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MICROBIOLOGICAL QUALITY OF LEAFY GREEN VEGETABLES GROWN IN THE TOLUCA VALLEY

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ABSTRACT

The intake of fresh fruits and vegetables has increased in recent years, as have outbreaks of foodborne illnesses associated with these products. The risk of microbiological hazards in vegetables can occur from the field to the table. The main sources of contamination are agricultural soil and irrigation water, which can affect product quality and be a reservoir of foodborne pathogenic bacteria. This research analysed the microbiological quality of green leafy vegetable crops: spinach (*Spinacia oleracea* L.), lettuce (*Lactuca sativa* L.) and coriander (*Coriandrum sativum* L.), grown in the valley of Toluca. The presence of microorganisms indicating microbiological contamination (mesophilic bacteria, total coliforms, faecal coliforms and *Escherichia coli*, *Salmonella* and *Listeria*) was evaluated in plants, water and soil. Sampling, processing, isolation and bacterial identification were performed in accordance with Mexican Official Standards and the FDA Bacteriological Analytical Manual. The recovered bacteria were subjected to biochemical tests, serotyping and PCR. Microbial counts were present in 100 % of the samples tested. The spinach culture had higher recovery of mesophilic bacteria and total coliforms, while the lettuce culture presented higher values for the faecal coliform group. The results indicated higher microbiological contamination in water and soil > 250 CFU for mesophilic bacteria, total coliforms and faecal coliforms, respectively. The microbiological counts of the three cultures were found within the maximum limits established by Mexican regulations. However, the presence of faecal coliforms, which included *E. coli* bacteria of serotype O105 ab flagellar, compromises the quality of the product and poses a risk to the health of the consumer.

Keywords: contamination, irrigation, mesophilic bacteria, coliforms, *Escherichia coli*.

INTRODUCTION

Vegetables constitute an important food source worldwide that provides carotene, vitamin C, calcium, iron and other minerals (SIAP, 2018). Due to production characteristics, they can be contaminated at any point in the development of the crop, harvesting, processing, distribution and final preparation (Maffei *et al*., 2016). Organic

fertilizers, irrigation water quality and soil are important sources of contamination (Rajwar *et al*., 2016). For example, Rodriguez *et al*. (2015) indicated that leafy vegetables allow microorganisms to be preserved in the wetter areas of the plants and remain protected from direct sunlight (Rodriguez *et al*., 2015). The consumption of fresh and minimally processed vegetables can pose a health risk and cause foodborne illness (Kuan *et al*., 2017). On this account, it is important to detect indicator organisms that may suggest exposure, inadequate handling and preservation of a food. Such organisms may indicate contamination of faecal origin or hygiene failure during the production chain (OPS, 2020).

The microbiological study of fresh products can be performed by phenotypic and molecular tests so that the pathogen can be properly identified (Rajwar *et al*., 2016). Among the most frequently identified indicator organisms in food and water are aerobic mesophilic bacteria, total coliforms and faecal coliforms that represent a health risk, including *Listeria* spp., *Salmonella* spp. and *Escherichia coli* (NOM-210-SSA1-2014). Jeamsripong *et al*. (2019) reported the transfer of *E. coli* from animal faeces to lettuce through irrigation. Iwo and Okoh (2019) found that vegetable production is exposed to different sources of contamination during primary production. Likewise, the outbreaks reported by the Center for disease Control and Prevention (CDC) from the USA since 2020 have been due to the presence of *Salmonella* Montevideo and *E. coli* O157:H7 in the consumption of sprouts, romaine lettuce, baby spinach and some other leafy green vegetables; water and handlers have been identified as the sources of contamination (Iwu and Okoh, 2019; CDC, 2021).

All this emphasizes the importance of identifying and preventing contamination in vegetable production. Therefore, the objective of this study was to determine the microbiological quality through the quantification of aerobic mesophilic organisms (AM), total coliforms (TC) and faecal coliforms (FC) in lettuce (*Lactuca sativa*), coriander (*Coriandrum sativum*) and spinach (*Spinacia oleracea*) grown in the valley of Toluca, to determine if the evaluated foods pose a risk for consumers.

MATERIALS AND METHODS

Study area and samples

Sampling was conducted in the municipalities of Tenango del Valle (18° 39′ 07′′ 99° N; 99° 31′ 37′′ W), Toluca de Lerdo (19° 16′ 41′′ N; 99° 39′ 23′′ W), and Calimaya (19° 10′ 25″ N; 99° 37′ 02″ W) which belong to the State of Mexico (INEGI, 2022).

