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Survival of *Salmonella* Enteritidis and *Escherichia coli* in cactus cladodes under domestic marketing conditions in Mexico

José Luis Zárate-Castrejón[1], Talina Olivia Martínez-Martínez[1]*, Juan Gabriel Angeles-Núñez[1], Fanny Guadalupe Concha-Valdez[2], José Ramón Verde-Calvo[3] and Martha Elva Ramírez-Guzmán[4]

[1]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Campo Experimental Bajío. Km. 6.5 Carretera Celaya-San Miguel de Allende, C.P. 38110, Celaya, Guanajuato, México
[2]Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Universidad Autónoma de Yucatán. Mérida, Yucatán Avenida Itzáes # 490 x Calle 59, Colonia Centro C.P. 97000 Mérida, Yucatán, Mexico.
[3]Universidad Autónoma Metropolitana. Depto. de Biotecnología, Laboratorio de Enología y Alimentos Fermentados. México, D.F. México. Av. San Rafael Atlixco No. 186, Col. Vicentina, Del. Iztapalapa. C.P. 09340. México, D.F. México.
[4]Colegio de Postgraduados, Campus Montecillo, Colegio de Postgraduados, Carretera México-Texcoco, Km. 36.5, Texcoco, México.

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In Mexico, during domestic marketing of cactus cladodes, called "nopalitos", there is a tendency not to provide refrigerated storage, and sometimes dealers do not take care of the hygienic conditions of the product during commercialization. Therefore, the objective of this study was to evaluate the survival of *Salmonella* Enteritidis and *Escherichia coli* on cladodes of *Opuntia ficus-indica* Var. Atlixco under conditions associated with domestic marketing in Mexico. Some phenolic compounds present in cladodes, which could influence the survival of these pathogens in the vegetable were analyzed at the same time. For the survival experiment, a 2x2 factorial design was used; treatments included two presentations of cactus cladodes (without spines and with spines) and two storage temperatures. Viable cells were counted during 16 days of storage using specific culture media. Phenolic compounds were determined using HPLC. *E. coli* did not survive on cactus without spines during 16 days at 4°C, while *S.* Enteritidis was able to survive until 16 days in all the treatments. The analytic results obtained indicated higher contents of caffeic and protocatechuic acid. The results showed the importance of refrigerated storage of nopalitos during their commercialization to reduce the risk of presence of foodborne illness and provide good practices during marketing.

Key words: *Opuntia ficus-indica*, human pathogens, handling, storage temperature.

INTRODUCTION

Cactus cladodes (*Opuntia ficus-indica*) are a crop of great economic importance for Mexico; the species is cultivated in an area of 12,038 ha, with a production value of $1,617.645 MXN (SIAP, 2015). Most of the fresh cactus cladodes are sold with spines in domestic markets; their storage time is longer than for cladodes without spines,

where periods of commercialization are about three to five days (Valencia-Sandoval et al., 2010). Although, there is no information on outbreaks of foodborne illnesses associated with the consumption of cactus cladodes, some bad practices in the handling of this vegetable, especially in storage and elimination of spines, can involve the risk of product contamination by foodborne pathogens (Angeles-Núñez et al., 2014). The survival and growth of these pathogens in other vegetables have been associated with temperature, time and presentation of product (Corbo et al., 2005).

Foodborne pathogens have mechanisms to protect themselves within the plant and continue their proliferation. Studies have demonstrated the ability of *Salmonella* to survive in cactus leaves through the formation of biofilms after 24 h of incubation (De los Santos et al., 2012). This bacterium was found in the tissue of the cactus leaf cladodes, persisting for up to 14 days at room temperature (Landa-Salgado et al., 2013). Particularly, *Salmonella* was able to survive and proliferate under refrigerated storage conditions, increasing its population up to 3 log CFU at 4°C for periods longer than three weeks (Kroupitski et al., 2009; Liao et al., 2010); while *Escherichia coli* increased its population up to 5 log CFU at < 8°C for more than three weeks (Corbo et al., 2005, Liao et al., 2010).

