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Profile and Antibiotic Resistance Genes of *Escherichia coli* Strains Isolated from Ready-to-Eat Raw Mixed Vegetables Salads in Collective Catering in Abidjan, Côte D'Ivoire

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Abstract In recent years many cases of food poisoning due to the consumption of vegetable salads contaminated with antibiotic-resistant strains of *E. coli* have been reported. The objective of the study was to detect the profile and the genetic factors of antibiotic resistance of *Escherichia coli*, isolated from ready-to-eat raw mixed vegetable salads, in catering. A total of 218 *E. coli* strains isolated from salads were confirmed by identification of *iudA* gene. Antibiotic resistance profile was determined by the agar diffusion method and by the detection of resistance genetic supports. Prevalence of *E. coli* resistant to antibiotics in vegetable salads was 70.2% with 28.4% of multi-resistant. Antibiotic resistance particularly concerned tetracycline (52.3%), streptomycin (38.5%) and to a lesser extent, nalidixic acid (15.6%). The genes aaa [3] -IV, CIMT, QnrA, tetA, tetB, cmlA and cat1 respectively conferring resistance to gentamicin, ampicillin, quinolones, tetracycline and chloramphenicol were highlighted. The study reveals that the risk of contamination by strains of *E. coli* resistant to antibiotics exist and require healthy control measures.

Keywords: *vegetable salads, Escherichia coli, antibiotic resistance, collective catering, Abidjan*

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1. Introduction

Antibiotic resistance is a growing threat to public health worldwide [1]. It compromises the prevention and effective treatment of an increasing number of infections caused by bacteria. In addition, it increases the cost of health care by extending the length of hospital stays, requiring more intensive care and more expensive drugs [1].The numbers of infections due to these resistances are estimated at 2000000 in the United States with 23 000 deaths per year [2] and 386 000 in Europe in 2007 with 25 000 deaths per year [3]. In Africa, the few available data indicate that the region shares the global trend of increasing resistance [4]. In Ivory Coast, for some years, the National Research Center (CNR) of antibiotic resistance and the observatory of the resistance of microorganisms to anti-infective in Côte d'Ivoire (ORMICI) have sounded the alarm over the emergence of multi-resistant bacteria and the consequences that could result [5,6]. Enterobacteriaceae resistant to antibiotics including E. coli were isolated from: human biological products (faeces, urine, blood) [7,8,9], products from livestock [10,11,12], food [13], water [14], soil [15,16], faeces of domestic animals (dogs and cats) and birds of national park Thaï [17]. In some cases, studies have shown that resistances were carried by plasmids, assuming a horizontal transfer of resistance [18,19].

E. coli is a very important bacterium in public health, responsible for intestinal and extra-intestinal infections. Each year, *E. coli* strains are responsible for 2 million deaths worldwide, whether through intestinal or extra-intestinal infections [20]. Strains involved in intestinal infections are responsible for gastroenteritis and those responsible for extra-intestinal infections are associated with sepsis, urinary tract infections and neonatal meningitis [21]. In 2011, following the consumption of vegetables contaminated with a strains of Shiga toxin producing *E. coli* (STEC) serotype O104: H4 and resistant

to several families of antibiotic, 3816 cases of bloody diarrhea, 845 cases of Hemolytic Syndrome and Uremic (SHU) and 54 deaths occurred [22].

Foods such as ready-to-eat raw mixed vegetable salads, usually eaten without prior cooking process, are frequently offered in catering in Abidjan. The situation of antimicrobial resistance and of the corresponding genetic supports in the bacteria carried by these vegetables remains very poorly documented.

The objective of the study was to determine the profile and genetic factors of antibiotic resistance among E. coli strains isolated from ready-to-eat raw mixed vegetable salads, served in collective catering.

2. Material and Methods

2.1. Sampling and Origin of *E. coli* Strains

A total of 218 strains of *E. coli* were isolated from ready to eat raw mixed vegetable salads in collective catering in Abidjan. Salads were collected from February to November 2015 in different restaurants in five municipalities (Abobo, Adjamé, Yopougon, Treichville, Cocody). After collection, the samples were transported in a cooler with ice packs.

