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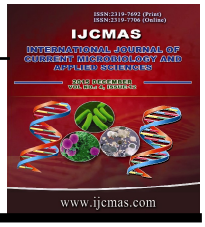

Original Research Article

A Study on the Contamination Routes of Leafy Greens and Onion Plants by *Escherichia coli* and *Listeria innocua*

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Keywords

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A B S T R A C T

Fresh vegetables have been the source of recent outbreaks of food borne illnesses due to contamination by human pathogens such as *E. coli* O157:H7 and *Listeria monocytogenes* in the field. The objectives of the present study were to investigate (i) the potential uptake of bacterial surrogates of *E. coli* O157:H7 and *Listeria monocytogenes*, *E. coli* (EC) and *L. innocua* (LI) respectively, from soil into leafy greens (lettuce and spinach) and onion (green onion and yellow onion) plants, and (ii) the survivability of EC and LIonleafy greens. Mature plants were soil-inoculated with a bacterial suspension(ca. 10^8 cfu/ml) of EC or LI. Spinach and lettuce leaves were also surface inoculated with 1 ml of a culture of EC and LI (ca. 10^8cfu/ml). Vegetable samples were microbiologically analyzed by plating on Eosin Methylene Blue Agar and Listeria Identification Agar to recover EC and LI respectively. Soil-inoculated *E. coli* and *L. innocua* were recovered from green onions, yellow onions and lettuce at internalization frequencies of 14-61%, 26-57% and 0-25% respectively. Moreover, when the leaves of lettuce and spinach were surface-contaminated with EC or LI, they were found to harbour the microorganisms for > 48h and ≤ 24 h respectively.

Introduction

Fresh vegetables such as raw onions and leafy greens contain rich sources of nutrients and provide numerous health benefits, so nutritionists and health professionals highly recommend increasing consumption of these important foods (Garrow et al., 2000). Onions are widely consumed especially in recognition of their multi-faceted health benefits (Brewster, 2008) and their versatility in culinary applications. Leafy vegetables such as lettuce and spinach are also popular salad vegetables given that they are rich sources of fiber, vitamins and minerals (Anderson et al., 1994). Unfortunately, onions and leafy greens represent one of several high-risk commodity groups that have caused some major historical foodborne disease outbreaks in other countries and together with other produce commodities contribute to approximately 75% of produce-related illnesses in developed countries

(Sivapalasingam et al., 2004). The unique morphology of green onions, characterized bytheir moist hollow tube leaves, provides ample opportunity for amplification of microbial hazards (FAO, 2008). Also, if a pathogen is inside this tube, little can bedone to remove it as it is protected from washing (FAO, 2008).Yellow onions are also susceptible to contamination by pathogens in the soil since they are underground vegetables. Pathogens known to contaminate yellow and green onions include hepatitis A virus (Lynch et al., 2009), *Shigella flexneri* (Lynch et al., 2009), *Salmonella* (Lynch et al., 2009), *Campylobacter* spp (McMahon and Wilson, 2001), *Clostridium botulinum*(Lily et al., 1996) and*E. coli* (Mukherjee et al., 2004). Bohaychuk et al. (2009) isolated *E. coli* from 55 (8.2%) of 673 intact samples of green and yellow onions with bacterial load ranging from0.48 to 3.04 log MPN/g. Calvin et al. (2004) also observed that green onions grown in agricultural settings are quite prone to contamination by microbial pathogens. In addition to alliums, there have also been large outbreaks of foodborne illness associated with leafy green vegetables. In 2005, bagged salad containing romaine lettuce were linked to an outbreak of *E. coli* O157:H7 foodborne illness (Smith DeWaal and Bhuiya, 2008) in the United States. Small-scale (< 50 cases) outbreaks involving *E. coli* O157:H7-contaminated lettuce have also occurred in countries such as the United States despite improved production and handling practices. In Europe, outbreaks have been attributed to both locally produced and imported leafy greens. In 2004, an outbreak of *Salmonella* Thompson infections reported in Norway, Sweden, and England were linked to the consumption of contaminated rucola lettuce imported from Italy (Nygard et al., 2008). In 2005, a nationwide outbreak of *S.typhimurium* var Copenhagen DT104B occurred in Finland due to contaminated lettuce imported from Spain. In Sweden, a total of 135 cases, including 11 cases of hemolytic uremic syndrome (HUS), were linked to the consumption of locally produced lettuce that was contaminated with *E. coli* O157. Spinach contaminated with *E. coli* O157:H7 was also at the center of a large outbreak in 2006, resulting in 205 confirmed illnesses and three deaths in the United States (Smith DeWaal and Bhuiya, 2008). In Mauritius, there is a lack of surveillance and active monitoring for the presence of microbiological contaminants in fresh vegetables. Nevertheless, the scope for pre-harvest contamination of fresh produce should not be underestimated.

