Research Article


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Received 26 March 2019; Revised 5 September 2019; Accepted 14 September 2019; Published 16 December 2019

Academic Editor: Haile Yancy

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Foodborne infections, mainly those attributable to Campylobacter, are one of the most common causes of intestinal diseases, of bacterial origin in humans. Although the vehicle of transmission is not always identified, the most common vehicles are poultry, poultry products, and contaminated water. In Southern Benin, an excessive use of poultry manure as fertilizer in vegetable farms was noted. This survey aimed to determine the prevalence and concentration of Campylobacter spp., especially Campylobacter jejuni and Campylobacter coli, in selected environmental samples (poultry manure, and irrigation water) and freshly harvested leafy vegetables in two (Houeyiho and Sèmè-Kpodji) vegetable farms in southern Benin. To achieve this objective, we analyzed 280 samples, including 224 samples of leafy vegetables (Solanum macrocarpon and Lactuca sativa capita), 28 samples of irrigation water, and 28 samples of poultry manure. The analysis of the samples taken was carried out according to the modified NF EN ISO 10272-1 standard. Of the 280 samples analyzed in this survey, 63 were positive for Campylobacter contamination. For leafy vegetable samples analyzed in this survey, the contamination rate was of 15.63%. 60.71% of poultry manure samples analyzed were contaminated with Campylobacter spp. and 39.29% of irrigation water samples were contaminated. The statistical analysis of these results showed that there is a correlation between the contamination of leafy vegetables, poultry manure, and irrigations (p < 0.01). Campylobacter jejuni (53.97%) was more involved in contaminations than Campylobacter coli (36.57%). This study has shown that there is a real risk of food poisoning by Campylobacter jejuni and Campylobacter coli among consumers of leafy vegetables in southern Benin. The origin of contamination of these leafy vegetables is poultry manure used as fertilizer in vegetable gardens and irrigation water used.

1. Introduction

In Sub-Saharan Africa, the products of urban agriculture are considered to be one response to the shortage of foodstuffs [1]. In addition to the contributions of urban agriculture to urban food security, nutrition, and local economies, farming also affects urban water management, sanitation, and health services [2]. In that context, urban production of vegetables is increasing rapidly but, in both Africa and Asia, faces many constraints, especially land pressure, access to water, and low soil fertility [3, 4]. In Benin, West Africa, the same problems have been identified in periurban and urban gardening areas, where irrigated vegetable production developed rapidly after 1990, coinciding with the drastic drop in fish resources in the
Atlantic Ocean and the rivers [5]. The main leafy vegetables grown are \textit{Lactuca sativa} and \textit{Solanum macrocarpon} L.

The emergence of coastal soil poverty and land pressure is leading farmers to intensify production using inorganic and organic fertilizers and pesticides. This in order to satisfy the growing demand for vegetables. Today, animal manure (60% poultry manure and 40% cattle manure) is frequently used as fertilizer in Southern Benin. Animal manures have been used as effective fertilizers for centuries [5, 6]. Brooks et al. [7] investigated potential microbial runoff associated with the application of poultry litter on the soil. Several other studies pointed to pollution and health risks caused by lack of knowledge and bad practices in the management of livestock manure and chemical fertilizers [1, 3, 8].

Excessive use of fertilizer at each agricultural campaign has been reported in both Africa and Asia, particularly the use of poultry manure at rates of 20–50 t·ha^{-1} and the use of mineral fertilizers, such as urea and NPK (10–20–20 nitrogen, phosphorus, and potassium fertilizer), at rates of 1.2–2 t·ha^{-1} [8, 9]. Unfortunately, the intensive use of organic matter like cow dung and poultry manure and other animal feces are a significant environmental risk to soils, waters, and crops, including fecal contamination [8].

Researchers at Emerging Pathogens Institute (EPI) at the University of Florida in the United States recently focused on Infectious Diseases of Food Origin. They estimated that 31 food-borne pathogens are responsible for 9.4 million human infections each year in the United States, resulting in 55,961 hospitalizations and 1,351 deaths. Of all these cases, 39% are associated with bacteria, including \textit{Campylobacter}, \textit{Salmonella}, and \textit{Clostridium perfringens}, which occupy the top three positions in the ranking [10].

