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Original article – Thematic Issue

Survival and internalization of *Escherichia coli* in baby chard subjected to ozone applications during hydroponic system cultivation

M.A. Faicán-Benenaula[1,2,3], C. Hernández-Adasme[3], A. Machuca[3] and V. Escalona Contreras[3]

[1] Instituto de Tecnología de Alimentos, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe, Argentina
[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Santa Fe, Argentina
[3] Centro de Estudios Postcosecha (CEPOC), Facultad de Ciencias Agronómicas, Universidad de Chile, Santiago, Chile

Summary

***Escherichia coli* (*E. coli*) has been linked to multiple outbreaks of foodborne illness associated with the consumption of leafy vegetables. Typically, sanitizers only reduce microbiological load up to 2 log units, suggesting that pathogens survive inside the leaves. Contamination can occur in different crop production systems, such as field cultivation, cultivation in closed production systems, and hydroponic cultivation. Without proper safety measures, pathogenic microorganisms can contaminate leafy vegetables in hydroponic production systems. This study aimed to evaluate the survival of *E. coli* in chard plants (*Beta vulgaris* L. cv. SCR 107) whose nutrient solution was subjected to different doses of ozone (O_3) (0, 0.5, and 2 mg L^{-1}) for 3 min in floating root hydroponic culture. Furthermore, the study aimed to investigate the possible internalization of this bacteria in chard plants. Two experiments were conducted; the first consisted of applying O_3 to the nutrient solution inoculated with 10^8 CFU mL^{-1} of *E. coli*. Survival and internalization of bacteria from the nutrient solution to the roots and leaves were measured by plate counts. In the second experiment, *E. coli* was introduced into the nutrient solution (10^8 CFU mL^{-1}) and leaves (10^6 CFU mL^{-1}) separately; subsequently, bacterial internalization was assessed by microscopy. The results indicated that, although the application of O_3 reduced *E. coli* counts, the bacteria resisted the treatment and successfully penetrated the leaves of chard plants.**

Keywords
bacteria, *Beta vulgaris*, hydroponic system, nutrient solution, pathogen, sanitizers

Significance of this study

What is already known on this subject?

- Previous research has noted that ozone application during the cultivation of baby chard affects the survival and internalization of *E. coli*. The impact of ozone on contamination, penetration, and plant responses is explored, but further research is essential to fully evaluate the efficacy and mechanisms.

What are the new findings?

- Recent research reveals the impact of ozone on *E. coli* survival in chard, suggesting the potential of ozone to reduce contamination. Ozone alters surface occurrence and infiltration, with possible safety implications. Further research is required.

What is the expected impact on horticulture?

- Ozone applications in chard cultivation offer transformative potential for horticulture, enhancing safety by reducing the risk of *E. coli* contamination and reshaping cultivation practices for improved leafy vegetable quality and consumer health. Ozone is a naturally occurring gas in the environment, making it a sustainable alternative as a non-polluting sanitizer.

Introduction

Due to their vitamins, minerals, and dietary fiber content, regular consumption of vegetables plays an important role in human nutrition. Consumers' search for a healthy diet has increased the demand for vegetables, including leafy vegetables (Finger et al., 2023). However, with the increasing global demand for fresh vegetables, foodborne pathogen contamination related to their consumption continues to persist (Castro-Ibañez et al., 2017; Sela Saldinger et al., 2023; Zhao et al., 2022). Vegetables, including leafy ones, are usually grown in open lands and prone to microbial contamination at pre- and post-harvesting stages (Finger et al., 2023). Contamination sources with foodborne pathogens include contaminated water, soil, and soil amendments, such as untreated or partially treated manure fertilizer, the proximity of poultry and livestock facilities, and contact with wildlife. Contaminated irrigation water is considered the most important risk factor for preharvest produce contamination (Delaquis et al., 2007; Harapas et al., 2010; Gil et al., 2015; Sela Saldinger et al., 2023). In that sense, one of the most prominent enteric pathogens responsible for foodborne disease outbreaks associated with the consumption of leafy vegetables is *E. coli* (Warriner and Namvar, 2010; Sela Saldinger et al., 2023; Zhao et al., 2022).

