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Short communication

Behavior of shiga toxin-producing *Escherichia coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli* and enterotoxigenic *E. coli* strains on whole and sliced jalapeño and serrano peppers

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ABSTRACT

The behavior of enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and non-O157 shiga toxin-producing *E. coli* (non-O157-STEC) on whole and slices of jalapeño and serrano peppers as well as in blended sauce at 25 ± 2 °C and 3 ± 2 °C was investigated. Chili peppers were collected from markets of Pachuca city, Hidalgo, Mexico. On whole serrano and jalapeño stored at 25 ± 2 °C or 3 ± 2 °C, no growth was observed for EPEC, ETEC, EIEC and non-O157-STEC rifampicin resistant strains. After twelve days at 25 ± 2 °C, on serrano peppers all diarrheagenic *E. coli* pathotypes (DEP) strains had decreased by a total of approximately 3.7 log, whereas on jalapeño peppers the strains had decreased by approximately 2.8 log, and at 3 ± 2 °C they decreased to approximately 2.5 and 2.2 log respectively, on serrano and jalapeño. All *E. coli* pathotypes grew onto sliced chili peppers and in blended sauce: after 24 h at 25 ± 2 °C, all pathotypes had grown to approximately 3 and 4 log CFU on pepper slices and sauce, respectively. At 3 ± 2 °C the bacterial growth was inhibited.

1. Introduction

In recent years, an increase of foodborne illness from diarrheagenic *Escherichia coli* pathotypes (DEP) infection have been reported. A recent foodborne outbreak involving enteroaggregative–haemorrhagic *E. coli* (EAHEC) originating from sprouts in several European countries highlights the importance of screening for DEP in fresh vegetables (Buchholz et al., 2011). Different studies have documented the importance of DEP as agents associated with acute diarrhea illness in Mexican people (Cravioto et al., 1991; Estrada-Garcia et al., 2005, 2009; Paniagua et al., 2007). Furthermore DEP have been identified in diarrheal stool samples of US travelers to Mexico (Adachi et al., 2002; Paredes-Paredes et al., 2011). Four DEP (EPEC, ETEC, EIEC and non-O157-STEC) have been isolated from different foods and beverages in México (Castro-Rosas et al., 2012; Cerna-Cortes et al., 2012a, 2012b, 2013; Estrada-García et al., 2002; Torres-Vitela et al., 2013).

The EPEC, ETEC, EIEC and non-O157-STEC are an important cause of diarrhea in developed countries and visitors from regions where DEP are not endemic (Cravioto et al., 1991; Estrada-Garcia et al., 2005, 2009; Nataro and Kaper, 1998; Paniagua et al., 2007; Trabulsi et al., 2002). EPEC strains have been isolated from a variety of animal species, such as cattle, goats, sheep, chickens, pigeons, and gulls (Cortes et al., 2005). Pigs and cattle are the predominant reservoir of ETEC (Nagy and Fekete, 1999). Humans are a major reservoir for EIEC (Levine, 1987; Kaper et al., 2004). Non-O157-STEC strains are mostly commensal bacteria in animals, with a high potential for foodborne transmission to humans (Caprioli et al., 2005). Ruminants, primarily cattle, are the predominant reservoir of STEC (Caprioli et al., 2005). All these four pathotypes are usually transmitted by contaminated food. Fecal contamination of food and drinking water is the major route of infection of these pathogens for humans (Kuhnert et al., 2000).

Chili peppers are major commercial crops in Mexico, accounting for over 1,981,500 tons of production in 2009. Of these, 613,308 tons were jalapeño peppers and 216,617 tons were serrano peppers (SAGARPA, 2010). These two pepper species are the most commonly consumed in a raw state (*e.g.*, green salads, sauces), both in Mexico and other countries. Recently, we have isolated ETEC and

