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Potential enterobacteria risk factors associated with contamination of lettuce (*Lactuca sativa*) grown in the peri-urban area of Abidjan (Côte d'Ivoire)

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ABSTRACT

This study was conducted to determine the risk factors associated with the contamination of lettuce grown in the suburban area of Abidjan. A total of 216 samples were collected. Total aerobic bacterial counts and Enterobacteriaceae were investigated on soil, fertilizer, irrigation water and lettuce samples. Mean values of total aerobic count for sites 1 and 2 were 9.97 $\log_{10}$ cfu/g and 8.20 $\log_{10}$ cfu/g for the manure respectively, and 8.11 $\log_{10}$ cfu/g for the soil of both sites. Lettuce samples from the market were more contaminated by Enterobacteriaceae than those obtained from the production sites. The main enterobacteria isolated were *Salmonella gallinarum*, *Serratia marcescens* and *E. coli*. All the lettuce samples analysed were contaminated by *E. coli* and *Serratia*. The prevalences of *Serratia marcescens* on lettuce from the market and from the production sites 1 and 2 were 58.33%, 33.33% and 41.67% respectively while those of *E. coli* were 33.33%, 25% and 8.33% respectively. The most common *Salmonella* serotypes isolated was *Salmonella gallinarum* with prevalences of 50%, 41.67%, 58.33%. *Salmonella choleraesuis* and *Shigella sonnei* were weakly represented. The manure has the highest potential as a source of contamination and infection of lettuce followed by the soils.

Keywords: Lettuce, contamination, enterobacteria, soil, irrigation water, manure

INTRODUCTION

Socio-economic needs in African cities have rendered agricultural activities difficult and decreased agriculture in suburban areas. Urban and suburban (peri-urban) agriculture is an answer to these preoccupations as long as they contribute to the food security of urban populations and the creation of temporary or permanent jobs for many low income households. Several studies put emphasis on the contribution of peri-urban agriculture for the satisfaction of food demands (Dieye, 2006; Ouédraogo et al., 2008), especially fruits and vegetables.

Fresh fruits and vegetables are important components of a healthy and balanced diet; their consumption is encouraged in many countries by governmental health agencies to protect against a range of illnesses such as cancers and cardiovascular diseases. However, fruits and vegetables, and in particular leafy greens that are consumed raw, are increasingly being identified as important vehicles for the transmission of human pathogens that were traditionally associated with foods of animal origin (Xicohtencatl-Cortes et al., 2009; Berger et al., 2009).

Côte d'Ivoire is a sub-Saharan African country facing significant sanitation challenges. In Côte d'Ivoire, fresh salads are not part of the normal diet for low income households, but are a common supplement to urban fast food served by the street sides, in canteens and restaurants. Even though part of the vegetables consumed comes from other areas of the country, most of the lettuce used comes from the peri-urban agriculture. In Abidjan (Côte d'Ivoire), the urban and peri-urban agriculture is practiced most often on swampy sites. Thus, in the peri-urban areas of Abidjan, vegetables are grown not far from the lagoon banks and other swampy sites (Mattys et al., 2006). In this difficult context marked by the lack of financial means for clean irrigation water and synthetic manure for fertilization of the sandy soils, the market gardeners are pushed to use waste waters for irrigation and animal excrements as manure for soil enrichment (Amoah et al., 2007; Oliveira et al., 2010).