Campylobacteriosis is a serious global issue owing to the heavy economic burden caused by the disease. In the United States, as much as US$ 4.3 billion is estimated to be used in fighting against this disease in one year [11]. The epidemiology of \textit{Campylobacter} infections in humans is not well understood, yet campylobacteriosis is known to be sporadic and rarely associated with large outbreaks. Although the vehicle of transmission is not always identified [12], the most common vehicles are poultry, poultry products, raw milk, and contaminated drinking water [12, 13]. Produce as a potential route for transmitting foodborne diseases to humans has recently gained attention owing to changes in diet [14]. As the overall consumption of fresh produce, especially raw vegetables, has increased as a result of an increase in health consciousness, raw vegetables could serve as a potential vehicle for the transmission of foodborne pathogens to humans. Although \textit{Campylobacter} spp. are not usually detected in produce or other produce-related products [15, 16], the Centers for Disease Control and Prevention [17] has a record of 18 outbreaks of \textit{Campylobacter enteritis} associated with produce worldwide from 1990 to 1999, and the first reported \textit{Campylobacter} outbreak associated with fresh produce occurred in 1993 in the United States and was linked to melons and strawberries [17].

Accordingly, the present study was undertaken to evaluate the level of contamination of leafy vegetables produced in southern Benin by \textit{Campylobacter jejuni} and \textit{Campylobacter coli}.

2. Methodology

2.1. Choice of Sampling Sites. The samples were taken come from vegetable farms of the communes of Sémé-Kpodji and Cotonou. These are the two largest vegetable sites in southern Benin with an area of about 80 hectares for the Sémé-Kpodji vegetable site [18] and more than 15 hectares for the Houéyiho [19] in Cotonou.

2.2. Sampling

2.2.1. Sample Size. The minimum size (n) of the samples was estimated from Schwartz formula: \( n = \left( z^2 \times p \times q \right) / d^2 \) where \( n \) is the minimum size of the samples; \( p \) (prevalence) = 0.20 because the prevalence of \textit{Campylobacter} spp. in Benin is 20% in poultry meat samples [20]; \( q = 1 - p \) = probability that a sample is not contaminated with \textit{Campylobacter} spp.; \( z \) = confidence level according to the normal reduced centered law (for a 95% confidence level, \( z = 1.96 \), \( d \) = a margin of error tolerated for this survey equal to 0.05, the minimum size of the samples is therefore: \( n = \left( 1.96^2 \times 0.2 \times 0.8 \right) / 0.05^2 = 245.86 \). Let \( n = 256 \) samples.

The total number of samples selected for this study is \( N = 280 \). These samples consist of leafy vegetables (\textit{Solanum macrocarpon} L. and \textit{Lactuca sativa capitata}), irrigation water and poultry manure. \textit{Solanum macrocarpon} was chosen because it is the most widely grown leaf vegetable on both sampling sites. The choice of \textit{Lactuca sativa capitata} was motivated by its culinary technique.

2.2.2. Sampling Technique. Market gardens where poultry manure is used have been identified. At random, 30 gardens were selected on each of Sémé-Kpodji and Houéyiho vegetable farms, at a rate of one garden per hectare and at a distance of at least 20 m from each other. On Sémé-Kpodji vegetable farm, 8 gardens using poultry manure and 8 using NPK (10–20–20 nitrogen, phosphorus, and potassium fertilizer) as fertilizer were thus selected to undergo the sampling. On the other hand, on Houéyiho vegetable farm, for each type of garden, 6 gardens were selected.

By using garden using poultry manure as fertilizers, 4 samples of \textit{Solanum macrocarpon}, 4 samples of \textit{Lactuca sativa capitata}, 2 poultry manure samples and 1 irrigation water samples were taken. The same was true for gardens using only NPK as fertilizer (Table 1). The leafy vegetables were cut with a pair of sterile scissors and forceps about 5 cm from the root. A mass of about 300 g of fresh leaves was thus taken from the vegetable plants and introduced into two sterile plastic bags.

Using a sterile spoon, about 300 g of poultry manure was collected in sterile plastic bags. Irrigation water samples were collected by immersing one-liter sterile glass bottles in water.