2.2. Isolation of *E. coli* strains

The *E. coli* isolation was carried out on RAPID'*E.coli* 2 selective chromogenic medium (Bio-rad, France) according to ISO 16140. A subculture was carried out on tryptic soy (Sigma Aldrich, Canada) and incubated for 24 hours at 37°C. The biochemical characteristics were determined according to the method of Le Minor and Richard [20].

2.3. Confirmation of the Identification of Strains

Confirmation of the identification of the strains was made by carrying out a polymerase chain reaction (PCR), according to the protocol of Maheux et al. [24]. It consisted in the identification of iudA gene, following the steps of extraction of DNA, amplification and revelation of amplification products.

2.3.1. Extraction of DNA

DNA was extracted by the heat shock method. Three to four colonies of 24h on trypticase soy agar (BBL, Canada) were bubbled through 200 µL of milli-Q water contained in Eppendorf tubes (Sigma Aldrich, Canada). The suspension obtained was heated for 10 minutes in the Marie bath (Fisher Scientific, USA) at 100°C. and transferred to ice for 1 min. After centrifugation at 6000 rpm for 10 min, 100 µl of the supernatant was recovered in an Eppendorft tube containing 100 µl of milli-Q water, homogenized and stored at 4°C. The concentration and purity of DNA was determined by measuring the absorbance ratio of A260 / A280 with a spectrophotometer (Ultrospec 2000 Techne genius, USA). The extracted DNA was used both for confirmation of the identification of strains and for the detection of virulence genes. The extracted DNA was used both for confirmation of the identification of strains and for the detection of antibiotic resistant genes.

2.3.2. Amplification

Amplifications were performed in a final reaction volume of 25µl containing different reagents (Sigma Aldrich, Saint Louis, USA): 10x buffer solution (10 mM Tris-HCl, pH 8.3 at 25 °C, 50 mMKCl) , $MgCl_2$, 1.5 mM, deoxyribonucleotides (dNTPs), 200 µM, each primer, 0.4 µM (Table 1), 0.5 µ of the Taq DNA polymerase and 1 µl of extracted DNA. Amplification was performed following an initial denaturation at 95 °C for 3 min, followed by 30 cycles of 94 °C for 45s, 58 °C for 30s and 72 °C for 5 min and a final step of 72 °C for 5 min, and storage at 4 °C.

2.3.3. Visualization of Amplification Products

Visualization of the amplification products was done by electrophoresis in an agarose gel (Sigma Aldrich, Saint Louis, Canada) at 2%, in the presence of 0.5 mg / ml ethidium bromide (Sigma Aldrich, Canada). The migration was carried out at 100 volts for 45 minutes and gels were visualized under UV light. The sizes of the amplification products were estimated by comparison with a molecular weight marker (Sigma Aldrich, Saint Louis, USA) used as a standard.

2.4. Determination of Antibiotic Resistance Profile

The antibiotic resistance profile of the strains was determined using the diffusion method in agar medium according to Bauer et al. [25] and the interpretation was carried out according to the recommendations of the antibiogram committee of the French company of Microbiology [26]. Thirteen (13) antibiotics from different families were tested (Table 1). From 24-day pure colonies obtained by subculture on trypticase agar (Sigma Aldrich, Canada), a standardized 0.5 Mc Farland inoculum corresponding to 10^8UFC/ml was prepared. From this inoculum, 100 µL were taken and added to 10 µL of physiological water (9 g NaCl + 1000L H_2O) so as to obtain a final inoculum with a concentration of 10^6UFC/ml. This inoculum was used to inoculate Mueller Hinton agar (Sigma Adrich, canada). Antibiotic discs (Bio-Rad, Manne, France) were conventionally deposited on the surface of the agar. These include ampicillin (10 µg), amoxicillin + clavulanic acid (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), astreonam (30 µg), cefepime (30 µg), chroramphenicol (30 µg), tetracycline (30 µg), nalidixic acid (30µg), ciprofloxacin (5µg), streptomycin (10 µg), gentamicin (10 µg), and cotrimozaxole (30µg). Incubation was carried out 24 hours at 37°C. The inhibition diameters around the antibiotic discs were estimated and the sensitive or resistant category interpretation was performed according to the CASFM [26]. The *E. coli* strain ATCC 25922 was used for the quality control of the method according to the CASFM [26]. The *E. coli* strain ATCC 25922 was used for the quality control of the method method according to the CASFM [26]. The *E. coli* strain ATCC 25922 was used for the quality control of the method.

