

Antibiotic Enteric Resistant Bacteria are Abundant on Lettuce from Urban Agriculture in the City of Yaounde

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ABSTRACT

The presence of antibiotic resistant bacteria on lettuce from wastewater irrigated fields in the town of Yaounde was investigated. Lettuce samples were collected from three agricultural fields located in three districts (Ekounou, Ezazou and Nkolbisson), over a period of four months. Resistance to amoxicillin, tetracycline, ciprofloxacin, sulfamethoxazole / trimethoprim and kanamycin were determined. Isolated bacteria presented highest resistance to amoxicillin (95.8%) and lowest resistance to ciprofloxacin (89.2%). The resistance rate of bacteria was the highest in March (93.1%), and lowest in May (89.9%). Antibiotic resistant bacteria identified were belonged to the genus *Salmonella*, *Bacillus*, *Staphylococcus*, *Shigella*, *klebsiella*, *Pseudomonas*, *citrobacter*, *Micrococcus*, *Enterococcus*, *Lactobacillus*, *Escherichia*, and *Streptococcus*. *Bacillus cereus* was the most abundant (17.5%). The identified isolates were all multidrug resistant, except *Lactobacillus* which was only resistant to amoxicillin. The obtained results demonstrate the health risk posed by the cultivation and consumption of lettuce from urban agriculture in the city of Yaounde, which could constitute a strong vector for antibiotic resistance dissemination.

KEYWORDS: Urban Agriculture; Lettuce; Antibiotics; antibiotic resistant bacteria

INTRODUCTION

Since the introduction of antibiotics for the treatment of bacterial infections decades ago, bacteria have developed sophisticated resistance mechanisms to avoid their effect. Antibiotic resistance has led to the need for more effective and therefore more expensive molecules beyond the reach of poor populations, thus increasing the rate of morbidity and mortality (Laxminarayan et al., 2016). Bacterial resistance is widespread and is found in several species of commensal bacteria in the environment which are today considered to be reservoirs of resistance genes (Zhang et al., 2008). In 2015, antibiotic resistant bacteria were responsible for 23,000 and 25,000 deaths in the United States and Europe respectively (Laxminarayan et al., 2016). The situation is similar worldwide and even worse in low- and middle-income countries (LMICs) where a high infectious disease burden is coupled with rapid emergence and spread of microbial drug resistance (Bougnom and Piddock, 2017). Approximately 20-80 % of antibiotics are excreted in urine or faeces from people or animals, either in their original formulation, partly degraded or as metabolites (Kümmerer, 2009). To survive in the presence of antibiotics, bacteria have evolved a plethora of different antibiotic resistance genes (ARGs) many of which are easily transmitted between bacteria via horizontal gene transfer (HGT).

Pressure on water resources in low and middle-income countries (LMICs) is increasing due to urbanization associated with population growth and increased urban food

demand (Yoveva et al., 2000). The urban population in Sub-Saharan Africa is expected to increase by 3.5% per year by 2030, and the number of city dwellers is expected to increase from 400 million in 2010 to 1.26 billion in 2050 (UN, 2012). The rapid increase in the urban population in LMICs is associated with increasing urban poverty, food insecurity and environmental pollution (Drechsel and Dongus, 2010). Urban agriculture is therefore a source of employment and income. It is fully integrated in cities and is generally practiced in wetlands. To be able to produce year-round, farmers use wastewater because of its easy access and low cost. When wastewater is used for agricultural irrigation, contaminants in the water can be transferred from soil to crops, this represents a health risk when fresh produce is consumed raw (Abaidoo et al., 2010). The risk is probably higher in developing countries where populations are often directly exposed to untreated wastewater due to a limited water supply and a lack of an effective sanitation system (Jekel et al. 2013).