Vegetables can be frequently contaminated in the field during irrigation with water contaminated with these pathogens. There are two common types of irrigation systems: sprinkler and drip irrigation. The type of irrigation system greatly influences the degree of crop contamination that occurs during irrigation (Matthews et al., 2014). Research in vegetable production systems suggests that overhead irrigation leads to higher contamination levels on the crop compared with drip irrigation (Keraita et al., 2007). However, the effect of irrigation practice on the transfer of pathogens to vegetables has not been examined in detail. There is clearly a need to better understand the role of different irrigation methods on the fate of human pathogens in production systems of high-risk vegetable crops. *E.coli*O157:H7is one of the most common zoonotic enteric pathogens associated with vegetables given its widespread presence in feces of livestock and feral animals (Cooley et al., 2013). *Listeria monocytogenes* on the other hand, is a common geophilic (soil-borne) bacterium and is ubiquitous in vegetation (Critzer and Doyle, 2010). The objectives of the present study were

therefore to: (i) investigate the potential uptake, infiltration or internalization of *E. coli* and *L. innocua* from soil into the edible parts of onion, lettuce and spinachcrops and (ii) investigate the survivability of these bacteriaon the phyllosphere (leaves) of these plants.

Materials and Method

Assessing the potential for systemic uptake of *E. coli* and *L. innocua in* green onion, yellow onion, spinach and lettuce plants

Soil Sterilization

The oven was preheated to 82-88°C (180°-190°F). Ten kg of soil was spread evenly in a large pan to a maximum depth of 10 cm. The pan was sprayed with water to moisten slightly and then covered tightly with aluminum foil. At the center of the covered baking pan, a thermometer probe was inserted into the soil and the pan placed into the oven. Once the soil temperature reached 82-88°C, the temperature was maintained for 60 minutes following which the pan was removed from the oven and allowed to completely cool. Once cooled, soil was transferred to clean gunny bags. Given the limited capacity of the oven, multiple cycles were run to sterilize several batches of soil.

Plant Preparation

Green onion (*Alliumfistulosum*var. Commun Rouge), yellow onion (*Alliumcepa*var. de Brunswick), lettuce (*Lactuca sativa* var. Blonde Maraichere) and spinach (*Spinaciaoleracea*var. Junius) seeds were used. Briefly, seeds were disinfected with 70% ethyl alcohol (EtOH) for 3 min, rinsed in sterile water, and soaked in Javel commercial bleach (0.525% sodium hypochlorite) for 15 min. Seeds were then rinsed in sterile water three times (5 min each rinse). Subsequently, they were sowed in steam-sterilized soil contained in Styrofoam plug trays and grown in a Biosafety Level 1 (BSL-1) greenhouse located at the Mauritius Sugar Industry and Research Institute, Reduit.

Plants were watered on a daily basis with sterile water. Seedlings were transplanted at 2 weeks of age to potting bags containing steam-sterilized soil (1 kg) placed in plastic saucers to serve as a water reservoir for indirect irrigation. The pH and water activity of the soil were regularly monitored with a pH meter (Mettler Toledo) and a water activity meter (Novasina) respectively. Over the period of October 2013 to December 2014 chamber temperatures ranged from 21 to 32°C (daytime) and 12 to 23°C (night-time) and the relative humidity varied between 65 to 81%. The saucer was refilled with ca. 50 ml sterile water daily. Additionally, the soil was supplemented with ‘Terreau’ or peat (Stender) as per the manufacturer's instructions to maintain plant growth, to speed up harvest time and increase yields.

Experiment Design

Four plant types (green onion, yellow onion, spinach and lettuce) were investigated in this part of the study. The plants were given one of 3 treatments (Sterile water, EC and LI) where EC and LI stand for *Escherichia coli* and *Listeria innocua.* Each treatment was given in duplicates. The experiment was carried out in two independent replicates. A total of 48 plants (3 treatments x 4 plant types x 2 plants per treatment x 2 replicates) were considered. The different treatments given to the plants are summarized in the Table 1 below.