Table 1. Primers used for confirmation of strains and detection of antibiotic resistance genes

Genes	Sequence 5' to 3'	Size (bp)	Type PCR	Tm	Reference
iudA	(F)-AAAACGGCAAGAAAAAGCAG (R)-ACGCGTGGTTACAGTCTTGCG	147	sPCR	58	Malheux et al. 2012
TetA	(F)-GGTTCACTCGAACGACGTCA (R)-CTGTCCGACAAGTTGCATGA	577	mPCR	55	Shahrani et al. 2014
Tet B	(F)-CCTCAGCTTCTCAACGCGTG (R)-GCACCTTGCTGATGACTCTT	634			Shahrani et al. 2014
QnrA	(F)-GGGTATGGATATTATTGATAAAG (R)-CTAATCCGGCAGCACTATTTA	670	Spcr	55	Shahrani et al. 2014
aac[3]-IV	(F)-CTTCAGGATGGCAAGTTGGT (R)-TCATCTCGTTCTCCGCTCAT	286	sPCR	55	Shahrani et al. 2014
CITM	(F)-TGGCCAGAACTGACAGGCAAA (R)-TTTCTCCTGAACGTGGCTGGC	462	sPCR	55	Shahrani et al. 2014
cat1	(F)-AGTTGCTCAATGTACCTATAACC (R)-TTGTAATTCATTAAGCATTCTGCC	547	mPCR	55	Shahrani et al. 2014
cmlA	(F)-CCGCCACGGTGTTGTTGTTATC (R)-CACCTTGCCTGCCCATCATTAG	698		55	Shahrani et al. 2014

Note: Tm: hybridization temperatures; sPCR: simplex PCR; mPCR: multiplex PCR

2.5. Detection of Genetic Support for Antibiotic Resistance

For the detection of resistance genes, only strains with phenotypic resistance were taken into account. These strains were subjected to DNA extraction using the method described above and detection by PCR of resistance genes. These include genes conferring resistance to ampicillin (*CITM*), tetracycline (*tetA*, *tetB*), chloramphenicol (*cat 1*, *cmlA*), quinolones (*qnr*) and gentamicin (*aaa[3]-IV*). The protocol of Shahrani et al. [27] was used for this highlighting.

Amplifications were performed in a final reaction volume of 25µl containing different reagents (Sigma Aldrich, Saint Louis, USA): a 10x buffer solution (10 mM Tris-HCl, pH 8.3 at 25°C, 50 mMKCl), $MgCl_2$, 2.5 mM, deoxyribonucleotides (dNTPs), 200 µM, of each primer, 0.5 µM (Table 1), 0.5 µ of Taq DNA polymerase and 1 µl of extracted DNA. The amplification program consisted of initial denaturation at 95 °C for 8 min followed by 32 cycles of 94°C for 60 s, 5 °C for 70 s and 72°C for 2 min and a final stage of 72°C for 5 min followed by storage at 4 °C. After amplification, the products were visualized by agarose gel electrophoresis as described above but on an agarose gel (Invitrogen, Carlsbad, CA, USA) between 1.5% and 2% depending on the size of the gene of interest.

2.6. Statistical Analysis

Statistical analyzes were performed with the IBM SPSS statistical program for Windows version 20. Descriptive statistics were used to determine percentages of susceptibility to different antibiotics. Descriptive statistics (frequency, mean) were used for the quantitative variables.