Some authors have suggested that the survival of pathogens depends on the availability of nutrients in foods and the presence of secondary metabolites that may inhibit the survival of bacteria. Pad extracts of *Nopalea cochenillifera* have flavonoids and tannins that inhibit the growth of *E. coli* and *Salmonella Typhimurium* (Gómez-Flores et al., 2006). According to some studies with cladodes of *O. ficus indica,* the presence of protocatechuic, gallic, 4-hydroxybenzoic feluric, chlorogenic, syringic and sinapic acids and the epicatechin and quercetin flavonoids have been detected (Qiu et al., 2003; Guevara- Figueroa et al., 2010). These phenolic compounds have antimicrobial action through enzymatic inhibition processes and protein transport; some compounds and destabilization of cell membranes have the ability to inhibit biofilm formation (Othman et al., 2010).

In order to determine the survival ability of *S.* Enteritidis and *E. coli* in cactus cladodia under temperature conditions associated with marketing in Mexico, both bacteria were inoculated into cactus leaves with spines and without spines to assess survival for 16 days under two temperatures, refrigeration (4°C) and environmental (18°C). Also, the presence of phenolic compounds in cladodes with antagonistic potential for these bacteria was determined. The results of this study demonstrated higher survival of *S*. Enteritidis and *E. coli* on cactus cladodes at 18°C, suggesting the importance of refrigerated storage during commercialization to reduce the risk of the growth of foodborne pathogens. Additionally, the low survival of pathogens in cactus without spines suggests an antimicrobial effect provided by the leakage of phenolic compounds from plant tissue due to the peeling process.

MATERIALS AND METHODS

Inoculum

The *S.* Enteritidis isolate (C-4153) was obtained from the bacterial culture collection of the Regional Research Center Dr. Hideyo Noguchi of Universidad Autonóma de Yucatán (UADY); this isolate was serotyped by the Institute of Epidemiological Diagnosis and Reference "Dr. Manuel Martinez Baez", Secretary of Health of Mexico (INDRE-SSA). *E. coli* (ATCC 10536) was provided by the Microbiology Laboratory, Department of Agroindustrial Engineering, Universidad Autónoma Chapingo. All cultures were maintained in nutrient broth (Difco Laboratory, U.S.A) at 35°C for 27 h. Bacterial solution of *S.* Enteritidis was prepared at 2.96 log CFU mL^{-1}, while the *E. coli* solution was at 2.89 log CFU mL^{-1}.

Cactus cladode preparation and inoculation

Cactus cladores of var. Atlixco were purchased from a local market (State of Mexico), and carried to the laboratory in a cooler box. Cactus cladodes were immersed in 1% NaClO solution for two minutes to disinfect them, and then dried on a disinfected surface. The cactus cladodes were divided into two groups. In the first, spines were removed using sterile gloves and knives, while the second group was preserved with spines. Later, cladodes with and without spines were stored at 4 or 18°C; 4 lots with 36 cladodes each were used. Circles of 2 cm in diameter were inoculated with bacterial solutions. Cladodes were packed individually in plastic zipper bags. Survival evaluations of *S.* Enteritidis and *E. coli* were made by triplicate on days 0, 3, 6, 8, 10, 12, 14 and 16.

Bacterial enumeration

The inoculated area of the cactus cladode was cut and diluted with 50 mL of sterile peptone water (0.8%) in sterile plastic bags and homogenized with a stomacher for 1 min. Serial dilutions were prepared, the bacterial dilution 10^{-5} was inoculated (1 mL) onto a Petri dish containing specific media. Hektoen Enteric Agar (Difco, BBL) was used for identification of *S.* Enteritidis and Agar Eosin-methylene blue (Merck) for *E. coli*. Incubation was carried out at 37°C for 24 h. Colony forming units (CFU) were enumerated. The results were expressed in log CFU mL^{-1}.

Extraction of phenolic compounds for HPLC analysis

Physiologically mature cladode tissue (18-20 cm long) was used to prepare extracts by the method of conventional extraction (reflux distillation). Different conditions were evaluated to determine the optimal conditions for extraction of total phenols: amount of tissue

*Corresponding author. E-mail: martinez.talina@inifap.gob.mx.

