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Bacterial Assessment on Leaves of Green Vegetable Grown on Hydroponics and its possible Health Risks

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Abstract

The World Health Organization has classified food borne illness as a public health problem especially for high risk populations such as the very young, the elderly and immune compromised. The consumption of raw green leafy vegetables has been linked as a risk factor for contamination with pathogens such as *Salmonella* spp. and *Escherichia coli* O157: H7. In this study we analyzed irrigation water and leaf samples from *Lactuca sativa* a highly consume lettuce in Puerto Rico, using the EPA membrane filtration method 1600 in order to determine the presence of microbiological pathogens. Two hydroponic agricultural facilities in Puerto Rico where selected and results demonstrated the presence of various bacterial genera including the pathogen Enterococcus faecalis as the most important disease causing organism in our samples. Results reveal 70 CFU / ml, 13 CFU / ml, 4 CFU / ml 26 CFU / ml, 9 CFU / ml and 5 CFU / ml, in area 1. In contrast, 15 CFU / ml, 32 CFU / ml, 26 CFU / ml, 17 CFU / ml, 4 CFU / ml, 6 CFU / ml resulted from leafy samples for area 2. Results in water samples from both areas showed > 300 CFU/ml (TNTC) (n = 18).

Keywords: *Lactuca sativa*, pathogens, food borne illness, hydroponics, risk assessment.

1. Background

Biological contamination and food borne illnesses is a worldwide health concern (Havelaar et al., 2010) since about 1.8 million deaths worldwide have been attributed to the presence of *Escherichia coli*, *Salmonella* or *Shigella* (Newell et al., 2010). A variety of bacteria, parasites and virus can cause serious health problems after ingesting contaminated food or raw food products; these cases are known as food borne illnesses (FBA) (Barrantes&Achí, 2011). The main pathogens associated with food related disease outbreaks are *Escherichia coli* O157:H7 and *Salmonella* spp. Among the main food borne illnesses are diarrheal conditions such as gastroenteritis, typhoid fever and shigellosis among others (Rivera et al., 2009; Callejas et al., 2011; Beuchat, 1996). Diarrheal diseases alone can cause over 2.2 million human death globally every year (WHO, 2004); but the burden arising from all foodborne diseases is clearly larger.

Among recent cases in 2006 an outbreak of *Escherichia coli* from ingestion of spinach affected 26 states in the continental USA, resulting in a total of 200 reported cases, of which 183 were linked to direct contamination with *E. coli* (Abadías et al., 2008; Calvin, 2007).

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Moreover, there have also been cases reported of food poisoned with *Shigella*and *Salmonella* in the USA during 1996-2006 (CDC, 2007) including 4 outbreaks consumption of tomato (Lycopersiconesculentum), 459 confirmed cases of *Salmonella*, and in 2006 121 outbreaks caused by *Salmonella* with 3,300 confirmed cases (CDC, 2007).

In Europe, the presence of *Salmonella* in fresh vegetables was responsible for at least 87% of outbreaks by foodborne diseases (D'Aoust, 1994). Meanwhile, in Costa Rica, about 23 outbreaks were reported in 2005 by *Shigella* and *Salmonella* with diarrhea being the main cause of report. In 2006, were reported at least 1,267 cases by the presence of *Shigella* remains the leading cause with 33.4% above Salmonella was given a total of 5.2% (Barrantes& Achi, 2011). According to The Foodborne Disease Active Surveillance (FDAs) in 2012, 19,531 confirmed cases were identified pathogen infections caused by *Campylobacter* cases 6,793, 121 *Listeria* and 7,800 by *Salmonella*. Of these three, *Salmonella* was the cause of about 33 deaths (CDC, 2013). Recently as December 2012, it was reported that an outbreak caused by the presence of *E. coli* 0157: H7 associated with the consumption of organic spinach (Spinaciaoleracea) was over, leaving at least a total of 33 people infected in 5 states of the United States. These events are some of many events that occur by the presence of plant-pathogenic bacteria resulting in adverse effects on human health and therefore require attention and continue monitoring, especially in regard to raw foods (Barrantes& Achi, 2011).

Leafy green vegetables are some of the most beneficial and healthy foods for human consumption. However, their raw state presents a greater risk for contamination by microorganisms since no cooking steps are required (Barrantes&Achí, 2011; Heaton & Jones, 2007; Erkam&Vural, 2008; Bordini, Asturiano, Jakabi, &Gelli, 2007). In most cases bacterial contamination has been related to the water with which these plants are irrigated therefore possible bacterial contamination will depend on the quality of the water used (FAO, 1998). Other factors affect contamination such as the type of vegetable, soil management and handling after harvest (FAO, 1998). Today many of these vegetable products are cultivated applying alternative agricultural methods such as hydroponics techniques. These systems are excellent resources for rapid production, however little information about microbiological quality of harvested products such as the lettuce *L. sativa* is available from small growers.