Lettuce is a plant grown annually in urban agriculture in tropical countries. This urban lettuce production requires the use of wastewater, which presents risks of contamination by pathogenic, opportunistic, and antibiotic-resistant bacteria. In this study, we evaluated the presence of antibiotic bacteria resistant in lettuce grown in fields irrigated with wastewater in the city of Yaoundé.

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1. MATERIAL AND METHODS

Study sites description

Yaounde is the capital of Cameroon with a surface area of 180 km² and a population of about 2.8 million inhabitants. It is located at 3°52' N and 11°32' E. The average annual temperature is 23.5 °C and a rainfall of 1650 mm per year. The lettuce production rate was estimated at 1467.7 tonnes in 2015 (Temple, 2001). The first sampling sites located at Nkolbisson at the estuary of the meeting point of two rivers (Mfoundi and Mefou). This site is located at 3.86° N and 11.45° E. The second sampling sites located at Ekounoudownstream of a river which, receives household waste from surrounding homes, 3.82° N and 11.53°E. The third sampling sites located at Tropicana (Ezazou village), 3.83° N and 11.53°E. Farmers channel wastewater used to water crops once or twice a day.

Samples collection

Sampling was conducted nine times over a period of three months, three times each month, from February 2020 to May 2020. In each agricultural field, three lettuces were randomly collected at each sampling time. Nine lettuces were collected over a month; 27 lettuces were collected from each field over the sampling period. The samples were collected in sterile plastic bags and transported to the laboratory in an ice box.

Isolation and enumeration of total resistant bacteria

Lettuce was cut into pieces with sterile scissors, and 20 g suspended in 180 mL of a physiological saline solution (0.85% NaCl). Antibiotic resistant bacteria were isolated on Muller Hinton agar supplemented with the following antibiotic concentrations: 16 µg/ml of Tetracycline (TET, Sigma), 4 µg/ml of Ciprofloxacin (CIP, Sigma), 64 µg/ml, (8:1) Sulfamethoxazole / Trimethoprim (Sul / Trm, Sigma), 32 µg/ml of Amoxicillin (AMC, Sigma), and 4 µg/ml of Kanamycin. The plates were incubated at 37°C for 48hrs. The selected antibiotic concentrations were based on previous studies (Watkinson et al., 2007). All the procedures were done in triplicate. Grown colonies were counted and the percentage of resistance for each antibiotic corresponding to the ratio of CFU mL⁻¹ on the culture medium with and without antibiotic (Novo and Manaia, 2010) was reported. Colonies with different morphologies were observed on the plates and streaked out on Luria Bertani agar for purification and isolation. Colonies were then stored at 4°C in Muller Hinton broth supplemented with glycerol at 15% for further identification.

Identification of antibiotic resistant bacteria

Antibiotic resistant bacteria were identified by colony morphology observation, Gram staining, catalase test, coagulase test and growth on specific media. Gram negative bacteria were seeded on Eosin methylene blue agar (for isolation of *Escherichia coli*), *Salmonella-Shigella* agar (for isolation of *Salmonella* spp) and Cetrimide agar (for isolation of *Pseudomonas* spp), while Gram positive bacteria were separated with catalase test and coagulase test and were seeded on bile esculin agar (for isolation of *Streptococcus* spp.).

Data analysis

The different values obtained were reported as the mean of three replicates. The data were subjected to a two-way analysis of variance (ANOVA) followed by a Duncan's test at 5% level.

The data were analysed using SPSS Software Package 25.

RESULTS AND DISCUSSION

The microbiological analysis revealed that lettuce from all sites were highly contaminated by antibiotic resistant bacteria. The highest resistance ratio was obtained with amoxicillin (97.4%), the lowest with ciprofloxacin (89.2%) (Table 1).

Table 1: Resistance ratio (%) of bacteria to different antibiotics

Antibiotics	Mean resistance ratio
Ciprofloxacin	89.2(±1.4) ^a
Bactrim	90.3(±1.6) ^{ab}
Kanamycin	90.7(±2.1) ^{ab}
Tetracycline	91.8(±1.3) ^{ab}
Amoxicillin	95.9(±0.8) ^b

Means followed by different letters indicate significant differences between resistance rates $P < 0.05$, $n = 3$ (Duncan).