Table 1. Comparison of means of survival of *Salmonella Enteritidis* and *E. coli* in cactus leaf with and without spines to 18 and 4°C.

Storage (days)	*Salmonella* (log UFC)				*E. coli* (log UFC)			
	18°C		4°C		18°C		4°C	
	CE	SE	CE	SE	CE	SE	CE	SE
3	3.09^{e}	3.29^{c}	2.9^{c}	2.82^{b}	4.02^{b}	3.94^{g}	3.18^{a}	1.93^{c}
6	3.33^{c}	3.30^{c}	3.05^{c}	2.38^{d}	4.39^{a}	4.25^{c}	3.15^{a}	2.17^{b}
8	3.39^{b}	3.30^{c}	3.20^{b}	2.57^{c}	4.37^{a}	4.19^{b}	2.74^{b}	1.62^{d}
10	3.82^{a}	3.75^{a}	3.35^{a}	2.31^{d}	3.74^{c}	3.61^{d}	2.53^{c}	1.43^{e}
12	3.80^{a}	3.70^{b}	3.32^{a}	2.15^{e}	3.40^{d}	3.23^{e}	2.39^{d}	1.25^{f}
14	3.22^{d}	3.16^{d}	2.56^{d}	2.05^{f}	3.30^{e}	3.08^{f}	2.28^{e}	0.21^{g}
16	2.29^{g}	2.15^{b}	1.75^{e}	1.45^{g}	2.53^{f}	2.20^{h}	1.82^{f}	0.00^{h}

CE=With spines, SE=Without spines, same letters are not significantly different.

(2, 6 and 10 g), 30 mL of solvent (water, methanol and ethanol), extraction temperature (40 and 60°C) and time (1, 2, 3, 4 and 5 h). Quantification of total phenols was performed in triplicate by the Folin–Ciocalteu method (Kuskoski et al., 2005) and expressed in terms of equivalent amounts of gallic acid.

HPLC analysis of phenolic compounds

The phenolic compounds in the extracts were determined by HPLC using a UV detector (Thermo Separations Products, USA) at 280 nm (Agilent 1100 series, Hewlett Packard Co., USA). The separation was conducted in an Alltech Lichrosorb C18 column (250 x 4.6 mm), all extracts and solvents were filtered through a 0.47 µm filter (Varian) prior to analysis. In accordance with the methodology of Ndhlala et al. (2007), two mobile phases we used: A: water : acetic acid (98:2 v/v) and B: water : acetonitrile : acetic acid (68:30:2). The flow rate was 2 mL min^{-1} and 20 µL of each sample were injected. Standard solutions (0.02 mg mL^{-1}) of gallic, protocatechuic, 4-hydroxybenzoic, caffeic, feluric, chlorogenic, syringic, ρ-coumaric and sinapic acids, and (-) epicatechin and quercetin were dissolved using methanol as the solvent (HPLC degree). All phenolics were identified by comparing the UV spectral properties and retention times to those of authentic standards.

Experimental design and statistical analysis

Two statistical test were performed, a 2x2 factorial design was used to determine the influence of the cladode cactus presentation (without spines and with spines), and temperature storage (4 and 18°C) on the survival of *S.* Enteritidis and *E. coli* every other day for 16 days (three repetitions in three individuals each time), an analysis of variance for repeated measures was used ($p \leq 0.05$) and Tukey Mean Difference tests. A 3x3x2x6 factorial design was used to determine the optimal conditions for the extraction of phenolic compounds, using ANOVA ($p \leq 0.05$) and Tukey mean difference tests. The SAS 9.1 program was used to perform the analyses.

RESULTS

Evaluation of *S.* Enteritidis and *E. coli* survival

The populations (CFU) of *S.* Enteritidis and *E. coli* on cactus cladodes stored at 4°C were lower than at 18°C; the minimal survival was on cactus cladodes without spines stored at 4°C. The analysis of variance showed interaction between storage time, temperature and presentation of cactus cladodes in the survival of *S.* Enteritidis and *E. coli* (Table 1). The maximum growth of *S.* Enteritidis was observed at 10 days after inoculation; the population of *Salmonella* in cactus cladodes without spines at 4°C declined with respect to the initial concentration 0.15 log CFU after 3 days of storage and 1.5 log CFU at final storage (16 days) (Figure 1). In cactus cladodes with spines, the population of *S.* Enteritidis increased approximately 0.21 log CFU from 3 to 10 days, and declined 1.2 log CFU at the end of storage (Figure 2).

