81 (± 0.10) with 6 to 14 or 40.0% to 100.0% of tomato fruits with counts. Biodegradable mulch dropped tomatoes ranged from 8 to 14 tomato fruits or 53.3% to 100% of tomato fruits positive post-enrichment. Varying differences occurred between all drop heights and biodegradable mulch dropped tomato surface transfer.

Tomato samples dropped onto biodegradable mulch had significantly higher log percent transfer for all drop heights than tomato samples dropped onto plastic mulch (0, 1, 2, 4, and 6 ft; $P < 0.05$) (Table 6). Plastic mulch and biodegradable mulch surface transfer counts of dropped tomato ranged from 2 to 4 and 6 to 14 tomato fruits and positive enrichment counts of dropped tomato fruits ranged from 3 to 6 and 8 to 14 tomatoes, respectively. In the case of 4 ft, generic *E. coli* was transferred to 100% of the biodegradable mulch dropped tomato samples with a minimum of ca. 2.95 log CFU/tomato. Tomato samples dropped on plastic mulch had generic *E. coli* transfer to 23.1% of samples with a maximum of ca. 2.20 log CFU/tomato.

Discussion

Factors Affecting Bacterial Transfer

Bacterial transfer is affected by many factors including the type of surface, fresh produce, and bacteria, contact and residence time, pressure, and moisture (6, 11, 20, 23, 30). In this study, the type of mulch and produce commodity affected bacterial transfer. Biodegradable mulch transferred more generic *E. coli* to tomato fruits than plastic mulch. Across all comparable height treatments, cucumber samples experienced higher transfer than jalapeño samples. Due to cucumber size and texture, these fruits are assumed to have a higher surface area and roughness compared to jalapeño, and higher surface roughness has been shown to increase bacterial adhesion (8). However, cucumber and tomato generic *E. coli* transfer did not differ at 0 and 6 ft and tomato transfer did not differ from jalapeño at all drop heights. Cherry tomatoes, like jalapeños, are smaller and less rough than cucumbers, indicating surface roughness may not be a factor contributing to variations in transfer observed by commodity in this present study. In general, natural crop features, such as crevices or stem scars, can affect pathogen transfer and proliferation (13, 16, 21, 24). Nevertheless, generic *E. coli* transfer occurred when dropping produce independent of commodity or surface type, suggesting neither is the primary factor dictating bacterial transfer.

This present study assumes that dropped produce would detach during harvesting activities and remain minimally in contact with the ground as 3 s of contact with simulated contaminated mulch was evaluated. Bhuller et al. (6) found no effect on contact time between 5 s, 1 min, 10 min, 1 h, and 4 h when dropping apples, lettuce, and peaches onto carpet and tile surfaces, rather transfer occurred independent of contact time. While Miranda and Schaffner (23) showed increasing contact times of <1, 5, 30, and 300 s increased the amount of transfer to

watermelon flesh, bread, and gummy candy when contacting stainless steel, ceramic, tile, wood, and carpet, the bacteria transferred at all tested times (23). Similarly, Dawson et al. (11) found contact times of 5, 30, and 60 s to increase bacterial transfer to bologna and bread, but only when residence time on the inoculated surfaces exceeded 8 h. While contact time and residence time may have an impact on the transfer of bacteria, bacteria were transferred at all contact times in previous studies; thus, 3 s contact time and 24 h coupon dry time tested may be effective in determining the microbial risk of dropped produce.

Effect of Drop Height

The removal of visibly damaged dropped cucumber and tomato samples from 4 and 6 ft, indicates damage from dropping is likely to occur. Damage was not assessed past visible breaking or cracking, however, that does not mean nonvisible damage did not occur at lower drop heights. Damage alone indicates that dropped produce may not be of marketable quality despite the microbial risk. Generally, across all commodities and mulch types, no effect of drop height (1, 2, 4, and 6 ft) on generic *E. coli* transfer was seen. More importantly, if the fresh produce was dropped, it became contaminated. The dropping of cucumbers and jalapeños significantly increased the log percent transfer compared to non-dropped fruits (0 ft). In the case of cucumbers, while significantly lower, generic *E. coli* transfer still occurred when not dropped (0 ft). Similarly, un-dropped tomatoes showed transfer; however, transfer was not significantly different across all treatments, indicating contact alone with the contaminated mulch, contaminated tomatoes independent of dropping. Transfer at no drop (0 ft) for cucumber and tomato indicates that dropping may play less of a role and instead contact with any contaminated surface may cross-contaminate fresh produce.

However, counts of generic *E. coli* were below the limit of detection for jalapeño with no fruit positive post-enrichment at 0 ft. This contradicts the findings of cucumber and tomato, as contact alone did not cause contamination of jalapeño samples. Contact with a contaminated source does not always mean fruit contamination; however, fresh jalapeño has previously been implicated with speculation of contaminated water as a causative agent proving contamination without dropping can occur (5, 25). Additionally, this experiment was conducted in a laboratory setting; thus, different results in the production field may be observed. Contamination and damage of two out of the three dropped commodities indicate that harvesting dropped produce is a potential risk. Contamination, even when minimal, has the capability of creating food safety issues as produce is unlikely to be inactivated at a later point, especially if improper prevention and mitigation practices are implemented (1). Excluding dropped produce from harvest, despite commodity, is a simple mitigation practice to decrease potential downstream contamination risks, as required by the FSMA PSR.

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Tables

Table 1. The data for dropped cucumbers onto plastic mulch.