Samples were collected randomly from plots of 500 m^2 planted with coriander (Hercules variety), lettuce (Romain) and spinach (Viking). A total of 180 plant samples were analysed; 60 per vegetable, 10 per commercial plot (two per municipality). Similarly, two samples of 1000 mL of water from the wells used for irrigation of each plot were obtained. The substrate was taken at a depth of 20 cm in five points of the entire planted area to form a composite sample of each plot.

Microbiological analysis

Microbiological analyses were performed at the Food Safety Laboratory of the Faculty of Agricultural Sciences of the Universidad Autónoma del Estado de México. Sampling was done in accordance with Mexican Official Standard NOM-109-SSA1-1994, which establishes the collection, transport, and handling of samples for microbiological analysis. For sample preparation, 10 g of plant material and soil were weighed (separately) under aseptic conditions. Then each sample was blended and homogenized in 90 mL of sterile peptonized saline water (0.15 % peptone + 0.85 % NaCl) for 1 minute in an Osterizer blender. From this suspension, 1 mL was added to tubes with 9 mL of peptonized water to compose serial decimal dilutions (10^{-1}, 10^{-2}) (NOM-110-SSA1-1994) for subsequent plate seeding. The water samples were seeded directly, with the addition of 1 mL of water in the corresponding culture media for each microorganism.

Aerobic mesophilic bacteria

For the quantification of mesophilic bacteria, the procedure described in NOM-092-SSA1-1994 and FDA (2022) was followed. An aliquot of 1 mL of each previously prepared dilution was taken and placed separately in the centre of a sterile Petri dish to which the standard count agar (BD BIOXON®) was added; the dishes were incubated for 48 h at 35 ± 2 °C; each sample was processed in triplicate. Once the incubation time had elapsed, all the colonies were quantified, following the guidelines of this same standard; the results are reported in CFU mL^{-1}.

Total and faecal coliforms

Coliform quantification was performed according to NOM-113-SSA1-1994; 1 mL was taken from the serial dilutions and incorporated in red violet bile lactose agar (BD BIOXON®). The dishes for total coliforms were incubated at 35 ± 2 °C for 48 h; and for faecal coliforms at 45 ± 2 °C for 24 h. Afterwards, colony reading and counting was performed; all samples were seeded in triplicate, and a Petri dish without inoculum was included as a negative control.

Results for each microbial group were reported in Log CFU mL^{-1} (for better handling and understanding); and were also compared with the maximum permissible limits (MPL) established by the Mexican Standard NOM-093- SSA1-1994.

If faecal coliforms (FC) were present, we proceeded to the analysis for the identification of enterobacteria; a Gram stain was performed to observe whether the isolates corresponded to the group of Gram-positive or negative bacteria. When only Gram-negative colonies were present, they were inoculated on selective media for each bacterium of interest. For the identification of *E. coli*, colonies were seeded on Eosin and Methylene Blue agar (BD BIOXON®) and *Escherichia coli* O157:H7 agar (DIBICO®). In the case of *Salmonella*, pre-enrichment was performed in universal enrichment broth (DIBICO) to proceed to seeding on Salmonella-Shigella agar (BD BIOXON®), Brilliant green (BD BIOXON®) and Xylose Lysine Deoxycholate Agar (DIBICO). Since

there was only growth in the selective media for *E. coli*, the microorganisms were identified by biochemical tests at species level by using indole, methyl red, Voges-Proskauer, citrate, TSI (Triple sugar iron), urea hydrolysis, ornithine decarboxylation and mobility reactions (Table 1).

Confirmation of the identity of *E. coli* was performed by the serotyping procedure using the method updated by Scheutz (Scheutz *et al*., 2004). The procedure was performed through agglutination assays in 96-cavity micro titre plates and rabbit antisera against 181 somatic antigens (O) and 65 flagellar antigens (H) prepared in rabbits (SERUNAM® Mexico), according to the procedure described by Orskov and Orskov (1984).

The strains characterized as *E. coli* had their phylogenetic group determined with multiplex PCR described by Clermont (Clermont *et al*., 2000). The presence of the genes *ChuA*, *YjaA* and the DNA fragment TspE.4C2, which define the four phylogenetic groups established for the bacteria (A, B1, B2 and D), was analysed.

The PCR was performed using a standard protocol. Each reaction was carried out in a final mixture of 20 µL under the following conditions: denaturation for 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30s at 72 °C, concluding with a final extension of 7 min at 72 °C. The primers used were ChuA (F:5′-GAG GAA CCA ACG GTC AGG AT-3′, and R: 5′-TGC CGC CAG TAC CAA AGA AC-3′); YjaA (F:5′-TGA AGT GTC AGG AGA CGC TG-3′, and R:5′-ATG GAG AAT GCG TTC CTC AA-3′), and the TspE.4 fragment. C2 (F:5′-GAGTAATGT CGG GGC ATT CA-3′, and R:5′-CGC GCC AAC AAA GTA TTA CG-3′), which generated fragments of 279, 211, and 152 bp, respectively.

