



BRAZILIAN JOURNAL OF MICROBIOLOGY

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Food Microbiology

Growth kinetics of *Escherichia coli* O157:H7 on the epicarp of fresh vegetables and fruits



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ARTICLE INFO

Article history:

Received 17 September 2015

Accepted 20 October 2016

Available online 30 August 2017

Associate Editor: Beatriz Ernestina Cabilio Guth

Keywords:

E. coli O157:H7

Adhesion

Predictive growth

Low selectivity

Fruit and vegetables

ABSTRACT

Despite the increasing reports on the incidence of fresh vegetables and fruits as a possible vehicle for human pathogens, there is currently limited knowledge on the growth potential of *Escherichia coli* O157:H7 on different plant substrates. This study analyzed the selective adhesion and growth of *E. coli* O157:H7 on chili habanero (*Capsicum chinense* L.), cucumber (*Cucumis sativus*), radish (*Raphanus sativus*), tomato (*Lycopersicon esculentum*), beet (*Beta vulgaris* subsp. *vulgaris*), and onion (*Allium cepa* L.) under laboratory conditions. The Gompertz parameters were used to determine the growth kinetics. Scanning electron microscopy was used to visualize the adhesion of *E. coli* O157:H7 on the epicarp of the samples. Predictive models were constructed to compare the growth of *E. coli* O157:H7 on the samples with different intrinsic factors and to demonstrate the low selectivity of the pathogen. No significant difference was observed in the lag-phase duration (LPD), generation time (GT), and exponential growth rate (EGR) of the pathogen adhered to the samples. The interaction between the microorganism and the substrate was less supportive to the growth of *E. coli* O157:H7 for onion, whereas for tomato and cucumber, the time for the microorganism to attain the maximum growth rate (M) was significantly longer than that recorded for other samples.

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Introduction

Escherichia coli O157:H7 is a foodborne zoonotic pathogen that serving as the causative agent of hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans. This microorganism possesses a remarkable capability to get transmitted

from animal reservoirs (cattle, goats, and sheep) to humans through a variety of food vehicles that plays a critical role in its pathogenicity. *E. coli* O157:H7 employs multiple mechanisms for colonizing vegetables through adhesion to cell surfaces. The microorganism adheres strongly to tomato skin, spinach leaves and alfalfa sprout roots by curli,^{1,2} which is mediated by the filamentous type III secretion system composed of EspA filaments.³ Flagella also play a key role in the leaf attachment process in *E. coli* O157:H7.⁴ The internalization of *E. coli* O157:H7 has been demonstrated under controlled environmental conditions in the seedlings of temperate

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<https://doi.org/10.1016/j.bjm.2017.08.001>

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vegetable crops such as lettuce, tomato, cress, radish, alfalfa, and mung bean,^{5–8} and the adhesion and internalization of *E. coli* O157:H7 in mature leafy vegetables have been demonstrated both experimentally and in field conditions.^{9–13}

Despite the increasing incidence of fresh produce as a vehicle for human pathogens, there is a dearth of information on the growth potential of *E. coli* O157:H7 on different plant substrates. Although most of the outbreaks involving the occurrence of *E. coli* O157:H7 on leafy vegetables have been associated with cross-contamination^{14–17} there are very few studies that have investigated the adhesion and growth of *E. coli* O157:H7 in other mature vegetables.^{18–22} Interestingly, many of these vegetables possess a variety of intrinsic factors that can considerably reduce the growth of foodborne microorganisms; however, *E. coli* O157:H7 manages to successfully survive under these varying conditions.

Assuming that the degree of mutual interaction between bacterial strain and the specific type of plant affects their association, this study aimed to investigate the capacity of *E. coli* O157:H7 to colonize freshly consumed vegetables and fruits under experimental conditions. The growth of the pathogen on different substrates was compared using the Gompertz equation to obtain bacterial growth curves and linear regression-derived parameters such as the lag-phase duration, maximum microbial population, and specific microbial growth.