Soil Inoculation

Bacterial Cultures

E. coli ATCC 25922 strain was provided by the Food Technology Laboratory of the Ministry of Agro-Industry and Food Security of Mauritius. The strain was plated onto Eosin Methylene Blue medium (HiMedia) and incubated for 24 h at 37°C for confirmatory identification of *E. coli*. *Listeria innocua* ATCC 33090 (Microbiologics Ltd) and was revived on *Polymyxin* Acriflavin Lithium-Chloride Ceftazidime Aesculin Mannitol (PALCAM) medium (HiMedia). Olive green colonies with dark sunken centers and black haloes were confirmed to be *L. innocua. L. innocua* hydrolyzes aesculin to form aesculetin and dextrose. Aesculetin reacts with ammonium ferric citrate and forms a brown-black complex seen as a black halo around colonies. Strains were stored at -80°C in glycerol stocks.

Inoculum Preparation

The cells of the two cultures were adapted to grow on Plate Count Agar (PCA)supplemented with 100 μg/ml of Nalidixic acid (Sigma) (PCA-N) to select for Nalidixic-acid (NA) resistant strains of E. coli andL. innocua.NA-resistant mutant strains were subsequently transferred on fresh Plate Count Agar supplemented with 100 μg/ml of NAand plates incubated overnight at 35°C to yield solid cultures. Stock cultures of NA resistant strains of E. coli andL. innocuawere also stored in TSB-N broth containing 25% glycerol (Sigma) at −18°C. To prepare liquid cultures, a single colony of each NA-resistant strain was transferred to 200 ml of tryptic soy broth (TSB-N) and placedon an orbital shaker at 35°C for 18 h.

Soil Inoculation of Plants

On the day of inoculation of the plants, 100 ml of each culture was mixed with 900 ml of sterile water (10-fold dilution of an overnight culture) to serve as the inoculum for the plants. The concentration of each culture was determined by serial dilution and plating on PCA-N. In addition, the population density of *E. coli* and *L. innocua*r ecovered from the soil immediately after inoculation was also determined. Various treatments were given to the plants as indicated in Table 1. Plants serving as negative controls were treated with sterile water. All plants were watered once or twice daily as needed.

Microbiological Analysis of Vegetables at Harvest

Green and yellow onions reaching commercial maturity were harvested by aseptically uprooting the entire plants. Uprooted green onions and onion bulbs were washed with sterile water to remove adhering soil debris. Samples were then sanitized withJavel commercial bleach (0.525% sodium hypochlorite), rinsed in sterile water and then air-dried. An alcohol-sterilized knife was used to trim the roots to simulate commercial practice. For spinach and lettuce, 3 to 5 were plucked and pooled to form a composite sample weighing approximately 10 g. Vegetable samples were then mixedwith 0.1% Buffered Peptone Water at a 1:4 ratio, and macerated for 10 minutes to form a homogenate. The homogenate was serially diluted in 0.1% Buffered Peptone water following which the homogenate and serial dilutions were plated onto Eosin Methylene Blue agar or PALCAM agar supplemented with 100 μg/ml of Nalidixic acid. Plates were then incubated at 44 or 35°C respectively for 48 h. In addition, vegetable samples suspected

to be contaminated with *E. coli* or *L. innocua*were subjected to primary enrichment in LaurylTryptose broth (LTB)and Half-Fraser broth respectively and incubated at 44 and 35°C for 24 h. Broths were supplemented with NA to a final concentration of 100 ug/ml. Aliquots of LTB and Half-Fraser Broth were then transferred for secondary enrichment into EC and Fraser broths supplemented with NA, and incubated at 44°C and 35°C for 24 h respectively. A loopful of secondary enrichment broth was then streaked onto EMB-N or PALCAM-N and plates incubated at 44 or 35°C respectively for 24 h. Colonies with characteristic green metallic sheen on EMB-N or olive green colonies with a surrounding black halo on PALCAM-N were presumed to be Nalidixic-acid resistant *E. coli* or *L. innocua* respectively.

Assessing the survivability of *E. coli* and *L. innocua* on lettuce and spinach leaves

Spinach and lettuce plants were cultivated as described previously. Once they have attained commercial maturity, a spot inoculation method was used to artificially contaminate the leaves as would normally happen during overhead or spinkler irrigation with contaminated water. Briefly, leaves were spot-inoculated with 1000ul of late-log phase cultures of Nalidixic-acid resistant *E. coli* or *L. innocua* using an appropriate micropipettor. In addition, leaves of plants designated as "negative control" were spotted with sterile water. After 24h and 48h,leaves were aseptically plucked as described previously, placed in an individual sterile Whirl-Pak filter bag containing 40 ml of 0.1% BPW and stomached for 2 min. The homogenate was then diluted 10-fold in 0.1% Buffered Peptone Water, and 0.1-ml aliquots of the appropriate dilutions were spread-plated onto EMB-N or PALCAM-N. Plates were incubated and enumerated after 24h as described previously.

