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Internalization of enteropathogenic human bacteria in lettuce and coriander plant tissue

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The ability of plant rhizosphere and to some extent phyllosphere to support metabolism of some human enteric bacteria has been widely demonstrated. The nutrients provided by seedlings during germination support bacterial survival in tissue of growing plants. Plant rhizosphere has been described as being high in nutrients, and rhizosphere microbiomes are well adapted to this environment, enteric human pathogens when introduced to such environment face strong competition and their survival is depended on biofilm formation. *Coriandrum sativum* (coriander) and *Lactuca sativa* (lettuce) were transplanted in soil mixed with human excreta at a ratio of 40:1 containing $3\log_{10}$ cfu/g soil of a mixture of human enteric pathogens, consisting of enteropathogenic *Escherichia coli* (EPEC), *Campylobacter coli, Campylobacter jejuni* and *Salmonella enterica* and control pots (positive and negative) were included in the experiment. At harvest, which was carried out at seven weeks after planting, soil, roots, stems and leaves were assayed for presence of enteric pathogens both on surface and in the tissue. Pathogenic *E. coli* and *S. enterica* were isolated from soil and on the surface of coriander roots. *C. jejuni and C. coli* were isolated from all the plant tissues. Conclusively, this study demonstrated a rarely reported internalization of *C. jejuni* and *C. coli* in coriander at seven weeks post-inoculation. It is therefore evident that use of untreated human excreta contaminated with enteric pathogens to grow edible vegetables, could pose significant food safety hazard when consumed uncooked or undercooked.

Key words: Internalization, enteric pathogens, human excreta, coriander, rhizosphere.

INTRODUCTION

A major challenge experienced in informal urban settlements is sanitation which has negative impact on environment and public health. Human excreta have been shown to contain high amount of plant nutrients

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that can be used to boost crop production (Chebet, 2017; Kwamboka, 2015; Winker et al., 2009). However, excreta contain infectious microbial pathogens which must be destroyed or reduced to acceptable levels, before being applied on crops for supply of plant nutrients (Esrey, 2001). Some strains of the enteric bacteria have been shown to be resistant to common antibiotics due to presence of multidrug resistant genes (Christabel et al., 2012) posing even more complex risk with the use of excreta.

The ability of plant rhizosphere to support metabolism of *Escherichia coli* during lettuces seed germination has been reported by Ongeng et al. (2015). The organic secretions from roots of the seedlings do support bacterial multiplication and thus survival in plant rhizosphere. Regardless of the route that bacteria are introduced to the plant, including; irrigation water, soil or manure, the bacteria has the ability to establish and colonize the plants rhizosphere with the ability to migrate in plant system (Ongeng et al., 2015; Lim et al., 2014; Ongeng et al., 2011; Warrier et al., 2003). Some of these bacteria have the ability to withstand antibiotics and survive well in the plants rhizosphere as they utilize roots exudates as source of carbon which withstand osmotic pressure in rhizosphere environment (Berg et al., 2005; Ongeng et al., 2015; Yang et al., 2001).

Bacteria internalization is dependent on the type of bacteria and plant, age and health condition of the plant as well as the bacteria concentration and route of exposure. While some plants such as lettuce, cabbage and radish allowed internalization, others like spinach had no indication of bacteria internalization (Jablasone et al., 2005; Ongeng et al., 2011). The ability of different enteric pathogens to survive in the plant tissue and adopt effectively needs more study (Ongeng et al., 2015), as this would be a possible explanation of increased human enteric pathogens outbreaks in relation to freshly consumed produce. The objective of this study was to establish possibility of crop contamination (internally and on the surface) with *E. coli, Salmonella enterica*, *Campylobacter jejuni* and *Campylobacter coli* at harvest when fertilized with human excreta.

MATERIALS AND METHODS

Human excreta collection

The human excreta were collected from Kibera informal settlement which presents a population with diverse enteric infections (Karanja et al., 2002). Eleven schools were randomly selected out of 30 schools with pupil population ranging from 120 to 189. Pupils were provided with Peepoo bags (http://www. peepoople.com) without urea for three days of sampling in July 2015. Thirty used Peepoo bags were randomly selected among used bags, every day from each school for three consecutive days. Samples were placed in cool boxes and transported to University of Nairobi laboratory for analysis to establish the baseline information on EPEC, *C. jejuni, C. coli* and helminths among other parameters reported elsewhere.

