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FOOD MICROBIOLOGY - SHORT COMMUNICATION

# Absence of Shiga toxin–producing *Escherichia coli* (STEC) in organic leafy greens from the metropolitan region of São Paulo, Brazil

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## Abstract

Shiga toxin–producing *Escherichia coli* (STEC) is an important pathogen with public health implications, including its potential association with vegetables. In this study, we investigated the presence of STEC in vegetables obtained from organic producers located in São Paulo city, Brazil. As part of a routine surveillance study conducted over (years of isolation), a total of 200 samples of organic vegetables were screened using biochemical and PCR methods. Among the vegetable samples tested, 30 (15%) were positive for non-Shiga toxin–producing *E. coli*. While no STEC was detected in the organic vegetables in this study, the presence of non-STEC in vegetables raises concerns about the lack of proper hygiene practices during vegetable handling. This contamination represents a public health risk, particularly considering that these isolates can still be pathogenic, and vegetables are often consumed raw. To address this important issue, continuous monitoring of these farms is recommended to ensure the quality and safety of organic vegetables produced for both domestic consumption and exportation.

**Keywords** Leafy greens · Vegetables · STEC · Organic vegetables · Shiga-producing *Escherichia coli*

## Introduction

Organic food is widely recognized as a healthier alternative to conventional foods due to the implementation of alternative crop systems that exclude the use of additives, chemicals, and genetically modified ingredients [1]. Instead of relying on chemical fertilizers, organic producers utilize organic compost, often derived from animal manure. However, it is important to consider that animals serve as reservoirs for various pathogenic microorganisms, including Shiga toxin–producing *Escherichia coli* (STEC), which raises concerns regarding the potential contamination of organic produce through the use of animal manure [2, 3].

To mitigate the risk of food contamination, composting techniques are employed to treat manure and organic matter through fermentation, which results in temperature increase and pathogen elimination, while ensuring minimal environmental impact [4]. In addition to proper composting techniques, good agricultural practices encompass various aspects, including soil management, irrigation water quality, human health, and effective management strategies [5]. Failure to adhere to these practices can result in microbiological contamination, particularly in raw vegetable foods, making them potential sources of foodborne pathogens. Numerous outbreaks worldwide have been linked to fresh leafy green vegetables contaminated with *Escherichia coli* [6]. In addition, several high-profile foodborne illness outbreaks have occurred in North America, affecting leafy greens grown in the region. The Central Coast of California experienced a recurring series of outbreaks associated with leafy greens, including the 2020 STEC outbreak caused by *E. coli* O157:H7 [7].

STEC has been detected in both conventional and organic vegetables, as reported by several studies [6–10]. Furthermore, STEC has been implicated in various outbreaks associated with the consumption of leafy

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vegetables. For instance, watercress-related outbreaks occurred in England [11], and the USA [12, 13].

STEC is responsible for causing severe clinical syndromes in humans, including hemorrhagic colitis, hemolytic uremic syndrome (HUS) in young children, and thrombotic thrombocytopenic purpura in the elderly [14].

Given the limited availability of reports on the prevalence of STEC in vegetables cultivated in Brazil, this study aimed to examine the occurrence of STEC in organic leafy greens grown in the metropolitan region of São Paulo city, Brazil. Additionally, the study aimed to assess the virulence potential of the isolated strains to provide valuable information for evaluating consumers' exposure to this pathogen.

## Materials and methods

### Sample collection

A total of 200 vegetable samples were collected from three organic certified producers in Ibiuna, Sao Paulo, Brazil. Ibiuna is situated in the prominent vegetable production region of the State of Sao Paulo, which supplies products to São Paulo city, the largest metropolitan area in Latin America.

Three individual vegetable units were manually collected and combined to form each sample. These samples were then placed in plastic bags and promptly transferred to insulated boxes containing reusable ice for transportation to the Food Microbiology Laboratory of the University of Sao Paulo in Sao Paulo city. The samples were analyzed within 6 h of collection to ensure timely assessment.

Among the vegetables samples collected, we analyzed 47 lettuce (*Lactuca sativa L.*), 46 chicory (*Cichorium intybus* L.), 36 spinach (*Tetragonia tetragonoides* (Pall.) Kuntze), 27 endive (*Cichorium endivia* L.), 25 parsley (*Petroselinum crispum* (Mill.) Nyman), 7 arugula (*Eruca sativa* Mill.), 4 watercress (*Barbarea verna* (Mill.) Asch.), 4 cilantro (*Coriandrum sativum* L.), 2 sorrel (*Rumex acetosa* L.), 1 kale (*Brassica oleracea* L. var. *acephala* D.C.), and 1 chard (*Beta vulgaris* L.).