Materials and methods

Microorganism and preparation of inoculums

Escherichia coli O157:H7 strain (SS11) used in this study was isolated from the feces of pigs from Santa Elena in Yucatán, Mexico.²³ The strain was isolated after pre-enrichment with EC broth (Difco, Detroit, MI, USA) using the immunomagnetic separation procedure (IMS) employing anti-*E. coli* O157 Dynabeads (DynaL Biotech, Norway) as described by the manufacturer. The IMS-enriched bacterial culture (50 µL) was streaked onto Chromocult coliform agar (Merck, Darmstadt, Germany) and Sorbitol MacConkey agar (CM813; Oxoid Ltd, USA) supplemented with cefixime-potassium tellurite (SR172; Oxoid Ltd.). The phenotype of the microorganism was characterized with the API 20E system (BioMérieux, France). Strain serotype O157:H7 was identified with the immunocapture system and latex agglutination (Tecra Diagnostics, NSW, Australia). Polymerase chain reaction (PCR) was used to confirm the presence of *rfbO157* and *stx1* genes in the strain SS11 and to confirm the absence of *stx2* verotoxin gene. For this purpose, three pairs of oligonucleotide primers were synthesized for the amplification of *rfbO157* (420 bp), *stx2* (584 bp) and *stx1* (306 bp).^{24–26} The PCR products were detected by 2% agarose gel-electrophoresis at 100 V and staining with 10 µg/mL ethidium bromide.

The suspensions of *E. coli* (10⁴ CFU/mL) were prepared for the inoculation of the vegetables and fruits. A single colony of *E. coli* O157:H7 from a streak plate was suspended in 20 mL of EC selective enrichment broth (Difco) and incubated overnight at 37 °C (16–18 h) in a shaking incubator at 150 rpm

(Thermo Scientific, USA). The overnight culture (1 mL) was inoculated into 99 mL of EC broth and incubated at 37 °C till it attained the exponential phase (10⁵ CFU/mL). The bacterial cell concentrations were estimated by comparing the transmittance of the cell suspension with a previously determined standard curve at 540 nm using a UV-visible spectrophotometer. The final concentration of 10⁴ CFU/mL was obtained by diluting the bacterial suspension with 400 mL of EC broth, and the concentration of the inoculum was further confirmed by plating 0.1 mL of an appropriately diluted culture on TSA.

Selection and preparation of vegetables and fruits

The prepared *E. coli* O157:H7 suspension was inoculated in triplicate on three vegetables viz., radish (*Raphanus sativus*), beet (*Beta vulgaris* subsp. *vulgaris*), and onion (*Allium cepa* L.), and on three fruits viz. ripe red tomato (*Lycopersicon esculentum* L.), cucumber (*Cucumis sativus*), and green habanero chili (*Capsicum chinense* L.). Fresh and healthy-looking fruit and vegetable samples were purchased no more than one day prior to the inoculation from a local supermarket and stored at 10 °C till use. The samples with visible defects on the surface such as bruises, cuts, or abrasions were excluded from the experiment. The vegetables were decontaminated by immersing in 200 mg/L of chlorine solution (6.5 mg/L of free chlorine) for 2 min before inoculation. The decontamination procedures were previously standardized based on the absence of growth of Enterobacteriaceae on MacConkey agar. The samples were then rinsed with sterile deionized water for 1 min and air dried with paper towels in a biosafety cabinet (Esco, Infinity® Class II, Australia).

Growth of *E. coli* O157:H7 on the epicarp layer of fruits and vegetables

The samples were treated for five different periods (0, 3, 5, 7 and 9 h) of incubation to determine the adhesion capacity of the *E. coli* O157:H7. The samples were completely submerged into 400 mL of *E. coli* O157:H7 broth (10⁴ CFU/mL) for 30 min and gently shaken at 100 rpm at 37 °C. Then the samples were kept individually in sterile air bubble polyethylene bags (Stomacher® Bag, Seward, UK) for the defined period of time for each treatment. After incubation, the samples were washed thrice with sterile deionized water and air dried in a biosafety cabinet. In addition, two control samples were considered. The first one was an internal control of inoculated samples that were not subjected to the final washes, and second one was a negative control of inoculated samples that were decontaminated with 200 mg/L of chlorine solution in the final wash.

The adhesion of *E. coli* O157:H7 to the epicarp of vegetables and fruits was determined by spreading the samples on MacConkey agar. Three slices of the vegetable shell (10 g) were cut off aseptically and soaked in 90 mL of phosphate buffered saline (PBS, pH 7.1). After a five-fold serial dilution, 0.1 mL of the sample solution was inoculated on MacConkey agar in duplicate and incubated for 24 h at 35 ± 1 °C. The number of colonies observed in the plates was used to calculate the CFU/g of tissue.