During the minimal processing of vegetables, the primary strategy to mitigate the risk of contamination by pathogenic microorganisms is to sanitize by washing (Hinojosa et al., 2013). Various sanitizers are used by the industry, including sodium hypochlorite, hydrogen peroxide, and UV-C radiation, among others (Silveira et al., 2008; López-Gálvez et al., 2009; Silveira et al., 2015; Green et al., 2024). Nonetheless, these sanitizers exhibit limited efficacy in terms of redu-

cing the microbiological load by no more than 2 log CFU g^{-1} (Gómez-López et al., 2009; Hinojosa et al., 2013; Escalona et al., 2015). This observation suggests the presence of a subset of these pathogens capable of internalizing and surviving within plant tissue despite the utilization of sanitizers (Deering et al., 2012).

Previous studies demonstrated that foodborne bacteria, including *Salmonella* and *E. coli,* can be internalized into plants by two major routes: (1) bacteria can move into plants through physical opening sites in the plant surface (stomata, lenticels, and root) or mechanically damaged areas of the plant surface; and (2) bacteria have the motility ability for penetrating the epidermis of plants (Koseki et al., 2011; Saldaña et al., 2011; Olaimat and Holley, 2012; Gil et al., 2015; Sela Saldinger et al., 2023; Kim et al., 2023). When considering leafy vegetables, the surface properties are critical in influencing bacterial cell adhesion to plant tissue. Studies conducted with iceberg lettuce (Kroupitski et al., 2009) and basil (Golberg et al., 2011) have noted negligible adherence of *Salmonella* spp. on leaves. In contrast, arugula and parsley leaves exhibited notable adherence of this bacteria in regions encompassing the cuticle and beneath the stomata (Golberg et al., 2011). Nevertheless, there are no studies on the adherence of human pathogens in chard. Hence, understanding the contamination pathways and the interaction between enteric pathogens and plant tissues is essential as a fundamental prerequisite for formulating innovative intervention strategies to ensure the safety of fresh salads (Gil et al., 2015).

Hence, applying safe food handling practices and procedures at every stage of the food production chain reduces the risk of contamination and prevents harm to consumers. Unlike traditional farming methods, soilless indoor production, such as hydroponics, provides a highly regulated environment that is easier to manage and avoids the growth of harmful microorganisms in the cultivation facility, as long as careful cultivation practices are followed (Sela Saldinger et al., 2023). However, certain authors consider that hydroponic cultivation may pose risks due to inadequate disinfection and/or contamination within the production units (Park et al., 2012). Although this cultivation system efficiently optimizes available resources, it may introduce a risk of pathogen spread due to the accumulation and dissemination of disease vectors in the recirculated nutrient solution (Johansson et al., 2002; Hong and Moorman, 2005). To mitigate this problem, different technologies were employed to prevent degradation and the proliferation of pathogenic microorganisms within the solution (Hong et al., 2003; Graham et al., 2011). Thus, ozone (O_3) is an alternative because it is a superior disinfectant powder to conventional disinfectants such as chlorine and its derivatives. Ozone uses can have many advantages in the food industry, such as surface sanitization, control of harmful microorganisms, and reuse of wastewater (Peng et al., 2018; Martínez-Sánchez and Aguayo, 2019; Chhem-Kieth et al., 2022; Risoli and Lauria, 2022). When microorganisms are exposed to O_3, their cell walls are destroyed, and internal organic matter is oxidized. The ozone could directly disrupt viral and bacterial metabolism and reproduction by breaking down macromolecule polymers (Peng et al., 2018). Moreover, O_3 is gaining attention because it is a 'zero residue' practice since O_3 naturally decomposes into hydrogen peroxide (H_2O_2) and hydroxide (OH)-radicals (Martínez-Sánchez and Aguayo, 2019; Risoli and Lauria, 2022).

O_3 bubbled into the water can be applied directly to the soil or by foliar spraying, while the nutrient solution can be ozonated and employed in hydroponic cultivation. These treatments are promising methods for improving soil physicochemical properties, inactivating a wide range of harmful microorganisms in the soil, controlling plant diseases, and avoiding chemical residues in vegetables (Risoli and Lauria, 2022). The aqueous decomposition reactions of O_3 also increase its pathogen elimination capacity during the treatment (Pinto et al., 2017). The transformation into hydroxyl radicals is crucial for its effectiveness in eliminating water pathogens and making it a safer and more effective method for disinfection in hydroponic systems. Given the above, this study aimed to assess the survival and internalization of *E. coli* in chard plants cv. SCR 107 exposed to O_3 applications during hydroponic cultivation.