A questionnaire was utilized to gather information regarding the origin of seeds, type of fertilizer used, composition of manure, duration of composting, frequency of fertilization, presence of animals, water source, frequency of microbiological analysis of irrigation water, source of pre-washing water, and locations where the vegetables are marketed. This comprehensive questionnaire aimed to obtain detailed data on various aspects related to the production and handling practices of the vegetables (Supplementary material).

### Isolation of Shiga toxin–producing *E. coli*

For each vegetable sample, the leaves from three individual units were combined, resulting in one composite sample. From this composite sample, 100 g of the leaf material was aseptically transferred into a sterile bag. Subsequently, 500 mL of modified tryptone soy broth supplemented with vancomycin (8 mg/L), cefixime (50 µg/L), and potassium tellurite (2.5 mg/L) was added to the bag following the method described by Catarame et al. [15]. The bags were then manually massaged for 5 min to facilitate the release of microorganisms attached to the leaves. The bags were incubated at 37°C for 24 h, and thereafter, two loopfuls of the enrichment broth were streaked onto MacConkey sorbitol agar (Oxoid) and CHROMagar STEC (CHROMagar Microbiology, Paris, France). Up to 30 colonies exhibiting typical characteristics of *E. coli* were selected for further testing using EPM, MIli, and Simmons Citrate tests [16, 17].

### Detection of STEC by PCR

Presumptive colonies confirmed as *E. coli* were subjected to PCR for the detection of virulence genes, including *uidA*, *rfbO157*, and *fliCH7*. DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Two multiplex PCR assays were conducted: one for the detection of *stx1*, *stx2*, and *eae* genes [18], and another for the detection of *rfbO157* [19] and *fliCH7* [20]. Simplex PCR assays for *ehx* and *uid* genes were conducted according to Feng and Monday [18]. The primer sequences are provided in Table 1.

For each sample, the PCR mixture had a final volume of 25 µL, comprising 12.5 µL of GoTaq Green Master Mix (Promega Corporation, Madison, USA), 300 nM of specific primers (IDT, USA), and 100 to 200 ng of DNA template. The thermal cycling conditions for amplifying the *stx1*, *stx2*, *eae*, *uid*, and, *ehx* genes were as follows: an initial denaturation step at 95°C for 3 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. The conditions for amplifying the *rfbO157* and *fliCH7* genes were as follows: an initial denaturation step at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 15 s, extension at 72°C for 30 s, and a final extension step at 72°C for 8 min.

As a positive control, *E. coli* O157:H7 strain (EDL933) was used, while distilled water served as the negative control in the PCR reactions. The PCR products were analyzed

**Table 1** Primer sequences of oligonucleotides used in PCR reactions

| Target gene | Primer | Product of PCR reaction size (bp) | Reference |
|---|---|---|---|
| *stx1* | F: cag tta atg tgg cga agg | 348 | Feng and Monday (2000) |
| | R: cac cag aca atg taa ccg ctg | | |
| *stx2* | F: atc cta ttc ccg gga gtt tac g | 584 | Feng and Monday (2000) |
| | R: gcg tca tcg tat aca cag gag c | | |
| *eaeA* | F: att acc atc cac aca gac ggt | 397 | Feng and Monday (2000) |
| | R: aca gcg tgg ttg gat caa cct | | |
| *ehx* | F: gtt tat tct ggg gca ggc tc | 158 | Feng and Monday (2000) |
| | R: ctt cac gtc acc ata cat at | | |
| *uidA* | F: gcg aaa act gtg gaa ttg gg | 252 | Feng and Monday (2000) |
| | R: tga tgc tcc atc act tcc tg | | |
| *rfb O157* | F: cgg aca tcc atg tga tat gg | 259 | Paton and Paton (1998) |
| | R: ttg cct atg tac agc taa tcc | | |
| *fliC H7* | F: gcg ctg tcg agt tct atc gagc | 625 | Gannon *et al. (*1997) |
| | R: caa cgg tga ctt tat cgc cat tcc | | |

*F* forward, *R* reverse

by gel electrophoresis on a 1% agarose gel and visualized using the Molecular Imager® Gel DocTM XR+ Imaging System (Bio-Rad Laboratories Inc., USA) after staining with ethidium bromide.

## Results

Out of the 200 samples, 30 samples (15%) tested positive for *E. coli* on CHROMagar. The distribution of the microorganism across the three properties was as follows: Property "A" had a detection rate of 29.2% (19/65), property "B" had a detection rate of 12.1% (7/58), and property "C" had a detection rate of 5.2% (4/77).