These cultural practices could favor a microbial contamination of the vegetables of which some can be dangerous for the consumer. The consumption of fruits and vegetables is commonly viewed as a potential risk of infection with enteropathogens such as *Salmonella* and *Escherichia coli* O157, but also with viruses as shown by the recent outbreaks linked to lettuce, spinach and tomatoes (Heaton and Jones 2008; Westrell et al., 2009; Petterson et al., 2010). Furtheremore, food-borne illnesses have been linked to the consumption of vegetables worldwide (Barak et al., 2008; Soderstrom et al., 2008; Wendel et al., 2009; Berger et al., 2009). A study of produce-associated outbreaks in the USA from 1973 to 1997 found that viruses accounted for only 20% of outbreaks in which an agent was identified, mostly due to hepatitis A. Parasites accounted for 16% of all produce-associated outbreaks. Bacterial pathogens continue to be a major contributor to produce-associated food-borne illnesses. Bacteria were responsible for 60% of the outbreaks in which an etiologic agent was identified (Sivapalasingam et al., 2004). *Salmonella* was the most commonly reported bacterial pathogen, accounting for nearly half of the outbreaks due to bacteria (Sivapalasingam et al., 2004) followed by *E. coli*. This means that enterobacteria account for more than half of the outbreaks due to bacteria. The factors generally cited in the contamination of vegetables are irrigation water, soil, manure, animals, conditions of crops, young plants… (Mootian et al., 1997; Heaton and Jones, 2008; Barak and Liang, 2009).

A study conducted in Côte d'Ivoire by Sackou et al. (2006) showed that 75% of the lettuce samples from the markets of Abidjan don't meet the criteria set up in microbiology for vegetables. These works also revealed the presence of thermo tolerant coliform on the quasi-totality of the lettuce samples analyzed. Because of the risks of health problems for consumers, the main objective of this work is to prevent food borne illnesses associated with lettuce consumption. Thus, the aim of this present study was to reveal some potential risk factors associated with the contamination of lettuce grown in the peri-urban areas of Abidjan (Côte d'Ivoire), by assessing the enterobacteria risk.

MATERIALS AND METHODS

Sampling sites

The study was carried out on commercial and farm lettuce (*Lactuca sativa*) samples, untreated irrigation water obtained from wells at a depth of 0,5 to 2 m, manure and soils from the two biggest peri-urban sites (1 and 2) of market gardening products of

Abidjan (RCI). The first site is located in the township of Port-Bouet, close to the International Airport Félix Houphouët Boigny with a surface of about 30 ha, and mainly used for the culture of lettuce. Other vegetables such as Bell pepper, cabbage, onion leaf etc… are also grown on that site. The second site is in the township of Cocody close to the Ebrié lagoon with a surface of about 20 ha, mainly used also for the culture of lettuce. The third site is a local wholesale and retail market named *Gouro* market in the township of Adjamé. It is the largest market where vegetables and raw edible products are stocked and sold. So, two (2) vegetables production sites and a market were investigated for this study.

Sampling

The sampling was realized from the two lettuce production sites and the market. A lettuce sample consisted of three lettuce plants. Water sample corresponded to 500 mL of water from a well, manure and soil sample consisted of 100 g of material. Two samples of each kind were taken from each production site every month. Therefore, 192 samples were collected from the production sites and 24 from the sale site over a period of 12 months, making it 24 samples for each factor studied and for each collecting site (production sites 1 and 2, market) and a total of 216 samples. Each sample was placed inside a sterile plastic bag, labelled and stored in an ice box and shipped to the laboratory for immediate analysis.

Microbiological analysis

Once in the laboratory, no more than 2 hours after the sampling, each sample of lettuce was mixed in a stomacher (laboratory blender Model 80 Seward Medical, London) for 30 seconds at normal speed. To analyze the samples, the methods stated in Compendium of Methods for the Examination of Foods (Vanderzant and Splittstoesser, 1992) were used. Total aerobic counts were numbered on Plate Count Agar (Oxoid CM 463) and incubated for 24-48 hours at 30 °C. Enterobacteriaceae were counted on Violet Red Bile Glucose Agar (Oxoid CM 485) and incubated at 37 °C for 24-48 hours. Pink-red color colonies with precipitation were taken into consideration. All the tests were carried out in duplicate. Duplicate agar plates of between 15 and 300 colonies were counted, and mean counts calculated. The counts for each plate were expressed as the log of the colony forming unit ($\log_{10}$ cfu/g or $\log_{10}$ cfu/mL) of the sample analysed.

Furthermore representative colonies of Enterobacteriaceae were identified by Gram staining reactions, catalase and oxydase tests and phenotypic tests using API 20E (Biomerieux, France). The isolates were identified by comparing their characteristics to those of known taxa, as described by Bergey's Manual for Determinative Bacteriology (Sneath et al., 1986).