Commodity	Height (ft)	Damaged Post-Drop	Surface Transfer		Enrichment		Log CFU/Produce Range		Surface Transfer[a]					
			Count	%	Count	%	min	max	n =	Log CFU/ Produce		Log Percent		Percent
Cucumber	0	0/15	9/15	60.0	10/15	66.7	<1.30,	3.19	9	1.87±0.72[b]	a[c]	0.68±0.74	a	1.97±2.10
	1	0/15	12/15	80.0	13/15	86.7	<1.30,	4.08	12	2.76±0.74	b	1.46±0.44	b	4.31±1.55
	2	0/15	10/15	66.7	11/15	73.3	<1.30,	3.76	10	2.78±0.77	b	1.32±0.59	b	3.74±1.80
	4	5/15	9/10	90.0	9/10	90.0	<1.30,	3.92	9	2.75±0.52	b	1.33±0.29	b	3.78±1.34
	6	15/15[d]							-					

[a] Counts below the limit of detection (< 1.30 log CFU/produce) were removed.
[b] Mean ± standard deviation of generic *Escherichia coli*
[c] Lowercase letters indicate a significant difference ($P < 0.05$) within each commodity
[d] All cucumbers dropped from 6 ft were visibly damaged and removed from further analysis post-drop.

Table 2. The data for dropped jalapeños onto the plastic mulch.

Commodity	Height (ft)	Damaged Post-Drop	Surface Transfer		Enrichment		Log CFU/Produce Range			Surface Transfer[a]					
			Count	%	Count	%	min	max	n =	Log CFU/ Produce		Log Percent		Percent
Jalapeño	0	0/15	0/15	0.0	0/15	0.0	<1.30,	<1.30	0			-[b]		
	1	0/15	1/15	6.7	2/15	13.3	<1.30,	1.30	1	1.30±0.00[c]	a[d]	-0.03±0.01	a	0.97±1.01
	2	0/15	2/15	13.3	3/15	20.0	<1.30,	1.30	2	1.30±0.00	a	0.10±0.25	a	1.11±1.28
	4	0/15	2/15	13.3	5/15	33.3	<1.30,	1.90	2	1.80±0.17	b	0.45±0.12	a	1.57±1.13
	6	0/15	1/15	6.7	3/15	20.0	<1.30,	1.90	1	1.90[e]	b	0.45	a	1.57

[a] Counts below the limit of detection (< 1.30 log CFU/produce) were removed.
[b] All counts were below the limit of detection for jalapeños at 0 ft.
[c] Mean ± standard deviation of generic *Escherichia coli*
[d] Lowercase letters indicate a significant difference ($P < 0.05$) within each commodity.
[e] No standard deviation could be calculated due to only 1 jalapeño sample with generic *Escherichia coli* counts.

Table 3. The data for dropped tomatoes onto the plastic mulch.

Commodity	Height (ft)	Damaged Post-Drop	Surface Transfer		Enrichment		Log CFU/Produce Range			Surface Transfer[a]				
			Count	%	Count	%	min	max	n =	Log CFU/ Produce		Log Percent		Percent
Tomato	0	0/15	2/15	13.3	4/15	26.7	<1.30,	1.60	2	1.40±0.17[b]	a[c]	0.46±0.16	a	1.58±1.17
	1	0/15	4/15	26.7	6/15	40.0	<1.30,	2.20	4	1.83±0.34	ab	0.71±0.36	a	2.03±1.43
	2	0/15	4/15	26.7	6/15	40.0	<1.30,	2.60	4	1.87±0.57	ab	0.76±0.61	a	2.14±1.84
	4	2/15	3/13	23.1	3/13	23.1	<1.30,	2.20	3	1.89±0.37	ab	0.71±0.37	a	2.03±1.45
	6	6/15	4/9	44.4	5/9	55.6	<1.30,	3.16	4	2.58±0.62	b	1.19±0.43	a	3.29±1.54

[a] Counts below the limit of detection (< 1.30 log CFU/produce) were removed.
[b] Mean ± standard deviation of generic *Escherichia coli*
[c] Lowercase letters indicate a significant difference ($P < 0.05$) within each commodity and mulch.

Table 4. The data for dropped cucumbers, jalapeños, and tomatoes onto the plastic mulch.

Commodity	Height (ft)	Surface Transfer[a]				
		n =	Log CFU/Produce		Log Percent	
Cucumber	0	9	1.87±0.72[b]	A[c]	0.68±0.74	A
Jalapeño		0		-[d]		
Tomato		2	1.40±0.17	A	0.46±0.16	A
Cucumber	1	12	2.76±0.74	A	1.46±0.44	A
Jalapeño		1	1.30±0.00	B	-0.03±0.01	B
Tomato		4	1.83±0.34	B	0.71±0.36	B
Cucumber	2	10	2.78±0.77	A	1.32±0.59	A
Jalapeño		2	1.30±0.00	B	0.10±0.25	B
Tomato		4	1.87±0.57	B	0.76±0.61	AB
Cucumber	4	9	2.75±0.52	A	1.33±0.29	A
Jalapeño		2	1.80±0.17	B	0.45±0.12	B
Tomato		3	1.89±0.37	B	0.71±0.37	B
Cucumber	6	0		-[e]		
Jalapeño		1	1.90[f]	A	0.45	A
Tomato		4	2.58±0.62	A	1.19±0.43	A

[a] Counts below the limit of detection (< 1.30 log CFU/produce) were removed.
[b] Mean ± standard deviation of generic *Escherichia coli*
[c] Capital letters indicate a significant difference ($P < 0.05$) across each commodity and height
[d] All counts were below the limit of detection for jalapeños at 0ft.
[e] All cucumbers dropped from 6ft were visibly damaged and removed from further analysis post-drop.
[f] No standard deviation could be calculated due to only 1 jalapeño sample with generic *Escherichia coli* counts.

Table 5. The data for dropped tomatoes on biodegradable mulch.

Commodity	Material	Height (ft)	Damaged Post-Drop	Surface Transfer		Enrichment		Log CFU/Produce Range			Surface Transfer[a]				
				Count	%	Count	%	min	max	n =	Log CFU/ Produce		Log Percent		Percent
Tomato	Biodegradable	0	0/15	6/15	40.0	8/15	53.3	<1.30,	3.99	6	3.27±0.51[b]	ab[c]	1.57±0.29	ab	4.81±1.34
		1	0/15	14/15	93.3	14/15	93.3	<1.30,	4.53	14	3.08±0.86	a	1.44±0.50	a	4.22±1.65
		2	0/15	13/15	86.7	13/15	86.7	<1.30,	4.54	13	3.40±0.91	ab	1.67±0.37	ab	5.31±1.45
		4	3/15	12/12	100.0	12/12	100.0	2.95,	4.68	12	3.85±0.53	b	1.81±0.10	b	6.11±1.11
		6	7/15	6/8	75.0	8/8	100.0	<1.30,	4.66	6	3.20±0.88	ab	1.57±0.39	ab	4.81±1.48

[a] Counts below the limit of detection (< 1.30 log CFU/produce) were removed.
[b] Mean ± standard deviation of generic *Escherichia coli*
[c] Lowercase letters indicate a significant difference ($P < 0.05$) within each commodity and mulch.