A total of 145 *E. coli* isolates were subjected to PCR analysis for the detection of various genes including *stx1*, *stx2*, *eae*, *rfbO157*, *fliCh7*, *ehx*, and *uid*. None of the isolates tested positive for any of these genes. However, the *fliCh7* gene was detected in seven isolates. Among these, six *E. coli* isolates were obtained from property "A," while one isolate was recovered from property "B." It is important to note that all DNA samples extracted from the enrichment broths were negative for the tested genes. The responses to the questionnaire provided by the participants are summarized in Table 2.

**Table 2** Replies to the questionnaire applied to organic producers A, B, and C on their farming practices

| | A | B | C |
|---|---|---|---|
| Types of vegetables grown in the farm | Cabbage, cucumber, potato, eggplant, string beans, turnip, green leafy vegetables, carrots, okra | Zucchini, carrots, chayote, tomato, cucumber, string beans, cabbage, green leafy vegetables | Eggplant, beets, leafy green, strawberry, celery, pepper |
| Source of the seeds | Commercial and previous cropping | Commercial and previous cropping | Previous cropping |
| Place of cropping | beds | beds | beds |
| Composition of organic fertilizer | Castor bean flour, fishmeal, chicken manure | Viscera and fishmeal, Castor bean flour, bone meal, molasses, coal dust, rice bran | Chicken and bovine manure, residues of vegetables |
| Period of composting | 3–4 months | 3 months | 2–3 months |
| Animals in the propriety | Chicken and mule | Dog | Chicken, cattle, dogs |
| Source of Irrigation water | River/creek | Pond, rain water | Pond |
| Microbiological analysis of water: frequency | Once a year | Once a year | Once a year |
| Source of water used for pre-washing vegetables | Well | Well | Well |
| Vegetables destination | Supermarkets and street markets | Street markets and restaurants in the area | Street markets |

## Discussion

The presence of STEC in vegetables is a significant concern for public health due to the potential risk of foodborne illness. In this study, we investigated the presence of STEC in organic vegetables obtained from producers in São Paulo city, Brazil. However, none of the tested samples were positive for STEC. This study corroborates the findings of Silva et al. [21], who also reported no detection of STEC in their analysis of 869 samples of vegetables (lettuce, chicory, and arugula) from three organic vegetable producers in the Campinas region of Sao Paulo State. The absence of STEC in both studies suggests that the prevalence of this pathogen in organic leafy greens in the specific regions investigated is low or non-existent.

The study by Rodrigues et al. [22] investigated the presence of enteric bacteria, *Salmonella* spp., and *Escherichia coli* O157:H7 in the supply chain of organic lettuce in the Southern region of Brazil. The study analyzed samples from irrigation water, water used for washing the vegetables, fertilizer, soil, lettuce seedlings, and lettuce obtained from three certified farms. Out of the 27 samples of irrigation and washing water, two were positive for *Escherichia coli* O157:H7. However, none of the 36 lettuce samples tested positive for this microorganism. These findings indicate a potential risk of contamination in the irrigation and washing water but suggest that the lettuce samples themselves were not contaminated with *Escherichia coli* O157:H7.

Characteristics such as soil composition, geography, climate, and rainfall intensity have an impact on the prevalence and survival of enteric pathogens. Higher temperatures and tropical climates have been associated with a lower survival ability of *E. coli* O157 [23, 24]. Latitude, which directly influences weather conditions, also seems to be related to the transmission and shedding of STEC by cattle feces. In fact, cattle are known carriers of STEC in their intestines, where the bacteria reside without causing harm to the animals themselves. However, during the process of manure production, STEC bacteria from the intestines can be present in the feces of cattle. If proper hygiene practices are not followed, such as in farming or agricultural settings, the bacteria can contaminate the environment and potentially spread to crops or other food sources [25]. In the USA, the frequency of outbreaks is higher in northern states compared to that in the southern states. Similarly, in Europe, the occurrence of cases is more commonly reported in northern countries like Germany and the Netherlands, as opposed to southern countries like Spain and Italy. In the southern hemisphere, the incidence of STEC isolation in human patients is higher in most southern countries such as Argentina and South Africa [26]. The warm and humid tropical climate in Brazil, particularly in certain regions, can create favorable conditions for the survival and growth of bacteria, including STEC. High temperatures and humidity levels can promote bacterial proliferation, increasing the risk of contamination in food production and distribution chains [27]. Studies have demonstrated the presence of STEC in various environmental sources, including cattle feces [28, 29], sheep feces [30], fertilizers [31], and irrigation water [22].