Human excreta samples (60) obtained from asymptomatic school going children, positive with pathogenic *E. coli, S. enterica*, thermophilic *Campylobacter spp.* and helminthes were pooled and mixed into slurry. Twenty five grams of the slurry preparations was mixed with 1000 g of pathogen free sub-soil in sodium hypochlorite disinfected planting pots. Nine grams of di-ammonium phosphate (DAP) fertilizer was added and lettuce or coriander (Dhania) planted in four replicates, one seedling per pot. Control pots were included at every testing and planting period; negative controls were made up of soil plus DAP without slurry while the positive controls were set up by planting coriander in separate pots with 1000 g pathogen free soil plus DAP. These were then inoculated with three suspensions of pathogenic *E. coli* isolate's numbers 91, 90, and 521, respectively and two suspensions of thermophilic *Campylobacter spp* isolates numbers 592 and 525, respectively at a concentration of 10^{10}cfu/ml each.

Lactuca sativa and *Coriandrum sativum* seeds were disinfected with sodium hypochlorite (commercially available at 3.85%) for 15 min and pre-germinated in sterile vermiculite in a 66 wells seed planter. They were kept in a closed, dust-free green house and watered with distilled water. Upon germination the seedlings were supplied with sterile nutrient solution twice daily (Broughton and Dillworth, 1970) till ready for transplanting. Two weeks old seedlings were transplanted in the pots containing soil mixed with contaminated human excreta as earlier indicated. Upon maturity of the plants (at seven weeks) analyses was carried out on soil, roots, stems and leaves to establish presences of the above mentioned enteric microorganisms on the surface and intracellular. A portion of vermiculite together with seedlings was also submitted to the laboratory for similar analysis. The pH and ammonia concentration of soil was determined at harvest.

Recovery of internalized bacteria and surface contamination

At harvest, leaves were cut aseptically with sterile blade and placed in sterile bags; the same was done for stems. The roots were extracted from pot and placed in sterile bags. About 30 g of soil was aseptically obtained from each pot after thorough mixing with a spatula and placed in a sterile tube. At the laboratory, the weight of each sample was determined using a digital balance (OHAUS CORP, Av8108, USA) depending on the weight of the recovered sample, an appropriate amount of sterile buffered peptone water (Oxoid) was poured into the bag to make a 10-fold dilution. The samples were then thoroughly cleaned using a laboratory Stomacher (Seward Medical London, SEI IPP UK) at moderate speed for 3 min to clean off surface contaminants.

The surface clean-up suspension from all the different plant parts as well as the soil suspension were separately placed in clean 15 ml tubes and assayed for presence of organisms as outlined above. The plant samples were further cleaned in running tap water and rinsed with sterile distilled water to remove all remaining surface bacterial contaminants. The cleaned plant samples were soaked for 30 min in 3.85% sodium hypochlorite to inactivate any remaining bacteria contaminant on the plants' surface. Excess hypochlorite was rinsed with sterile distilled water and the water tested for sterility. The mortar samples were ground separately on sterile frozen pestle and mottle. Ground preparations were suspended in sterile buffered peptone water then transferred in sterile 15 ml tubes to make a 10^{-1} dilution. All plant samples were then tested for viable *Ascaris lumbricoides* eggs according to modified McMaster method by Utzinger et al. (2010) and modified Bailenger method (WHO, 1996) with slight modifications.

Enumeration of thermo-tolerant coliforms, heat-stable *E. coli* and isolation of *Salmonella spp.* was carried out according to the WHO

Table 1. Presence of pathogenic bacteria and *A. lumbricoides* eggs in human excreta slurry and in soil mixed with human excreta slurry at the start of plants internalization experiment.