S. Enteritidis was able to survive in the storage times and temperatures of domestic marketing in cactus cladodes with and without spines. The ability to survive at 4°C has been observed from nine days to eight weeks of storage; in this time, the populations declined approximately 0.5 to 2 log CFU (Liao et al., 2010). *Salmonella* has been able to survive at temperatures lower than 4°C. Strawn and Dayluk (2010) demonstrated viability of the bacteria in papaya and mango after 180 days at -20°C, and Kimber et al. (2012) found *Salmonella* on almonds and pistachios stored at -19 and 4°C at least one year after inoculation. In contrast, at 18°C, *S.* Enteritidis increased by 0.45 log CFU after 3 to 14 days in both presentations of cactus cladodes (Figures 1 to 2). Liao et al. (2010) observed a similar situation in jalapeño pepper, where populations of *Salmonella* Saintpaul increased around 3 log CFU at 20°C in just 48 h. *E. coli* presented a similar behavior to *S.* Enteritidis; the population of *E. coli* decreased significantly ($p < 0.001$) at 4°C in both presentations of cactus cladodes. However, the population of this bacterium decreased to 0.94 log CFU at 3 days, and viable cells were not found at 16 days on cactus cladodes without spines (Figure 3). On cladodes with spines, the population increased by 0.3 log CFU on days 3 and 5, followed by a gradual decrease:

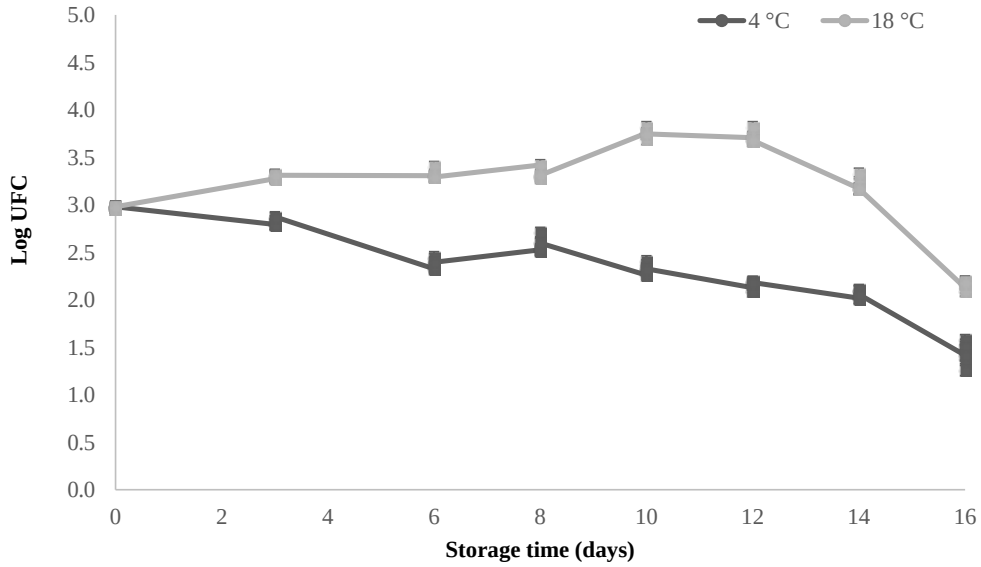

Figure 1. Survival of *S.* Enteritidis in cactus cladodes without spines, stored at 4 and 18°C. Bars denote standard deviation, n = 9.