Results and Discussion

Choice of Bacterial Strains for the Study

In this part of the study, *E. coli* ATCC 25922 and *L. innocua* ATCC 33090, non-pathogenic surrogate microorganisms were used in lieu of the enteric pathogens *Salmonella* or

E. coli O157:H7 and the ubiquitous soil-borne pathogen *L. monocytogenes* respectively, to avoid introduction of pathogenic agents in the BSL-1 greenhouse. Other authors including Ingham et al. (2004, 2005) and Wood et al. (2010) have also resorted to non-pathogenic surrogates to circumvent this limitation. Examples of surrogates that have been used*in planta* studies include *E. coli* Shiga toxin-negative *E. coli* O157:H7 (Islam et al., 2004, 2005; Erickson et al., 2010), *Listeria innocua* (Girardin et al., 2005), and avirulent *Salmonella* (Islam et al., 2004). In using these surrogates, the assumption has been made that they would respond similarly as the pathogenic agent.

Choice of Growing Media used for the Study

In this study, the growing media and cultivation conditions were optimized to promote the uptake of inoculated bacteriain the plant to simulate a worst-case scenario. This was achieved by sterilizing the soil used as a growing medium to get rid of indigenous microorganisms present. Indeed, the high complexity of interactions between the inocula of interest and background microflora have often prompted plant physiologists and microbiologists to use

simple 'gnotobiotic' type models, namely sterile or sub-sterile growing media (Erickson, 2012). However, it is worth mentioning that these simplistic models also have some limitations since they tend to mask the role of interactions among the different groups of microorganisms present in the soil and in the rhizosphere. Indeed, plant roots and soil are never sterile; rather they are surrounded or invaded by large numbers of microorganisms with potentially intense biochemical activity.

Uptake of *E. coli* and *L. innocuainalliums* (Green onions and Yellow onion)

In our study, *E. coli* ATCC 25922 and *L. innocua* ATCC 33090 were used as non-pathogenic surrogates to mimic *Salmonella* spp. or *E. coli* O157:H7and *L. monocytogenes* respectively. *E. coli* and *L. innocua* were detected in green onion at a variable rate of 14-61% and 42-52% and at a mean population density of 0.80 and 0.85 log cfu/g respectively (Table 2) after 1 day post-inoculation. *E. coli* and *L. innocua* were also detected after enrichment of yellow onions at a variable rate of 26-57% and 29-47% respectively 1 day post-inoculation (Table 3). Armon et al. (1994) previously reported that onion fields that were irrigated with highly contaminated water, harbored significant cell numbers of indicator microorganisms upon harvest. Chancellor et al. (2005) investigated the systemic uptake of fluorescent microspheres (1.0 to 10 µm) in green onions, and observed greater fluorescence in the roots, with the intensity decreasing with increasing vertical distance from the roots. Since the microspheres used in the study were comparable in size to bacteria, findings of the study could provide indirect evidence for root uptake and translocation of bacteria from soil to the aerial parts of green onions. In a previous study, we demonstrated that soil-inoculated green onions harboured *E. coli* O157:H7and *Salmonella enteric* at an approximate population density of 6 log cfu/g (Neetoo et al. 2012). Furthermore, we observed that the population within the edible foliar portion of green onions was 4.9-5.9 log CFU/g, demonstrating significant internalization. Our findings also indicated differential abundance of internalized pathogens along the length of the plant with preferential localization of bacteria in the order of roots > bulbs ~ stem > foliage. However, the strikingly low population density of *E. coli* or *L. innocua* in green and yellow onions noted in the current study is worth commenting. This disparity between the current findings and the previous study could partly be explained by the rapid loss of viability of the inocula in the soil due to choice of different strains. In the current study, we resorted to pathogen surrogates since we were confined to the requirements of a BSL-1 green house. In contrast, in the previous study (Neetoo et al., 2012), we tested the internalization potential of actual pathogenic strains. Indeed, some human pathogens strains have been demonstrated with greater environmental fitness making them better colonizers of produce surfaces than others (Jablasone et al., 2005). Moreover, it is possible that the surrogates inoculated into soil were more sensitive to the phenolic compounds released by onion plants. Islam et al. (2004, 2005) noted more rapid death of *E. coli* in soil in which onions were grown than in soil in which carrots were grown (Islam et al., 2004, 2005) possibly due to a higher phenolic content of onions compared to carrots. The discrepancy between our two studies could also be attributed to the environmental parameters of the growing chamber; while in the previous study, the temperature, relative humidity and sunlight were controlled, in the current experiment, there was large daily and seasonal

fluctuation in temperature, relative humidity and sunlight/UV exposure. Henries et al. (2012) mentioned that internalization is less frequently observed in field experiments where daily variations in humidity, temperature and UV light intensity vary.