Table 6. The data for dropped tomatoes on plastic mulch and biodegradable mulch.

Commodity	Height (ft)	Material	Surface Transfer[a]				
			n =	Log CFU/Produce		Log Percent	
Tomato	0	Plastic	2	1.40±0.17[b]	A[c]	0.46±0.16	A
		Biodegradable	6	3.27±0.51	B	1.57±0.29	B
	1	Plastic	4	1.83±0.34	A	0.71±0.36	A
		Biodegradable	14	3.08±0.86	B	1.44±0.50	B
	2	Plastic	4	1.87±0.57	A	0.76±0.61	A
		Biodegradable	13	3.40±0.91	B	1.67±0.37	B
	4	Plastic	3	1.89±0.37	A	0.71±0.37	A
		Biodegradable	12	3.85±0.53	B	1.81±0.10	B
	6	Plastic	4	2.58±0.62	A	1.19±0.43	A
		Biodegradable	6	3.20±0.88	A	1.57±0.39	B

[a] Counts below the limit of detection (< 1.30 log CFU/produce) were removed.
[b] Mean ± standard deviation of generic *Escherichia coli*
[c] Capital letters indicate a significant difference ($P < 0.05$) across each height and mulch.

Supplemental Materials

Table 1. The weight data for produce dropped onto plastic mulch (n=15).

Commodity	Material	Height (ft)	Weight (g)[a]
Cucumber	Plastic Mulch	0	124.70±49.02
		1	116.39±30.15
		2	114.96±40.01
		4	116.00±40.33
		6	113.76±40.42
Jalapeño		0	10.77±2.88
		1	11.30±3.78
		2	10.43±2.69
		4	11.00±3.90
		6	10.88±3.86
Tomato		0	9.23±2.29
		1	10.58±3.19
		2	9.94±3.02
		4	10.63±2.67
		6	10.08±2.06

[a] Mean ± standard deviation

Table 2. The weight data for tomatoes dropped onto biodegradable mulch (n=15).

Commodity	Material	Height (ft)	Weight (g)[a]
Tomato	Biodegradable Mulch	0	7.87±2.63
		1	7.54±2.06
		2	7.49±1.46
		4	7.63±2.17
		6	8.41±2.95

[a] Mean ± standard deviation

Table 3. Mean ± standard deviation of log CFU/cm^2 of generic *Escherichia coli* on mulch pre-drop per commodity (n=15).

Commodity	Material	Log CFU/cm^2
Cucumber	Plastic	5.43±0.30
Jalapeño		5.33±0.25
Tomato		5.54±0.25
	Biodegradable	5.69±0.21

Table 4. Mean ± standard deviation of log CFU/cm^2 of generic *Escherichia coli* on plastic mulch post-drop per commodity.

Commodity	Material	Height (ft)	n =	Log CFU/cm^2
Cucumber	Plastic	0	15	5.48±0.24
		1	15	5.56±0.14
		2	15	5.45±0.20
		4	10	5.37±0.25
		6	0[a]	-
Jalapeño		0	15	5.29±0.41
		1	15	5.27±0.31
		2	15	5.20±0.30
		4	15	5.13±0.33
		6	15	5.10±0.28
Tomato		0	15	5.59±0.24
		1	15	5.63±0.24
		2	15	5.56±0.28
		4	13	5.60±0.28
		6	9	5.50±0.25

[a] All cucumbers were damaged from 6ft.

Table 5. Mean ± standard deviation of log CFU/cm^2 of generic *Escherichia coli* on biodegradable mulch post tomato drop.

Commodity	Material	Height (ft)	n =	Log CFU/cm^2
Tomato	Biodegradable	0	15	5.74±0.24
		1	15	5.66±0.29
		2	15	5.76±0.13
		4	12	5.76±0.20
		6	8	5.80±0.25

Chapter 6: Effect of Contact Time on Transfer of Generic *Escherichia coli* to Fresh Cucumber, Jalapeño, and Tomato

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Abstract

Dropped produce is excluded from harvest per the US FDA Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR). Dropped produce includes fresh produce that is detached from the plant and unintentionally contacts the ground and produce attached to the plant and unintentionally contacts the ground. The exclusion of produce attached to the plant in contact with the ground indicates ground contact is a food safety risk, however, the impact of contact time has been minimally researched regarding this scope. This study aims to characterize the risk of produce (cucumber, jalapeño, and tomato) growing in contact with plastic mulch over 7 days. Plastic mulch coupons were spot inoculated with a green-florescent protein tagged generic *Escherichia coli* at ca. 5 log CFU/cm^2 and dried for 24 h. After 24 h, coupons were transported to the production field. Fresh cucumber, jalapeño, and tomato fruit attached to the plant were placed in contact with contaminated generic *E. coli* mulch and enumerated at 0 (3 s), 1, 3, 5, and 7 days post-contact. If coupon or fruit counts fell below the limit of detection, samples were enriched following a modified FDA BAM method. Generic *E. coli* transferred to the fruits of cucumber and tomato across all contact times. No differences were observed in the contact time of tomato and log percent transfer ($P > 0.05$). Jalapeño counts were below the limit of detection on days 3 and 5 with no differences observed in log percent transfer and all contact times with transfer ($P > 0.05$). A higher percentage of cucumber samples were contaminated compared to other tested commodities. In general, transfer occurred with no trend in increasing contact time, showing ground contact alone may not be the sole factor contributing to contamination. Instead, food safety efforts should focus on reducing contact with a contamination source in the pre-harvest environment, as already outlined in the FSMA PSR, rather than ground contact alone.