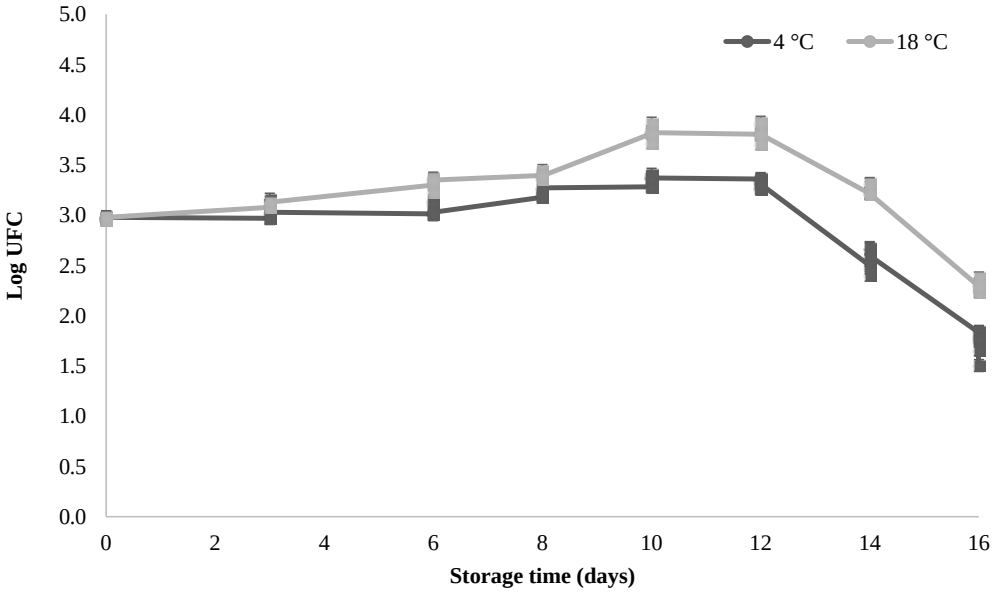

Figure 2. Survival of *S.* Enteritidis in cactus cladodes with spines, stored at 4 and 18°C. Bars denote standard deviation, n=9.

1.06 log CFU was detected on day 16, nearly the initial concentration (Figure 4). At 18°C, in cactus cladodes without spines and with spines, the bacterial population had increased at day 3; maximum growth was found on day 6, after this time the population decreased considerably, with remnants of 2.20 and 2.53 log CFU without and with spines, respectively, on day 16. The survival ability of *E. coli* on cactus cladodes has not been documented; however, on fruits of prickly pear without peel, *E. coli* O157: H7 was able to survive and increase its population to 4.5 and 5 log CFU g^{-1} in storage at 4 and 8°C, respectively (Corbo et al., 2005). In other vegetables at <8°C, this bacterium showed decreased growth but was able to survive (Khalil and Frank, 2010; Liao et al., 2010; Corbo et al., 2005). The ability of both pathogens to survive at the same time was studied by Hsu et al. (2006) at 4°C on aromatic herbs; the populations of both bacteria decreased to about <0.8 log at 5 days of

