Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.10222

Microbiological quality and presence of foodborne pathogens in fresh-squeezed orange juice samples purchased from street vendors and hygienic practices in Morelia, Mexico

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Abstract

Few studies have screened fresh orange juice for the presence of foodborne pathogens. This study therefore evaluated the microbiological quality and occurrence of *Salmonella*, *Shigella*, *Escherichia coli*, and diarrheagenic *E. coli* pathotypes (DEP) in fresh-squeezed orange juice purchased from street vendors in Morelia, Michoacan, Mexico. The general hygienic practices of street vendors were poor. All 100 samples analyzed were positive for aerobic mesophilic bacteria, with concentrations ranging from 2.41 to 6.03 log CFU/mL. A total of 90 (90%), 35 (35%), and 14 (14%) samples were positive for total coliforms, fecal coliforms, and *E. coli*, respectively, present with concentrations ranging from < 1 to 4.84 log CFU/mL, < 3 to > 1,100 MPN/mL, and < 3 to 210 MPN/mL, respectively. *Salmonella* was not detected in any of the samples, whereas *Shigella sonnei* was isolated from one sample. Two samples were positive for DEP; one harbored ETEC strains and the other STEC strains. Our results highlight the elevated risk for consumer health associated with the ingestion of fresh-squeezed orange juice.

Keywords: street-vended orange juice; microbiological quality; foodborne pathogens; hygienic practices.

Practical Application: Fresh orange juice made by street vendors harbors foodborne pathogens; therefore, its consumption may represent a public health risk. Much of this risk could be reduced through proper hygienic practices.

1 Introduction

Street foods are defined as ready-to-eat foods and beverages sold on the streets by vendors who cook, transport, and display these items in a variety of ways, including in pushcarts, modified bicycles, tricycles, and stationary stalls (Rosales Chavez et al., 2021). In recent years, street foods have assumed important cultural, economic, and social dimensions. Civil and public health administrators have come to realize that street foods have significant economic potential, where food is made available prices at affordable to lower- and middle-income groups, particularly the urban middle classes. The selling of food by street vendors also generates employment for the unskilled and unemployed sections of societies (World Health Organization, 2011).

Street vendors in Mexico provide ready-to-eat food to a high proportion of the inhabitants. In a national food intake survey assessing food expenditure and food consumption away from home, 19% of the respondents reported consuming a meal at a restaurant at least once a month, whereas 60% reported consuming a meal, snack, or drinks from street vendors at least once a month (Langellier, 2015). The consumption of fresh-squeezed orange juice is part of the traditional breakfast in Mexico. Hence, as expected, it is one of the most popular beverages sold by street vendors. Fresh-squeezed orange juice is preferred by consumers because of the "fresh flavor" and contains several micronutrients, antioxidants, and polyphenolic compounds. Additionally, orange juice is also rich in vitamin C and provides appropriate amounts of folate, potassium, niacin, and riboflavin. In this way, orange juice might improve human health and decrease the risk of cardiovascular and degenerative diseases (Motallaei et al., 2021). Also, 100 g of natural orange juice contains about 8.4 g of sugars, of which the main ones are sucrose, fructose, and glucose (Motallaei et al., 2021). Thus, orange juice contributes to the daily intake of simple carbohydrates for consumers (Chanson-Rolle et al., 2016).

The feature of fresh-squeezed orange juice that sets it apart from processed fruit juice products is the lack of pasteurization, and street-vended orange juice sometimes is prepared, handled, and served in unhygienic conditions; therefore, it is not surprising that unpasteurized orange juice has been identified as the vehicle of foodborne pathogens in several outbreaks. Pathogens involved in these outbreaks include enterotoxigenic *Escherichia coli* (Singh et al., 1995), *Shigella* (Thurston et al., 1998), *Salmonella* (Vojdani et al., 2008; Jain et al., 2009; Noel et al., 2010), and hepatitis A virus (Frank et al., 2007). In Mexico, street-vended fresh-squeezed orange juice also has been shown to harbor human pathogens (Castillo et al., 2006; Cerna-Cortes et al., 2016). Because in Mexico, few studies have screened fresh orange juice for the presence of specific pathogenic bacteria, and due to the hygienic conditions in which the street vendors produce