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Non-O157-STEC from jalapeño and serrano pepper samples in Mexico: STEC were isolated from 36% of serrano samples and 14% of jalapeño samples. ETEC were isolated from 12% of serrano samples and 2% of jalapeño samples (Cerna-Cortes et al., 2012a). The ETEC (Estrada-García et al., 2002) and EIEC (Lopez-Saucedo et al., 2003) strains have also been identified in chili sauces sold on the street in Mexico City. A recent *Salmonella* Saintpaul outbreak in the USA was linked to jalapeño and serrano peppers (CDC, 2008) highlights the importance of know the presence and behavior of different pathogenic bacteria as DEP on these peppers. Although consumer demand for jalapeño and serrano peppers continues to grow, no data exists on EPEC, ETEC, EIEC and non-O157-STEC behavior on fresh or minimally processed jalapeño and serrano peppers or chili sauces. The objective was to determine the behavior of ETEC, EPEC, EIEC and non-O157-STEC on fresh whole chili peppers and slices and sauce.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains comprised three strains of each DEP: non-O157-STEC, EIEC, ETEC, and EPEC (Table 1). Mutant strains resistant to rifampicin (Rif; Sigma–Aldrich St. Louis, MO, USA) were obtained from all 12 native strains (Castro-Rosas et al., 2010). These strains were streaked onto trypticase soy agar (TSA) slants and maintained at 3–5 °C, with weekly transfers onto TSA.

2.2. Raw material preparation

Jalapeño and serrano peppers were purchased in the central market of the city of Pachuca, Hidalgo State, Mexico. After purchasing, peppers were placed in sterile bags, transported to the laboratory and processed within 1 h. In the laboratory, peppers were manually cleaned with a cloth to remove dust. Only the peppers free of visible defects (*e.g.*, bruises, cuts, abrasions, etc.) were used. Before inoculation, the peppers were maintained at room temperature (approx. 25 °C). Slices (approx. 0.5 cm thick) were taken from clean peppers free of visible defects, and only those slices of similar size were used in the trial.

2.3. Ingredients and preparation of the blended sauces

The blended sauces were prepared as we previously described (Castro-Rosas et al., 2011). Briefly, saladette tomatoes, coriander, jalapeño and serrano peppers, onion, and salt were purchased from a local supermarket. All raw produce were soaked in tap water (*ca.* 20 °C) for 5 min and then manually washed using gloves. The washed raw produce was rinsed three times with tap water and then drained in strainers at room temperature before being used for blended sauce preparation. Onions were peeled and collected into sterile containers. Two types of sauce were prepared, one with jalapeño and another one with serrano peppers. Sauce formulations were: tomatoes (100 g), onion (4 g), coriander (2 g), salt (0.5 g), and jalapeño (20 g) or serrano (20 g) peppers. All ingredients were liquidized in a blender for 1 min. 100 ml of blended sauce from each pepper was collected in a separate sterile stomacher bag. Each sterile bag was one sample.

Table 1
Diarrheagenic *E. coli* pathotypes strains used in the trials.

Pathotype	Bacterial strain	Source	Reference
Non-O157-STEC	STSP41	Serrano pepper	Cerna-Cortes et al., 2012a
	STJP6	Jalapeño pepper	Cerna-Cortes et al., 2012a
	STSCM23	Ready-to-eat salad	Castro-Rosas et al., 2012
ETEC	ETSP7	Serrano pepper	Cerna-Cortes et al., 2012a
	ETJPI	Jalapeño pepper	Cerna-Cortes et al., 2012a
	ETSAS22	Ready-to-eat salad	Castro-Rosas et al., 2012
EIEC	EISCM13	Ready-to-eat salad	Castro-Rosas et al., 2012
	EIMS79	Mung bean sprout	Cerna-Cortes et al., 2013
	EICJB121	Carrot juice	Torres-Vitela et al., 2013
EPEC	EPRJ5	Fresh-cut jicama	Gómez-Aldapa et al., 2013
	EPRJ87	Fresh-cut jicama	Gómez-Aldapa et al., 2013
	EPCSA225	Ready-to-eat cooked salad	Bautista-De León et al., 2013