Statistical analysis

All values were expressed as the mean of three measurements. The data collected were subject to one-way analysis of variance (ANOVA). With respect to the factors of lettuce contamination investigated during risk assessment by Enterobacteriaceae (soil, manure, untreated water, lettuce), the mean values were compared. In order to determine which enterobacteria mean values for the different factors studied were significantly different from others, the differences between the means were assessed by Duncan's multiple range test at $\alpha = 0.05$.

RESULTS

The distribution patterns of total aerobic count and enterobacteria count from the two vegetable production sites are shown in Figures 1 and 2. The microbial load of the manure and the soil was significantly ($p<0.05$) higher than the one from the irrigation water and the lettuce for the two production sites. The mean values of total aerobic count for site 1 were 9.97 $\log_{10}$ cfu/g and 8.11 $\log_{10}$ cfu/g for the manure and the soil respectively. For site 2, these values were 8.20 $\log_{10}$ cfu/g and 8.11 $\log_{10}$ cfu/g for the manure and the soil respectively. The mean values for total count were 6.64 $\log_{10}$ cfu/ml and 7.32 $\log_{10}$ cfu/g for the irrigation water and the lettuce from site 1 respectively, and 6.34 $\log_{10}$ cfu/ml and 6.34 $\log_{10}$ cfu/g for site 2 respectively. There were no significant differences ($p<0.05$) between enterobacteria load from the two production sites. The irrigation water seems less contaminated by enteric bacteria. the values were similar for the sites 1 and 2, 4.98 $\log_{10}$cfu/ml and 4.97 $\log_{10}$ cfu/ml respectively. The total aerobic and enterobacteria counts of the lettuce samples from the market and the two production sites are shown in Figure 3. The lettuce samples from the market were more contaminated than those from the production sites. The total aerobic and enterobacteria load of lettuce samples from the market revealed 8.15 $\log_{10}$ cfu/g and 6.5 $\log_{10}$ cfu/g respectively; 7.32 $\log_{10}$ cfu/g and 5.32 $\log_{10}$ cfu/g for site 1 samples; 6.34 $\log_{10}$ cfu/g and 5.23 $\log_{10}$ cfu/g for the site 2 samples.

The prevalences of the enterobacteria strains from the production sites are presented in Table 1. The species of enterobacteria identified were nearly the same excepted for *Providencia alcalifasciens* and *Proteus vulgaris* which were detected only on the production sites (Table 2). Three serovars of *Salmonella enterica* have been identified: *Salmonella gallinarum*, *Salmonella arizonae* and *Salmonella choleraesuis*. In addition, 3 species of *Enterobacter* (*Ent. aerogenes, Ent. agglomerans, Ent. cloacae*), 2 species of *Citrobacter* (*C. freundii*, *C. diversus*), 2 species of *Serratia* (*S. marcescens, S. plymutica*), one species of *Escherichia* (*E. coli*), *Shigella* (*S. sonnei*), and *Afnia* (*A. alvei*) were recorded.

Among the isolated strains, *Salmonella gallinarum* was the predominant with a frequency of isolation of 41.67%, 50%, 66.67%, and 25% for lettuce, irrigation water, manure and soil samples respectively from site 1. For site 2, the prevalences of *Salmonella gallinarum* were 58.33%, 41.67%, 33.33% and 75% for lettuce, water, manure and soil samples respectively, followed by *Serratia marcescens* for which the frequency of isolation seems similar, except for the soil samples with a frequency of 50% and 33.33% for sites 1 and 2 respectively. *Serratia* was absent in the manure from site 2. The serovar *Salmonella arizonae* was detected with a high prevalence in the irrigation water (58.33%) and the soil (50%) samples from site 2. Prevalences of 33.33% and 8.33% were recorded for soil and lettuce samples from site 1 respectively. *E. coli* was isolated with a prevalence of 66.67% in the water samples from site 1 and detected with low concentrations in the water samples (8.33%) from site 2. Therefore, the irrigation water was more contaminated by *E. coli* than the other factors investigated. *E. coli* was detected in manure and soil samples from site 2. *Salmonella choleraesuis* and *Shigella sonnei* were randomly detected. *Salmonella choleraesuis* was isolated with a frequency of 8.33% in the manure samples from site 2 and the lettuce samples from site 1. *Salmonella choleraesuis* was not detected in the other factors studied.