All samples thus taken were numbered and immediately sent to a laboratory in a cooler containing cold accumulators for analysis. Microbiological analyses were performed within 4 hours after sampling.

2.2.3. Enrichment, Isolation, and Purification of \textit{Campylobacter} spp. Strains. The analysis of the samples taken were carried out according to the modified NF EN ISO 10272-1 standard.
described by Bankolé et al. [20]. 25 g of sample was taken in a sterile bag containing 225 mL Preston broth (Oxoid, England) enriched with fresh sheep blood and Preston supplement (Oxoid CM0689, England). After homogenization with Stomacher, the bag was then hermetically closed.

Assembly obtained was incubated at 42°C ± 1°C under microaerophilic condition (incubation in a jar containing a lit candle) for 48 hours ± 2 h. Subsequently, 48 h ± 2 h subculture was streak-seeded on Preston-Campylobacter (PC) and Karmali-Campylobacter (KC) agar plates. These plates were incubated microaerophilic condition at 42°C ± 1°C for 48 h ± 2 h. After incubation, a characteristic Campylobacter colony was taken from PC and KC agars respectively and seeded on nutrient agar (NG) enriched with fresh sheep blood. These agar plates then were incubated microaerophilic condition at 37°C for 36 h ± 2 h. The pure cultures obtained were stored in glycerol MH broth (30%) at −37°C (for two weeks) for further analyses.

2.2.4. Phenotypic Identification of Campylobacter spp. Strains. Identification of Campylobacter spp. strains was carried out based on bacterial strain morphology, Gram stain, biochemical characterization tests (catalase, oxidase, hydrolysis of hippurate, nitrate production reductase, fermentation of sugars, production of hydrogen sulphide and gas and growth at 25°C and 42°C and antibioticype). These biochemical tests was carried out according to NMKL 119 [21]. Campylobacter spp. isolates were identified as C. jejuni, C. coli or Campylobacter spp. Reference strains of Campylobacter (Campylobacter jejuni ATCC 29428, Campylobacter coli ATCC 33559) and other bacteria (Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 29213, Enterococcus coli ATCC 25922) were used to validate the tests and techniques used.

3. Results

3.1. Samples Contamination of Sémè-Kpodji Vegetable Farm. The samples of the garden using poultry manure as fertilizer had a contamination rate of 27.3%. Of the 64 leafy vegetable samples, 12 (18.8%) were Campylobacter spp. positive, and of the 16 poultry manure samples, 9 (56.3%) were Campylobacter spp. The bivariate correlation analysis between the contamination rate of poultry manure and leafy vegetables showed a significant correlation at the 0.01 level (p-value ≤ 0.001). Samples from gardens using NPK fertilizer had a contamination rate of 11.1%. The leafy vegetable and irrigation water samples had a contamination rate of 7.8% and 37.5%, respectively. There is a significant correlation at 0.01 level between the contamination of irrigation water and that of leafy vegetables (p-value ≤ 0.001) (Table 2).

3.2. Contamination of Samples from Houeyiho (Cotonou) Vegetable Farm. 25.8% (31/120) of samples taken from Houeyiho vegetable farm were contaminated with Campylobacter spp. Contamination rates of samples from gardens using poultry manure and NPK as fertilizer were, respectively, 33.3% and 16.7%. On gardens using poultry manure, 25% (12/48) and 66.7% (8/12) of leafy vegetables and poultry manure were positive for Campylobacter spp. Analysis of these results showed a significant correlation at 0.01 level between contaminated poultry manure and contaminated leafy vegetables (p-value ≤ 0.001). For gardens using NPK, contamination rate of leafy vegetable and irrigation water samples were respectively 33.3% (6/18) and 66.7% (8/12) of leafy vegetables and poultry manure were positive for Campylobacter spp. Analysis of these results showed a significant correlation at 0.01 level between contaminated poultry manure and contaminated leafy vegetables (p-value ≤ 0.001) (Table 3).
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Of leafy vegetable samples were contaminated by Campylobacter jejuni, 33.3% by Campylobacter coli and 16.7% by Campylobacter spp. Of the poultry manure, 55.6% were contaminated with Campylobacter jejuni, 33.3% with Campylobacter coli and 11.1% with Campylobacter spp. For the irrigation water samples, 66.7% were contaminated with Campylobacter jejuni and 33.3% with Campylobacter coli (Figure 1). Bivariate correlation analysis showed a significant correlation at 0.01 level of Campylobacter species distribution between leafy vegetable and poultry manure samples contaminated ($\chi^2$ = 0.875, $p$-value = 0.002).