Highlights

- Generic *E. coli* transferred to cucumber and tomato at all tested contact times.
- More cucumber samples were contaminated than other tested commodities.
- Transfer of generic *E. coli* was variable across tested commodities and contact time.
- Contact with contaminated mulch generally resulted in contaminated produce samples.

According to Subpart K of the US FDA Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR), dropped produce is prohibited from distribution (27). Dropped produce is produce that drops to the ground before harvest (27). The FDA has further clarified that dropped produce includes produce that is detached from the plant and unintentionally contacts the ground as well as produce that is attached to the plant and unintentionally contacts the ground (26, 28). Root crops that grow underground, crops that grow on the ground, or produce intentionally dropped for harvest are excluded from Subpart K of the FSMA PSR (27). Detached dropped produce is prohibited from harvest due to potential microbial contamination incurred from damage, as structurally damaged produce has been shown to increase the proliferation of bacteria (1-3, 5, 8, 11, 13, 21, 24).

Exclusion of produce attached to the plant and unintentionally contacting the ground indicates that ground contact alone is a food safety risk regardless of damage. Cantaloupes growing in contact with soil had higher counts of bacteria on the under surfaces touching soil compared to upper surfaces not touching soil (6). However, cantaloupes naturally grow on the ground and would be excluded from the FSMA PSR dropped produce requirement, providing non-applicable reasoning for the exclusion of ground contacted produce. Tomato and cucumber fruits have also shown higher counts of bacteria when contacting ground surfaces of soil and mulch than those avoiding ground contact (9, 16, 19). While simulated contaminated mulch has been shown to transfer *Salmonella* to fresh tomato samples, the fruit tested had been harvested from the plant and detached when evaluating transfer (25). The impact of ground contact time over set time-points for attached fresh produce has been minimally studied.

Ground has not been exclusively defined by the FSMA PSR and may include various surfaces (e.g., soil, mulch) depending on the growing environment and horticultural practices

used. In open-field production, plastic mulch is a common horticultural practice due to weed control, increased soil temperature, reduced water loss, fertilizer leaching, and soil compaction (15, 23). Specifically, when cucumber, tomato, and pepper plants are grown with plastic mulch, early harvest, yield, and quality increases have been reported (15). Trellising is often accompanied by plastic mulch, as trellises can support fruit reducing disease pressure and space requirements, increase photosynthetic rates due to increased light exposure, increase yield and fruit quality, and protect from ground contact (16). Depending on the produce commodity and variety, trellising and natural vertical growth protect from ground contact by lifting the plant off the ground; however, unintentional contact with the ground can still occur due to naturally low-growing fruit or as fruits increase in size.

A Northeast Center to Advance Food Safety unpublished survey found that handling dropped produce was a commonly misunderstood practice, which may be attributed to several factors (7). For example, trellised cucumber plants may produce more uniform fruit, increase yields, and reduce disease and pest pressure compared to ground grown (10, 14, 20, 22). Despite trellising benefits, depending on the size of the operation, cucumber plants are frequently left to grow and trail the ground due to the labor and cost required to trellis fruit (14, 16, 17, 22). If left to grow intentionally and naturally on the ground, cucumber fruits would then be excluded from Subpart K of the FSMA PSR (e.g., crops that grow on the ground) and thus acceptable to harvest (27). Tomato presents a similar challenge to cucumber when interpreting the harvesting requirements of FSMA PSR, as tomato plants are also vining crops that would naturally grow on the ground if not staked. To help clarify the FSMA PSR dropped produce harvesting requirements, this study aims to evaluate the transfer of generic *Escherichia coli* to three fresh produce commodities (cucumber, jalapeño, and tomato) over the course of 0 to 7 days.

Materials and Methods

Fresh Produce Commodities

All fresh produce used in this study was grown locally in an open field at the Virginia Tech Urban Horticulture Center (Blacksburg, Virginia, USA). Cucumber (*Cucumis sativus* var. *Picolino*; Johnny's Selected Seeds, Winslow, ME, USA), jalapeño pepper (*Capsicum annum* var. *Early Jalapeño*; Johnny's Selected Seeds, Winslow, ME, USA), and cherry tomato (*Solanum lycopersicum* var. *Sakura*; Johnny's Selected Seeds, Winslow, ME, USA) were the commodities of interest. All fresh produce was grown in individual rows grouped by commodity laid with embossed white-on-black plastic mulch (1.25 mil, Ginegar Plastics, Inc., Santa Maria, CA, USA) with the white side facing up. Cucumber plants were left to trail on the plastic mulch and jalapeño plants were left unsupported for natural vertical growth. Tomato plants were provided support for vertical growth using a basket weave system. Five contact times (0, 1, 3, 5, and 7 d) were selected with 15 fruits per time-point for all commodities.

Inoculum Preparation

Rifampicin-resistant (80 ppm), green-fluorescent protein-tagged generic *Escherichia coli* was prepared using methods outlined by Blessington et al. (4). The generic *E. coli* frozen stock culture (-80°C) was streaked onto tryptic soy agar (TSA; Bacto, BD, Franklin Lakes, NJ, USA) containing 80 ppm rifampicin (TSAR; TCI America, Inc., Portland, OR, USA) and incubated for 24 h at 37 ± 2°C. After 24 h incubation period, a single colony was transferred to 10 mL of tryptic soy broth (TSB; Bacto, BD, Franklin Lakes, NJ, USA) containing 80 ppm rifampicin (TSBR) and incubated for 24 h incubation at 37 ± 2°C. 10-µL of overnight TSBR was transferred to 10 mL of fresh TSBR and incubated following the same conditions. The second overnight TSBR was spread in the amount of 1 mL over a large, 150x15 mm, TSAR petri plate

(Corning, Inc., Corning, NY, USA) and incubated for 24 h at 37°C ± 2°C. Using 9 mL 0.1% peptone water (Fisher BioReagents, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a sterile spreader, the resulting bacterial lawn was harvested and pipetted into a sterile tube. The generic *E. coli* slurry was diluted in 0.1% peptone water to reach a final concentration of ca. 8 log CFU/mL with verification via standard spread plating on TSAR.