Materials and methods

Bacterial strain and inoculum preparation

The non-pathogenic strain *E. coli* ATCC® 35218TM obtained from the bacterial strain collection at the Postharvest Studies Center (CEPOC) was used (strain origin: canine feces, detailed strain information available at http://www.atcc.org/products/all/35218.aspx). The strain was stored at -80°C in tryptic soy broth (Merck, Darmstadt, Germany)

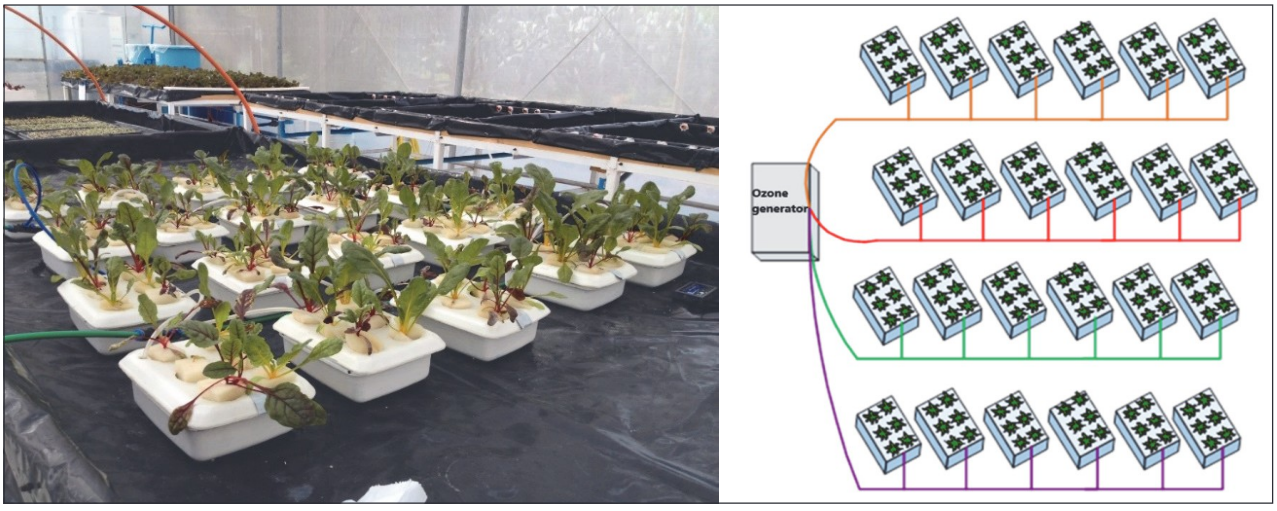

Figure 1. Closed floating root hydroponic cultivation system. Each cultivation unit corresponds to the experimental unit with 6 plants (below). Four treatments were applied (O_3 doses: 0, 0.5, and 2 mg L^{-1}).

Table 1. Composition of Hoagland II nutrient solution (Hoagland and Arnon, 1950).

Macronutrients	mM
N	6.0
P	1.0
K	3.0
Ca	2.0
Mg	1.0
S	1.0
Micronutrients	**µM**
B	25.0
Mn	5.3
Zn	3.8
Cu	4.0
Mo	0.26
Cl	60.7
Na	50.0
Fe	53.7

with 10% glycerol until use. To activate the strain, it was incubated in 50 mL of CASO broth (Merck, Santiago, Chile) with constant agitation at 150 rpm for 12 to 24 h at 37°C.

Plant material and cultivation conditions

Chard plants (*Beta vulgaris* L. var. *cicla* cv. *SCR 107*) were used in the study. The chard seeds were sown in polyethylene trays of 200 alveoli of 18 mL (Protekta®) containing an inert substrate mixture composed of granulated rockwool (Agrolan®, Compañía Industrial El Volcán S.A., Santiago, Chile) and expanded perlite A6 (Harbolite Chile Ltda., Santiago, Chile) hydrated in a volumetric ratio of 2:1. The seedlings were watered daily with tap water until the cotyledons were fully expanded. Then, they were rinsed with a modified Hoagland II nutrient solution (Table 1) (Hoagland and Arnon, 1950) diluted to 50%.