2.4. Inocula preparation and inoculation

Trypticase soy broth (TSB; 3 ml) was inoculated with individual rifampicin resistant EPEC, ETEC, EIEC and STEC strains and incubated at 35 °C for 18 h. The cultures were washed twice in sterile isotonic saline solution (ISS) by centrifugation at 3500 rpm for 20 min, and then the pellets were resuspended in sterile peptone water at about 10^9 CFU/ml. Regarding each one of the three EPEC, ETEC, EIEC and STEC strains, an inocula cocktail was prepared from the same pathotype by mixing 1 ml of each washed suspension. Whole peppers were inoculated with approximately 5 log CFU of a cell suspension from each cocktail (EPEC, ETEC, EIEC and STEC cocktails) by placing 10 μl inside a circle (approx. 0.5 cm diameter) marked on the external surface. Slices were inoculated with approximately 1 log CFU of a cell suspension by placing a single drop (10 μl/drop) on each. Both whole peppers and slices were placed on a sterile stainless steel tray and stored at 3 ± 2 °C or at 25 ± 2 °C and 90% of relative humidity; the incubation was in a humidity chamber with constant temperature (Model LHT-0250E, Daihan Labtech Co., LTD, Korea). Sterile bags containing blended sauce (100 ml) from each pepper were inoculated with approximately 1 log CFU of microorganisms/ml of sauce and stored at 3 ± 2 °C or 25 ± 2 °C.

2.5. Microbiological counts

In whole peppers, quantification of strain survival was carried out by removing the inoculated area (marked circle) with a sterile knife to a depth of approximately 0.5 cm. Each extracted piece was placed in a sterile bag containing 10 ml of 0.1% sterile peptone water and manually rubbed for 2 min while inside the sealed bag. Slices were placed individually in a sterile bag containing 10 ml of 0.1% sterile peptone water and rubbed manually for 2 min while inside the sealed bag. The stomacher bags containing the inoculated sauce were pummeled in a stomacher for 1 min, and sample dilutions, for microorganism counts, were prepared using peptone as a diluent (0.1%). Counts were performed by plate count using appropriate dilutions of the bacterial suspensions spread on TSA plates containing 100 mg of Rif/liter. Plates were incubated at 35 ± 2 °C for 24–48 h. In order to confirm the presence of rifampicin resistant DEP strains, three to five colonies from some Rif plates were transferred to eosin methylene blue agar plates and incubated at 35 ± 2 °C for 24 h. The colonies that were isolated from eosin methylene blue agar were confirmed by IMViC tests.

2.6. Experimental design

Each experiment (type of pepper, whole pepper, slice pepper and sauce) with each DEP was performed in three independent trials, and each trial was done in triplicate (three inoculated whole peppers, slice peppers or sauce for each DEP were analyzed in each sampling time). Counts were performed with two replicate plates from each sample.

2.7. Statistical analyses

Statistically significant differences ($p < 0.05$) were calculated with an analysis of variance (ANOVA) and a Duncan's test, using the Statistica 8 program (StatSoft, Inc., Tulsa, version 8).

3. Results and discussion

All EPEC, ETEC, EIEC and STEC cocktails exhibited a similar behavior on whole serrano and jalapeño peppers. After inoculating with approximately 5 log CFU of EPEC, ETEC, EIEC and STEC cocktails of strains, on both types of whole chili peppers, all DEP strains decreased over time at 25 ± 2 °C (Fig. 1). The numbers of all DEP were not significantly different ($p > 0.05$) from each other until day 12 on each type of chili pepper (Fig. 1). A similar behavior was observed at 3 ± 2 °C after inoculation of chili peppers with approximately 5 log CFU of microorganisms (Fig. 2); no differences ($p > 0.05$) were observed between behavior of all DEP on each type of chili pepper (Fig. 2). On serrano DEP strain populations decreased faster ($p < 0.05$) than on jalapeño peppers at both temperatures (Figs. 1 and 2). On serrano peppers, after one day at 25 ± 2 °C, all DEP strains had decreased by approximately 1.8–2.4 log and on jalapeño by 0.86–1.4 log per chili pepper (Figs. 1 and 2). After twelve days at 25 ± 2 °C, on serrano peppers DEP strains had decreased by a total of approximately 3.7 log, whereas on jalapeño peppers the strains had decreased by approximately 2.8 log. Inactivation of all DEP strains on both, serrano and jalapeño peppers, were slower overall at 3 ± 2 °C (Fig. 2). At day twelve, on serrano peppers all DEP strains had decreased by a total of approximately 2.5 log, whereas on jalapeño the microorganisms had decreased by 2.2 log per chili pepper. In general, survival among EPEC, ETEC, EIEC and STEC strains on whole serrano or jalapeño peppers did not significantly differ ($p > 0.05$) at room temperature (25 ± 2 °C) or refrigeration (3 ± 2 °C).