Received 15 Feb., 2022
Accepted 05 May, 2022
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orange juice being practically unknown in Mexico, the aims of this study were (i) to determine the microbiological quality of fresh-squeezed orange juice purchased from street vendors in Morelia, Michoacan, Mexico, ii) to evaluate the presence of *Salmonella*, *Shigella*, *E. coli*, and diarrheagenic *E. coli* pathotypes (DEP) in the samples, and iii) to identify poor hygiene practices associated with orange juice contamination at the point of sale.

2 Materials and methods

2.1 Study area and sample collection

The area selected for this study was Morelia, a city located in the north-central part of the state of Michoacan in central Mexico (Figure 1). Morelia is the capital and largest city of the state, with a large urban area of 1,192.4 km^2 with 849,053 registered inhabitants, which account for 17.9% of the total inhabitants of the state (Instituto Nacional de Estadistica y Geografía, 2020), and a total of 707,482 national and international visitors per year (Instituto Nacional de Estadistica y Geografia, 2016).

2.2 Hygienic practices of street vendors

The hygienic practices of the street vendors were recorded by direct observation while the product was manufactured by the vendor at the sales point and the use of a quick-assessment questionnaire that considered five cleanliness criteria: 1) cleanliness of the stall, 2) cleanliness of the food-handler, 3) cleanliness of the oranges, 4) garbage disposal, and 5) cleaning of work utensils. A score of "1" was assigned for the correct practice and score "0" for the wrong practice.

2.3 Sample collection

Given that in Morelia, most street vendors do not register their business with the local government, it was not possible to use a business directory to draw a random sample of street stalls manufacturing orange juice. Therefore, we performed an inspection throughout the city to locate street vendors. Based on our inspection, most of the localized street stalls were included in this study, and a total of 100 samples of fresh-squeezed orange juice were purchased from street vendors located throughout Morelia City (Figure 1). Eight to nine samples per month were collected, between March 2019 and February 2020. The sampling schedule was from 7 to 10 am, when there is a greater influx of people who go to work and buy orange juice as their breakfast. Each sample consisted of 1 L of orange juice, which was poured by the vendor into a disposable plastic cup. After purchasing, the juice was immediately poured into a sterile polypropylene bag (Whirl-Pak, Nasco, USA), which was placed in a rack with frozen gel packs for transportation to the laboratory. Samples were analyzed no more than 2 hours after purchase. The pH of the orange juice samples was determined using a pH meter (model pH 209, HANNA Instruments, Sarmeola di Rubano-PD, Italy).

2.4 Microbiological analysis

From each orange juice sample, 50 mL was placed in a sterile plastic bag (Whirl-Pak, Nasco, USA), and sterile phosphate-buffered saline (PBS) was added to obtain a final dilution of 1:10 (10^{-1}). Samples were homogenized for 1 min in a Stomacher 400 circulator (Seward, Norfolk, England). Of this homogenized solution, 1 mL was used for preparing serial dilutions (10^{-2} to 10^{-4}) in 9 mL of sterile PBS solution. These dilutions were used