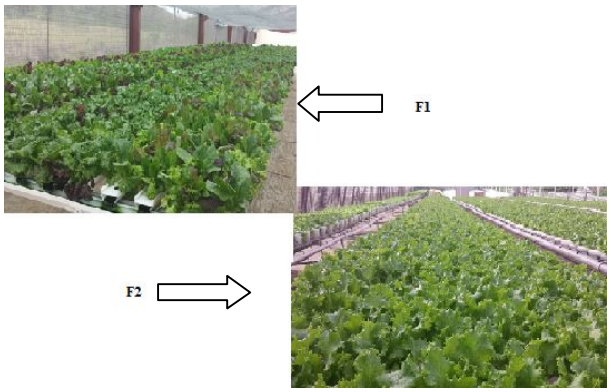
This study aims to identify possible biological contaminants, therefore aiding in addressing corrective measures for future production. This study is the first report on the assessment of the presence of pathogenic bacteria on leaves of green vegetable grown on hydroponics agriculture systems in Puerto Rico and its possible health risks.

2. Methodology

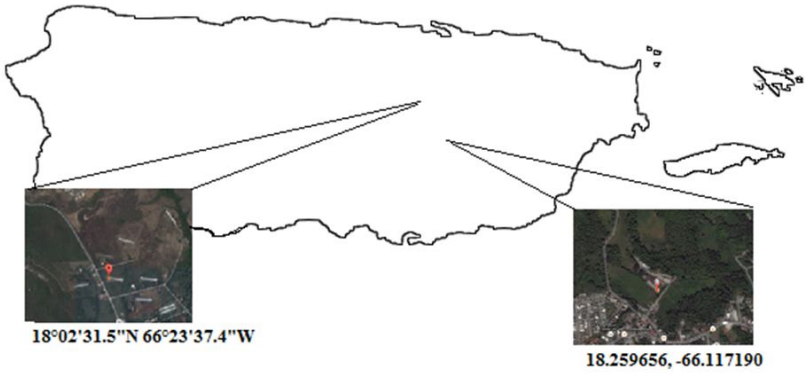
2.1 Sampling

Two in Puerto Rico were selected and labeled as F1 and F2. Samples from the lettuce *L. sativa* leaves and hydroponic system irrigation water (100ml) were selected using aseptic techniques and the method EPA 1600 for collection and sample handling. A total of 3 – 4 leaves per lettuce head were selected. In both hydroponic facilities, selected 3 tubes on the pipe location; In tubes 1 and 2 selected approximately (20g) of leaves were selected from (2) different sites (internal and external, the external leaves are those with more proximity to the water).

In tube 3 only external leaves were included for a total of 9 leaf samples and 9 water samples per agricultural facility and a final total of 36 samples. Three (3) points in the irrigation pipes were selected and sampled at site A (beginning of system), B (middle) and C (end) based on the pipe location and distance to water system. Sampling was performed using the Whirl-pack®Nasco system as per described in Abadías, Usall, Anguera, Solsona, y Vinas (2008).



Facilities Location



2.1.1 Handling and process of samples

Irrigation water samples were filtered (a total of 100ml) using a 0.45 µm cellulose membrane, which was used for further bacteriological propagation. Leaf samples were washed with 25ml of sterile distilled water and agitated delicately for 10 minutes to allow for microorganism detachment from surface. The resulting wash water was filtered in the same manner as the irrigation water samples.

2.2 Bacteriological analysis

EPA method 1600 was used to determine growth and colony count of bacteria. Membranes were inoculated on 35mm Petri dishes on various media: Tryptic Soy Agar, McConkey Agar o Mannitol Salt Agar. Samples were incubated for 72 hours at 28ºC then growth was documented. Colonies that were isolated in the differential media were selected for identification.

2.3 Microbial identification

Discrete cultures were selected and inoculated in 20ml of inoculating fluid (IF) and placed on turbidometer to achieve 80% turbidity. This solution (10 µl) was placed on the Biolog (Hayward, CA) Gen 3 Microarray panel. Samples were incubated for 18 hours prior to analysis using the Biolog system.

2.4 Statistical Analysis

The MiniTab 14 (Minitab, State College, PA) program was used for descriptive analysis and paired t- test significant values were selected at a P value of $p< 0.05$.

3. Results

Leaves and water samples were organized as per sampling point and agricultural facility. Table 1 presents the colony forming units per milliliter (CFU/ml) for leaves and water samples on both sampled facilities.