Coupon Inoculation and Produce Treatment

Embossed white-on-black plastic mulch, obtained from the same roll used in the field, was constructed to fit either a 150x15 mm Petri dish for cucumber samples or a 100x15 mm Petri dish (Corning, Inc., Corning, NY, USA) for tomato and jalapeño samples. All coupons were affixed to Petri plates using double-sided mounting tape with the black side facing up. Before inoculation, mulch coupons were sprayed with 70% ethanol (VWR International, LLC, Radnor, PA, USA) and air-dried. Mulch coupons were spot inoculated with 100-µL of the diluted generic *E. coli* in the form of twenty 5-µL spots evenly dispersed. After inoculation, coupons were air-dried in a biosafety cabinet for 90 minutes and then relocated to a growth chamber with conditions set to 23°C, 55% RH for 24 h. After 24 h, coupons were transported in covered bins to the planting site for use. 24 h dried coupons were enumerated to determine starting generic *E. coli* levels as described in the enumeration procedure (Supplemental Materials, Table 1). In the field, a single fruit of each commodity was laid on a dried inoculated mulch dish and left until removed for enumeration.

Enumeration Procedure

Fruits and coupons of each commodity were enumerated at 0, 1, 3, 5, and 7 d post field placement. At 0 d, the produce contacted inoculated mulch for 3 s and was immediately enumerated. All additional produce was left in the field and randomly enumerated at the selected

time-points. To enumerate, the produce was placed in a filtered sterile sampling bag (Nasco Whirl-Pak, Thermo Fisher Scientific, Inc., Waltham, MA, USA) washed with 20 mL of 0.1% peptone water and hand-massaged for 30 s, shaken for 30 s, and hand-massaged for 30 s. The inoculated coupon was placed in a 7 in x 12 in non-filtered sterile sampling bag (Fisherbrand, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and enumerated following the same procedure for produce samples (Supplemental Materials, Table 2). Produce samples were weighed in the sterile bags with wash water, and the weight of the sterile bag and wash water was subtracted from the total weight (Supplement Material, Table 3). Sample wash water was serially diluted in 0.1% peptone water and 0.1 mL was plated in duplicate to TSAR. When produce or coupon sample counts were low, 0.1 mL or 0.25 mL to 1 or 4 plates in duplicate were directly plated from the sampling bag to increase detection, respectively. All plates were counted manually for generic *E. coli* using a UV light box for confirmation of green-fluorescent protein. Only colonies that fluoresced in the light box were included in the final counts.

Enrichment Procedure

If produce (< 1.30 log CFU/produce) or coupon (< -0.05 log CFU/cm^2, cucumber; < 0.12 log CFU/cm^2, jalapeño and tomato) samples fell below the limit of detection, samples were enriched following a modified Chapter 4 FDA BAM Method for *E. coli* (29). Produce samples were stomached for 90 s (4 strokes/s), hand macerated for 90 s, and stomached for 90 s (4 strokes/s), and enriched with 200 mL of *E. coli* broth. Stomached produce samples were hand-massaged for 30 s, shaken for 30 s, and hand-massaged for 30 s, and incubated for 24 h at 37 ± 2°C. The overnight produce samples were streaked onto TSAR using a 10-µL loop and incubated for 24 h at 37 ± 2°C. For coupon samples, 1 mL of rinsate was transferred to 1.0% buffered peptone water (BPW; Difco, BD, Franklin Lakes, NJ, USA) and incubated for 24 h at 37 ± 2°C.

After 24 h, 0.1 mL of the overnight BPW was transferred to 10 mL of *E. coli* broth and incubated for 24 h at 37 ± 2°C. A 10-µL loop was used to streak from the overnight *E. coli* broth to TSAR and incubated for 24 h at 37 ± 2°C. Plates streaked from produce and coupon samples were examined under UV light for the presence or absence of generic *E. coli*. If the growth fluoresced in a UV light box, growth was recorded as positive.

Statistical Analysis

All generic *E. coli* counts were log-transformed (mulch samples: log CFU/cm^2 = log (CFU_{mulch} x 20 mL wash water / $cm^2{}_{mulch}$); produce samples: log CFU/produce = log ($CFU_{produce}$ x 20 mL wash water)). Produce samples below the limit of detection (< 1.30 log CFU/produce) were removed from analysis to calculate mean log CFU/produce and mean log percent transfer. For plastic mulch coupons, a value of 0.5 CFU was provided when coupon counts were below the limit of detection (< -0.05 log CFU/cm^2, cucumber; < 0.12 log CFU/cm^2, jalapeño and tomato). Adapted from Todd-Searle et al. (25), log percent transfer to produce was calculated using the following equation: Log Percent Transfer (%) = $Logarithm_{10}$ ($CFU_{produce}$ / ($CFU_{produce}$ + $CFU_{plastic\ mulch}$) x 100%). Log transformation and log percent transfer calculations were performed in Microsoft Excel. Significant differences ($P \leq 0.05$) were determined using Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) Test in RStudio version 4.2.3.

Results and Discussion

Generic Escherichia coli Transfer to Cucumbers

Between 26.7% (4/15) and 100% (15/15) of cucumber fruits showed transfer of generic *E. coli* and 46.7% (7/15) and 100% (15/15) of cucumber fruits positive post-enrichment with counts ranging from below the limit of detection (< 1.30 log CFU/cucumber) to ca. 4.85 log

CFU/cucumber across all contact times (Table 1). If generic *E. coli* transferred to the surface of cucumber samples, the mean log CFU/cucumber ranged from 2.95 (± 1.10) to 3.85 (± 0.65), and the mean log percent transfer from 3.82 (± 0.49) to 4.02 (± 0.59). Notably, 100% (15/15) of cucumbers had generic *E. coli* transfer at day 1 with log CFU/cucumber ranges from ca. 2.08 to 4.63 with a mean log percent transfer of 4.02 (± 0.59). While the cucumber log percent transfer of day 1 was significantly different from day 3 ($P < 0.05$), generic *E. coli* transferred to cucumber samples at all contact times whether it was 3 s (0 d) or 7 days. Micallef et al. (16) suggested trellised cucumbers had lower bacterial counts than non-trellised cucumbers in contact with soil or mulch. These findings and the present study indicate contamination from ground contact is likely, but Micallef et al. (16) also concluded that when factors that supported bacterial proliferation were present (e.g., increased moisture from rainfall), bacterial counts of production systems did not differ. Therefore, elimination of ground contact does not necessarily eliminate contamination.