Experimental design and statistical analysis

A completely randomized experimental design was followed, for which samples were taken from three vegetable crops (spinach, lettuce, and coriander), from two plots per municipality in three different municipalities with a total of 180 samples. Similarly, we collected water samples (2 samples of 1000 mL per plot) and soil (composite sample per plot) used in each crop. Each sample was collected and analysed in triplicate.

The results were evaluated by analysis of variance ($p \leq 0.05$) to detect differences between municipalities and by vegetable for the different microbial groups. When there was significant difference, the Tukey test ($p \leq 0.05$) was applied; the SAS® statistical program (SAS Institute, Inc., 2002) was used for both procedures.

Table 1. Biochemical tests for the identification of *Escherichia coli.*

	I	M	O	Vp	Rm	Cs	U	TSI	H_2SO_3	Result
Escherichia coli	+	+	+	-	+	-	-	A/A	-	Positive
Salmonella	+	+	+	-	+	-	-	A/A	-	Negative

I: indole, M: mobility, O: ornithine, Vp: Voges-Proskauer, Rm: methyl red, Cs: Simmons citrate, U: urea, H_2SO_3: hydrogen sulphide production; TSI: Triple sugar iron.

RESULTS AND DISCUSSION

In the three municipalities and in all the crops, microorganisms were found, which indicated the microbiological quality of those crops. There were significant differences ($p \leq 0.05$) among the cultures analysed (Table 2).

In the municipality of Tenango del Valle, the recovery of aerobic mesophilic bacteria in lettuce was 10 % higher with a significant difference compared to Calimaya and Toluca. For the total coliform group, the highest recovery was obtained in Tenango del Valle. The same was observed in Calimaya for the faecal coliforms group.

In the case of spinach, aerobic mesophilic bacteria and total coliforms showed significant differences among municipalities, with the highest counts in Tenango del Valle and Toluca, respectively. Similarly, in the municipality of Calimaya there was a higher presence of faecal coliforms.

For the coriander crop, the three municipalities showed significant statistical differences for aerobic mesophilic bacteria, total and faecal coliforms, with the highest recoveries in Tenango del Valle and Toluca.

Crop contamination contrasts with the statement of Maffei *et al*. (2016) who emphasized that microbiological contamination does not always occur in fresh produce cultivation. In addition, it is related to cultural practices, such as the use of animal manure, which can act as a vehicle for pathogen transmission to the crop (Kuan *et al*., 2017).

It is worth noticing the characteristics observed in the production units of the different crops. These were in the open and the water was stagnant; there were also farm and domestic animals; thus, the lack of better agricultural practices (BAP) was notable. Agroecosystem production niches may contribute to the dissemination of bacterial pathogens associated with produce consumed fresh (Iwu and Okoh, 2019).

Aerobic mesophilic bacteria, total coliforms and faecal coliforms in lettuce, spinach and coriander at the sampled locations were found to be within the maximum permissible

Table 2. Quantification of microorganisms present in vegetables in three municipalities of the Valley of Mexico.

Vegetable	Microorganisms	Municipalities		
		Calimaya	Toluca	Tenango del Valle
Lettuce	AM¶	†3.28 ± 1.82 a	3.26 ± 1.07 a	3.60 ± 1.72 b
	TC§	2.51 ± 1.18 a	3.05 ± 1.08 b	3.17 ± 1.34 c
	FC¤	1.97 ± 0.60 a	1.89 ± 0.61 b	0.63 ±1,52 c
Spinach	AM	4.26 ± 2.07 a	3.97 ± 1.40 b	4.58 ± 1.63 c
	TC	3.66 ± 0.67 a	4.00 ± 2.07 b	3.91 ± 1.52 b
	FC	1.32 ± 0.40 a	0.31 ± 0.18 b	0.41 ± 0.18 b
Coriander	AM	3.23 ± 1.56 a	4.09 ± 1.66 b	4.41 ± 2.05 c
	TC	3.00 ± 1.73 a	3.64 ± 1.67 b	3.27 ± 1.63 c
	FC	1.59 ± 0.35 a	1.32 ± 0.23 b	1.76 ± 0.02c

†Average bacterial counts expressed in logarithms of colony forming units (CFU mL^{-1}); ¶AM: aerobic mesophilic bacteria, §TC: total coliforms, ¤FC: faecal coliforms. Means with different letters indicate significant differences (Tukey; $p \leq 0.05$).

limit (MPL) established by Mexican Official Standard NOM-093-SSA1-1994. However, bacterial recovery in 100 % of the samples reflect the need to include better agricultural practices, as high microbial counts are fertile ground for field-originated contamination to increase at any of the stages through which the product must pass, from harvest to fresh consumption (Iwu and Okoh, 2019).