The prevalences of the enterobacteria strains in the lettuce samples from the production sites and market site are shown in Table 2. The different species identified on the lettuce samples from the production sites matched those from the market samples. The prevalence of *Salmonella gallinarum* was 50% for the market samples, 41.67% and 58% for the production sites 1 and 2 respectively. *Salmonella gallinarum*, *Salmonella arizonae*, *E. coli* and *Serratia* were identified on all the sampling sites. The market lettuce was more contaminated by *E. coli* and *Serratia marcescens* than the one from production sites. The frequencies of isolation of *E. coli* were 33.33%, 25% and 8.33% for the market site and the production sites 1 and 2 respectively. The prevalences of *Serratia marcescens* were 58.33%, 33.33% and 41.67% for the market site, and the production sites 1 and 2 respectively. *Afnia alvei* was identified only on lettuce samples from production site 2. *Enterobacter cloacae*, *Citrobacter freundii*, *Citrobacter diversus*, *Providencia alcalifasciens* and *Proteus vulgaris* were not detected on any lettuce samples.

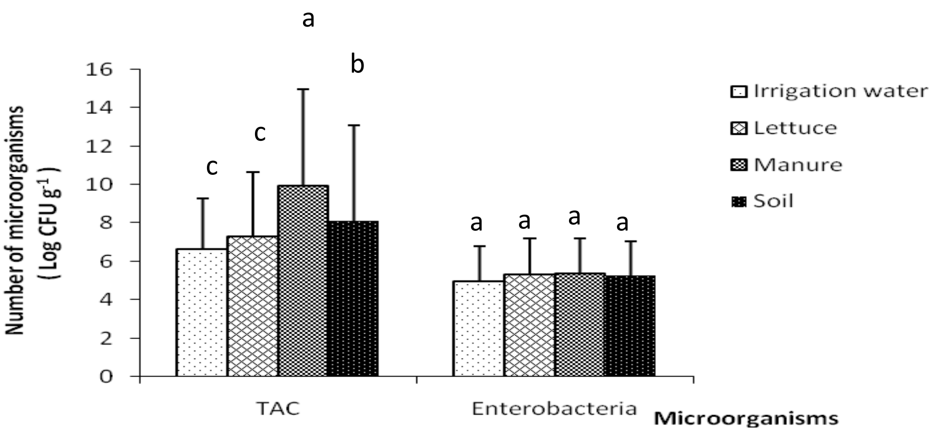

Figure 1: Mean values of total aerobic (TAC) and enterobacteria counts from production site 1 samples (**a, b, c** : mean with the same letter are not significantly different).

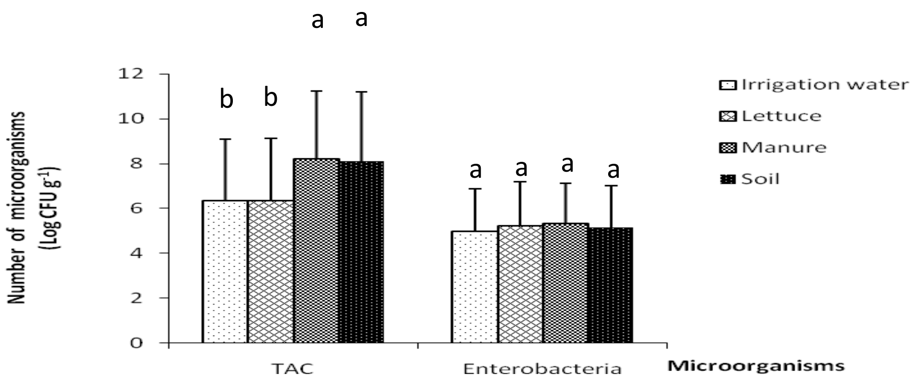

Figure 2: Mean values of total aerobic and enterobacteria counts from production site 2 samples (**a, b, c** : mean with the same letter are not significantly different).