Uptake of *E. coli* and *L. innocua in lettuce*

In this study the systemic uptake of*E. coli* and *L. innocua,*were studied in two leafy vegetables: lettuce and spinach. We observed a relatively low frequency of internalization of bacteria in lettuce leaves at a rate of 8-25% 24 h after soil inoculation (Table 5).Congruent with our findings, Bernstein et al. (2007) also found that *Salmonella enterica* serovar Newport, member of the same family (*Enterobacteriaceae*) as *E. coli*, became internalized within the aerial parts of romaine lettuce via the root in 33-day old plants. Mootian et al. (2009) studied the transfer of *E. coli* O157:H7 at low numbers from soil, manure-amended soil and water to growing young (12 d) or mature (30 d) lettuce plants; *E. coli* O157:H7 was detected by enrichment in 30% (36 of 120) of mature plants that were harvested at 15d. Zhang et al. (2009) on the other hand, reported that *E. coli* O157:H7 was not internalized within intact lettuce leaves and roots, regardless of the variety of lettuce, age of plants or strain of *E. coli* O157:H7. The large disparity in the different researchers' findings could be due to differences in strains used, environmental parameters of the growing chambers or growing seasons. Golberg et al. (2011) indicated that internalization of *Salmonella* in iceberg lettuce varied widely (0-100%), with a higher incidence occurring mainly in summer.

Although a few authors have been able to demonstrate internalization of enteric pathogens in lettuce, their viability was shown to decrease rapidly. For example, Bernstein et al. (2007) observed internalized *Salmonella* in lettuce leaves at 2 days post-inoculation but not 5 days later. This is comparable with our findings where 25% and 0% of lettuce samples tested positive for *E. coli* after 24 and 48 h respectively (data not shown). Similarly, no lettuce samples were found to test positive for *L. innocua* after 48h (data not shown).

Finally, it is worth mentioning that although caution was exercised to avoid splash of soil particles onto the lettuce leaves in the current study, the possibility is not ruled out that capillary action on the exterior stem could have resulted in movement of the bacterial inoculum from the soil to the aerial leafy parts of the plants.

Uptake of *E. coli* and *L. innocuain* spinach

As indicated in Table 5, we did not observe any instance of internalization of *E. coli* or

L. innocua in spinach leaves (0%). The difference in the rate of bacterial uptake between the two leafy green vegetables (spinach vs. lettuce) in our study is worth noting. Dong et al. (2003) and Golberg et al. (2011) also indicated that the frequency of internalization of human pathogens in leafy greens often varies with the type of leafy vegetable. Golberg et al. (2011) examined the frequency of *S. typhimurium* internalization in different types of intact leafy vegetables during a 2-year study. The frequency varied significantly among the different inoculated plants. The highest frequencies were observed in aragula leaves (88%) and iceberg lettuce (81%) followed by basil (46%), red lettuce (20%), romaine lettuce (16%) and parsley (1.9%). Lack of uptake of *E. coli* and *L. innocua* in spinach observed in the study is comparable findings

of Pu et al. (2009) who found that internalization of *E. coli* O157:H7 occurred only in 1 out of 120 intact spinach samples (0.8%) inoculated at 3 or 7 log cfu/ml after seed germination. Similarly, Jablasone et al. (2005) showed that internalization of *E. coli* O157:H7, *S. typhimurium* and *L. monocytogenes* did not occur in mature plants of spinach following inoculation of the spinach seeds. Mitra et al. (2009) also found that there was no evidence of internalization of *E. coli* O157:H7 within intact spinach leaves. Sharma et al. (2009) also studied the translocation of *E. coli* O157:H7 from soil to different parts of the spinach plant; the author reported being unable to recover *E. coli* O157:H7 from shoot tissues although they did observe the cells in root tissues.

Taken together, the internalization frequency of the bacterial surrogates in the two leafy vegetables, was surprisingly low (0-25%). It is quite possible that the two bacterial species used in our study are inherently poor plant colonists. Dong et al. (2003) studied the abilities of different strains of *E. coli*, *S. enterica* and *K. pneumoniae* to internalize from the rhizosphere to the interior of alfalfa sprouts and found that these strains differed substantially in their invasiveness and *E. coli* was shown to be the poorest colonizer.If the bacteria are unable to establish themselves as successful epiphytes in the rhizosphere, they are unlikely to be able to survive, penetrate any root openings and effectively infiltrate the root tissue.