Time (weeks)	Pathogens	Slurry	Slurry + soil + DAP	Soil + DAP (Negative control)
Day 0	*Ascaris lumbricoides* (100% viable) eggs/gram ± SD	4600±707	3250±70.7	0
	Coliforms (log_{10}cfu/g)± SD	9.27±0.14	8.01±0.94	0
	E. coli (log_{10}cfu/g)± SD	9.14±0.19	7.31±0.04	0
	Campylobacter coli and *C. jejuni* (log_{10}cfu/g) ± SD	1.55±0.07	1.15±0.07	0
	Salmonella enterica (log_{10}cfu/g)	1.55±0.07	1.15±0.07	0

Table 2. Remaining pathogen contamination at harvest (seven weeks, after planting) of coriander and lettuce grown in soil fertilised with human excreta slurry.

Pathogens isolated from coriander and lettuce	Mean concentrations ±SD (cfu/g) recovered at harvest (7 weeks)						
	Soil	Roots		Stem		Leaves	
		Surface	Tissue	Surface	Tissue	Surface	Tissue
Viable *A. lumbricoides* eggs/g	200±1.00	0	0	0	0	0	0
E. coli (EPCC) (log_{10} cfu/g) ± SD	0.50±1.00	0	0.67±0.78	0	0.57±0.66		0.57±0.66
C. jejuni and E. coli (log_{10} cfu/g) ± SD	1.45±0.07	1.3±0.14	1.2±0.14	0	1.2±0.14	0	1.15±0.07
Salmonella enterica (log_{10} cfu/g) ± SD	1*	0	1*	0	0	0	0

*Pathogenic bacteria isolated and not enumerated.

laboratory protocol (2010) for *Salmonella spp.* and *E. coli* from food and animal faeces. Presence of thermophilic *Campylobacter spp.* in soil and plant samples was carried out according to FDA bacteriological analytical manual (1998) and molecular characterization according to CDC global Salm-Surv laboratory protocol for *C. coli* and *C. jejuni*, with some modifications.

RESULTS

Internalization of microorganisms via plant roots from pathogen spiked soil

At the onset of the experiment (t=0), the *A. lumbricoides* eggs recovered from the human excreta slurry and soil-slurry mix were 100% viable. *Enteric E. coli* in the human excreta slurry was at average 9.27±0.14log_{10}cfu/g total solids. The concentrations were reduced to an average of 8.01±0.94 cfulog_{10}/g when mixed with 1000 g of soil. *C. jejuni, C. coli* and *S. enteric* were isolated from all the excreta slurry and soil mix preparations except for the negative controls (Table 1).

Presence of the pathogens from human excreta on plants surface and tissue

When human excreta slurry was mixed with soil and used to grow coriander, *A. lumbricoides* (200 eggs/g of soil) at viability of 20% were recovered from the soil at harvest; however none was found attached to any of the tested plants' parts. Enteropathogenic *E. coli* (EPEC) were isolated from soil and on the surface of coriander roots, and in the tissues of coriander and lettuce stem and leaves at varying concentrations but no EPEC was isolated from the surface of the plant parts. *C. jejuni and C. coli* were found in all the plants tissues including the root, stem and leaves. *S. enterica* was only isolated in coriander and lettuce roots and in soil mixed with excreta slurry (Table 2).

In the control containing bacteria isolate suspensions at 10^{10}cfu/ml, internalized enteropathogenic *E. coli (*EPEC) were recovered from tissue of plant parts (roots, stem and leaves) of coriander and lettuce as well as from the soil. Likewise *C. jejuni and C. coli* were internalized and recovered at harvest from roots, stem and leaves tissues. There was however no bacterial pathogen isolates recovered from the negative control (soil plus sterile distilled water) Table 3.

DISCUSSIONS

In this study, it was evident from control experiment that at concentration of 10^{10} cfu/ml *Campylobacter spp, E. coli* and *S. enterica* can internalize in plant tissue. Bernstein et al. (2007) demonstrated *Salmonella* internalization through roots at 500 cfu/g and identified it in leaves at 130 cfu/g of 33 day old lettuce, 2 day post-inoculation.

Table 3. Internalization of known bacteria pathogens into coriander and lettuce via roots planted in spiked replicates of potted soil.