Proximate analysis

AOAC (Association of Analytical Communities) official methods²⁷ were used for analysis of moisture (Method 925.09), total protein (Method 950.48), fat (Method 983.23), and ash contents of the samples (Method 930.05). pH values were measured by placing the flat electrode (Sensorex, Stanton, USA) directly against the surface of the exposed tissue at three different points. Proximate analysis of the epicarp was performed using the skin of the fruits and vegetables.

Scanning electron microscopy (SEM)

The adhesion of *E. coli* O157:H7 to the exocarp of the samples was visualized using a scanning electron microscope (Philips model XL 30 SEM microscope) operating at an accelerating voltage of 15 kV. A final pressure of 4 Torr (533.3 Pa) was gradually generated in the chamber in order to prevent the samples from drying, prior to performing the analysis.²⁸

Modeling of microbial growth

All the experiments were performed in triplicate. The microbial counts per gram of the sample were converted to $\log_{10}$ values before the calculations. The mathematical modeling of the microbial growth (predicted data) was performed by employing the modified Gompertz equation:

$$L(t) = A + C e^{-e(-B(t-M))},$$

where $L(t)$ is the log of the number of bacteria at time 't' (in hour). A is the asymptotic log of number of bacteria as 't' decreases indefinitely; C is the asymptotic amount of growth (log number) that occurs as 't' increases indefinitely; M is the time (in hour) at which the absolute growth rate is at maximum; and B is the relative growth at time 'M'.^{29,30}

The equation was fitted using a nonlinear regression routine of the SYSTAT statistical computer system package (SYSTAT Inc., Evanston, IL, USA). The algorithm was selected from the set of parameters with the lowest residual sum of squares with a 95% confidence interval. The Gompertz parameters (A , B , C , and M) were subsequently used to calculate the growth kinetics, including the exponential growth rate, EGR ($\log CFU/g/h$) = BC/e ; the lag-phase duration, LPD (h) = $M - 1/B$; the generation time, GT (h) = $\log(2)e/BC$; and the maximum population density, MPD ($\log CFU/g$) = $A + C$.

Statistical analysis

An analysis of variance (ANOVA) was performed to compare the growth parameters of *E. coli* O157:H7 obtained from the growth curves. The ANOVA and least significance difference (LSD) between the means were calculated at 95% confidence level using the SYSTAT software. The means that differed from the calculated LSD were considered statistically significant ($\alpha = 0.05$).

Results and discussion

This study investigated the quantification of the growth of *E. coli* O157:H7 on vegetables and fruits. Although the major disease outbreaks attributed to this pathogen have been associated with leafy vegetables, the results of this study showed that *E. coli* O157:H7 is also capable of adhering to vegetables with different physicochemical characteristics (Fig. 1). The methodology used in this study for the adhesion of *E. coli* O157:H7 was validated through internal controls. The bacterial cell that adhered to the surface of the samples formed a matrix with the epidermal layer (biofilm), which provided them adequate support to counter the effects of washing. *E. coli* O157:H7 strain possesses several fimbrial and non-fimbrial adhesins which usually do not play any role in its pathogenesis, but are involved with its adhesion to surfaces.³¹ For example, curli-expressing thin-aggregative fimbriae are responsible for binding to eukaryotic extracellular matrix proteins and promoting the formation of *E. coli* O157:H7 biofilms on inert surfaces.^{32,33} According to Torres et al.,³¹ the formation of biofilms increases the probability of survival of *E. coli* O157:H7, and hence, facilitates its protection against adverse environmental conditions. The samples from the vegetables and fruits that were not subjected to the final wash prior to plating (internal control) recorded higher bacterial concentration than those that were washed, suggesting the presence of both adhered and free microorganism on the surface of the samples. The SEM images of the samples obtained after the final washing clearly showed a layer of capsule bound to the cell wall of the samples that could possibly be associated with the exopolysaccharides secreted by the microorganism to form the matrix structure (Fig. 1D and E).