Once the plants reached 3 to 4 true leaves, they were transplanted to a floating root hydroponic system (Figure 1). This system consisted of 9 polystyrene trays measuring 20 × 16 × 7 cm, each containing 1 L of 100% Hoagland II nutrient solution, with 6 plants per tray.

After 3 days from transplantation, the nutrient solution was inoculated with 1 mL of *E. coli* ATCC® 35218TM at doses of 10^6–10^8 CFU mL^{-1}, confirmed through plate counting on eosin methylene blue (EMB) culture medium (Merck, Darmstadt, Germany) at 37°C. The temperature of the nutrient solution fluctuated between 22–25°C. Throughout the 10-day experiment, chard plants were grown at 22 to 27°C and 85% relative humidity.

Experiment 1. Survival and internalization of *E. coli* from nutrient solution and O_3 application

The nutrient solution in the floating root trays was inoculated at 10^8 CFU mL^{-1} of *E. coli*. After 2 days from inoculation, a single application of O_3 was injected directly into each tray for 3 min, using the doses according to the designated treatment. To generate O_3, the methodology described by Beltrán et al. (2005) was used. An O_3 generator (Atlas 30g, Ecozone, Alberta, Canada) that obtained the gas from dry air and an electrical discharge generator was used. The doses used were 0, 0.5, and 2 mg L^{-1}, and they were monitored using a portable O_3 analyzer (Chemetrics, model I-2019, New York, U.S.A.). Additionally, a negative control treatment was performed without inoculation and O_3. The treatments were: without inoculum + 0 mg L^{-1} of O_3 (D0); 0 mg L^{-1} of O_3 + *E. coli* (D0Ec); 0.5 mg L^{-1} of O_3 + *E. coli* (D05Ec), and 2 mg L^{-1} of O_3 + *E. coli* (D2Ec). The duration of this experiment was determined according to the harvest criterion of baby leaves, which reached 8 to 13 cm in length after 10 days of cultivation.

Microbiological analysis

Microbiological counts were performed using plate counting to determine the survival and internalization of *E. coli*. Roots and leaves were washed twice with sterile distilled water by dipping to remove non-adherent bacteria and then disinfected by immersion in 100 mg L^{-1} sodium hypochlorite at a pH of 6.5 for 1 min. Ten grams of leaves per replicate were used and placed in sterile bags (Bags 6141, London, England). Samples were macerated in 10 mL of sterile peptone water and then plated in an EMB culture medium for two days at 37°C. For the roots, 1 g of tissue was used. Subsequently, serial dilutions were made and plated under the same conditions described for leaves. After inoculation, analysis was performed on days 1, 2, 3, 5, and 10.

Experiment 2. Internalization of inoculated *E. coli* in the nutrient solution and chard tissue

This experiment was performed to verify the internalization of *E. coli* through chard leaves and roots, inoculating the pathogen in the leaves and the nutrient solution. Chard plants were grown under the same conditions as described in Experiment 1, but this time individually in glass jars containing 200 mL of nutrient solution. *E. coli* was dyed using the reagent 5-(4, 6-dichlorotriazinyl aminofluorescein) (5-DTAF) (Merck, Darmstadt, Germany). For this purpose, 30 mL of the broth containing *E. coli* ATCC® 35218TM was centrifuged at 2,700 *g* for 10 min at room temperature, forming a bacterial pellet. The supernatant was discarded, and the pellet was re-suspended in 10 mL of phosphate-buffered saline. Then, 2 mg of 5-DTAF was added, homogenized, and incubated in a water bath at 37°C for 2 h.