Amoxicillin was the least effective antibiotic with an average percentage resistance ratio of 95.9%. This high rate of resistance is similar to those reported by Adebayo (2012) in Nigeria where bacteria exhibited 80% resistance to Amoxicillin in lettuce. Amoxicillin is a beta-lactam antibiotic that has been used for longer time as the first resort against bacterial infections; this could explain the high rate of resistance observed. Beta lactams are the most imported and used antibiotics in Cameroon (Mouiche et al., 2020). High level resistance to tetracycline (91.8%) obtained in our study is similar to the one reported by Adebayo (2012) in Nigeria (72%), and higher than the one reported by Hassan (2011) in Saudi Arabia (17.2%) of resistance to tetracyclines in lettuce. Tetracyclines are among the most prescribed antibiotics in human and animal medicine in Cameroon for the treatment of many infections (Vougat et al., 2017). Additionally, tetracyclines are highly persistent, further increasing the risk of the development of antibiotic resistance (Fredrick et al., 2020). High Resistance ratios to kanamycin (90.7%) is a serious public health issue because kanamycin is a broad-spectrum antibiotic of last generation that is used in severe aerobic Gram-negative infections (Peterson, 2008).

Bactrim (Sulfamethoxazole / Trimethoprim) is widely prescribed for the treatment of prostatitis, sinusitis, urinary tract infections, typhoid fevers, digestive infections, ear infections, and bronchopulmonary infections which would promote its accumulation in the environment (Xu et al., 2014). This explains the high resistance rates observed (90.3%). Our results revealed that bacteria showed lower resistance ratios with ciprofloxacin (89.2%) compared to other antibiotics tested. In fact, mechanisms of resistance to these antibiotics described are essentially chromosomal (have a probability of very weak appearance: occurs on average every 10⁶ to 10⁹ divisions), though recently described mechanisms such as active efflux emerge but still confer low levels of resistance to this family (Muylaert et al., 2013). However, resistance ratio to ciprofloxacin (73.6%) is greater than that reported by other authors in lettuce samples in Saudi Arabia (Hassan, 2011). This is a serious public health concern because ciprofloxacin is a broad-spectrum antibiotic of last generation against various bacterial infections such as salmonellosis (Iroha et al., 2016).

Higher resistance ratios were observed during the month of March (93.2%), and lowest during May (89.9%) (Table 2).

Table 2: Resistance ratio (%) of bacteria at different months

Sampling dates	Meanresistance ratio
May	89.9(±1.8) ^a
February	91.7(±1.9) ^{ab}
March	93.2(±0.5) ^b

Means followed by different letters indicate significant differences between resistance rates $P < 0.05$, $n = 3$ (Duncan).

The variation of the resistance rates over the sampling period could be due to the modification of physical and chemical parameters of the waters. These variations were also reported by Diwan et al., (2018) in river water in central India. The high abundance of drug resistant bacteria on lettuce is consequent to the high abundance of drug resistant bacteria in wastewater that is used to irrigate the agricultural fields. Wastewaters receive antibiotic resistant bacteria from all sources including soil, water and inputs. Resistance ratios were higher in March, and lower in May. This could be explained by the fact that during the dry season, water sources dry up and stagnate, favoring the multiplication, accumulation of bacteria, and acquisition of antibiotic resistance genes by HGT (Zhang et al., 2008).

There was no significant difference among average resistance rates at the three sites (Table 3).

Table 3: Comparison of resistance ratio (%) of bacteria at sampling sites

Sampling Sites	Meanresistance ratio
Ezazou village	90.2(±1.2) ^a
Ekounou	92.2(±1.4) ^a
Nkolbison	92.2(±1.7) ^a

Means followed by different letters indicate significant differences between resistance rates $P < 0.05$, $n = 3$ (Duncan).