The presence of mesophilic bacteria (this study). as well as high microbial counts can lead to decomposition, post-harvest losses and outbreaks of foodborne infections in case of contamination with pathogens in consumed products (Praeger *et al*., 2018). Of much greater concern is the presence of microorganisms harmful to health, not visible to the naked eye or detectable through changes in appearance, taste, colour or other external characteristic. Certain pathogens have been shown to have the ability to persist on produce long enough to constitute a hazard to humans (Ocaña *et al*., 2019). The recovery of faecal coliforms points to failures in production and different routes of contamination (water, soil, insects, animals, handlers). Therefore, there is a health risk for consumers (FDA, 2020).

The water used for irrigation in each municipality presented counts of the three microbial groups evaluated with the highest CFU in Toluca for aerobic mesophilic bacteria, and Tenango del Valle for coliforms (Figure 1). One of the Tenango del Valle sampling sites exceeded the World Health Organization recommendation, which states that the water used for irrigation of fresh produce must not exceed 100 CFU mL^{-1} (2 Log CFU mL^{-1}) of faecal coliforms (WHO, 2019). The water consumed at the different production sites was from surface wells, which makes them susceptible to microbiological contamination (Iwu and Okoh, 2019). Globally one of the main irrigation systems used to apply to plots is rolling or gravity irrigation. This irrigation

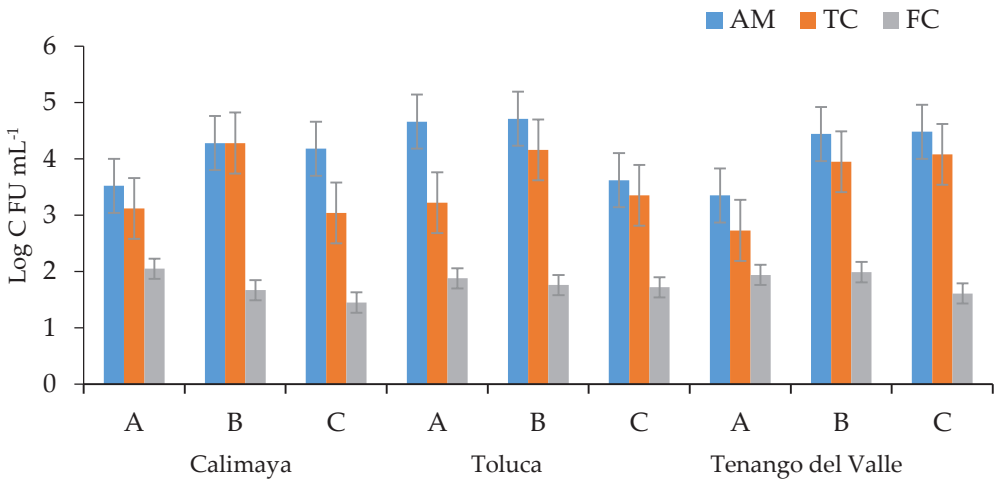

Figure 1. Average bacterial counts (CFU mL^{-1}) of aerobic mesophilic bacteria, total coliforms and fecal coliforms in water from production units in the three municipalities of the Valley of Mexico. A, B, C: evaluated plots.

is characterized by taking advantage of the slope to transport water, so there is infiltration, evaporation, leakage and runoff (SIAP, 2018). *E. coli* and *Salmonella* have been detected in water bodies exploited for agricultural use (Benjamin *et al*., 2013; Castro *et al*., 2015).

Microbiological contamination of each microbial group evaluated was found in the soil in which each crop was grown (Figure 2). It is of the utmost importance that irrigation water and agricultural soil present a good microbiological quality, since there are reports of transfer and survival of enteropathogenic bacteria to the plant through irrigation (Ocaña *et al*., 2018). Soil-to-plant contamination can occur, as noted by Lee *et al*. (2019), through splash transfer of *Salmonella* from soil to crops; this poses a hazard to product safety. In addition, these factors can raise the bacterial levels in harvested products (Benjamin *et al*., 2013).