Each facility obtained 66% of samples with over 300 CFU/ml which is classified as too numerous to count (TNTC). Water samples from both facilities resulted in high levels of bacterial counts (TNTC) for water samples used to irrigate and grow lettuces. In term of leaf samples both facilities reported the presence of bacterial counts at the external, middle or internal points. After statistical analysis a significant difference (p=0.004) was obtained between the bacterial concentration from the leaf and water samples, indicating that water samples had a much higher level of bacterial contamination than leaves.

Table 1: Results obtained in colonies per milliliter (CFU/ml) on leaves and irrigation water

Sampling point	CFU/ml			
	F-1		F-2	
	Leaves	Water	Leaves	Water
A1	70.0	TNTC	15.0	TNTC
A2	13.0	TNTC	32.0	TNTC
A3	4.0	TNTC	TNTC	TNTC
B1	TNTC	TNTC	26.0	TNTC
B2	26.0	TNTC	17.0	TNTC
B3	TNTC	TNTC	TNTC	TNTC
C1	9.0	TNTC	4.0	TNTC
C2	TNTC	TNTC	6.0	TNTC
C3	5.0	TNTC	TNTC	TNTC

.

Numbers (1, 2 and 3) represent the irrigation tubes, letters (A, B and C) represent sampling points: Beginning (A), middle (B) and End (C). TNTC = CFU/ml > 300. When comparing CFU of leaves against water a significant difference was observed (p = 0.004).

Table 2 presents samples that presented fermentation on selective media. These results indicate that the isolated organisms are able to ferment lactose and survive in acidic environments providing an identification of such bacteria. The Mannitol Salt Agar (MSA) is selective and differential media usually for Staphylococcus. This media allows for the growth of organisms in high salt content which ferment mannitol. The McConkey culture media (MC) is selective for Gram negative bacteria which are lactose fermenters. Facility 1 presented 33% of fermenting growth on leaf samples for both subcultures on MC and TSA media while MSA presented 100% fermentation on leaf samples. Water samples for this facility presented 66% for both subcultures on MC agar while TSA did not present fermentative growth; MSA samples presented 33% and 66% for both subcultures respectively.

Facility 2 presented 0% growth on leaf samples for both subcultures on MC agar while presenting 33% for all subcultures for TSA media and 100% growth on MSA media. The water samples for this facility presented 100 % and 33% growth on the two subcultures for MC agar while 33% for both subcultures on TSA media and 100% and 66% for the two subcultures on MSA agar. On both leaf and water samples we can see evidence of Gram negative bacteria which will account for the observed fermentation. These results reflect the possibility that isolated organisms from the samples belong to fecal coliform groups.

Table 2. Percentage of leaf and water samples from first and second subcultures which revealed fermentation on various culture media.

Facility	Percentage of fermentation by culture media subculture 1 and 2							
	MC			TSA			MSA	
	1	2		1	2		1	2
F-1 leaf	33	33		33	33		100	100
water	66	66		0	0		33	66
F-2 leaf	0	0		33	33		100	100
water	100	33		33	33		100	66

McConkey(MC),*Tryptic Soy Agar* (TSA) *y Mannitol Salt Agar*(MSA)

The identification of the recuperated microorganisms did not reveal the presence of *E. coli* O157:H7 or *Salmonella* spp., one of the major organisms suspected for FBA however the presence of *Enterococcus faecalis* a fecal coliform organism was identified. This organism which is associated with FBA was present in 11% of the leaf samples but not in the irrigation water. Table 3 presents all the genera isolated from samples in each facility. Table 3 presents the genera isolated from both facilities. A total of 78% of samples presented bacterial growth including the following genera: *Bacillus*, *Mycobacterium*, *Aeromonas*, *Pseudomonas*, and *Enterobacter* among others. Although none of the more alarming genera such as *E. coli* O157:H7 nor *Salmonella* spp., were detected the presence of a large variety of different genera including possible pathogens suggest a contamination source for the irrigation water.

Table 3: Isolated microorganisms from each facility and growth media used Samples were obtained from lettuce leaves and irrigation water

F-1			F-2	
Bacteria	Media		Bacteria	Media
Corynebacterium mycetoides	MSA		*Methylobacterium mesophilicum*	MC/TSA
Paenibacillus thiaminolyticus	MSA		*Aeromonas bestiarum*	MC
Pseudomonas stutzeri	MSA		*Cupriavidus necator*	TSA
Enterobacter aerogenes	MC		*Bacillus horti*	MC
Pantoea agglomerans bgp 6	MC		*Providencia rettgeri*	MC
Serratia liquefaciens/grimesii	MC		*Pediococcus acidilactici*	MC
Raoultella terrigena	MSA		*Mycobacterium phlei*	TSA
Enterococcus faecalis	MSA		*Cupriavidus gilardii*	TSA
Bacillus halodurans	MSA		*Aeromonas* DNA group 11	MC/TSA
Pseudomonas syringae pv pisi	MSA		*Bacillus pseudofirmus*	MC
Shewanella algae	TSA			
Exiguobacterium undae	TSA			
Serratia odorifera	TSA			
Mycobacterium senegalense	TSA			