Generic Escherichia coli Transfer to Jalapeños

Between 0.0% (0/15) and 20.0% (3/15) of jalapeño fruits had transfer of generic *E. coli* and 6.7% (1/15) and 20.0% (3/15) of jalapeño fruits positive post-enrichment with counts ranging from below the limit of detection (< 1.30 log CFU/jalapeño) to ca. 3.47 log CFU/jalapeño (Table 2). All counts were below the limit of detection with 1/15 jalapeño sample positive post-enrichment for days 3 and 7. Day 7 plastic mulch coupons were also below the limit of detection (< 0.12 log CFU/cm^2); therefore, it is not surprising that jalapeño samples were below the limit of detection at this contact time (Supplemental Materials, Table 2). If generic *E. coli* transferred to the surface of jalapeño fruit, the mean log CFU/ jalapeño ranged from 2.40 (± 1.27) to 3.46 (± 0.01), and the mean log percent transfer from 3.10 (± 1.27) to 4.64 (± 0.01). No

differences were observed between jalapeño contact times 0, 1, and 5 days (contact times with transfer) to log CFU/produce or log percent transfer ($P > 0.05$). On day 5, 1/15 jalapeño fruits had counts of generic *E. coli* with a mean log CFU/ jalapeño of 3.46 (± 0.01) and mean log percent transfer of 4.64 (± 0.01). Plastic mulch coupons were below the limit of detection on day 5 indicating either die-off occurred on the plastic mulch but not the jalapeño fruit or generic *E. coli* grew on the surface of the jalapeño post transfer.

In Olaimat and Holley's (18) review of the microbial safety of fresh produce, their findings suggest fresh produce contact with pathogens does not always result in contamination. Day 3 plastic mulch had counts of generic *E. coli*; however, all jalapeño counts were below the limit of detection, supporting that contact with a contaminated source does not always lead to contamination; thus, other factors besides contact alone should be considered in the assessment of risk, such as moisture, crop features, and ground type (25). However, 1/15 jalapeños on day 3 were positive post-enrichment indicating while levels may be low generic *E. coli* was present.

Generic Escherichia coli Transfer to Tomatoes

Between 6.7% (1/15) and 66.7% (10/15) tomato fruits had transfer of generic *E. coli* and positive post-enrichment with counts ranging from below the limit of detection (< 1.30 log CFU/tomato) to ca. 3.52 log CFU/tomato (Table 3). If generic *E. coli* transferred to the surface of tomato samples, the mean log CFU/tomato ranged from 1.30 (± 0.00) to 2.96 (± 0.01), and the mean log percent transfer from 2.08 (± 0.06) to 3.67 (± 0.01). No difference was observed between all contact times to log CFU/tomato or tomato log percent transfer ($P > 0.05$), with transfer occurring at sampled time-points. Pagadala et al. (19) found tomato fruits growing in contact with the ground (soil or plastic mulch) had higher levels of total coliforms. Gu et al. (9) determined tomato fruit may become contaminated when contacting soils contaminated with

Salmonella leading researchers to discourage tomato production without staking and mulching. This supports findings from Honjoh et al. (12) that plastic mulch may provide a barrier and protection from contaminated soil. However, Todd-Searle et al. (25) as well as this present study have shown that contaminated mulch can contaminate tomato fruits.

Generic Escherichia coli Transfer Across Tested Commodities

Generic *E. coli* was transferred to more cucumber samples than other tested commodities at all contact times (Table 4). At day 0, no significant differences were observed between tested commodities and log percent transfer ($P > 0.05$). On days 3 and 5, no differences were observed between cucumber and tomato samples with transfer and log percent transfer ($P > 0.05$). However, day 1 and day 7 cucumber log percent transfer was higher than that of tomato when generic *E. coli* transfer occurred ($P < 0.05$). Cucumber log percent transfer was also higher than jalapeño on day 1 ($P < 0.05$). At day 5, only 1 jalapeño had counts of generic *E. coli* compared to 10 cucumbers; however, log percent transfer was not significantly different ($P > 0.05$). Day 5 tomato log percent transfer was lower than jalapeño ($P < 0.05$). Contact time caused variable differences in log percent transfer across all tested commodities. Across all contact times when transfer occurred, the log percent transfer of cucumber was always equivalent to or higher than other tested commodities, and the log percent transfer of jalapeño was always equivalent to or higher than tomato. In general, transfer occurred showing contamination may occur despite differences in commodity. As ground contact time increased to day 7, contamination did not increase, indicating food safety efforts should be focused on minimizing contact with contaminated sources regardless of the plant-growing nature (e.g., contact with the ground). Ground contact alone may not increase the food safety risk of fresh produce; however, contact with a contaminated source is likely to.

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Tables

Table 1. The data for drooped fresh cucumbers onto plastic mulch.

Commodity	Time (d)	Surface Transfer		Enrichment		Log CFU/Produce Range			Surface Transfer[a]				
		Count	%	Count	%	min	max	n =	Log CFU/Produce		Log Percent		Percent
Cucumber	0	6/15	40.0	7/15	46.7	<1.30,	4.39	6	3.56±0.82[b]	ab[c]	3.82±0.49	ab	45.60±1.63
	1	15/15	100.0	15/15	100.0	2.08,	4.63	15	3.85±0.65	a	4.02±0.59	a	55.70±1.80
	3	12/15	80.0	12/15	80.0	<1.30,	4.82	12	2.95±1.10	b	3.43±0.71	b	30.88±2.03
	5	10/15	66.7	12/15	80.0	<1.30,	4.85	10	2.93±1.17	b	3.55±0.67	ab	34.81±1.95
	7	4/15	26.7	8/15	53.3	<1.30,	5.44	4	3.70±1.07	ab	3.65±0.23	ab	38.47±1.26

[a] Counts below the limit of detection (< 1.30 log CFU/produce) were removed.
[b] Mean ± standard deviation of generic *Escherichia coli*
[c] Lowercase letters indicate a significant difference ($P < 0.05$) within each commodity.