3. Results

3.1. Confirmation of the Identification of E. *coli* Strains

Figure 1 shows the product of the amplification of the *iudA* gene of 147 bp common to all strains of *E. coli* and demonstrated in isolated *E. coli* strains of ready-to-use vegetable salads in collective Abidjan. The presence of the *iudA* gene was demonstrated in the 218 *E. coli* strains isolated

3.2. Prevalence of *E. coli* Resistant to Antibiotics

Among the isolated *E. coli* strains from ready-to-eat raw mixed vegetable salads, 70.2% showed resistance to at least one of the antibiotics tested. Resistance percentages differ depending on the type antibiotic. No resistance to cephalosporins was found, contrary to penicillin resistance, in particular ampicillin (22%) and ampicillin + clavulanic acid (2.3%). The percentages of resistance to ciprofloxacin and nalidixic acid are respectively 8.3% and 15.6%. Resistance to tetracycline was 57.3%, the highest followed by resistance to streptomycin of 38.5% (Figure 2). The prevalence of multi-resistant *E. coli* is 28.4%.

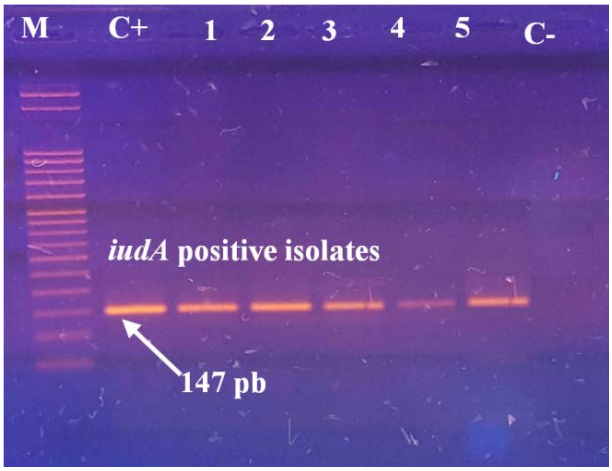

Figure 1. Electrophoretic profile of *iudA* gene amplification products in *E. coli* isolated from ready-to-eat raw mixed vegetable salads (M: Molecular marker of 50 bp (Sigma Aldrich, Saint Louis, USA) ; C+: Positive control *E. coli* ATCC 25922; Line 1, 2, 3, 4: *iudA* positive isolates; C-: Negative control).

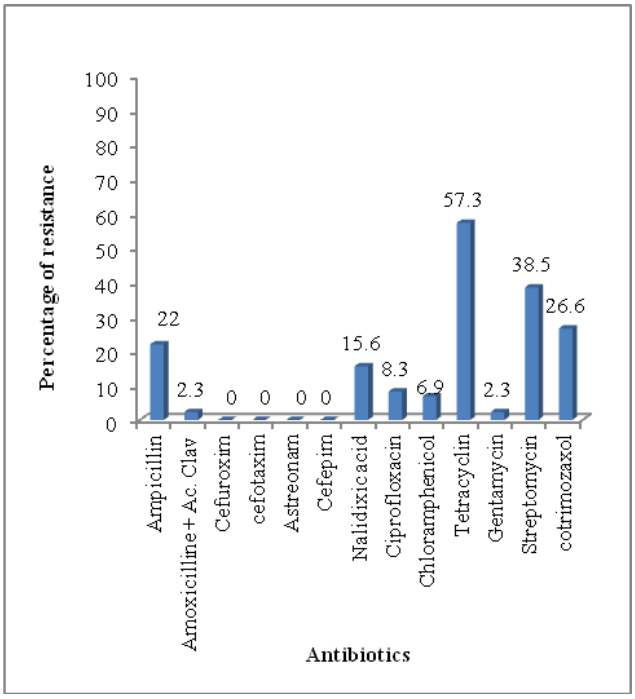

Figure 2. Antibiotic resistance profile of *E. coli* strains

3.3. Prevalence of Antibiotic Virulence Genes in *E. coli* Strains Isolated

The genes *CIMT* (Figure 3), *qnr* (Figure 4) and *aac [3] -IV* (Figure 5) were found respectively in 14.9%, 16.7% and 100% of the ampicillin, Quinolones and gentamicin resistant strains. The *tetA* and *tetB* genes (Figure 6) were identified respectively at 6.4% and 8.8% of strains resistant to tetracycline. The genes *cm1A* and *cat1* (Figure 7) were found in 33.3% and 20.0% of strains resistant to chloramphenicol, respectively. Table 2 shows the prevalence for each gene.