In gardens where NPK is used as a fertilizer, Campylobacter jejuni was present in 60% of leafy vegetable samples contaminated and 40% Campylobacter coli contamination. For with regard to contamination of two leafy vegetable species contaminated by Campylobacter spp., the contamination rates of 10.7% (12/112) and 20.5% (23/112) were recorded for Solanum macrocarpon and Lactuca sativa capitata, respectively. This contamination of these leafy vegetable species does not depend on the type of garden nor vegetable farms (Table 4).

3.3. Distribution of Campylobacter Species to Contaminated Samples from Sémé-Kpodji Vegetable Farm. Of the 32 contaminated samples from Sémé-Kpodji vegetable farm, 53.1% were contaminated with Campylobacter jejuni, 37.5% with Campylobacter coli and 9.4% with other Campylobacter species. Regarding the numbers of contaminated samples from gardens using poultry manure as fertilizer, we noted that 50% of leafy vegetable samples were contaminated by Campylobacter jejuni, 33.3% by Campylobacter coli and 16.7% by Campylobacter spp. Of the poultry manure, 55.6% were contaminated with Campylobacter jejuni, 33.3% with Campylobacter coli and 11.1% with Campylobacter spp. For the irrigation water samples, 66.7% were contaminated with Campylobacter jejuni and 33.3% with Campylobacter coli (Figure 1). Bivariate correlation analysis showed a significant correlation at 0.01 level of Campylobacter species distribution between leafy vegetable and poultry manure samples contaminated ($PC = 0.875, p$-value = 0.002).

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### Table 2: Contamination rate of samples from Sémé-Kpodji vegetable farm.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Analysis results</th>
<th>Number of samples</th>
<th>$PC$</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leafy vegetables</td>
<td>+</td>
<td>12 (25%)</td>
<td>36 (75%)</td>
<td>48 (72.7%)</td>
</tr>
<tr>
<td>Poultry manure</td>
<td>+</td>
<td>8 (66.7%)</td>
<td>4 (33.3%)</td>
<td>12 (18.2%)</td>
</tr>
<tr>
<td>Irrigation water</td>
<td>-</td>
<td>2 (33.3%)</td>
<td>4 (66.7%)</td>
<td>6 (9.1%)</td>
</tr>
<tr>
<td>Total number of samples</td>
<td>-</td>
<td>22 (33.3%)</td>
<td>44 (66.7%)</td>
<td>66 (100%)</td>
</tr>
</tbody>
</table>

### Table 3: Contamination rate of samples from Houeyiho vegetable farm.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Analysis results</th>
<th>Number of samples</th>
<th>$PC$</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leafy vegetables</td>
<td>+</td>
<td>6 (12.5%)</td>
<td>42 (87.5%)</td>
<td>48 (88.9%)</td>
</tr>
<tr>
<td>Poultry manure</td>
<td>+</td>
<td>3 (50%)</td>
<td>3 (50%)</td>
<td>6 (11.1%)</td>
</tr>
<tr>
<td>Irrigation water</td>
<td>-</td>
<td>9 (16.7%)</td>
<td>45 (83.3%)</td>
<td>54 (100%)</td>
</tr>
<tr>
<td>Total number of samples</td>
<td>-</td>
<td>22 (33.3%)</td>
<td>44 (66.7%)</td>
<td>66 (100%)</td>
</tr>
</tbody>
</table>