These results are in agreement with our previous reports (Castro-Rosas et al., 2011) working with *Salmonella* and generic *E. coli* on jalapeño and serrano peppers and with the study reported by Huff et al. (2012), for *E. coli* O157:H7 on jalapeño peppers. In our previous reports no growth was observed for *Salmonella* and generic *E. coli* strains on whole serrano and jalapeño peppers stored at 25 ± 2 °C or 3 ± 2 °C; after 6 days at 25 ± 2 °C, the tested *Salmonella* serotypes and generic *E. coli* had decreased from an initial inoculum level of 5 log CFU to 1 and 2.5 log on serrano and jalapeño, respectively; and at 3 ± 2 °C they decreased to approximately 1.8 and 1.2 log respectively, on serrano and jalapeño. Huff et al. (2012), observed that intact external surface of jalapeño peppers did not support growth of *E. coli* O157:H7 under 12 and 7 °C; populations of *E. coli* O157:H7 declined in both temperatures throughout the length of storage.

All EPEC, ETEC, EIEC and STEC cocktails grew after inoculation onto serrano or jalapeño slices and incubation at 25 ± 2 °C (Fig. 3).

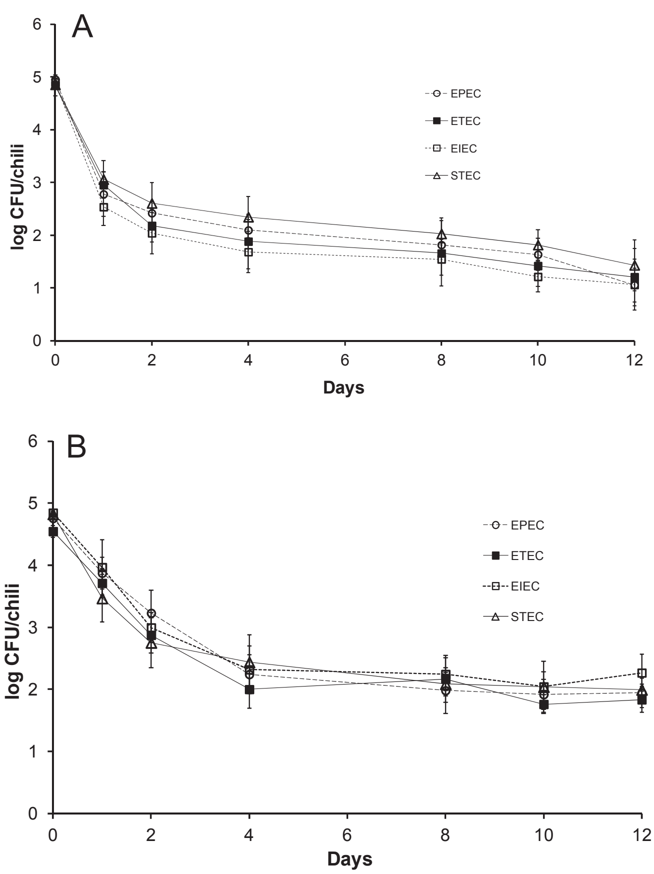

Fig. 1. Behavior of enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and shiga toxin-producing *E. coli* (STEC) strains on whole serrano (**A**) and jalapeño (**B**) peppers at 25 ± 2 °C and 90 ± 2% of relative humidity.