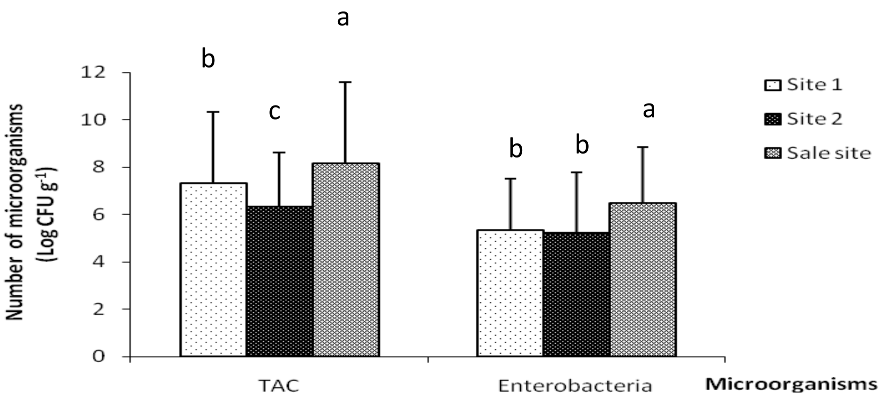

Figure 3: Mean values of total aerobic and enterobacteria counts of lettuce samples from the market and the production sites (**a, b, c**: mean with the same letter are not significantly different).

Table 1: Frequency of occurrence (%) of enterobacteria from the production sites samples.

Samples / Sites / Enterobacteria	Lettuce		Irrigation water		Manure		Soil	
	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2
Salmonella gallinarum	41.67^{c}	58.33^{b}	50^{b}	41.67^{c}	66.67^{a}	33.33^{c}	25^{d}	75^{a}
Salmonella arizonae	08.33^{e}	16 .67^{d}	-	58.33^{b}	-	08.33^{e}	33.33^{c}	50^{b}
Salmonella choleraesuis	08.33^{e}	-	-	08.33^{e}	-	-	-	-
Shigella sonnei	08.33^{e}	08.33^{e}	-	-	-	16.67^{d}	-	08.33^{e}
Escherichia coli	25^{d}	08.33^{e}	66.67^{a}	08.33^{e}	33.33^{c}	-	33.33^{c}	-
Enterobacter aerogenes	16.67^{d}	25^{d}	-	25^{d}	08.33^{e}	16.67^{d}	-	-
Enterobacter agglomerans	08.33^{e}	-	-	-	08.33^{e}	-	-	-
Enterobacter cloacae	-	-	-	-	-	-	08.33^{e}	08.33^{e}
Serratia marcescens	33.33^{c}	41.67^{c}	33.33^{c}	33.33^{c}	25^{d}	-	50^{b}	33.33^{c}
Serratia plymutica	16.67^{d}	41.67^{c}	16.67^{d}	16.67^{d}	50^{b}	16.67^{d}	08.33^{e}	-
Citrobacter freundii	-	-	-	16.67^{d}	-	08.33^{e}	08.33^{e}	08.33^{e}
Citrobacter diversus	-	-	-	08.33^{e}	16.67^{d}	16.67^{d}	-	-
Afnia alvei	-	08.33^{e}	-	-	25^{d}	-	16.67^{d}	-
Providencia alcalifasciens	-	-	-	-	08.33^{e}	-	25^{d}	-
Proteus vulgaris	-	-	-	08.33^{e}	-	-	-	-

- : Not identified. a ; b ; c : means with the same letters in the same line are not significantly different.

Tableau 2: Frequency of isolation of Enterobacteria species on lettuce samples from the market and production sites.