M: Molecular marker of 50 bp (Sigma Aldrich, Saint Louis, USA); Figure 3: Line 1, 2, 3, 4, 5, 6: *CIMT* positive isolate (462 bp); Figure 4: Line 1, 2, 3: *qnrA* positive isolate (670 bp); Figure 5: Line 1, 2, 3: *aac[3]-IV* positive isolates (286 bp); Figure 6: Line 1, 2, 3: Strains *tetB* positive isolates (634 bp) ; Line 4: *tetA* positive isolates (577 bp); Figure 7: Line 1, 2, 3: *cmlA* positive isolates (698 bp); Line 4, 5, 6: *cat1* positive isolates (547 bp); C-: Negative control.

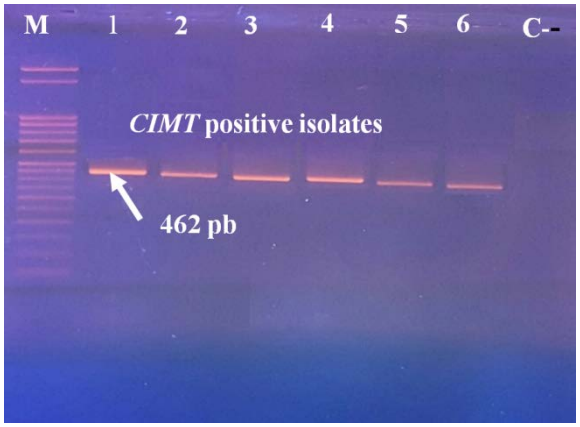

Figure 3. Electrophoretic profile of amplification products of the ampicillin resistance gene (*CIMT*)

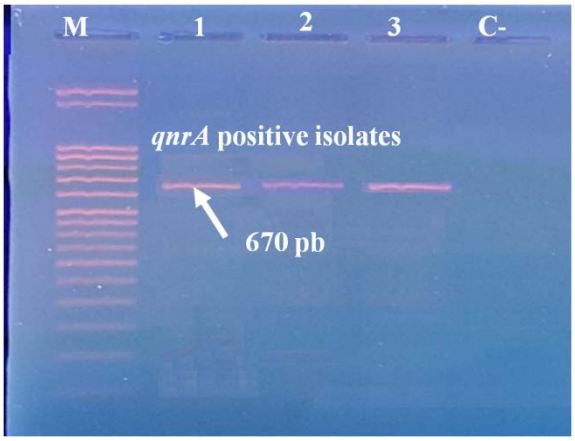

Figure 4. Electrophoretic profile of the amplification product of quinolone resistance gene (*qnrA*)

Table 2. Prevalence of antibiotic resistance genes

Antibiotique	Genes	*E. coli* tested	Number of genes detected	Prevalence of resistance genes (%)
Ampicillin	CIMT	47	7	14,9
Tetracyclin	*Tet A*	125	8	6,4
	Tet B	125	11	8,8
Chloramphenicol	*cmlA*	15	5	33,3
	Caat 1	15	3	20
Gentamicin	*aaa [3]-IV*	5	5	100
Quinolone	*QnrA*	18	3	16,7

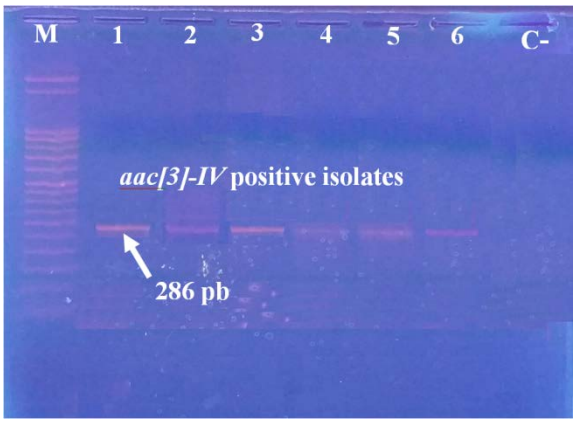

Figure 5. Electrophoretic profile of the amplification product of the gene for resistance to gentamicin (*aac[3]-IV*)