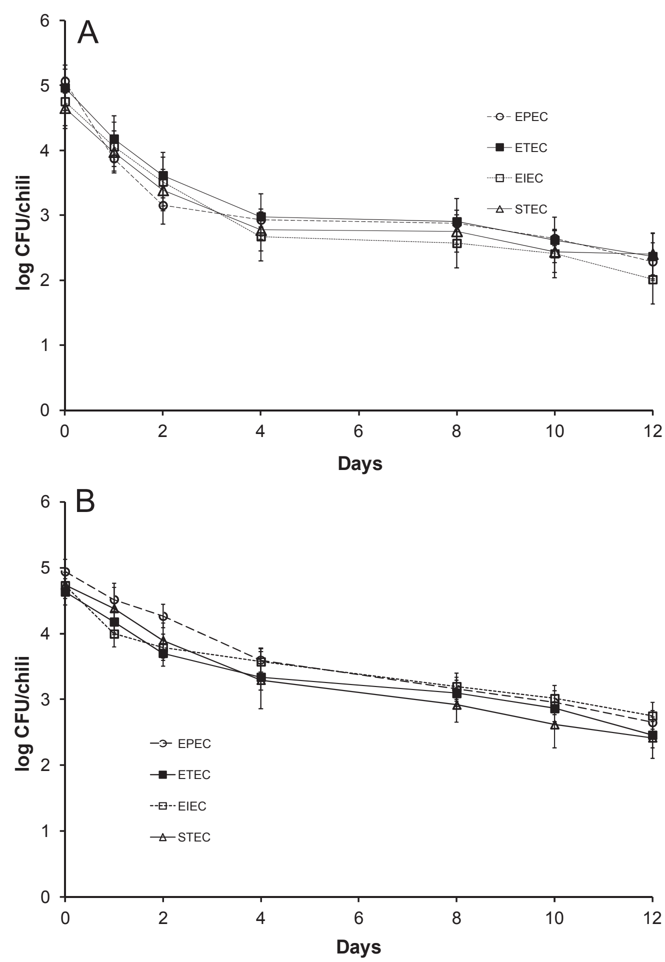

Fig. 2. Behavior of enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and shiga toxin-producing *E. coli* (STEC) strains on whole serrano (**A**) and jalapeño (**B**) peppers at 3 ± 2 °C and 90 ± 2% of relative humidity.

The DEP populations increased from approximately 1 log up to approximately 4 log CFU/slice in both type of chili pepper after 24 h of storage. No differences ($p > 0.05$) were observed between behavior of all DEP on each type of chili pepper slices (Fig. 3). These results are in agreement with our previous results with generic *E. coli* (Castro-Rosas et al., 2011). We observed growth of generic *E. coli* on slices of jalapeño and serrano peppers; after 24 h at 25 ± 2 °C *E. coli* had grown to approximately 4 log CFU on both type of slice peppers (Castro-Rosas et al., 2011).

Growth of EPEC, ETEC, EIEC and STEC strains on pepper slices was inhibited at 3 ± 2 °C (Fig. 4); these DEP decreased over time at 3 ± 2 °C. On both type of chili pepper slices all DEP strains had decreased by 0.3–1 log CFU/slice after six days. Nonetheless, survival of even a small concentration of all EPEC, ETEC, EIEC and STEC under refrigeration implicates a serious health hazard to consumers. Similarly, we did not observe growth of generic *E. coli* on sliced jalapeño and serrano at 3 ± 2 °C (Castro-Rosas et al., 2011).

The consumption of chili in the form of a blended sauce is very common in Mexico. However, diarrheagenic *E. coli* pathotypes have been isolated from restaurant sauces (Adachi et al., 2002) and from street-vended sauces (Estrada-García et al., 2002). Because of its typically low pH then, such characteristic of sauces, would suggest an adverse environment for survival and growth of pathogenic bacteria as EPEC, ETEC, EIEC and STEC. However, recently, we observed growth of *Salmonella* serotypes and generic *E. coli* in blended sauce from jalapeño and serrano peppers (Castro-Rosas et al., 2011). For this reason, we decided to investigate the behavior of EPEC, ETEC, EIEC and STEC strains in blended sauces of jalapeño and serrano peppers.