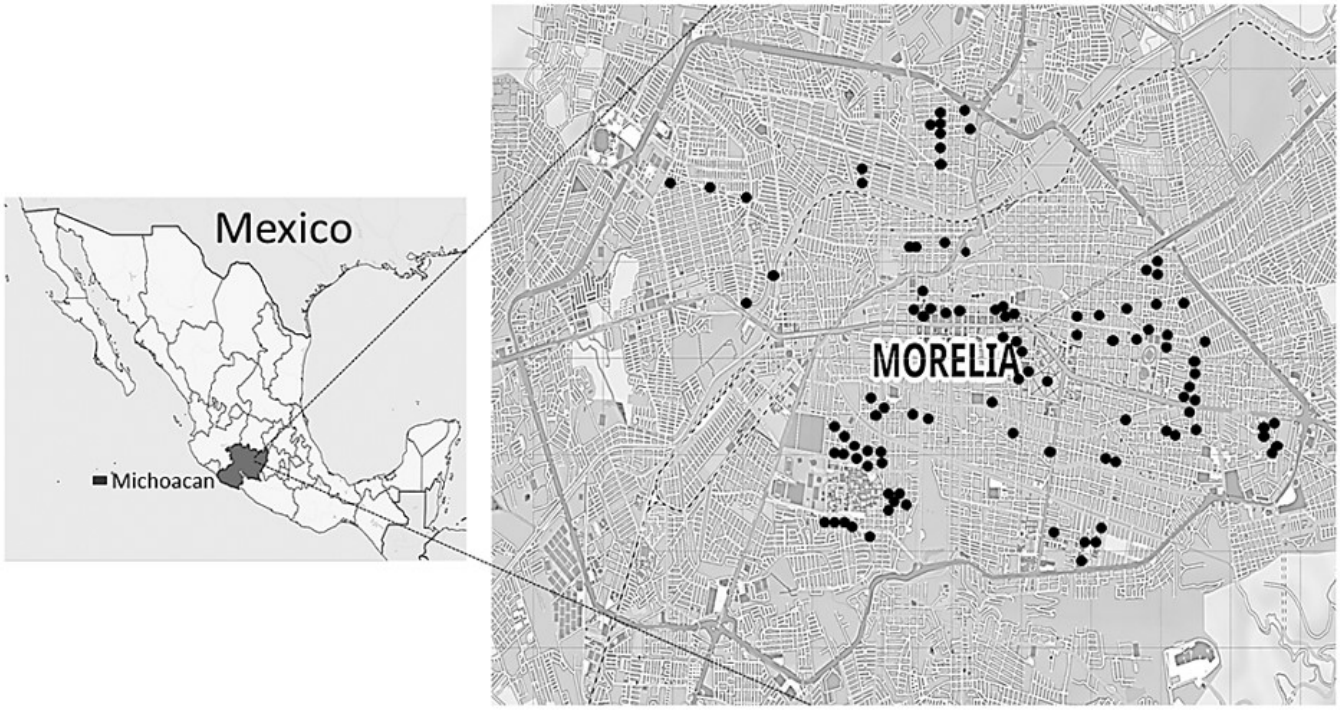

Figure 1. Places in Morelia, Michoacan where fresh-squeezed orange juice samples were collected (●).

for the quantification (CFU/mL) and estimation (MPN/mL) of various microorganisms. Each sample was tested for the presence of aerobic-mesophilic bacteria (AMB), total coliforms (TC), fecal coliforms (FC), *E. coli*, *Salmonella*, and *Shigella*, following the methods approved by the Bacteriological Analytical Manual of the U.S Food & Drug Administration (2019). Two to three confirmed *E. coli* strains per sample were streaked on trypticase soy agar slants, incubated at 37 °C for 24 h, and maintained at 3–5 °C until they were used for polymerase chain reaction (PCR). In Table 1, we list the methods of detection, identification, confirmation, and reporting of the results for the microorganisms found in fresh-squeezed orange juice samples.