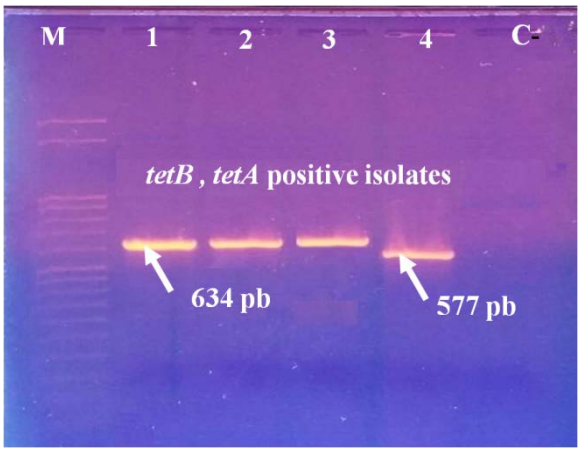

Figure 6. Electrophoretic profile of the amplification product of tetracycline resistance gene (*tetA*, *tetB*)

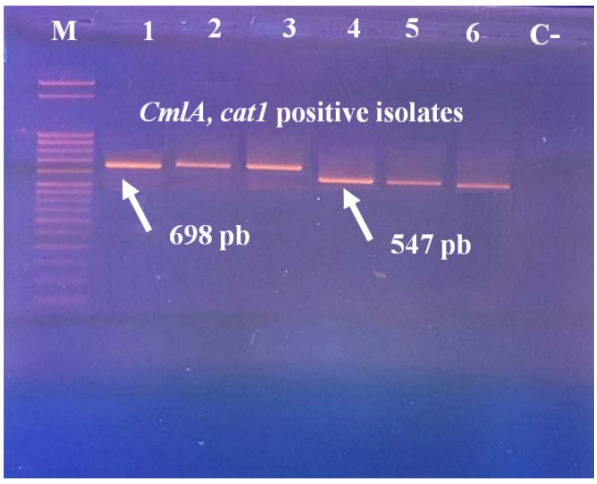

Figure 7. Electrophoretic profile of the amplification product of tetracycline resistance gene (*cmlA, cat1*)

4. Discussion

In this study, 70.2% of *E. coli* strains isolated from vegetable salads in collective catering in Abidjan showed resistance to at least one antibiotic. The presence of antibiotic-resistant *E. coli* strains in ready-to-eat raw mixed vegetable salads may be due to residual strains of contamination possibly related to agricultural practices. It has been shown that vegetable growing areas use poultry manure as the main [15,28] In addition, strains of *E. coli* resistant to antibiotics were also found in soil, irrigation water, manure and vegetables [15,28,29,30].The presence of *E. coli* in ready-to-eat raw mixed vegetable salads can also be due to the poor preparation practices according to the observation made by Verraes et al. [31]. Cross-contamination in the preparation of salads by contact with strains from the manipulator or from fresh food animal origin is a factor which has already been mentioned by these authors.

The prevalence of antibiotic-resistant *E. coli* in vegetable salads obtained in our study is similar to those of Adeshina et al. [32] in Nigeria (75%) and Hassan et al. [33] in Saudi Arabia (76.5%). By cons, Rasheed et al. [34] in India (20%) and Holvoet et al. [35] in Belgium (11.4%) were found more reduced rates. Significant levels of presence in vegetables *E. coli* resistant to antibiotics have been reported previously by several authors [36,37,38,39,40].

Antibiotic resistance strains differs depending antibiotics tested, but no resistance to cephalosporins was observed in this study. The results obtained are in agreement with those of Holvoet et al. [35] and Gritli et al. [39] and differ from those of the studies by Falomir et al. [41] and Annapurna et al. [42]. In a descending order of the importance of antimicrobial resistance, the study revealed resistance to tetracycline (57.3%), streptomycin (38.5%), cotrimozaxole (26.6%) and ampicillin (22%). These results are similar to those obtained by Sheeren et al. [37] in Jordan, a resistance of 41% tetracycline and 31% cotrimozaxole but different from those of Klingbeil et al. [40] in Lebanon with higher resistance levels, estimated at 80% for tetracycline, 72.7% for cotrimozaxole, 46.7% for streptomycin and 40.4% for ampicillin. According to Holvoet et al. [35], tetracycline resistance is alarming in developing countries and may reflect contamination of raw vegetables by irrigation water or contaminated manure.