Table 2. The data for drooped fresh jalapeños onto plastic mulch.

Commodity	Time (d)	Surface Transfer		Enrichment		Log CFU/Produce Range			Surface Transfer[a]				
		Count	%	Count	%	min	max	n =	Log CFU/Produce		Log Percent		Percent
Jalapeño	0	2/15	13.3	3/15	20.0	<1.30,	3.52	2	2.40±1.27[b]	a[c]	3.10±1.27	a	22.20±3.56
	1	3/15	20.0	3/15	20.0	<1.30,	2.82	3	2.41±0.52	a	3.11±0.51	a	22.42±1.67
	3	0/15[d]	0.0	1/15	6.7	<1.30,	<1.30	0			-		
	5	1/15	6.7	1/15	6.7	<1.30,	3.47	1	3.46±0.01	a	4.64±0.01	a	103.54±1.01
	7	0/15	0.0	1/15	6.7	<1.30,	<1.30	0			-		

[a] Counts below the limit of detection (< 1.30 log CFU/produce) were removed.
[b] Mean ± standard deviation of generic *Escherichia coli*
[c] Lowercase letters indicate a significant difference ($P < 0.05$) within each commodity.
[d] All counts were below the limit of detection (< 1.30 log CFU/produce).

Table 3. The data for drooped fresh tomatoes onto plastic mulch.

Commodity	Time (d)	Surface Transfer		Enrichment		Log CFU/Produce Range			Surface Transfer[a]				
		Count	%	Count	%	min	max	n =	Log CFU/Produce		Log Percent		Percent
Tomato	0	1/15	6.7	1/15	6.7	<1.30,	2.34	1	2.30±0.06[b]	a[c]	3.00±0.06	a	20.08±1.06
	1	10/15	66.7	10/15	66.7	<1.30,	3.53	10	2.34±0.81	a	3.05±0.81	a	21.12±2.25
	3	1/15	6.7	2/15	13.3	<1.30,	2.97	1	2.96±0.01	a	3.67±0.01	a	39.25±1.01
	5	5/15	33.3	7/15	46.7	<1.30,	3.25	5	2.60±0.62	a	3.41±0.59	a	30.26±1.80
	7	1/15	6.7	4/15	26.7	<1.30,	1.30	1	1.30±0.00	a	2.08±0.06	a	8.00±1.06

[a] Counts below the limit of detection (< 1.30 log CFU/produce) were removed.
[b] Mean ± standard deviation of generic *Escherichia coli*
[c] Lowercase letters indicate a significant difference ($P < 0.05$) within each commodity.

Table 4. The data for drooped fresh cucumbers, jalapeños, and tomatoes onto plastic mulch.

Commodity	Time (d)	n =	Surface Transfer[a] Log CFU/Produce		Log Percent	
Cucumber	0	6	3.56±0.82[b]	A[c]	3.82±0.49	A
Jalapeño		2	2.40±1.27	A	3.10±1.27	A
Tomato		1	2.30±0.06	A	3.00±0.06	A
Cucumber	1	15	3.85±0.65	A	4.02±0.59	A
Jalapeño		3	2.41±0.52	B	3.11±0.51	B
Tomato		10	2.34±0.81	B	3.05±0.81	B
Cucumber	3	12	2.95±1.10	A	3.43±0.71	A
Jalapeño		0			-[d]	
Tomato		1	2.96±0.01	A	3.67±0.01	A
Cucumber	5	10	2.93±1.17	A	3.55±0.67	AB
Jalapeño		1	3.46±0.01	A	4.64±0.01	A
Tomato		5	2.60±0.62	A	3.41±0.59	B
Cucumber	7	4	3.70±1.07	A	3.65±0.23	A
Jalapeño		0			-	
Tomato		1	1.30±0.00	B	2.08±0.06	B

[a] Counts below the limit of detection (< 1.30 log CFU/produce) were removed.
[b] Mean ± standard deviation of generic *Escherichia coli*
[c] Capital letters indicate significant differences ($P < 0.05$) across each commodity and time.
[d] All counts were below the limit of detection (< 1.30 log CFU/produce).

Supplemental Materials

Table 1. Mean ± standard deviation log CFU/cm^2 of generic *Escherichia coli* on plastic mulch before produce droop (n=15).

Commodity	Log CFU/cm^2
Cucumber	5.56±0.28
Jalapeño	5.41±0.16
Tomato	5.43±0.50

Table 2. The data for used plastic mulch after produce droop.

Commodity	Time (d)	Surface		Enrichment		Log CFU/cm^2 Range		Log CFU/cm^{2a}
		Count	%	Count	%	min	max	
Cucumber	0	15/15	100.0	15/15	100.0	3.69,	4.98	4.46±0.38[b]
	1	15/15	100.0	15/15	100.0	1.79,	4.82	3.44±0.75
	3	12/15	80.0	12/15	80.0	-0.05,	4.35	2.02±1.25
	5	9/15	60.0	9/15	60.0	-0.05,	4.45	1.74±1.40
	7	4/15	26.7	6/15	40.0	<-0.05,	4.83	2.88±1.32
Jalapeño	0	15/15	100.0	15/15	100.0	4.80,	5.49	5.22±0.16
	1	12/15	80.0	15/15	100.0	<0.12,	4.35	1.60±1.31
	3	1/15	6.7	1/15	6.7	<0.12,	0.90	0.86±0.06
	5	0/15	0.0	1/15	6.7	<0.12,	<0.12	-[c]
	7	0/15	0.0	1/15	6.7	<0.12,	<0.12	-
Tomato	0	15/15	100.0	15/15	100.0	2.87,	5.06	4.12±0.69
	1	15/15	100.0	15/15	100.0	<0.12,	3.23	2.33±0.90
	3	5/15	33.3	7/15	46.7	<0.12,	2.17	1.30±0.69
	5	6/15	40.0	6/15	40.0	<0.12,	2.94	1.79±1.05
	7	1/15	6.7	1/15	6.7	<0.12,	1.12	0.86±0.37

[a] Counts below the limit of detection (< -0.05 log CFU/cm^2, cucumber; < 0.12 log CFU/cm^2, jalapeño and tomato) were removed.
[b] Mean ± standard deviation of generic *Escherichia coli*
[c] All counts were below the limit of detection.