2.5 Multiplex PCRs for DEP locus identification

We prepared bacterial lysates by resuspending single colonies in 1 mL of deionized water (Milli-Q System, Millipore, Bedford, MA, USA), boiling them for 1 min, and then freezing them until needed. All *E. coli* strains were characterized for the presence of genes that define DEP by two PCRs. The first multiplex PCR assay as described by Lopez-Saucedo et al. (2003) identifies the following loci: heat-stable and heat-labile enterotoxins (*st*, *lt*) for enterotoxigenic *E. coli* (ETEC), intimin (*eae*A) and bundle-forming pilus (*bfp*A) for enteropathogenic *E. coli* (EPEC), Shiga toxin 1 and 2 (*stx*1, *stx*2) for Shiga toxin-producing *E. coli* (STEC), and invasion-associated loci (*ial*) for enteroinvasive *E. coli* (EIEC). Each PCR tube contained 23 µL of reaction mix, comprised (in final concentrations) of Tris-HCl (10 mM, pH 8.3), KCl (50 mM), $MgCl_2$ (2 mM), gelatin (100 µg/mL), glycerol (5% v/v), dATP, dCTP, dGTP, and dTTP (200 µM each), AmpliTaq polymerase (Gibco, BRL) (0.5 U/25 µL), a mixture of the 14 primers (Table 2), and 2 µL of bacterial lysates. The solutions were then subjected to the following cycling conditions: 95 °C (5 min, 1 cycle); 95 °C, 50 °C, and 72 °C (45 s each temperature, 40 cycles); and a final extension step (10 min, 72 °C). The reference strains for the multiplex PCR were ETEC H10407, EPEC E2348-69, EHEC EDL933, and EIEC E11. The second multiplex PCR assay targets three enteroaggregative *E. coli* (EAEC) plasmid-borne virulence genes, the master regulon (*agg*R), dispersin (*aap*), and anti-aggregation transporter (*aat*A) (Cerna et al., 2003), as well as the Afa adhesin usher (*afa*C) characteristic of diffusely adherent *E. coli* (DAEC) strains (Patzi-Vargas et al., 2013). Each PCR tube contained 23 µL of reaction mix comprised of (final concentrations): Tris-HCl (10 mM, pH 8.3), KCl (50 mM), $MgCl_2$ (2 mM), gelatin (100 µg/mL), glycerol (5%, vol/vol), dATP, dCTP, dGTP, and dTTP (200 µM each); AmpliTaq polymerase (Gibco, BRL) (1 U/25 µL), a mixture of the eight primers (Table 2), and 2 µL of bacterial lysates. The solutions were then subjected

Table 1. Methods of detection, identification, confirmation, and reporting of results for microorganisms in fresh-squeezed orange juice samples.

Microorganisms	Presumptive test or primary enrichment	Confirmatory test or secondary enrichment	Selective medium for isolation	Identification test	Results reported as
Aerobic mesophilic bacteria	NR[a]	Plate count agar (Bioxon™, BD, State of Mexico, Mexico) for 48 h at 35 °C	NR	NR	CFU/mL[b]
Total coliforms	NR	Violet red bile agar (Difco™, BD, Sparks, MD, USA) for 48 h at 35 °C	NR	NR	CFU/mL
Fecal coliforms	Lactose broth (Difco™, BD, Sparks, MD, USA) for 48 h at 35 °C	*E. coli* broth (Difco™, BD, Sparks, MD, USA) for 48 h at 44.5 °C	NR	Gas production	MPN/mL[c]
Escherichia coli	Lactose broth (Difco™, BD, Sparks, MD, USA) for 48 h at 35 °C	*E. coli* broth with MUG (4-methylumbelliferyl-β-D-glucuronide) (Difco™, BD, Sparks, MD, USA) for 48 h at 44.5 °C	Eosin methylene blue agar and MacConkey agar (Bioxon™, BD, State of Mexico, Mexico) for 24 h at 35 °C	Indole production, methyl red test, Voges–Proskauer test, and citrate utilization (Bioxon™, BD, State of Mexico, Mexico) for 24 h at 35 °C	MPN/mL
Salmonella/ *Shigella*	225 mL of Universal Pre-Enrichment broth for 24 h at 35 °C	Rappaport–Vassiliadis broth and Muller–Kauffmann tetrathionate broth (Difco™, BD, Sparks, MD, USA) for 24 h at 42 and 43 °C, respectively	Hektoen enteric agar (Difco™, BD, Detroit, MI, USA), *Salmonella–Shigella* agar, xylose lysine desoxycholate agar, and MacConkey agar (Bioxon™, BD, State of Mexico, Mexico) for 24 h at 35 °C	Indole production, methyl red test, Voges–Proskauer test, motility test, and citrate utilization. Urease production, lactose and glucose utilization, H_2S production, and lysine decarboxylase production (Bioxon™, BD, State of Mexico, Mexico) for 24 h at 35 °C	Presence/absence in 25 mL

[a]NR: not required; [b]CFU/mL: colony-forming units per mL; [c]MPN/mL: most probable number per mL.