Survivability of *E. coli* and *L. innocua* on the Phylloplane of Lettuce

In Mauritius, overhead or sprinkler irrigation is widely practiced by small, medium and large-scale farmers. However, the risk of food crop contamination by contaminated overhead irrigation water is evident. This risk is further compounded by the inconsistent quality or quantity of water distributed in certain agricultural regions of Mauritius, which can potentially lead to the reuse of non-potable (waste) water of uncertain quality for irrigation. Moreover, after the advent of tropical cyclones, the chance of cross-contamination of clean municipal water with sewage water is increased.

A number of studies have additionally reported that poor quality water has been used for agricultural purposes in developing countries (WHO/FAO 2006). Hence the likelihood of contamination of leafy greens by overhead (sprinkler) irrigation and persistence of these bacteria on the surface of leafy greens has been the subject of active research.

In the current study, lettuce and spinach leaves were artificially contaminated with *E. coli* or *L. innocua* by carefully depositing a defined inoculum (10^9 cells) on the foliage of plants to mimic contamination during splash dispersal or overhead (or sprinkler) irrigation in the field. Our findings revealed that the inocula failed to grow on the leaves and survived poorly beyond 48h. In fact, the population rapidly declined to below detectable levels after 24 or 48 h as indicated in Table 6.

It is well acknowledged that the quality of irrigation water and type of irrigation system influence the microbial safety of leafy greens (Aruscavage et al., 2006; Warriner at al. 2009). Solomon et al. (2002) studied the effect of spray irrigation on the presence of *E. coli* O157 and found that 90% of lettuce plants which had been spray-irrigated with water containing 7 log cfu/ml of *E. coli* O157 were contaminated, while only 19% were contaminated when drip irrigation was

used with the same concentration of *E. coli* O157. This is in agreement with our findings where we observed that 90% of spot-inoculated lettuce samples tested positive for *E. coli* after 24h compared with only 25% of soil-inoculated lettuce samples. Indeed, spray irrigation represents the greatest risk because contaminated water can be directly deposited onto the edible leaves of produce (FDA, 1998).

Harris et al. (1999) also observed a rapid decline in total populations of *E. coli* O157:H7 immediately after spraying lettuce plants in the field with a bacterial population of 7 log CFU/plant (Harris et al., 1999). Within seven days after application, 82% of the plants had already decreased to populations that were below the enumeration detection limit (10 cells/plant) but 93% of the plants were positive by enrichment. Three weeks later, 33% of the plants were still positive by enrichment. Contrary to Harris et al. (1999), our findings revealed a faster decline in the bacterial population on the lettuce phyllosphere; the proportion of lettuce samples testing positive for *E. coli* dropped from 90% to 20% within 24h. Similarly, the percentage of lettuce samples testing positive for *L. innocua* decreased drastically from 90% to 30% within 1 day.

Survivability of *E. coli* and *L. Innocua* on the phylloplane of Spinach Leaves

As far as persistence of the bacterial surrogates on the spinach phyllosphere, no evidence of survival of *E. coli* was observed 24h post-inoculation. *L. innocua* was detected in 1 out of 9 samples after 24h but undetectable in all samples after 48h. Overall, *E. coli* and *L. innocua* were undetectable in 100% of samples 48h post-inoculation(Table 7). The poor survival of bacteria on the phyllosphere of leafy greens has been documented elsewhere. Erickson et al. (2010) inoculated spray irrigation water with *E. coli* O157:H7 and detected the organism on the surface and within tissues of spinach plants immediately after irrigation; however 7days after spraying, all spinach leaves tested negative for surface contamination. Zhang et al. (2009) demonstrated that when lettuce leaves were spot inoculated with an *E. coli* O157:H7 inoculum at 7 log CFU/ml, all 424 surface-sanitized leaf samples were negative for internalized *E. coli* O157:H7.