The Gompertz function fits well for the observed data with the sum of squares being less than 1 in all the cases (Fig. 2). Despite of the different intrinsic factors of the vegetables and fruits used in this study that could influence the growth of human pathogenic microorganisms, no significant difference was found in the lag-phase duration ($LPD = 2.17 \pm 1.03$ h; mean $\pm$ S.E.), generation time ($GT = 0.47 \pm 0.07$ h), or exponential growth rate ($EGR = 0.72 \pm 0.12 \log CFU/mL/h$) of the *E. coli* O157:H7 adhered to the samples (Table 1). Additional information on the proximate composition of the vegetables and fruits under investigation are shown in Table 2. These values are comparable to those reported by Kim et al.,³⁴ for perilla leaves inoculated with *E. coli* O157:H7 at 36 °C ($LPD = 2.40 \pm 0.32$ h; $EGR = 0.55 \pm 0.11 \log CFU/mL/h$). The growth of the *E. coli* O157:H7 strain on the epicarp of the vegetables and fruits was slower than that observed under controlled laboratory conditions. The growth model of Buchanan and Klawitter,³⁵ which considered the same temperature and pH conditions employed in this study (35 ± 1 °C; pH 6.0) displayed faster kinetic values than those observed in this study ($LPD = 1.54$ h; $GT = 0.33$ h; and $EGR = 0.92 \log CFU/mL/h$). This could be an outcome of the variation in the interactions that occur between the substrate and pathogen which might not necessarily occur in a growth medium. Similarly, the duration of the lag phase is dependent on a wide variety of factors, including the initial concentration of the inoculum, the time required to recover from the physical damage or shock due to the transfer, and the time required for