Enterobacteria identified	Production Sites		Sale site (%)
	Site 1 (%)	Site 2 (%)	
Salmonella gallinarum	41.67^{c}	$58\ .33^{a}$	50^{b}
Salmonella arizonae	08.33^{e}	16.67^{d}	08.33^{e}
Salmonella choleraesuis	08.33^{e}	-	-
Shigella sonnei	08.33^{e}	08.33^{e}	-
Escherichia coli	25^{d}	08.33^{e}	33.33^{c}
Enterobacter aerogenes	16.67^{d}	25^{d}	-
Enterobacter agglomerans	08.33^{e}	-	16.67^{d}
Enterobacter cloacae	-	-	-
Serratia marcescens	33.33^{c}	41.67^{c}	58.33^{a}
Serratia plymutica	16.67^{d}	41.67^{c}	58.33^{a}
Citrobacter freundii	-	-	-
Citrobacter diversus	-	-	-
Afnia alvei	-	08.33^{e}	-
Providencia alcalifasciens	-	-	-
Proteus vulgaris	-	-	-

- Not identified ; - Same letters in the same line are not significantly different

DISCUSSION

The high level of total aerobic and Enterobacteriaceae counts obtained in the different samples that were analyzed could be explained by the fact that vegetables from the peri-urban areas of Abidjan are grown on sandy soils generally poor in mineral content and requiring organic manure composed mainly of raw animal manures. The water used for irrigation is from uncovered and unprotected wells. The immediate environment to the growing sites is marked with poor sanitary conditions. All the vegetable producers in Côte d'Ivoire use the same practices for soil fertilization that consists mainly of pouring the manure between the lettuce plants. The drop of the manure on the leaves during fertilization could explain the presence of some Enterobacteriaceae. The use of untreated water from wells or from the lagoon for irrigation increases the load of these bacteria on the vegetables. In this study, the manure was identified as the main factor of contamination of lettuce, and then followed by the soil. This result agrees with the one reported by Pell (2008), indicating that manure was the main factor of contamination of vegetables.

In this work, the lettuce samples were contaminated with *Enterobacter aerogenes, Enterobacter agglomerans, Serratia marcescens,* and *Serratia plymutica.* These enterobacteria were found with different prevalences in the factors studied. The poor sanitary conditions in the areas where lettuce is grown in Abidjan could explain the presence of these species of enterobacteria (Natvig et al., 2002; Amoah et al., 2007; Elouennass et al., 2008). According to Beuchat and Ryu, 1997) enteric pathogens are mainly introduced in vegetable as a result of bad hygiene. The species of enteric bacteria detected on the lettuce samples from the production sites could be due to the non treated irrigation water, the manure and the soil whose loads of total aerobic and enterobacteria counts were high. Numerous field studies have revealed that irrigation water can contaminate the surface of the plants (Petterson et al., 2010; Nikaido et al., 2010). Contamination of vegetables by the application of inadequate raw animal manures or sewage, contaminated soil, irrigation with faecal material, direct contamination by livestock, wild animals and birds and post-harvest issues such as worker hygiene is well documented (Natvig et al., 2002); Heaton and Jones, 2008; Barak and Liang, 2009; Mitra et al., 2009). According to Beuchat and Ryu (1997), the use of manure rather than chemical fertilizer, as well as untreated sewage or irrigation water containing pathogens, viruses, or parasites, undoubtfully contributes to the risk of contamination. Amoah et al. (2007) reported that in addition to the irrigation water, contamination was also attributed to manure application and contaminated soils.

Three serovars of *Salmonella enterica* have been identified: *Salmonella gallinarum*, *Salmonella arizonae* and *Salmonella choleraesuis*. *Salmonella enterica* are some of the most commonly known bacterial pathogens which cause human illness. Many human pathogenic organisms have been recognized to exist on plant root or leaf surfaces (Sivapalasingam et al., 2004; Brandl, 2006; Barak and Liand, 2009; Berger et al., 2010), and even inside plant tissues (Rosenblueth and Martinez-Romero, 2006; Klerks et al., 2007). Sivapalasingam et al. (2004) concluded that the association of *Salmonella* with fresh produce appears to be serovar-specific involving flagella, curli, cellulose, and O antigen capsule. Klerks et al. (2007) also described an interaction of *Salmonella enterica* with commercially available lettuce cultivars while Berger et al. (2009) stated that different *Salmonella* serovars use strain-specific mechanisms to attach to salad leaves.