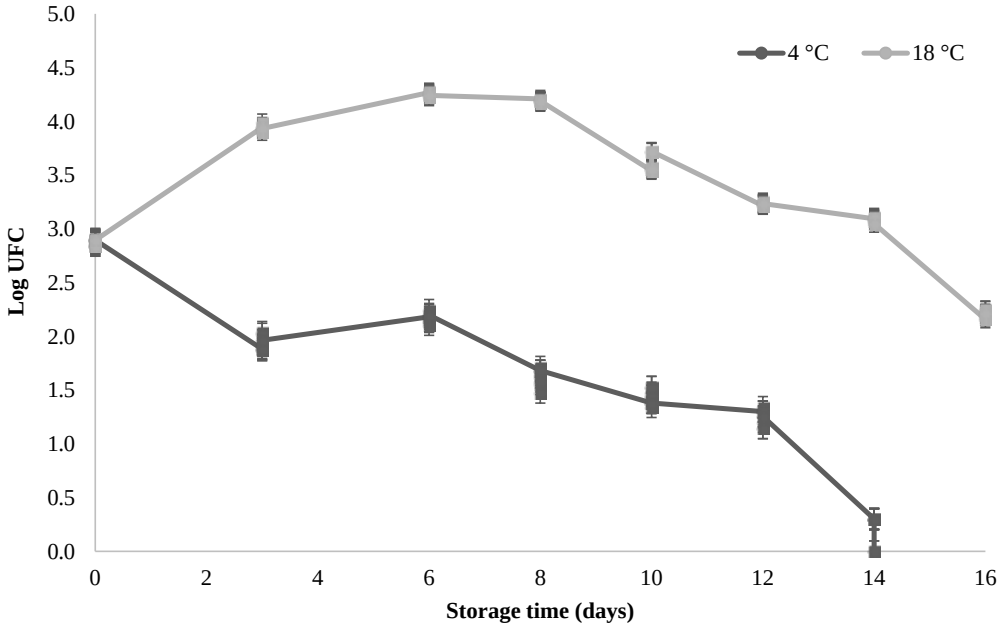

Figure 3. Survival of *E. coli* in cactus cladodes without spines stored at 4 and 18°C. Bars denote standard deviation, n = 9.

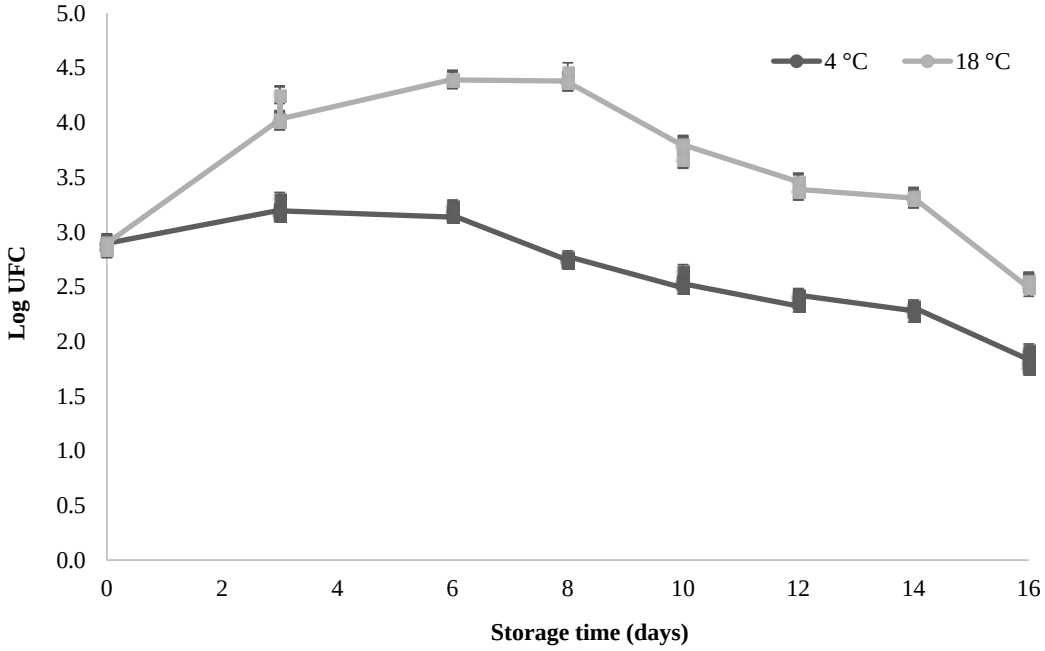

Figure 4. Survival of *E. coli* in cactus cladodes with spines stored at 4 and 18°C. Bars denote standard deviation, n = 9.

storage; however, *E. coli* O157: H7 decreased rapidly over time; bacteria were detected even after 24 days on rotting tissue. Other studies reported that the ability to survive was related to type of tissue. Khalil and Frank (2010) observed major growth on spinach leaves at 8°C (1.18 log CFU), while in lettuce, cilantro and parsley leaves, the bacterium did not grow at 8°C.

With the information obtained in this study on

Table 2. Comparison of means from the content of total phenols of aqueous and methanolic extracts. The means of total phenolics are shown according to statistical analysis.