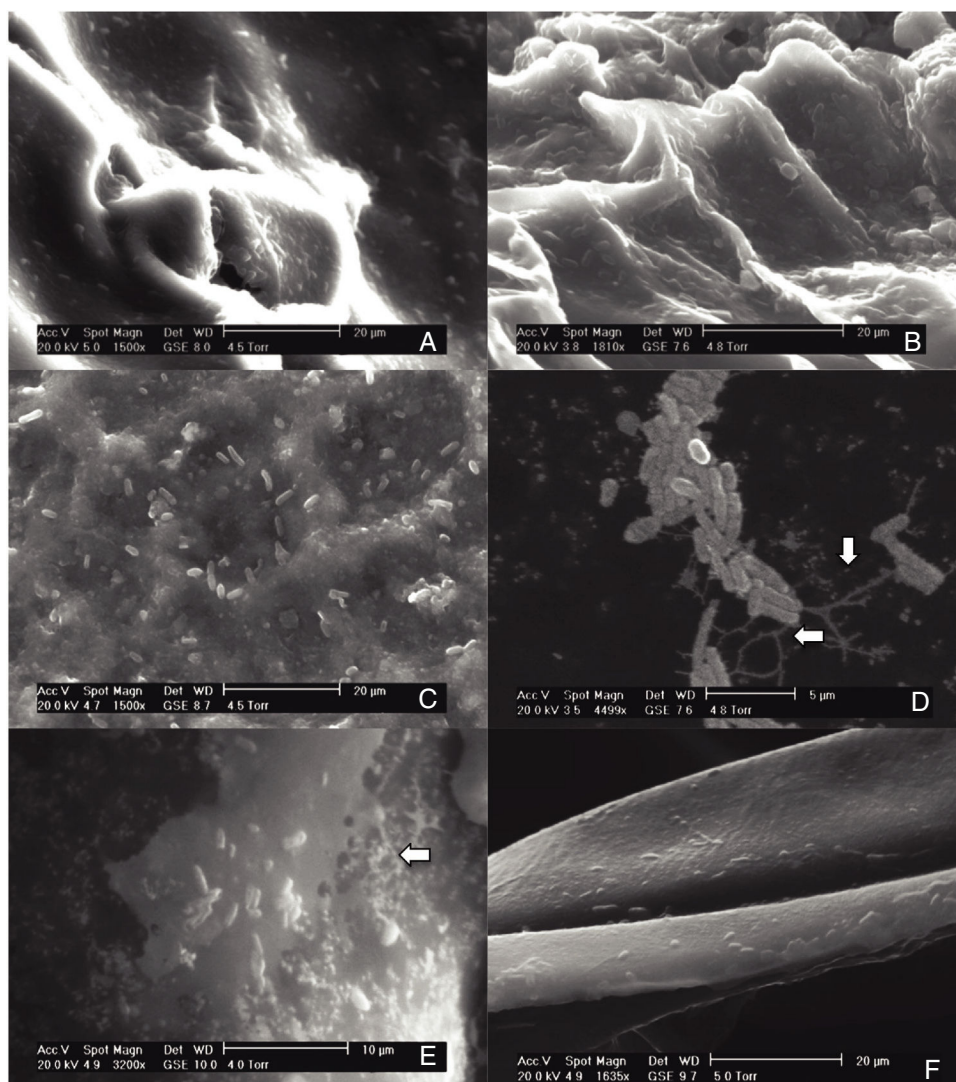


Fig. 1 – Scanning electron micrographs demonstrating the adhesion of *Escherichia coli* O157:H7 on the epicarp of vegetables and fruits, (A) beet, *Beta vulgaris* subsp. *vulgaris* (1550 $\times$); (B) habanero Chili, *Capsicum chinense* L., (1810 $\times$); (C) tomato, *Lycopersicon esculentum* (1500 $\times$); (D) radish, *Raphanus sativus* (4499 $\times$); (E) cucumber, *Cucumis sativus* (3200 $\times$); (F) onion, *Allium cepa* L. (1635 $\times$).

the synthesis of essential coenzymes or division factors and other enzymes involved in the metabolism of the substrates present in the medium.³⁶ In general when pathogens colonize plant surfaces, they are required to evade various defense mechanisms of the host plants. Plants possess a variety of complex immune responses, which enable them to detect and initiate counter measures to control undesired proliferation of foreign substances.³⁷ In addition, ethylene, a plant hormone, plays a vital role in the defense against plant pathogens.³⁸ Many microorganisms considerably increase the expression of ethylene; however the transcription level of ethylene induced by *E. coli* O157 were ten-fold lower, suggesting that either the plants do not sense the presence of the microorganism or that the microorganism evade the defense mechanisms of the plants consequently inhibiting the activation of ethylene.³⁹

E. coli O157:H7 growth was lower in onion than other samples suggesting an interaction that is not completely

favorable between the microorganism and the epicarp of the vegetable. In the case of tomato and cucumber, the time for the microorganism to reach the maximum growth rate (*M*) was significantly longer than that observed for other samples; the maximum population densities (MPD) for these vegetables were also the lowest (Table 1). The variation in the size of the bacterial populations among the different species has been attributed to a range of plant characteristics, including the leaf water content, pH, and the presence of bacterial inhibitory compounds. In addition, it occurs only when the water pressure on the surface of the product overcomes the internal gas pressure, and also it depends on the hydrophobic nature of the surface.^{40–42} *E. coli* grows in a broad pH range of 4.4–10.0 with an optimum pH of 6–7.⁴³ Specifically, *E. coli* O157:H7 can tolerate harsh acidic conditions, and few other strains have been reported to be able to survive even at pH 2.5–3.0 for over 4 h.⁴⁴ Arnold and Kaspar,⁴⁵ found that *E. coli* O157:H7 becomes