McConkey(MC),*Tryptic Soy Agar* (TSA) *y Manitol Salt Agar*(MSA)

3.1 Discussion

The results from both facilities reveal great diversity of organisms isolated including the presence of pathogenic microorganisms. All irrigation water samples presented values over 300 total CFU/ml which is over local state regulation thresholds of 200 CFU/ml. These high values suggest the irrigation water is the main source of contamination in both facilities. Our sampling selection indicated that the leaves in closest contact with the irrigation water yield the highest amount of bacterial contamination, these are the external leaves. Of particular interest are the types of possible pathogenic bacteria isolated from the samples. Table 4 presents pathogens isolated and their possible health effects. A total of 78% of samples presented bacterial growth including the following genera: *Bacillus*, *Mycobacterium*, *Aeromonas*, *Pseudomonas*, and *Enterobacter* among others. Thirty-one percent (31%) of isolated organisms are possible causative agents of bacteremia whereas other 69% can cause conditions such as meningitis, pneumonia, gastroenteritis urinary tract infection among others. It is worth noting that these organisms are not present in all sampling areas.

The quality of agricultural used water can vary depending on its source. Surface water can be influenced by runoff and illegal sanitary discharges, spring water can be tainted by damaged sanitary systems contaminating the downstream usage. One example from our samples is the presence of *Enterococcus faecalis* in samples from site 1. The presence of these organisms associated with fecal contamination led us to analyze the surrounding areas from the water source, a near lake. Evidence of livestock operations in the immediate areas close to the water source was obtained which suggests a possible source of contamination from livestock.

Table 4: Pathogens detected on *Lactuca sativa* leaves or irrigation water and their possible health effects.

Organism	Health Effect
Pseudomonas stutzeri	Septicemia and pneumonia
Pantoeaagglomeransbgp 6	Septic Arthritis bacteremia, pneumonia
Enterococcus faecalis	Bacteremia, diarrhea, fever, gastroenteritis
Serratiaodorifera	Urinary infection, septicemia
Mycobacterium senegalense	Bacteremia
Shewanella algae	Osteomyelitis, bacteremia, y emphysema/ cellulitis
PaenibacillusthiaminolyticUs	Bacteremia
Methylobacteriummesophilicum	Bacteremia
Aeromonasbestiarum	Gastroenteritis y diarrhea
Providencia rettgeri	Cholera and ocular infection
Mycobacterium phlei	Bacteremia
Aeromonas DNA group 11	Diarrhea, gastrointestinal infection and gastroenteritis
Cupriavidusgilardii	septicemia , pulmonar edema, anemia
Raoultellaterrigena	Sepsis, diarrhea, fever
Enterobacter aerogenes	Bacteremia, meningitis
Serratialiquefaciens/grimesii	bacteriemia, Urinary infection

Although farmers have limited control of the treatment of irrigation water, several measures such as good manufacturing practices (GMP) and good agricultural practices are recommended to reduce the possibility of contamination and accountability for the quality of the product through water quality testing and product monitoring. The water used for these purposes must comply with EPA and state quality limits to minimize the risk of product contamination. EPA regulations state that fecal microbial contamination must not be present in food products (EPA, 2000).

4. Conclusions

There are various ways raw vegetables can become contaminated with pathogenic microorganisms increasing the risk of food borne illnesses throughout all the steps required for processing foods such as growing, harvesting, processing and distribution. This study examined the presence of microbiological food contaminants in lettuce and irrigation water of two hydroponic facilities in Puerto Rico. Results clearly indicated the presence of high counts of bacterial growth in leaves and water samples; however, their exact identification was not possible in all samples. Bacteriological analysis with selective growth agar indicated no detection of the most known food contaminants *E. coli* or *Salmonella* spp., however the amount of other microorganisms such as *E. faecalis* found in about 11% of leaf samples imply a risk to consumer's health. Interestingly the presence of *E. faecalis* was not detected on the water samples which can suggest this organism contaminates leaves in some other stage of the handling process of the plant or a mechanical vector (Pesquero, Carneiro, &Pires, 2012). Water samples however did present a high content of other microorganisms some of which are known pathogens. The results of this study provide substantial evidence for the need of increased water quality monitoring, standards and handling practices for the hydroponics industry in order to eliminate health risks to consumers.

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