All EPEC, ETEC, EIEC and STEC strains grew on both, serrano and jalapeño sauces at 25 ± 2 °C (Fig. 5). No differences ($p > 0.05$) were observed between behavior of all DEP on each type of chili sauce (Fig. 5). After a short lag period (a proximately 4 h), the EPEC, ETEC, EIEC and STEC populations increased from approximately 1.2 log to approximately 5 log CFU/ml in both serrano and jalapeño sauces (Fig. 5). At 3 ± 2 °C, cell growth of the EPEC, ETEC, EIEC and STEC strains was inhibited (Fig. 6); the EPEC, ETEC, EIEC and STEC populations decreased over time at 3 ± 2 °C. After six days, EPEC, ETEC, EIEC and STEC strains had decreased approximately 0.6 log CFU/ml in both serrano and jalapeño sauce (Fig. 6). The numbers of all DEP were not significantly different ($p > 0.05$) from each other until day six in each type of chili sauce (Fig. 6). Not surprisingly, EPEC, ETEC, EIEC and STEC growth in sauce is highly temperature dependent. In the restaurant setting, it is usual for sauces to be held at room temperature for many hours (Adachi et al., 2002).

In this study, EPEC, ETEC, EIEC and STEC strains were able to survive on fresh whole jalapeno and serrano peppers. The surface moisture of chili peppers might be a factor that would provide a protective environment to EPEC, ETEC, EIEC and STEC strains. Many microorganisms have developed mechanisms to attach, survive, or grow in microniches on different vegetables (Mandrell et al., 2006). On vegetable surfaces the microorganisms are interacting in aggregates and possibly competing for the limited nutrients available

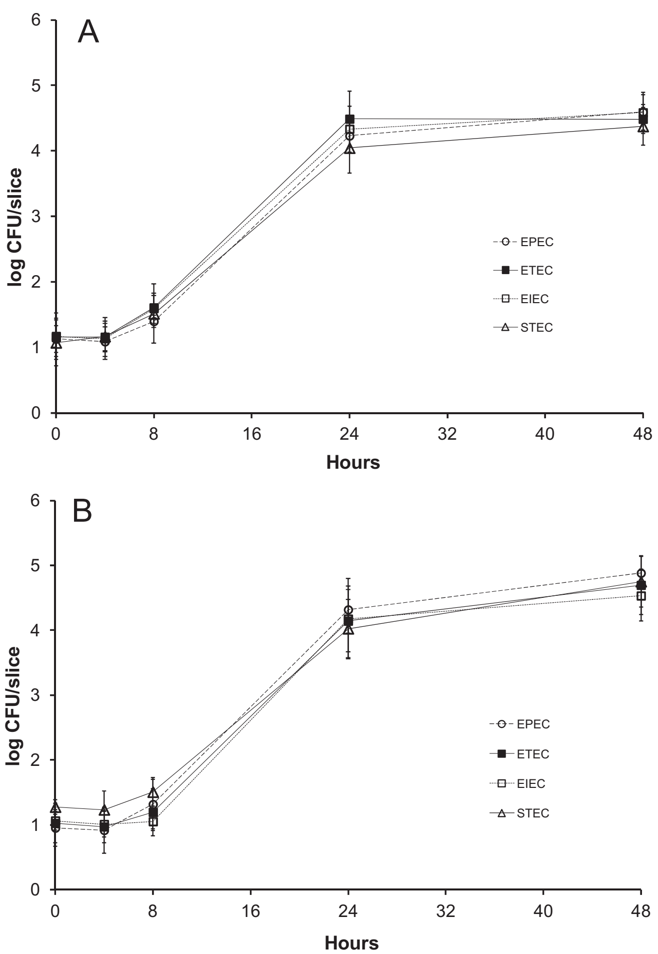

Fig. 3. Behavior of enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and shiga toxin-producing *E. coli* (STEC) strains in serrano (**A**) and jalapeño (**B**) peppers slices at 25 ± 2 °C and 90 ± 2% of relative humidity.