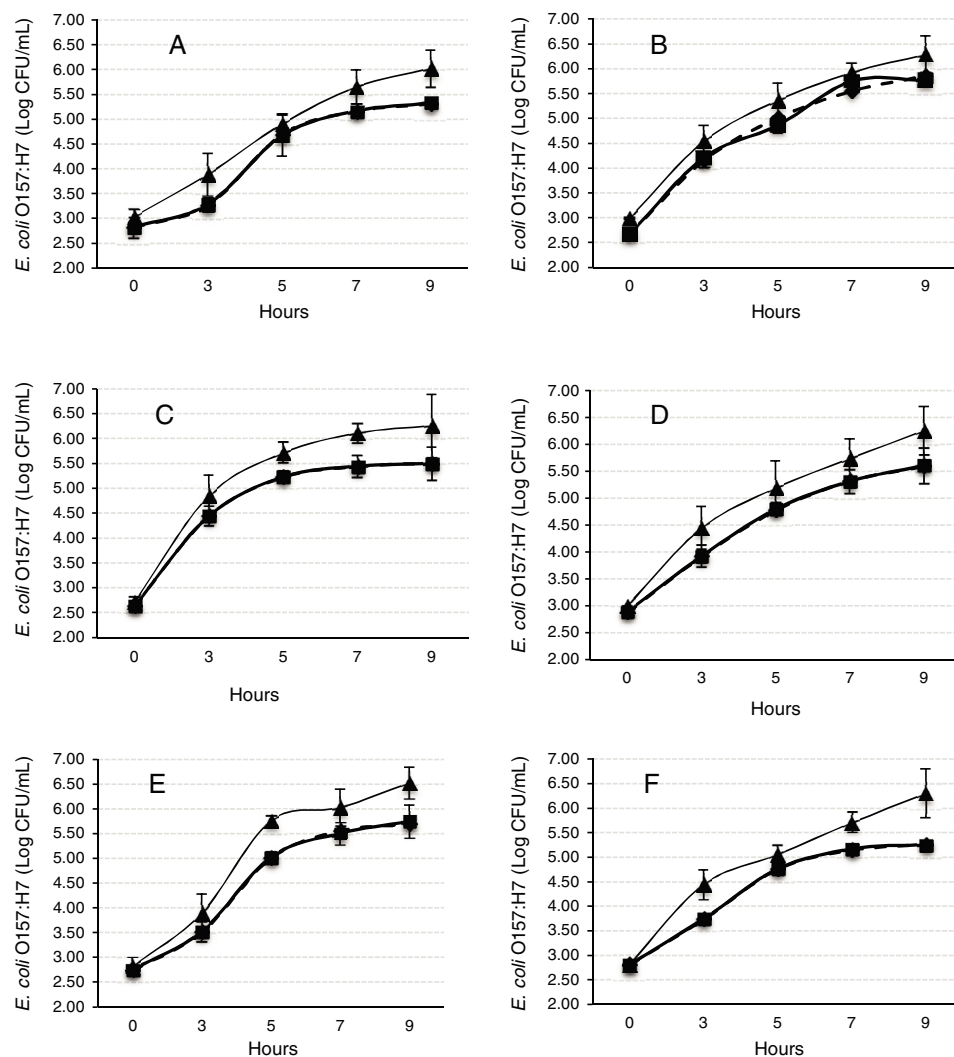


Fig. 2 – Observed and predicted data for *Escherichia coli* O157:H7 adhered to the epicarp of vegetables and fruits, (A) tomato; (B) radish; (C) cucumber; (D) beet; (E) habanero chili; (F) onion (▲: internal control; ◆: predicted data; ■: observed data).

more tolerant to acid when it is in the stationary growth phase or is starved during the log-phase of growth. The systemic pH of the vegetables and fruits used in this study is in a suitable range suitable for the growth of the pathogenic microorganism (Table 2), tomato and onion possess the lowest pH (4.77 and 5.40, respectively). This suggests that the pH in tomatoes might have influenced the growth rate of *E. coli* O157:H7 during the log-phase, as the pathogen took longer to reach the maximum growth rate (M) in tomatoes (Fig. 2, Table 1). This finding is in agreement with a study by Beuchat⁴⁶ with ripe tomatoes, which reported that the pH range of 3.9–4.4 present in the ripe tomatoes prevents or retards bacterial growth. Del Rosario and Beuchat⁴⁷ also suggested that vegetables and fruit juices with a pH value less than 4.0 are, in general, not good substrates to support the growth of *E. coli* O157:H7.

Other researchers have shown that MPD decreases significantly when the concentration of preservatives increases or when natural microbial compounds are present.⁴⁸ As in the case of cucumber the volatile oils and the methanolic

and dichloromethane extracted from the peel of *C. sativus* exhibited antimicrobial activity.⁴⁹ Cho et al.,⁵⁰ reported that the high content of the volatiles such as 2,6-nonadienal and 2-nonenal show strong activity against *E. coli* O157:H7. In contrast to this, there are no reports on the presence of antimicrobial compounds against human pathogens in the skin of tomato and onion. Although not specifically against *E. coli* O157:H7, but still some studies have reported antimicrobial activity of onion aqueous extracts against gram-negative microorganisms.⁵¹ However, Rounds et al.,⁵² showed that powdered onion induced the growth of *E. coli* by 1.2 log CFU/g, indicating that onion, like other vegetables, provides nutrients that enhance the growth of bacteria. Nevertheless, there is no conclusive explanation on the delay of the growth rate of *E. coli* O157:H7 on those vegetables.