Treatment	Total phenols[&] gallic acid mg g^{-1}	Tukey mean separation			
60°C, water, 10 g, 6 h	1.127[a]	A			
60°C, water, 10 g, 3 h	1.084[b]	A	B		
40°C, water, 10 g, 5 h	1.058[c]	A	B	C	
40°C, water, 10 g, 6 h	1.051[c]	A	B	C	
60°C, water, 10 g, 5 h	1.039[c]	A	B	C	D
60°C, methanol, 10 g, 6 h	1.039[c]	A	B	C	D
60°C, methanol, 10 g, 4 h	1.031[c]	A	B	C	D
60°C, water, 10 g, 4 h	1.026	A	B	C	D

Means with the same letter are not significantly different

temperature and time of survival, there is a latent risk of the presence of foodborne pathogens in cactus cladodes, particularly when they are transported or kept in poor hygienic conditions or unrefrigerated, and consumed fresh in salads and juices.

The presentation of cactus cladodes during storage was another important factor in the survival of both bacteria; survival was significantly greater ($p \leq 0.001$) in cactus cladodes with spines. The surface wax of the cladodes controls the transpiration and reflects solar radiation, and prevents the penetration of microorganisms into the surface tissue. The bacteria probably produce biofilms that allow them to survive on this wax. Some bacteria have the ability to form biofilms on the surface of the epidermis of fruits, leaves, stems and flower organs, as an adhesion and protection mechanism, also to trap nutrients for feeding (Ávila-Quezada et al., 2010). Hernandez et al. (2009) documented biofilm formation by *Salmonella* Typhimurium and *S.* Javanica 24 h after inoculation; their results showed faster adhesion. Few studies have been conducted to estimate the survival of both bacteria on the surface of this vegetable; however, in other products such as cucumber, mango, guava and tomato, *S.* Enteritidis was capable of developing biofilms on surfaces after inoculation (Tang et al., 2012). If these bacteria are established on the cuticle of cactus cladodes with biofilms, there is a risk of internalization of foodborne pathogens into tissue during the removal of the spines, which would remain there until consumption. The survival of *S.* Enteritidis and *E. coli* in spineless cactus cladode was significantly less at 4°C; this behavior is probably attributable to the decrease in bacterial growth influenced by the temperature; however, the hypothesis of liberation of metabolites as result of mechanical damage in the spine removal process should not be rejected. The production of these compounds is activated as a defense mechanism against a wide variety of microorganisms and increases their survival. The presence of metabolites with microbiological properties has been documented in several species of the genus *Opuntia.* Some extracts of *O. cochenillifera* (Syn.: *Nopalea cochenillifera*) showed *in vitro* inhibition of the growth of *Candida albicans, Candida glabrata*, *E. coli*, *Salmonella* Typhimurium, *S.* Typhi, *Micrococcus* sp., *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Saccharomyces cerevisiae* and others (Gomez-Flores et al., 2006; Necchi et al., 2012). Also, extracts of *O. stricta* presented antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans*, *Bacillus* sp., *Pseudomonas aeruginosa* and *Enterococcus faecalis* (Koubaa et al., 2015), while extracts of *O. ficus-indica* presented bactericidal activity against *Campylobacter jejuni*, *Campylobacter coli*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *Proteus mirabilis*, *Salmonella* spp., *E. faecalis*, *Citrobacter freundii*, *Acinetobacter baumannii*, *Streptococcus pneumoniae*, *Enterococcus faecium* and *Enterobacter cloacae* (Wasnik and Tumane, 2016). Hayek and Ibrahim (2012) determined the antimicrobial potential of *Opuntia matudae* (xoconostle) against *E. coli* O157: H7. The authors attributed this inhibition to organic acids and polyphenols, especially flavonoids and tannins. Phenolic compounds caused cell wall degradation and disruption of the cytoplasmic membrane (Cetin-Karaca and Newman, 2015). Other mechanisms are enzyme inactivation, inhibition of DNA and RNA synthesis, electron transport chain and biofilm formation, and neutralization of toxins (Gutiérrez-Larraínzar et al., 2012).