Table 2. Primers used to identify diarrheagenic *Escherichia coli* pathotypes (DEP).

DEP	Target gene	Primers (5´- 3')	Amplicon size (pb)	Multiplex PCR assay
ETEC	*lt*	F: GGC GAC AGA TTA TAC CGT GC	450	1
		R: CGG TCT CTA TAT TCC CTG TT		
	st	F: ATT TTT CTT TCT GTA TTG TCT T	190	1
		R: CAC CCG GTA CAA GCA GGA TT		
STEC	*stx*1	F: CTG GAT TTA ATG TCG CAT AGT G	150	1
		R: AGA ACG CCC ACT GAG ATC ATC		
	*stx*2	F: GGC ACT GTC TGA AAC TGC TCC	255	1
		R: TCG CCA GTT ATC TGA CAT TCT G		
EPEC	*bfp*A	F: AAT GGT GCT TGC GCT TGC TGC	324	1
		R: GCC GCT TTA TCC AAC CTG GTA		
	*eae*A	F: GAC CCG GCA CAA GCA TAA GC	384	1
		R: CCA CCT GCA GCA ACA AGA GG		
EIEC	*ial*	F: GGT ATG ATG ATG ATG AGT CCA	650	1
		R: GGA GGC CAA CAA TTA TTT CC		
EAEC	*agg*R	F: CTA ATT GTA CAA TCG ATG TA	457	2
		R: AGA GTC CAT CTC TTT GAT AAG		
	aap	F: CTT GGG TAT CAG CCT GAA TG	310	2
		R: AAC CCA TTC GGT TAG AGC AC		
	*att*A	F: CTG GCG AAA GAC TGT ATC AT	629	2
		R: CAA TGT ATA GAA ATC CGC TGT T		
DAEC	*afa*C	F: GGC TTT TCT GCT GAA CTG G	809	2
		R: CGG TCT CAT AAT CAT GTC C		

ETEC: enterotoxigenic *E. coli*; STEC: Shiga toxin-producing *E. coli*; EPEC: enteropathogenic *E. coli*; EIEC: enteroinvasive *E. coli*; EAEC: enteroaggregative *E. coli*; DAEC: diffusely adherent *E. coli*. The sequences of primers for the loci *lt*, *st*, *stx*1, *stx*2, *eae*A, *bfp*A, and *ial* were obtained from Lopez-Saucedo et al. (2003), for *agg*R, *aap*, and *att*A from Cerna et al. (2003), and for *afa*C from Patzi-Vargas et al. (2013).

Table 3. Aerobic-mesophilic bacterial (AMB), total coliform (TC), fecal coliform (FC), *E. coli*, and diarrheagenic *E. coli* pathotypes (DEP) concentrations, and number of positive samples in fresh-squeezed orange juice samples.[a]

Microorganisms	Minimum	Median	Maximum	Number of positive samples (%)
AMB	2.41	4.51	6.03	100 (100)
TC	< 1	3.69	4.84	90 (90)
FC	< 3	< 3	> 1,100	35 (35)
E. coli	< 3	< 3	210	14 (14)
DEP	< 3	< 3	9.2	2 (2)

[a]n = 100. Minimum, median, and maximum values are in $\log_{10}$ CFU per mL for AMB and TC, and in most probable number (MPN) per mL for FC, *E. coli*, and DEP.

to the following cycling conditions: 95 °C (5 min, 1 cycle); 95, 55.5, and 72 °C (45 s each temperature, 40 cycles); and a final extension step (10 min, 72 °C). The reference strains for this multiplex PCR were EAEC 042 and DAEC C18451-A. Both multiplex PCR assays were performed in a Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR products of both assays (5 µL) were visualized by ethidium bromide staining after electrophoresis on a 2.5% agarose gel in Tris-borate-EDTA buffer (Invitrogen™). The gels were visualized under a UV transilluminator. Only the presence of the correct-sized gene product(s) was interpreted as a positive test.