### Table 4: Contamination rate of two leafy vegetable species sampled.

<table>
<thead>
<tr>
<th>Vegetable farms</th>
<th>Type of garden</th>
<th>Number of samples contaminated by Campylobacter spp. according to each leafy vegetables</th>
<th>Contamination rate of leafy vegetables by type of gardens</th>
<th>Contamination rate of leafy vegetables by farms</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sémé-Kpodji</td>
<td>Manure-garden</td>
<td>3 (25%)</td>
<td>9 (75%)</td>
<td>12 (18.75%)</td>
<td>17 (13.28%)</td>
</tr>
<tr>
<td>NPK-garden</td>
<td>2 (40%)</td>
<td>3 (60%)</td>
<td>5 (7.81%)</td>
<td>0.875</td>
<td>0.001</td>
</tr>
<tr>
<td>Houeyiho</td>
<td>Manure-garden</td>
<td>4 (33.33%)</td>
<td>8 (66.67%)</td>
<td>12 (25%)</td>
<td>18 (18.75%)</td>
</tr>
<tr>
<td>NPK-garden</td>
<td>3 (50%)</td>
<td>3 (50%)</td>
<td>6 (12.50%)</td>
<td>0.875</td>
<td>0.001</td>
</tr>
<tr>
<td>Total</td>
<td>12/112 (10.71%)</td>
<td>23/112 (20.54%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4. Discussion

The contamination rate of 22.5% obtained in this study was a relatively high contamination rate; nevertheless, it indicates the presence of Campylobacter on these two vegetables farms. This rate of Campylobacter contamination is somewhat similar to that of imported poultry meat (20%) in Benin [20].

The contamination rate of vegetables samples analyzed (15.6%) seems high to that obtained by Chai et al. [23] on Malaysian farms where 6.3% of vegetables were contaminated with Campylobacter spp. This contamination rate, nevertheless confirms Jacobs-Reitsma hypothesis, which at the end of its

contaminated irrigation water samples, there is a high proportion of Campylobacter coli (66.7%) and a low proportion of Campylobacter jejuni (33.3%). The samples from these gardens are only contaminated by these two species of Campylobacter (Figure 2). But there is no significant correlation between these samples at 0.01 level ($P = 0.500$, $p$-value $= 0.667$).

3.4. Distribution of Campylobacter Species according to Contaminated Samples from Houehiyo (Cotonou) Vegetable Farm. Seventeen contaminated samples from Houehiyo vegetable farm were by Campylobacter jejuni (54.8%). Campylobacter coli contamination accounted for 35.5% and that for other unidentified Campylobacter species accounted for 9.68%. In gardens using poultry manure as fertilizer, contaminated leafy vegetable samples were 50% Campylobacter jejuni, followed by 33.3% Campylobacter coli and Campylobacter spp. at 16.7%. 62.5% of the contaminated poultry manure were Campylobacter jejuni, 25% by Campylobacter coli and 12.5% by Campylobacter spp. Contaminated irrigation water samples were 50% Campylobacter jejuni and Campylobacter coli (Figure 3). There is a significant correlation at 0.01 level of distribution of Campylobacter species between contaminated leafy vegetable and poultry manure samples ($PC = 1$; $p$-value $\leq 0.001$).

With regard to contaminated samples from gardens using NPK fertilizer, Campylobacter jejuni, and Campylobacter coli are the only species present. The contaminated leafy vegetable samples were 50% Campylobacter jejuni and Campylobacter coli. As for the irrigation water samples, 66.7% were contaminated with Campylobacter jejuni and 33.3% with Campylobacter coli (Figure 4). The statistical analysis of these results showed that there is a significant correlation at 0.01 level between leafy vegetable contamination by Campylobacter jejuni, Campylobacter coli and that of irrigation water ($PC = 1$, $p$-value $\leq 0.001$).

4. Discussion

The contamination rate of 22.5% obtained in this study was a relatively high contamination rate; nevertheless, it indicates the presence of Campylobacter on these two vegetables farms. This rate of Campylobacter contamination is somewhat similar to that of imported poultry meat (20%) in Benin [20].