Due to the presence of aerobic mesophilic bacteria and coliform groups, both the soil and the water used in the crops can be identified as possible sources of contamination of the vegetables analysed. The microbiological quality of the water used for irrigation is very important for the safety of horticultural products such as lettuce, spinach, coriander, among others, since these products are consumed fresh. However, in Mexico and in some other countries, water quality is not defined at regulations in the production of vegetables marketed in popular markets and supply centres. The poor microbiological quality of water and soil found coincides with a study where *E. coli* was detected in baby spinach grown in three regions of Spain, where those authors reported that soil and irrigation water are important factors of microbial contamination affecting food safety (Castro *et al*., 2015). Manure-based fertilizer, flies, vegetable

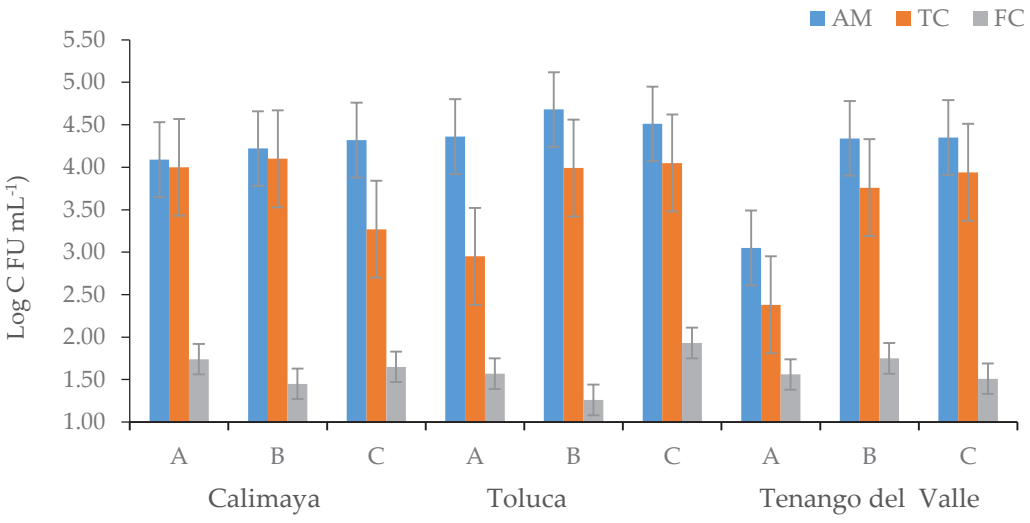

Figure 2. Average bacterial counts (CFU mL^{-1}) of aerobic mesophilic bacteria, total coliforms and faecal coliforms in agricultural soil of the production units in three municipalities of the Valley of Mexico. A, B, C: evaluated plots.

cultivation near livestock, transportation and handling in the production chain have been identified as vectors and sources of faecal and pathogen contamination for plants (Benjamin *et al*., 2013; Castro *et al*., 2015).

Bacterial colonies subjected to staining grouped the bacteria within the Gram-negatives, while growth on specific agars and complementary biochemical tests were positive only for *E. coli*. The serotyping results classified the *E. coli* isolates within the flagellar serotype O105ab from the coriander isolates, and the PCR reaction amplified 152 bp fragments that placed the bacteria in phylogenetic group A. However, there are studies highlighting that the O antigen gene cluster of *E. coli* O105 has the same genes and organization from *Shigella boydii* O11, which may influence the survival and invasion of the bacteria, which are presented as a risk factor, even when it is thought that washing can eliminate this type of contamination (Tao *et al*., 2004; Kniret *et al*., 2016). Likewise, several studies have identified faecal contamination and the presence of pathogenic microorganisms in vegetables such as spinach, lettuce and other crops for fresh consumption (Jiménez *et al*., 2010; Puig *et al*., 2014). In some cases, they did not exceed the maximum permissible limit; however, there were enteropathogenic microorganisms dangerous to humans. With proper monitoring of crop growing conditions, water quality and agricultural soil, the incidence of bacterial pathogens in fresh vegetal products could be minimized to try to ensure their safety.

CONCLUSIONS

The microbial counts obtained in this research, even when they place vegetables within the established limits, indicate that they may have contamination of faecal origin and act as vehicles for the transmission of faecal contaminants, such as *Escherichia coli*, to people through the daily diet. Although in Mexico this bacterium is only reported to be responsible for secretory diarrhoea and persistent diarrhoea, in European countries and the United States it has caused outbreaks and deaths. Therefore, it is important to constantly monitor the sources of microbiological contamination in products for fresh consumption, especially those in production units for exports.

The quality of the water used for irrigation and the soil where the crop is grown can affect the level of contamination present in vegetables and drastically reduce their quality. Surveillance during production, and the implementation of better agricultural practices programs for producers and workers are necessary, as well as the implementation of post-harvest treatment of the vegetables grown.

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