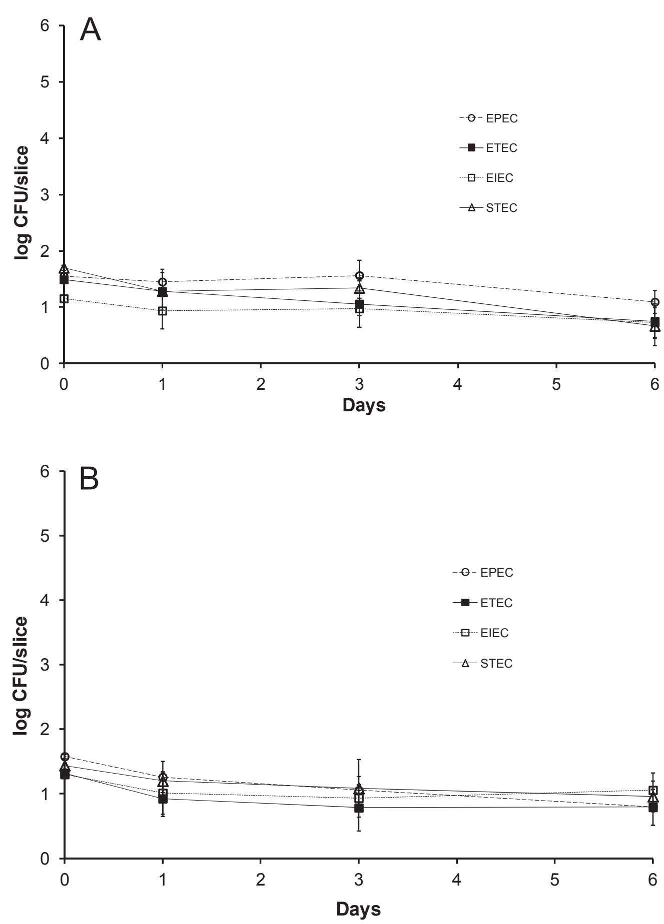

Fig. 4. Behavior of enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and shiga toxin-producing *E. coli* (STEC) strains in serrano (**A**) and jalapeño (**B**) peppers slices at 3 ± 2 °C and 90 ± 2% of relative humidity.

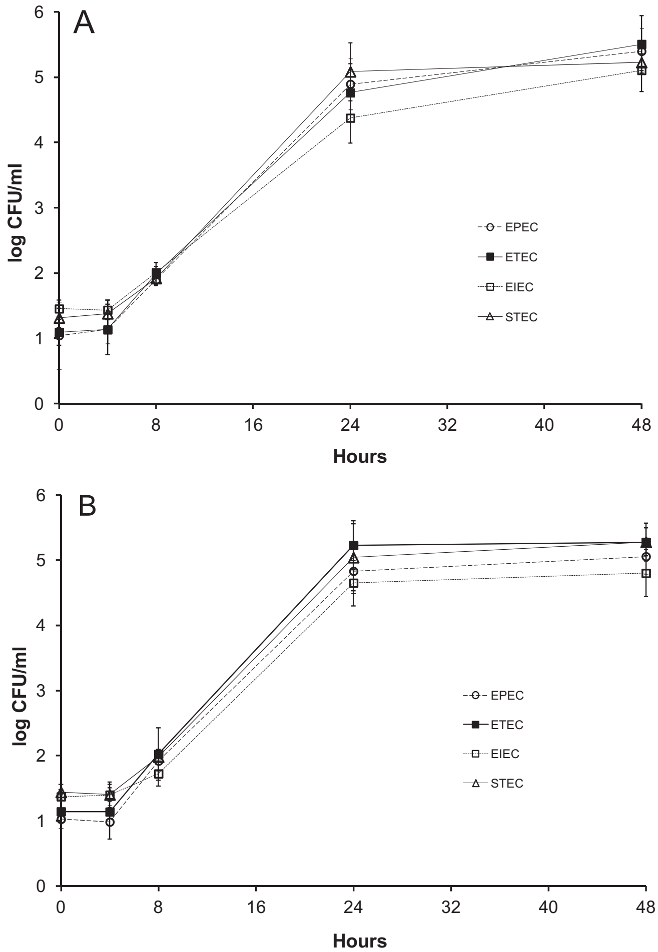

Fig. 5. Behavior of enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and shiga toxin-producing *E. coli* (STEC) strains in sauces from serrano (**A**) and jalapeño (**B**) peppers at 25 ± 2 °C.