Furthermore, these resistances can be potentially acquired by the food chain from human contamination that have adapted to therapeutic practices [43,44]. Low resistances in gentamycin (2.3%) and chloramphenicol (6.9%) were similar to those of Campos et al. [38] in Portugal (Chloramphenicol: 3%), Gritli et al. [39] in Tunisia (Gentamicin and chloramphenicol: 7.4%), Hasan et al. [33] in Saudi Arabia (Gentamicin: 4.7%) and Benzason et al. [45] in Portugal (Gentamicin: 2.5%). In contrast with our results and those previously mentioned, no resistance to gentamicin was observed in Bangladesh [38,46]. Resistance to nalidixic acid and ciprofloxacin was found to be 15.6% and 8.3%, respectively. Campos et al. [38] noted in their study resistance levels of 5% for ciprofloxacin and 36% for nalidixic acid. However, other authors found no resistance to ciprofloxacin [38,39,41,46]. According to Hasan et al. [33] resistance to fluoroquinolones, gentamicin and cephalosporins suggests sources of animal or human contamination because these classes are not used in vegetable farming, or plant-associated bacteria by horizontal transfer can also be assumed.

In this study, 24.6% of *E. coli* strains were found to be resistant to at least three families of antibiotics. Bacteria resistant to at least three families of antibiotics are referred to as Multi Resistant Bacteria (BMR) [47].This result is in agreement with those of Shereen et al. [37] (27.8%) in Jordan (27.8%), Nipa et al. [36] in Bangladesh (33.3%) and Odu et al. [48] in Nigeria (33%) and contrary to those obtained by Klingbeil et al. [37] in Lebanon and Adeshina et al. [32] in Nigeria (75%).

This study highlighted one of the genetic supports for antibiotic resistance in *E. coli* strains isolated from ready-to-eat raw mixed vegetable salads. Indeed, the presence of resistance genes to tetracycline (*tetA*, *tetB*), quinolones (*Qnr*), ampicillin (*CIMT*, gentamicin (*aaa [3] -IV*) and chloramphenicol (*cmlA*, *caat1*) have been highlighted. This result is consistent with studies carried out in Portugal by Campos et al., [38] Shakerian et al. [49] in Iran, Kim et al. [50] in Korea where *tetA*, *tetB*, *catA*, *aaa[3]-IV*, CIMT genes have also been demonstrated in *E. coli* strains isolated from vegetable salads. Sheeren et al. [37] also reported tetracycline resistance to the presence of *tetA* (64.7%) and *tetB* (5.9%) genes in *E. coli* strains from vegetables in Jordan. According to these authors, the rapid diffusion of tetracycline resistance genes to bacteria is due to the localization of the *tetA* gene on plasmids, transposons and integrons [51]. Also the *tetA* and *tetB* genes are usually found and maintained in soil and water for a long time [52]. In our study, quinolone resistance was associated with the presence of *qnr* gene to a percentage of 16.7%. This result is similar to other studies conducted in Côte d'Ivoire [5,17,19], which demonstrated the presence of *qnr* gene in strains enterobacteria of human, animal and environmental origins.

At present, multi resistance is frequently observed in *E. coli* isolates from human clinical cases worldwide, and this characteristic has an increasing impact on the treatment of community *E. coli* infections [53,54].

Previous studies have reported that *E. coli* isolates from animals and foodstuffs have determinants of resistance to many classes of antimicrobial agents constuting an important reservoir for transmissible resistance genes [55]. These resistant bacteria may enter the food chain, which is a food safety problem because they can transfer resistance genes to opportunistic pathogens [56,57].

5. Conclusion

This study shows that ready-to-eat raw mixed vegetable salads consumed in collective catering in Abidjan are contaminated with multi-resistant strains of *E. coli*. In some cases, this resistance is carried by resistance genes assuming a horizontal transfer of resistance to pathogenic bacteria. Vegetable salads can be used as a vehicle for the transfer of multi-resistant bacteria. Adequate hygiene measures should be taken when preparing to preserve the health of the consumer.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest associated with this work.

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