2.6 Statistical analysis

Descriptive statistics were used to summarize data in the form of frequencies and percentages, presented in Table 3. One-way ANOVA was also carried out to compare the degree of contamination (AMB, TC, FC, and *E. coli*) among the orange juice samples and corroborated by Tukey's method. A p-value < 0.05 was considered significant. All statistical analyses were run with the program IBM SPSS Statistics version 21.

3 Results and discussion

Regarding the hygiene practices of street vendors, 48% of vendors had a dirty stall, 30% wore unwashed clothes, 71% used dirty oranges (they washed the oranges with the same water contained in a bucket for hours), 20% had poor waste management, and 45% used unclean work utensils. This result shows that street vendors lack adequate appreciation of basic food safety issues. However, several studies on the hygienic practices of street food vending report that most street food vendors have knowledge of hygienic practices but concluded that most of them do not put this knowledge into practice (Alimi, 2016). Rane (2011) reported that improper food handling and improper waste disposal lead to the transference of pathogens such as *E. coli* and *Salmonella*.

Moreover, the use of dirty utensils leads to cross-contamination of food with *Staphylococcus aureus*, *E. coli*, and *Shigella* due to contaminated water, dishcloths, and handlers. Different studies performed in Mexico and other countries have shown that street vendors often use stands that are of inefficient construction; running water is not easily accessible, and hand and utensil washing are performed in the same bucket, sometimes without soap. Garbage is likewise "conveniently" discarded right next to the stands, attracting insects and rodents. Vendors handle money while serving food, and, in many cases, toilets are not available, thus forcing the vendors to eliminate their body waste also in areas close by and to return to their vending sites without washing their hands. Such conditions and practices are likely to lead to cross-contamination of street food (Estrada-Garcia et al., 2004; Rane, 2011; Alimi, 2016).

Orange juice pH values ranged from 3.0 to 4.7, with 80% of the samples showing a pH ≤ 4. This result coincides with previous studies carried out in Mexico (Castillo et al., 2006; Cerna-Cortes et al., 2016; Ocaña de Jesús et al., 2022).

All samples analyzed were positive for AMB (Table 3), with concentrations ranging from 2.41 to 6.03 log CFU/mL. These results are consistent with those reported by Castillo et al. (2006), Cerna-Cortes et al. (2016), and Ocaña de Jesús et al. (2022), who found similar AMB levels in fresh orange juice samples collected from street vendors and popular markets in Guadalajara, Mexico City, and Toluca. Unfortunately, in Mexico there is no national guideline that establishes the maximum permissible limits for microorganisms in fresh fruit juice; however, high AMB counts, as occurred in this study, are important since this indicator group is related to overall food quality and a lack of hygiene during the production process (Castillo et al., 2006). Therefore, proper raw material handling and sanitation practices need to be promoted and implemented.

In our study, a total of 90 (90%) orange juice samples were positive for TC, with concentrations ranging from < 1 to 4.84 log CFU/mL (Table 3). Our results are similar to those of Ocaña de Jesús et al. (2022), who reported that all fresh orange juice samples analyzed and collected from different popular markets in Toluca, Mexico, harbored TC at concentrations ranging 0.9 to 4.40 log CFU/mL. TC are a hygienic indicator, and a high coliform count generally indicates unsanitary conditions or poor hygiene practices during or after food production.

Fecal coliforms were present in 35 (35%) orange juice samples, with limits ranging from < 3 to > 1,100 MPN/mL (Table 3). Different from our results, Cerna-Cortes et al. (2016) showed that only 25% of the orange juice samples analyzed contained FC at concentrations of up to 460 MPN/mL. The presence of FC can be attributed to fecal contamination of the water used to wash utensils or fruits or transferred directly from the vendors and the environment in which the juice is prepared (Ocaña de Jesús et al., 2022).