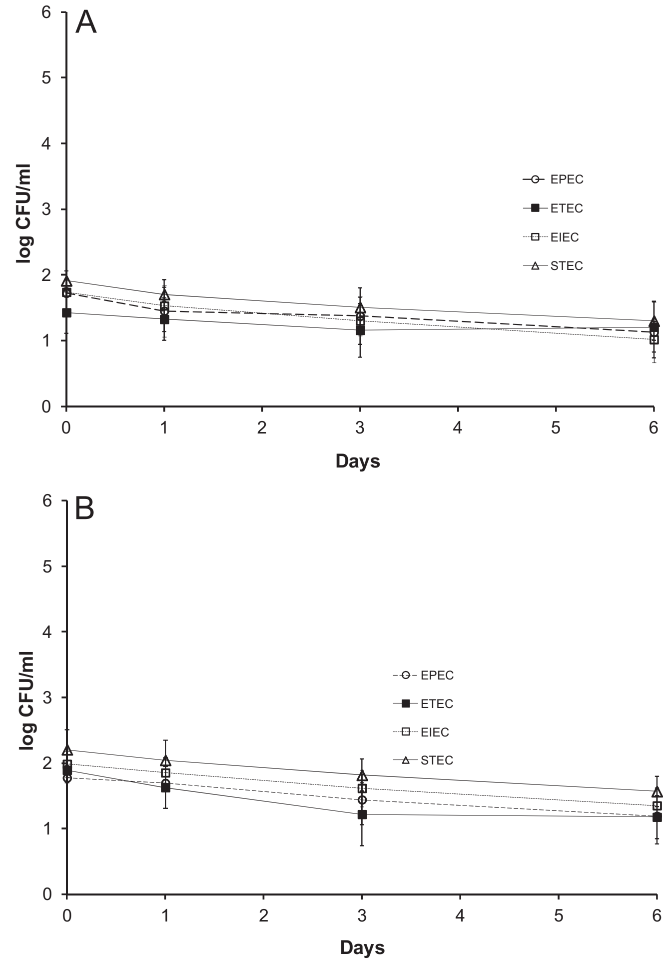

Fig. 6. Behavior of enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and shiga toxin-producing *E. coli* (STEC) strains in sauces from serrano (**A**) and jalapeño (**B**) peppers at 3 ± 2 °C.

in the microniches at the junction of epidermal cells where cuticular waxes are less dense, water accumulates, and nutrients are more available than in other sites (Mandrell et al., 2006). Free water in surface apertures of vegetables, such as stomata, constitutes a water channel that connects a plant's apoplast with its external environment (Bartz, 2006). Microorganisms can internalize into the vegetables through water channels in various ways. The internalized microorganisms are protected from environmental stress (Bartz, 2006).

The results of this study demonstrate that EPEC, ETEC, EIEC and Non-O157-STEC can survive on whole or sliced serrano and jalapeño peppers and in sauce made of raw chili peppers, making them effective transmission vehicles and posing a potential public health threat.

The presence of bacterial growth, on the slices of chili peppers and sauce made of raw chili peppers, shows that the minimally processed form of this product is an important and potential risk to consumers' health. Much of this risk can be mitigated through proper handling and correct food safety practices, such as thorough washing and disinfection, prevention of contamination by humans during preparation and storage, discarding of leftovers, cooking before consumption and storage under refrigeration (3–5 °C), or at room temperature for no more than 3 h. Avoiding temperature abuse may help to limiting the growth of pathogens in jalapeno and serrano slices and in sauce.

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