The contamination rate of vegetables samples analyzed (15.6%) seems high to that obtained by Chai et al. [23] on Malaysian farms where 6.3% of vegetables were contaminated with Campylobacter spp. This contamination rate, nevertheless confirms Jacobs-Reitsma hypothesis, which at the end of its
be said, like poultry manure, that irrigation water would also be a potential source of contamination by Campylobacter spp. This is especially true since most of the irrigation water used in all these gardens is marigot or wells water about 1 - 2 m deep, and therefore exposed to all kinds of pollution including Campylobacter contamination. This observation is similar to the results of a study conducted in 2001 by Savill et al. in New Zealand in groundwater [27]. Our results are in line with those obtained in 2002 by Schaffer and Parriaux who, at the end of their studies, revealed the presence of Campylobacter spp. in surface water and runoff [28]. The same observation was made by Lyngstad et al. [29] and Sparks [30] who also showed that poor quality water (untreated water from wells) may contain Campylobacter spp. Other authors, including Hanninen et al. have shown that water can also be an epidemic source of campylobacteriosis, particularly in countries where there is insufficient chlorination of drinking water [31]. In addition, several authors have shown through the results of their work that pigs are essential reservoirs of Campylobacter. This is the case of the work done by Weijtens et al. (2000), which showed on 10 pigs in 8 farms, that 85–90% of these animals, according to age, are positive with a level of excretion of Campylobacter by the sows which increases after the farrowing [32]. While in some vegetable farms, including Houeyiho site, some market gardeners practice pig farming, in addition, more seriously, some hen pens are near irrigation points. This would certainly be the cause of the high levels of contamination (50%) obtained in gardens where NPK is used as fertilizer.

The difference of contamination rate between the two leafy vegetable types (Solanum macrocarpon and Lactuca sativa capitata) could be explained by the particular architecture of Lactuca sativa capitata. A provision that gives them a high capacity for water retention, unlike Solanum macrocarpon. This arrangement also increases the water content of the lettuce and thus promotes the survival of Campylobacter spp. for several days. This hypothesis is supported by the work of several researchers. This is the case of Daczkowska-Kazon and Brzostek-Nowakowska who have shown that some Campylobacter species including Campylobacter jejuni and Campylobacter lari appear to be more resistant in river water [33] and Talibart et al. (2000) who revealed that survival times of Campylobacter spp. in water could be variable depending on the strains (from 6 to more than 60 days) [34]. In addition, Trigui et al. (2015) have shown that Campylobacter spp. can survive and remain viable in water for long periods of time (30 to 52 days) [35]. Often consumed in the "raw state" consumers of Lactuca sativa capitata, are truly more exposed to a Campylobacter infections than those who consume Solanum macrocarpon.

The statistical analysis of results showed that there is a significant correlation at 0.01 level of distribution of two Campylobacter species between leafy vegetable and poultry manure samples (PC = 0.875 and 1.000; p-value ≤ 0.001) and between leafy vegetable and irrigation water samples (PC = 1.000, p-value ≤ 0.001). There is strong contamination of leafy vegetables by Campylobacter jejuni than Campylobacter coli shares. But the statistical analysis of our data did not show a significant difference. Mohammadjour et al. (2018) [36] obtained results different from ours, where 18.2% and 2.5% fresh vegetables were contaminated, respectively, by Campylobacter jejuni and Campylobacter coli.

\[
\text{Yield (\%)}
\]

<table>
<thead>
<tr>
<th></th>
<th>Campylobacter jejuni</th>
<th>Campylobacter coli</th>
<th>Campylobacter spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Irrigation water</strong></td>
<td>60%</td>
<td>40%</td>
<td>20%</td>
</tr>
<tr>
<td><strong>Leafy vegetables</strong></td>
<td>70%</td>
<td>30%</td>
<td>10%</td>
</tr>
</tbody>
</table>

**Figure 4:** Distribution of Campylobacter species according to contaminated samples from NPK gardens.
5. Conclusion

The present work showed on all samples analyzed a contamination rate by *Campylobacter spp.* of 22.5%. The leafy vegetable samples analyzed were contaminated with *Campylobacter spp.* at a rate of 15.6%. This contamination rate is apparently low, but shows that there is a risk of food poisoning by *Campylobacter spp.* in consumers of these leafy vegetables. Even so, these leafy vegetables are often eaten raw. With regard to the origin of this contamination, poultry manure used as fertilizer and the irrigation water used in the gardens of the selected vegetable farms are incriminated. *Campylobacter jejuni* was much more identified (51.4%) as a species involved in the contamination of leafy vegetables, followed by *Campylobacter coli* (37.1%). However, this difference is not significant.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References