Spiked Controls	10log$_{10}$cfu/ml	Soil	Mean concentrations ±SD (cfu/g)recovered at harvest (7 weeks) controls					
			Roots		Stem		Leaves	
			Surface	Tissue	Surface	Tissue	Surface	Tissue
Positive	EPEC (isolates no 17, 18, 19)	2.23±4.09	1.08±0.32	0.7±0.41	0	0.68±0.38	0	0.96±0.95
	Campylobacter coli and *jejuni* (isolates no 22, 23)	1.44±0.01	1.24±0.01	1.18±0.01	0	1.22±0.01	0	0.68±0.38
Negative	Sterile saline solution	0	0	0	0	0	0	0

Franz et al. (2007) also reported internal colonized *Salmonella typhimurium* and *E. coli* O157:H7 in leaves of lettuces at 35 days post-inoculation in potted soil.

Ge et al. (2012) demonstrated internalization of *Salmonella spp* in lettuces and green onions leaves at 2 days post-inoculation. They also reported increased internalization in water stressed lettuce as opposed to green onions. Onions were not affected by water stress at a contamination of 10^3 cfu/g. Wright et al. (2013) contaminated growth media with 10^7 cfu/g *E. coli* and planted lettuce and spinach. The authors demonstrated internalization 10 days post-inoculation in 81% spinach and 31% lettuce roots and leaves, respectively. The authors observed formation of internal bacteria colonies in the plant apoplast at the 9th day of inoculation, which was as a result of bacteria association with the plant root's epidermal cells at day 6, progressing to bacteria penetration below root surface and formation of internal colonies.

In this study *C. jejuni and C. coli* were isolated at harvest from all the plant parts including roots, stem and leaves. *C. jejuni* has been isolated from plants species including spinach and radish (Branol et al., 2004). Though leaves environment is largely hostile to bacteria and particularly to human pathogens but there are cases where the same thrives in leaves (Kroupiski et al., 2009).

On the contrary, root tip forms energy-rich microenvironment supporting bacteria multiplication. Brandl et al. (2004) demonstrated *C. jejuni* in spinach roots and leaves upto 30 days post-inoculation, when temperatures were maintained at 10 to 16°C. Injured plants were shown to support survival of Campylobacter relative to healthy plants, implying post-harvest contamination of vegetables and fruits which play key role in food-safety. The bacteria was isolated from excreta contaminated soil and roots at seven weeks post-inoculation.

Survival of *C. jejuni* in faecal contaminated soil has also been reported by Brandl et al. (2004), who demonstrated *C. jejuni* in soil and roots rhizosphere, long after leaves internalization had stopped. The low oxygen tension resulting from both roots and bacteria respiration at the rhizosphere supports survival of Campylobacter cells. Humic acids abundantly found in soils are known to increase survival of laboratory culturable *Campylobacter spp* in soil (Weinrich et al., 1990). To address the challenge of effective pathogen destruction and hence preventing infectious disease transmission through human excreta, sanitisation technologies need to be diversified as well as refined to offer methods applicable in all the different contexts. The hygienic quality of fertiliser produced from excreta ought to be high enough, to avoid infection risks to both the handlers and end product consumer. Crop production can be increased by supplying a locally available fertilizer which improves yields by supplying limiting nutrients to soil as well as improving water holding capacity (Heinonen-Tanski and Wijk-Sijbesma, 2005).

Conclusion

Pathogenic bacteria contaminating soil at a minimum of 10^3 cfu/g having been diluted by mixing with soil allowed bacteria attachment to coriander roots with no evident internalization. This may remain as environmental contamination which later gets its way to edible crop parts at harvest.

However higher bacteria soil contamination (10^{10} cfu/g) allowed bacteria internalization through coriander and lettuce roots which were detected in the roots, stem and leaves. Pathogen reduction through treatment of human excreta as per the WHO guidelines (2006) on reuse of treated human excreta will thus stop plant internalization of pathogenic bacteria, as well as soil and plant contamination. Otherwise, the colonization of plants with human pathogens is a possibility and requires in depth understanding, since it possess significant food safety hazard.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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