High rates of antibiotic resistant bacteria in all sampling sites could be explained by the origin of the water used for irrigation which is highly polluted, and the use of chicken droppings for soil fertilization. It has been reported that 21.8% of antibiotics administered during poultry farming are found in droppings (Kabir, 2004).

A total of 67 drug resistant bacteria belonging to the following genera were isolated: *Bacillus*, *Staphylococcus*, *Enterococcus*, *Shigella*, *Pseudomonas*, *Klebsiella*, *Micrococcus*, *Staphylococcus*, *Citrobacter*, *Streptococcus*, *Escherichia*, *Lactobacillus* and *Salmonella*. The isolated bacteria were present in all the sampling sites and multi-resistant (Table 4).

Table 4: Frequency of antibiotic resistant bacteria isolated from lettuce.

Isolates	Total number	Number of antibiotic resistant bacteria (%)				
		AML	SUL/TRM	CIP	KAN	TET
<i>Bacillus cereus</i>	10(17.5 %)	10(100%)	5(50%)	1(10%)	3(30%)	4(40%)
<i>Staphylococcus aureus</i>	9(15.7 %)	6(66.7%)	2(22.2%)	2(22.2%)	3(33.3%)	5(55.5%)
<i>Enterococcus spp</i>	6(10.5 %)	4(66.7%)	2(33.3%)	1(16.7%)	2(33.3%)	3(50%)
<i>Shigella spp</i>	6(10.5 %)	5(83.3%)	2(33.3%)	2(33.3%)	3(50%)	0
<i>Pseudomonas spp</i>	5(8.77 %)	4(80%)	1(20%)	1(20%)	3(60%)	1(20%)
<i>Bacillus spp</i>	5(8.77 %)	2(40%)	2(40%)	0	1(20%)	0
<i>Klebsiella spp</i>	3(5.26 %)	1(33.3%)	1(33.3%)	0	2(66.7%)	1(33.3%)
<i>Micrococcus spp</i>	3(5.26 %)	3(100%)	0	1(33.3%)	0	2(66.7%)
<i>Staphylococcus spp</i>	3(5.26 %)	3(100%)	1(33.3%)	1(33.3%)	3(100%)	1(33.3%)
<i>Citrobacterspp</i>	2(3.50 %)	2(100%)	1(50%)	0	0	1(50%)
<i>Streptococcus spp</i>	2(3.50 %)	2(100%)	1(50%)	0	1(50%)	0
<i>Escherichia coli</i>	1(1.75 %)	1(100%)	0	1(100%)	1(100%)	1(100%)
<i>Salmonella spp</i>	1(1.75 %)	1(100%)	1(100%)	0	0	0
<i>Lactobacillus spp</i>	1(1.75 %)	1(100%)	0	0	0	0
Total	57	45(78.9%)	19(33.3%)	10(17.5%)	22(38.6%)	19(33.3%)

AML: Amoxicilline, TET: Tétracycline, CIP: Ciprofloxacine, KAN: Kanamycine, SUL/TRM: Sulfaméthoxazole/ Triméthoprime

Bacillus cereus (17.5%) was the most abundant antibiotic resistant bacteria, and resistant to all tested antibiotics. This could be justified by the fact that this germ is ubiquitous, acquire antibiotic resistance genes from the micro flora of the soil, and can produce spores, which can withstand extreme conditions. Adverse conditions and ultraviolet rays from the sun can kill and reduce the burden of other bacteria in lettuce (Owolabi and Ichoku, 2014). *Bacillus cereus* is responsible for food poisoning and opportunistic infections (Fiedler et al., 2019).