Despite the relatively low prevalence of STEC in food, it is essential to maintain vigilance and ensure effective monitoring systems to prevent any potential risks associated with the transmission of STEC. Continued research and surveillance efforts are necessary to assess the presence of STEC in different stages of the food production process and identify potential sources of contamination. This information can guide the implementation of appropriate preventive measures to ensure the safety and quality of food products, particularly those consumed raw or minimally processed, and protect public health.

The absence of STEC in the organic vegetables examined is encouraging from a food safety standpoint. It suggests that the production practices and measures implemented by the organic producers in this region may have been effective in preventing contamination by STEC. These findings could be attributed to the careful adherence to organic farming principles, such as the limited use of chemicals, genetically modified ingredients, and synthetic fertilizers. These practices enhance the microbial diversity and beneficial organisms in the soil, creating an environment that supports plant health and reduces the risk of pathogenic contamination.

Despite the absence of STEC, it is worth noting that 15% of the vegetable samples were positive for non-Shiga toxin–producing *E. coli*. This indicates the presence of other potentially pathogenic strains in the vegetables. The detection of non-STEC highlights the importance of maintaining good hygiene practices throughout the entire vegetable handling process, including cultivation, harvesting, processing, and distribution.

The consumption of raw or minimally processed vegetables carries an inherent risk of foodborne illness if proper hygiene practices are not followed. The presence of non-STEC in the organic vegetables underscores the need for continuous monitoring and improvement of hygiene practices in organic farming systems. This includes ensuring the cleanliness of equipment, implementing effective hand hygiene protocols, and proper sanitation of irrigation water and wash water used during vegetable processing.

Regular monitoring of organic farms is crucial to ensure the ongoing safety and quality of organic vegetables intended for domestic consumption and exportation. Ongoing surveillance, combined with education and training programs for farmers and workers, can further enhance the implementation of good agricultural practices and reduce the risk of contamination by pathogenic bacteria.

The findings of this research, which did not detect STEC in the tested vegetable samples, are consistent with previous studies conducted in different countries. Reports by Wood et al. [32] in Canada, Saeed et al. [33] in Iraq, Loncaveric et al. [34] in Norway, Ryu et al. [35] in Korea, and Marine et al. [36] in the USA have also shown the absence of STEC in vegetable samples. These results suggest that the prevalence of STEC in vegetables may vary across different regions and agricultural practices.

On the other hand, studies conducted in other countries have reported the presence of STEC, including serotype O157:H7, in vegetable samples. Kuan et al. [9] in Malaysia, Khalil et al. [37] in Egypt, Mazaheri et al. [38] in Iran, Özpınar et al. [39] in Turkey, and Pinaka et al. [40] in Greece have documented the detection of STEC in vegetables. These variations in findings could be attributed to differences in agricultural practices, environmental conditions, and prevalence of STEC in animal reservoirs, among other factors.

Agricultural practices vary between countries, including the use of fertilizers, irrigation methods, and crop management techniques. These practices can affect the overall microbial load and potential contamination of vegetables with STEC. For instance, the use of animal manure or certain irrigation water sources can introduce STEC into the agricultural environment, leading to potential contamination of vegetables during cultivation. Environmental conditions, such as temperature, humidity, and prevalence of natural reservoirs of STEC, can also play a role. STEC bacteria can survive and multiply under specific environmental conditions, and regions with higher prevalence of STEC in animal reservoirs may have an increased likelihood of contamination in vegetable production. Additionally, variations in detection methods and sampling strategies used in different studies can influence the reported prevalence rates. Differences in sample sizes, sampling locations, and laboratory techniques can lead to variations in the detection and identification of STEC. It is important to consider these factors when interpreting the findings of studies conducted in different countries. Each region has its unique agricultural practices, environmental conditions, and prevalence of STEC, which can contribute to variations in the presence of STEC in vegetable samples [41].

The contrasting results between studies highlight the importance of region-specific assessments and continuous monitoring of food safety. While this research in Brazil did not find STEC in organic vegetable samples, it is crucial to consider the potential risks associated with STEC contamination in vegetables, as demonstrated by studies conducted in other parts of the world. Stringent food safety measures, including good agricultural practices, proper handling, and effective surveillance systems, are essential to minimize the risk of STEC contamination in vegetables and ensure consumer safety.

In conclusion, while the absence of STEC in the organic vegetables investigated in this study is encouraging, the presence of non-STEC and the potential for contamination highlight the importance of maintaining rigorous hygiene practices throughout the organic vegetable supply chain. Continued efforts in monitoring and improving hygiene practices are essential to ensure the safety of organic vegetables and protect public health.

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## Declarations

**Conflict of interest** The authors declare no competing interests.

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