In contrast, various authors have demonstrated long-term persistence of human pathogens on the foliage of food crops. Cooley et al. (2003) found that cells of *E. coli* O157:H7 and *S. enteric* actually grew and their numbers increased to 7 log cfu/g on intact leaf tissue of *Arabidopsis thaliana* (thale cress) at 100% humidity. Islam et al. (2004) indicated that enteric pathogens survived on parsley in the field for up to 231 d (Islam et al., 2004). Zhang et al. (2009) studied the survival of inoculated *E. coli* O157:H7 on intact lettuce and found that the organism survived for at least 25d on leaf surfaces, with greater survival on the abaxial (lower) side of the leaves than on the adaxial (upper) side. It is thought that enteric pathogens such as *E. coli* O157:H7 and *Salmonella* have the ability to interact with plant surfaces leading to long-term colonization through development of biofilms (Aruscavage et al., 2006).

Biofilms on fresh produce occur as groups of bacterial cells aggregate in exopolysaccharide materials that serve to protect the cells from environmental stresses including dessication (Morris and Monier, 2003). Formation of biofilms on surfaces of spinach and lettuce, Chinese cabbage, celery as well as other leafy greens has been demonstrated (Morris and Monier, 2003).

Morris et al. (1998) estimated that 10-40% of bacteria on the surface of intact parsley and endive leaves were associated in biofilms. In addition to *E. coli*, Olmez and Temur (2010) observed the initiation of biofilm formation by *L. monocytogenes* on intact lettuce surfaces after 24h of incubation at 10°C. Likewise, Niemira and Cooke (2010) reported that *E. coli* O157:H7 had the ability to form biofilms on intact spinach and lettuce after 24 h of storage at 4°C. The short-lived survival of inoculated bacteria on the leaves of lettuce and spinach suggest that the conditions were not conducive to biofilm development under the current conditions of the study.

In addition to interaction of the microorganisms with the plant surfaces (biotic factor), there can be various abiotic factors that affect the survival of microorganisms on fresh produce during the pre-harvest phase. These include nutrient availability, UV radiation, toxic compounds which are released by the plant, competition from other microorganisms or dessication (Whipps et al., 2008). Extensive research has shown that the leaf surface is a particularly hostile environment for bacterial colonists due in part to the low availability of carbon nutrients on uninjured leaves (Lindow and Brandl, 2003). Brandl et al. (2004) mentioned that readily available nutrients in the form of leaf exudates from injured tissues are a key factor in the persistence of *E. coli* on the foliage of leafy vegetables. The poor survivability of *E. coli* and *L. innocua* noted in our study could be attributed to the harsh environmental conditions prevailing in the green house characterized by moderate to high air temperatures in the range of 27-32°C during daytime, low relative humidity of 60-70% and long photoperiod of 11-13 hours. Stine et al. (2005) pinpointed exposure to UV solar radiation as one of the major factors affecting bacterial survival on the phyllosphere. Johannessen et al., (2008) speculated that decreased exposure to sunlight could be partly responsible for the increased survival of *C. jejuni* and *E. coli* on leaves on the exterior leaves of lettuce heads. Another critical abiotic factor is the relative humidity; in field conditions of low relative humidity (38%), *L. innocua* decreased by 9 log CFU/leaf within 2 days on parsley leaves (Dreuz et al., 2007). Similarly, survival of *S. enteric* subsp. *enterica* was enhanced when lettuce was grown under humid conditions (85 to 90% relative humidity) compared with dry conditions (45–48% relative humidity) (Stine et al., 2005).

Pre-harvest microbial contamination of vegetable plantsis an area of research that has already been extensively studied in countries having a temperate climate. However, to our knowledge, no studies have been published on the ability of human pathogens to infiltrate, translocate and/or internalize in the edible parts of vegetables grown in a tropical climate such as that of Mauritius. In the current study, two vegetables belonging to the *Allium* family (green and bulb onions) and two leafy green vegetables (lettuce and spinach) were used as model host systems to study their susceptibility to uptake and persistence of bacterial human pathogen surrogates. Findings of this research revealed *E. coli* and *L. innocua* have the potential to be systemically taken up into food crops such as green onions, yellow onions and lettuce at internalization frequencies of 14-61%, 26-57% and 0-25% respectively. Moreover, lettuce and spinach leaves were found to harbour the microorganisms for > 48h and ≤ 24 h respectively when artificially contaminated with the same bacteria. Taken together, findings of this study point to the microbiological and public health risks

associated with consumption of raw green onions, yellow onions, lettuce and spinach due to the possibilities of pre-harvest microbial contamination by human pathogens. In the light of these results, there is an urgent need to review the current agronomic practices in Mauritius and implement measures for improved farm- to-table strategies to ensure the safety of these commodities.