The high abundance of antibiotic resistant *Staphylococcus aureus* (15.7%) could be attributed to the contamination from skin of humans and animals. *S. aureus* is a normal flora of the skin of healthy mammals and could have acquired antibiotic resistance genes under the pressure of the continued use of antibiotics on animals as growth promoters or for the treatment of bacterial infections. Likewise, isolates

of *S. aureus* from Africa show a high degree of resistance to penicillin, tetracycline and sulfamethoxazole / trimethoprim, indicating the wide use of these drugs in African countries (CDDEP, 2015). This germ is responsible for food poisoning, localized infections and in some cases of fatal infections (Fiedler et al., 2019). *Enterobacteriaceae* members were the most prevalent, namely *Shigella spp* (10.5%), *Klebsiella spp* (5.26%), *Citrobacterspp* (3.50%) *Escherichia coli* (1.75%) and *Salmonellaspp* (1.75%). This can be explained by intense faecal contamination of irrigation water, and by the fact these bacteria resist in the environment, survive, adapt to a wide range of niches, and have the ability to exchange both virulence and resistance traits (Costa et al., 2013). These germs are responsible for gastroenteritis and urinary tract and pulmonary infections (Adzitey, 2018). *E. coli* showed 100% resistance to amoxicillin, tetracycline and ciprofloxacin. These results are similar to those reported by Adebayo (2012) in Nigeria with 100% resistance to

amoxicillin and ciprofloxacin and 75% to tetracycline; and higher than those reported by Toe (2018) in Ivory Coast where with 38.9% resistance to tetracycline and 5.6% to Ciprofloxacin in lettuce samples. This could be consequent to the high fecal contamination of water used for irrigation in all sampling sites. Enterococci represented 10.3% (n = 6) of isolated drug resistant bacteria. This could be explained by the contamination of wastewater used for irrigation of agricultural field with livestock and human faeces (Holvoet et al 2013). Water used for irrigation of lettuce is therefore a source of direct contamination by antibiotic resistant enterococci. The genetic plasticity (transfer of genetic elements) of these bacteria allows them, to adapt to many ecosystems, and to be vectors of antibiotic resistance and bacterial virulence. *Enterococcus* spp showed 33.3% resistance to sulfamethoxazole / trimethoprim and kanamycin, 50% to tetracycline and 16.7% to Ciprofloxacin. These results are different from those reported in Tunisia by Ben (2015) where *Enterococcus* spp isolated from lettuce samples presented 17.6% resistance to kanamycin, 11.7% to tetracycline, 76.5% to ciprofloxacin and 100% to sulfamethoxazole. / trimethoprim. *Pseudomonas* spp represented 10.3% (n = 6) of all antibiotic resistant bacteria isolated. The intrinsic and acquired resistance of *Pseudomonas* to many antibiotics is due to several adaptations, including active efflux systems, reduced cell wall permeability, acquisition of plasmids, expression of various enzymes and formation of biofilms (Deplano et al 2005).

Antibiotic resistant genes contaminating food constitute an indirect risk for public health, as they increase the gene pool from which pathogenic bacteria can acquire resistance traits (Verraes et al., 2013). Observation of resistance to several antibiotics by bacteria isolated from lettuce suggests a substantial risk of transfer of antibacterial resistance to humans as they are often eaten without further treatment. Consequently, transfer of antibiotic resistance genes between bacteria after ingestion in the gastrointestinal tract in humans can occur. Therefore, the consumption of raw lettuce from urban agriculture in the city of Yaoundé represents a serious public health concern.

CONCLUSIONS

The determination of the profile of antibiotic resistant bacteria present on irrigated lettuce from urban agriculture in the city of Yaoundé, revealed the presence of a diverse genera of enteric drug resistant bacteria. The evolutionary advantage of these bacteria to mutate rapidly, their ability to develop a multi-resistant phenotype and to adapt to antibacterial threats in their environment make the treatment of infectious diseases caused by these potential pathogenic bacteria a major public health concern. These results showed the danger faced by populations who consume raw or untreated lettuce. The consumption of lettuce from urban agriculture in the city of Yaoundé could represent a strong vector for the dissemination of antibiotic resistance among the populations.

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