Determination of phenolic compounds in cladodes of cactus *O. ficus-indica* var. Atlixco

The comparison of means showed water as the best solvent to extract phenolic compounds. However, this solvent yielded an excessive amount of mucilage which obstructed the passage of the sample in the HPLC columns; therefore, the second option was methanol as solvent. The phenolic compound extraction used 10 g of tissue, solvent methanol, extraction time and temperature of 60°C for 6 h (Table 2).

Gallic (tR=3.8), protocatechuic (tR=6.6), 4-hydroxybenzoic

Table 3. Concentration of phenolic compounds analyzed by HPLC in extracts of cactus cladodes var. 'Atlixco'.

Phenolic Compounds	Cactus cladodes var. Atlixco (mg 100 g^{-1})
Gallic acid	1.58±0.05
Protocatechuic acid	24.03±0.21
Hydroxybenzoic acid	0.02±0.01
Caffeic acid	41.32±0.28
Chlorogenic acid	0.12±0.01
Syringic acid	0.24±0.1
ρ-coumaric acid	0.33±0.7
Feluric acid	0.74±0.03
Sinapic acid	3.22±0.24
4-hydroxybenzaldehyde acid	0.16±0.01
Quercetin	5.31±0.61
Epicatechin	0.17±0.01

(tR=11.6), caffeic (tR=16.9), feluric, (tR=32.6), chlorogenic (tR=18.9), syringic (tR=19.9), ρ-coumaric (tR=26.7), sinapic (tR=37.2), 4- hydroxybenzaldehyde (tR=14.1) acids and (-) epicatechin (tR=24.4) and quercetin (tR=37.7) were detected (Table 3).

The major compound was caffeic acid (41.32 mg 100 g^{-1} fresh weight), followed by protocatechuic acid (24.03 mg 100 g^{-1}). Both acids are recognized to have anti-inflammatory, anti-glycemic, antioxidants, anti-cancer, anti-mutagenic and anti-microbial properties; they are also precursors of lignin formation in plant tissues. The concentrations of phenolic compounds were similar to those of Guevara-Figueroa et al. (2010) who determined the presence of protocatechuic (0.06-2.5 mg 100 g^{-1}), gallic (0.64 mg 100 g^{-1}) 4-hydroxybenzoic (0.5 -3.19 mg 100 g^{-1}), feluric (0.56-4.32 mg 100 g^{-1}) acids and quercetin (iso-quercetin form: 22.9-32.21 mg 100 g^{-1}) in the Blanco, Manso, Amarillo and Cristalino varieties. Ginestra et al. (2009) documented quercetin (isoquercetin form: 7 mg 100 g^{-1}), traces of 4-hydroxybenzoic, trans-ferulic and trans and cis p-coumaric acids in a mix of Surfarina, Muscaredda and Sanguigna cultivars of *O. ficus-indica*. Qiu et al. (2003) determined protocatechuic (0358 mg 100 g^{-1}), 4-hydroxybenzoic (2 mg 100 g^{-1}) and feluric (0.47 mg 100 g^{-1}) acids in *O. dillenii*,. The amount of phenolic compounds obtained in this study with the Atlixco variety was similar to other studies except for 4-hydroxybenzoic acid (0.02 mg 100 g^{-1}), known for its antimicrobial and antioxidant activity (Yang et al., 2009). In this study, chlorogenic, syringic, sinapic acids and the flavonoid (-) epicatechin were documented for first time in the Atlixco variety of *O. ficus-indica*; they are important due to their antioxidant, antibacterial, antiviral, anticancer, anti-mutagenic and anxiolytic properties (Othman et al., 2010).

Conclusions

The effect of temperature and presentation of cactus cladodes was significant on the survival of *S* Enteritidis and *E. coli*. *S.* Enteritidis and *E. coli* were able to grow and survive for 16 days at 4 and 18°C in cactus leaves with spines. In cactus leaves without spines, *S.* Enteritidis survived for 16 days at 4 and 18°C, while *E. coli* only survived during this period at 18°C. *Opuntia ficus-indica* (L.) Mill var. Atlixco presents phenolic compounds with antimicrobial potential that could reduce the pathogenicity of *S.* Enteritidis and *E. coli* associated with consumption of fresh cactus.

Conflict of Interests

The authors have not declared any conflict of interests.

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