Table.1 Inoculation Treatments of Plants

TREATMENTS	DETAILS OF INOCULATION OF POTTED VEGETABLE PLANTS
Negative control	Addition of 200 ml of sterile water to the potted vegetable
E. coli	Inoculation of each potted vegetable type with 200 ml of diluted suspension of overnight culture of *E. coli* with a cell density of ca. 10^8 cfu/ml; twice a week
L. innocua	Inoculation of each potted vegetable type with 200 ml of diluted suspension of overnight culture of *L. innocua* with cell density of ca. 10^8 cfu/ml; twice a week

Table.2 Internalization Frequency of *E. coli* (EC) and *L. Innocua* (LI) in Green Onions via Artificially Contaminated Soil

Inoculum	Inoculum level of culture (logcfu/ml)	Mean bacterial population density (log cfu/g)		% Positive Vegetable Samples
		Soil	Vegetable	
------	0.0	< 1.0 ± 0.00	< 0.5 ± 0.00 (0/28)	0
EC	8.3 ± 0.36	8.4 ± 0.71	0.80 ± 0.52 (30/79)	38.0
LI	8.1 ± 0.75	7.2 ± 0.93	0.85 ± 0.60 (33/70)	47.1

Table.3 Internalization Frequency of *E. coli* (EC) and *L. Innocua* (LI) in Yellow Onions via Artificially Contaminated Soil

Inoculum	Inoculum level of culture (logcfu/ml)	Mean bacterial population density (logcfu/g)		% Positive Vegetable Samples
		Soil	Vegetable	
------	0.0	< 1.0 ± 0.00	< 0.5 ± 0.00 (0/16)	0.0
EC	8.3 ± 0.36	8.1 ± 0.65	< 0.5 ± 0.00 (28/74)	37.8
LI	8.1 ± 0.75	7.9 ± 0.82	< 0.5 ± 0.00 (23/58)	40.0

Table.4 Internalization Frequency of *E. coli* (EC) and *L. Innocua* (LI) in Spinach via Artificially Contaminated Soil

Inoculum	Inoculum level of culture (logcfu/ml)	Mean bacterial population density (logcfu/g)		% Positive Vegetable Samples
		Soil	Vegetable	
------	0	< 1.0 ± 0.00	< 2.0 ± 0.0 (0/9)	0.0
EC	8.3 ± 0.36	6.9 ± 0.72	< 2.0 ± 0.0 (0/9)	0.0
LI	8.1 ± 0.75	7.1 ± 0.94	< 2.0 ± 0.0 (0/9)	0.0

Table.5 Internalization Frequency of *E. Coli* (EC) and *L. Innocua* (LI) in Lettuce via Artificially Contaminated Soil

Inoculum	Inoculum level of culture (logcfu/ml)	Mean bacterial population density (logcfu/g)		% Positive Vegetable Samples
		Soil	Vegetable	
------	0.0	< 1.0 ± 0.00	< 2.0 ± 0.0 (0/16)	0.0
EC	8.3 ± 0.36	7.3 ± 0.45	< 2.0 ± 0.0 (3/12)	25.0
LI	8.1 ± 0.75	7.2 ± 0.33	< 2.0 ± 0.0 (1/12)	8.3

Table.6 Survival of *E. Coli* and *L. Innocua*on Lettuce Leaves 24h and 48h Post-Inoculation (hpi)

Inoculum	Inoculum level (log cfu/ml)	Population density (log cfu/g) of *E. coli* on the surface of lettuce leaves		
		0 hpi	24 hpi	48 hpi
------	0.0	< 2.0± 0.0 (0/10)	< 2.0± 0.0 (0/10)	< 2.0± 0.0 (0/10)
EC	8.6± 0.59	8.2 ± 0.41	< 2.0± 0.0 (9/10)	< 2.0± 0.0 (2/10)
LI	8.0± 0.47	8.0 ± 0.55	< 2.0± 0.0 (9/10)	< 2.0± 0.0 (3/10)

Table.7 Survival of *E. Coli* and *L. Innocua* on Spinach Leaves 24h and 48h Post-Inoculation (hpi)

Inoculum	Inoculum level (logcfu/ml)	Population density (log cfu/g) of *E. coli* on the surface of spinach leaves		
		0hpi	24 hpi	48 hpi
------	0	< 2.0± 0.0 (0/10)	< 2.0± 0.0 (0/10)	< 2.0± 0.0 (0/10)
EC	8.5± 0.34	7.7 ± 0.48	< 2.0± 0.0 (0/9)	< 2.0± 0.0 (0/9)
LI	7.9± 0.84	7.9 ± 0.40	< 2.0± 0.0 (1/9)	< 2.0± 0.0 (0/9)

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