The use of poultry manure as fertilizer could explain the presence of *Salmonella gallinarum* from the growing sites since the ecological niche of this serovar is the digestive tract of poultry (Kokosharov and Phetisova, 2002). This result agrees with the one from the work of Beuchat and Ryu (1997) who indicated that the manure essentially from poultry droppings contributes strongly to the pollution of lettuce by *Salmonella gallinarum*. The presence of *Salmonella choleraesuis* suggests that pig feces are also used as organic manure for the fertilization of the soils since the digestive tract of pigs is the main reservoir of this serovar. The ecological niche of *Salmonella arizonae*, a telluric serovar, unlike the other two serovars is the intestine of cold blooded animals like lizards, geckos and frogs (Hardouin, 2008). Its presence in the soils could be linked to these animals in large number on the two production sites due to the high level of insalubrity. These results indicated diverse patterns of *Salmonella* contamination while the presence of *Shigella sonnei* may be explained by the presence of human wastes.

Both growing sites are sometimes used as defecation sites by the neighborhood. The unsanitary hands of the producers who have no knowledge of hygiene practices could also contribute to lettuce contamination by *Shigella sonnei*. *E. coli* was detected in all the samples examined. Its presence in the irrigation water and the soil samples is due to the fact that the production sites are located downhill, and collect all the running water from the rain, households and toilet water. Also, some evacuation canals of the waste waters are directed towards those areas so that the water from the wells used for gardening in Abidjan are polluted because they are subject to faecal contaminations. According to Mañas et al. (2009), the use of water from wells as irrigation water for lettuce production includes some risks because of the presence of many pathogenic agents such as *E. coli* and *Salmonella*. Our results confirm those of Wießner et al. (2009) who showed the presence of *Escherichia coli* in the soil, the manure and the lettuce samples analyzed. Harapas et al. (2010) reveal that the presence of *E. coli* on injured tissue is common to different vegetables such as celery, Cos lettuce and chive. Both manure and irrigation water contribute significantly to the spread of human pathogens onto fields and the crops growing there (Natvig et al., 2002).

The samples of lettuce sold on the market are more contaminated by the Enterobacteriaceae. Post-harvest handling and marketing could increase the farm-gate contamination levels of the vegetables. Indeed, the lettuce was displayed on the floor, not far from the mud, open gutters and public toilets. In addition, the influx of people on the markets could constitute a factor of pollution and could increase the level of contamination of the vegetables. In the field however, contamination of vegetable crops may occur via soil amended with manure from agricultural animals which are known reservoirs for *Salmonellae* (Viswanathan and Kaur, 2001; Natvig et al., 2002).

According to Berger et al. (2009), during the pre-harvest phase, pathogen populations can establish themselves on growing crops. The risk can be amplified after harvest either by further direct contamination or by proliferation of existing pathogen populations during processing and post harvest handling procedures.

The species of Enterobacteriaceae that contaminated the lettuce samples from the growing sites and the market site were similar. These results confirmed those of Sackou et al. (2006) on the sanitary quality of lettuce grown in Abidjan. These authors affirmed that most of the strains of enterobacteria found on the lettuce sold on the market are from the fields. For these authors, 75% of the lettuce samples from the markets of Abidjan don't meet the criteria set up in microbiology for vegetables.

According to the same authors, 44% of the consumers in Abidjan don't use any disinfectant to wash the raw edible vegetables.

The presence of bacteria responsible of food infections such as *Salmonella gallinarum*, *Shigella sonnei*, *Salmonella choleraesuis*, *E. coli*…, on the lettuce samples analyzed demonstrates that the poor sanitary conditions in which lettuce is produced, harvested, transported, and sold can be a risk for the consumers' health. The contamination of the lettuce can occur on the field, during harvesting, and post-harvesting, handling, processing, shipping, or marketing. It can also occur through cross-contamination (other food in storage, preparation, and display areas) or at home (improper handling after buying the products).

This study shows that lettuce grown and sold in Abidjan represents a risk for consumers and also for public health. To reduce the health risk associated with the consumption of contaminated lettuce, safer farming and irrigation practices are required and the remaining risk could best be addressed where the lettuce is prepared for consumption.

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