In conclusion, the predictive models presented in this study offer a good comparative account of the growth of *E. coli* O157:H7 on samples with different intrinsic factors demonstrating low selectivity of the pathogen. The data suggest that

Table 1 – Mathematical modeling of the growth of *E. coli* O157:H7 adhered to the epicarp of vegetables and fruits. The Gompertz (A, B, C, M) and derived parameters (GT, generation time; EGR, exponential growth rate; LPD, lag-phase duration; MPD, maximum population density) are shown. Data are presented as mean $\pm$ standard error ($n = 3$). The letters between the columns indicate significant differences ($p < 0.05$).

	A (log CFU/mL)		B_{growth} (h)		C (log CFU/mL)		M (h)		GT (h)		EGR (log CFU/mL/h)		LPD (h)		MPD (log CFU/mL)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Tomato	2.819	0.12 a	1.498	0.60 a	2.060	0.59 a	3.631	0.03 a	0.398	0.11 a	0.881	0.23 a	2.895	1.53 a	4.279	0.54 a
Beet	2.139	0.12 b	1.684	1.30 a	2.729	1.20 a	2.009	0.38 b	0.562	0.03 a	0.540	0.03 a	2.230	1.50 a	4.868	0.27 b
Radish	2.507	0.15 a	1.431	0.63 a	2.537	0.80 a	2.032	0.13 b	0.336	0.07 a	0.968	0.18 a	1.026	0.37 a	5.044	0.69 b
Chili	2.573	0.15 a	1.344	0.92 a	2.351	1.00 a	2.066	0.55 b	0.632	0.07 a	0.491	0.06 a	2.709	1.30 a	4.924	1.05 b
Cucumber	2.742	0.05 a	1.790	0.84 a	1.971	0.79 a	3.351	0.17 a	0.413	0.10 a	0.806	0.16 a	1.966	0.69 a	4.314	0.85 a
Onion	2.804	0.03 a	1.286	0.59 a	1.921	0.61 a	2.961	0.06 a	0.480	0.06 a	0.647	0.08 a	2.193	0.74 a	4.125	0.63 a
F-value	5.193		0.053		0.147		3.766		1.953		1.847		0.349		4.052	
p-value	0.009		0.998		0.977		0.045		0.159		0.178		0.873		0.048	

Table 2 – Epicarp proximal composition (%) of vegetables and fruits ($n = 5$).

	Moisture		Ash		Crude fiber		pH	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Tomato	95.4	0.23	0.74	0.04	0.57	0.22	4.77	0.08
Beet	81.6	0.25	1.69	0.13	1.11	0.24	6.44	0.07
Radish	90.0	0.76	1.34	0.37	0.65	0.08	7.03	0.04
Chili	92.3	0.35	0.76	0.01	2.98	0.19	6.30	0.40
Cucumber	94.9	0.17	0.64	0.03	1.25	0.07	7.08	0.11
Onion	95.1	0.27	0.27	0.03	0.47	0.04	5.40	0.60

selectivity of the bacterium, *E. coli* O157:H7 strain SS11 slightly depends on the intrinsic factors of the vegetables and fruits under study, with the clear exception of tomato, cucumber and onion, wherein the delay in the growth of the pathogen was significant. However, it is important to note that the growth models constructed under controlled environmental conditions offer limited insights and may vary widely from the observed profile of vegetables and fruits in the field, since this study was designed to inoculate the samples with *E. coli* O157:H7 without the presence of competitive microflora on the surface. A lower concentration of the microorganism could also be expected in the field than that was employed in the study in the laboratory. The results reported by Brackett,⁵² suggested that reducing the native microbial populations by washing and sanitizing or by controlled atmosphere storage, enables proliferation of human pathogens on the surface of vegetables. The reduction in the surface populations reduces competition for space and nutrients thereby providing growth potential for pathogenic contaminants. In theory, this scenario can result in an unspoiled product that is unsafe for consumption. Future research in this field should aim to verify the potential for adhesion and internalization of *E. coli* O157:H7 in the tissues of vegetables under realistic field conditions.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgement

The authors are grateful to Ana Ruth Christopher Ramos from CINVESTAV-Mérida (México) for technical assistance with the scanning electron microscopy. We also thank Daniela A. Acevedo Ojeda from UMM for assistance with PCR analysis. This project was supported by FOMIX-CONACYT – Gobierno del Estado de Yucatán (México), grant no. 108103.

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