Our results also show that 14 (14%) orange juice samples harbored *E. coli* at concentrations of < 3 to 210 MPN/mL (Table 3). Similar to our results, Bagci & Temiz (2011) showed that 10 (17%) of 60 orange samples harbored *E. coli* at concentrations of up to 15 MPN/mL. Our prevalence of *E. coli* is lower than that reported by Castillo et al. (2006) and Ocaña de Jesús et al. (2022), also in Mexico, who found that 75% and 85% of orange juice samples evaluated in Guadalajara and Toluca, respectively, contained *E. coli*. The presence of *E. coli* in orange juice samples is an indication of poor sanitation in the environment where juices are prepared. We found no difference of degree of contamination (AMB, TC and FC) among the orange juice samples analyzed ($p > 0.05$).

We did not find *Salmonella* in orange juice samples. Similarly, Bagci & Temiz (2011) showed that there was no *Salmonella* in fresh-squeezed orange juice samples purchased in Ankara, Turkey. Nevertheless, our results differ from those reported by Castillo et al. (2006) and Ocaña de Jesús et al. (2022) for Mexico, who found that 9% and 85% of fresh orange juice samples analyzed were *Salmonella*-positive. Regarding *Shigella*, in our study, one sample contained *Shigella sonnei*. *Shigella* has been previously reported in 5% of the orange juice samples studied in Guadalajara Mexico (Castillo et al., 2006). Rane (2011) informed that deficient personal hygiene of vendors leads to the transference of *Staphylococcus*, *Salmonella*, and *Shigella* into foods. Globally, *S. sonnei* is an emerging pathogen and the second most common infectious species of shigellosis (bloody diarrhea) in low- and middle-income countries and the leading one in the developed world (Shad & Shad, 2021). Also, *S. sonnei* has significantly contributed to foodborne outbreaks, highlighting food items as the major source (Shad & Shad, 2021).

In this study, 33 *E. coli* strains were isolated from 14 orange juice samples. All were genotyped for the presence of 11 characteristic DEP loci; two samples were contaminated with DEP, one being positive for the heat-labile enterotoxin locus (ETEC), and the other positive for the Shiga toxin 2 locus (STEC). The presence of ETEC and STEC strains has previously been reported in fresh juices made from carrot and beetroot in Mexico (Torres-Vitela et al., 2013; Gómez-Aldapa et al., 2014). STEC strains are also referred to as enterohemorrhagic strains and cause bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in humans, whereas ETEC strains are an important cause of diarrhea in resource-limited settings, particularly among young children. They are also a frequent cause of traveler's diarrhea (Schuetz, 2019). Therefore, it is necessary to implement good hygienic practices and effective sanitization of oranges. In a previous study, the treatment of oranges with hot water at 80 °C for 1 min or 70 °C for 2 min produced a 5-log CFU reduction in *E. coli* on the orange surfaces and a significant reduction of AMB on overall fruit surfaces and in juice, without altering the original sensory quality of the fresh juice (Pao & Davis, 1999). This sanitization method can be implemented by street vendors for the reduction/elimination of bacteria from orange peel surfaces.

4 Conclusions

Our results show that fresh-squeezed orange juice samples had poor microbiological quality. Some harbored foodborne pathogens, probably due to poor hygiene practices. Thus, the consumption of fresh orange juice can pose a potential risk of foodborne illness, mostly in immunodeficient individuals, that could be presented within the local population and among national and international visitors. Much of this risk could

be reduced through proper handling and correct food safety practices, effective sanitization of the oranges, and the prevention of contamination by humans. Street vendors should be trained in food safety, and it is recommended to create a register of all street vendors to ensure their adherence to food safety regulations. These recommendations may help to enhance the microbiological quality and safety of fresh orange juice sold by street vendors.

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