Table 3. The weight data in grams for produce drooped (n=15).

Commodity	Time (d)	Weight (g)[a]
Cucumber	0	143.81±35.88
	1	150.68±43.25
	3	153.98±45.14
	5	163.95±84.45
	7	126.70±83.02
Jalapeño	0	17.85±7.24
	1	16.98±8.82
	3	17.17±6.45
	5	15.05±7.65
	7	16.84±4.69
Tomato	0	18.55±4.60
	1	18.09±4.29
	3	19.38±5.10
	5	19.13±5.18
	7	17.51±4.37

[a] Mean ± standard deviation

Chapter 7: Conclusions and Future Research

The FDA Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) provides the fresh produce industry with standards for the growing, harvesting, packing, and holding of produce for human consumption. Specifically, the FSMA PSR subpart K prohibits dropped produce from harvest; however, the handling of dropped produce is a commonly misunderstood practice. To clarify harvesting requirements and provide more scientific research to explore the microbial risk of dropped produce, the four studies of this research thesis aimed to evaluate (i) the survival of *Salmonella* on biodegradable mulch, landscape fabric, and plastic mulch across 140 days (ii) the survival of generic *Escherichia coli* on plastic mulch held in environments of the growth chamber, greenhouse, and field over 7 days, (iii) the impact of drop height of 0, 1, 2, 4, and 6 ft on the transfer of generic *Escherichia coli* from mulch to fresh cucumber, jalapeño, and tomato, and (iv) the impact of contact time of 0, 1, 3, 5, and 7 d on the transfer of generic *Escherichia coli* from plastic mulch to fresh cucumber, jalapeño, and tomato.

In the first study, ground cover type impacted the survival of *Salmonella*. Over shorter time intervals (0-30 d), biodegradable mulch had the lowest reduction of *Salmonella*; however, by longer time intervals (60-90 d), reduction steadied across tested ground covers. By 140 days, landscape fabric had the lowest reduction of *Salmonella* and the highest number of coupons positive for *Salmonella* post-enrichment. Ground cover composition is speculated to play a role in the differing survival of *Salmonella,* indicating that ground cover type should be included in risk management. This study was completed in a growth chamber and established ground covers are a food safety hazard in fresh produce pre-harvest production. Ground covers should be further evaluated in production settings (e.g., greenhouse, field) that better mimic realistic growing conditions to determine a more applicable risk of ground cover type.

In the second study, the growing environment impacted the survival of generic *E. coli* on plastic mulch. The reduction of generic *E. coli* on plastic mulch was minimal in the growth chamber (<2 log CFU/cm^2) by 7 days compared to a 6-log reduction of field and greenhouse mulch by 0.17 and 7 days, respectively. As the environment became less controlled (i.e., field), the reduction of generic *E. coli* increased. These results indicate food safety risks differ based on the fresh produce production environment; thus, food safety risk management also differs. While this study begins to fill the research gaps of study one, only one ground cover material (i.e., plastic mulch) was evaluated with a non-foodborne pathogen. Further research should be done utilizing other commercial ground cover types (e.g., biodegradable mulch, landscape fabric) with a foodborne pathogen or foodborne pathogen surrogate in a realistic fresh produce pre-harvest environment.

In the third study, fresh produce commodity, drop height, and ground surface had variable effects on generic *E. coli* inoculated mulch transfer to controlled dropped produce. While the amount of transfer varied, generic *E. coli* transferred to cucumber, jalapeño, and tomato fruit from plastic mulch and tomato fruit from biodegradable mulch at all drop heights (1, 2, 4, and 6 ft). Even when not dropped (0 ft), generic *E. coli* transferred to cucumber and tomato samples. At 4 and 6 ft, dropped cucumber and tomato experienced damage, resulting in unmarketable produce, despite mulch type. Due to potential damage and microbial contamination, dropped produce could pose a potential downstream food safety risk. Excluding dropped produce from harvest is a simple risk-prevention practice to prevent microbial contamination from entering the production chain of fresh produce, as required by the FSMA PSR. Since this study was performed in a laboratory setting, fresh produce may need to be dropped in the production environment to confirm results apply to a realistic industry setting.

In the fourth and final study, fresh produce commodity and contact time had minimal effects on the transfer of generic *E. coli* from plastic mulch to tested drooped fruit. No trend in transfer and contact time could be concluded, as transfer within and across commodities varied. Generic *E. coli* was transferred to cucumber and tomato at all contact times (0, 1, 3, 5, and 7 d) and 3 of the 5 time-points for jalapeños (0, 1, and 5 d). Since contamination was not influenced by increasing plastic mulch contact time, results confirm cross-contamination poses a food safety risk in the production environment despite unintentional or intentional ground contact. Growing produce in contact with the ground alone may not be the sole factor in the contamination of fresh produce, as a contamination event is required. Food safety efforts should focus on minimizing visible contamination that may contaminate fresh produce in the production environment, as outlined in the FSMA PSR. Additional produce commodities, beyond those tested in this research thesis, should be drooped onto contaminated mulch to widen the commodity scope.

While limitations exist and further research may be needed to definitively conclude results, the studies present in this thesis help further the understanding of ground cover cross-contamination risks and microbial risks of harvesting dropped fresh produce whether detached or attached to the plant. This research provides science-based data that may be used to better inform fresh produce food safety regulations, like the FSMA PSR, and assist the fresh produce industry by providing science-based practices to prevent and mitigate potential food safety risks in fresh produce production.