To evaluate the internalization in the leaves, 100 µL of DTAF-stained *E. coli* ATCC 35218 (10^6 CFU mL^{-1}) was inoculated at five different points on the leaf surface. The third leaf of the plants was used for the inoculation. To determine internalization from the nutrient solution, 100 µL of DTAF-stained *E. coli* ATCC 35218 (10^8 CFU mL^{-1}) was inoculated into the solution. Two days after inoculation (both leaves and nutrient solution), samples of leaf lamina, petiole, and roots were taken and processed for microscopic observation. For the leaf lamina samples, two rectangular pieces were extracted from each side of the central vein in the middle zone of the leaf per repetition. Petiole samples were extracted by making a longitudinal cut. Three cuts were taken per petiole and repetition. In the root samples, 8 random root hairs were chosen for examination per each repetition. Images were captured using a trinocular microscope (Motic BA301, China) equipped with a blue-purple DAPI epifluorescence filter (wavelength range 358–492 nm) and 40×, 100×, 400×, and 1,000× objective zooms.

Experimental design and statistical analysis

The experiment was conducted in a completely randomised design ($n = 3$). In Experiment 1, each experimental unit consisted of a floating root tray holding six plants. Each tray constituted a single repetition, with three repetitions

per treatment. For Experiment 2, the experimental unit encompassed one plant per jar containing 200 mL of nutrient solution, with three repetitions per treatment. In both experiments, microbiological counts of *E. coli* were processed using Infostat statistics software (v. 2015, FCA, Universidad Nacional de Cordoba, Cordoba, Argentina) and subjected to a one-way analysis of variance (ANOVA). Given the significant differences observed, Tukey's multiple comparisons test was used at a significance level of 5%.

Results

Experiment 1. Survival of *E. coli* in nutrient solution and O_3 applications

All inoculated nutrient solution samples tested positive for *E. coli*, while the pathogen was not detected in D0. The initial average bacterial count in the nutrient solution was 7.82 log CFU mL^{1}, measured one day after inoculation. On day 2, D05Ec significantly decreased the *E. coli* counts (4.67 log CFU mL^{-1}) compared to D0Ec and D2Ec (Figure 2). The same trend persisted on day 3, but no differences were observed among the O_3 treatments on day 5. Ten days after inoculation, the bacterial counts were lower than the initial count and ranged from 5.0 to 5.8 log CFU mL^{-1} without significant differences among the O_3 treatments and control (Figure 2).

Experiment 1. Survival and internalization of *E. coli* in roots

Significant differences in *E. coli* counts were observed in all treatments compared to D0 on all days assessed (Table 2). Throughout the entire experiment, *E. coli* was not detected in non-inoculated roots (detection limit <1 log CFU mL^{-1}). No variations in *E. coli* counts were noted after one day of inoculation. However, on day 2, D05Ec and D2Ec treatments significantly reduced *E. coli* counts compared to D0Ec treatment by 23.0% and 22.3%, respectively. A similar pattern was observed on day 5. Specifically, treatments D05Ec and D2Ec significantly reduced *E. coli* counts compared to D0Ec by 12.1% and 9.3%, respectively. On day 3, treatment D05Ec significantly decreased the pathogen counts concerning D0Ec by 29.8%. On the final evaluation day (day 10), the D05Ec treatment significantly reduced the *E. coli* count compared to D2Ec by 15.4% (Table 2).

Experiment 1. Survival and internalization of *E. coli* in leaves

The non-inoculated treatment D0 consistently maintained non-detectable levels of *E. coli* (<1 log CFU mL^{-1}) throughout the entire experimental duration, significantly lower than the levels observed in the inoculated treatments. On day 1, inoculated treatments ranged similar *E. coli* counts from 3.21 to 3.72 log CFU mL^{-1} (Table 2). Between days 2 to 10 after inoculation, D05Ec and D2Ec significantly diminished the counts compared to D0Ec (Table 1). In the first two evaluations, D05Ec decreased *E. coli* counts by 8 to 9%, while on days 5 and 10, the reductions were 10 and 12%, respectively. The D2Ec reduced the presence of *E. coli* by 3 to 5% during the first two evaluations and, in the last two evaluations, decreased by 9 to 10% (Table 2).

Experiment 2. Internalization of inoculated *E. coli* in the nutrient solution and chard tissue

According to Figure 3A, a cluster of *E. coli* bacterial cells was detected attached and internalizing inside a root hair when the nutrient solution was inoculated with bacteria. Moreover, microscopic images obtained from the petiole revealed the presence of *E. coli*, particularly within the spongy parenchyma cells (Figure 3B). However, when the leaves were examined, the bacteria were absent.

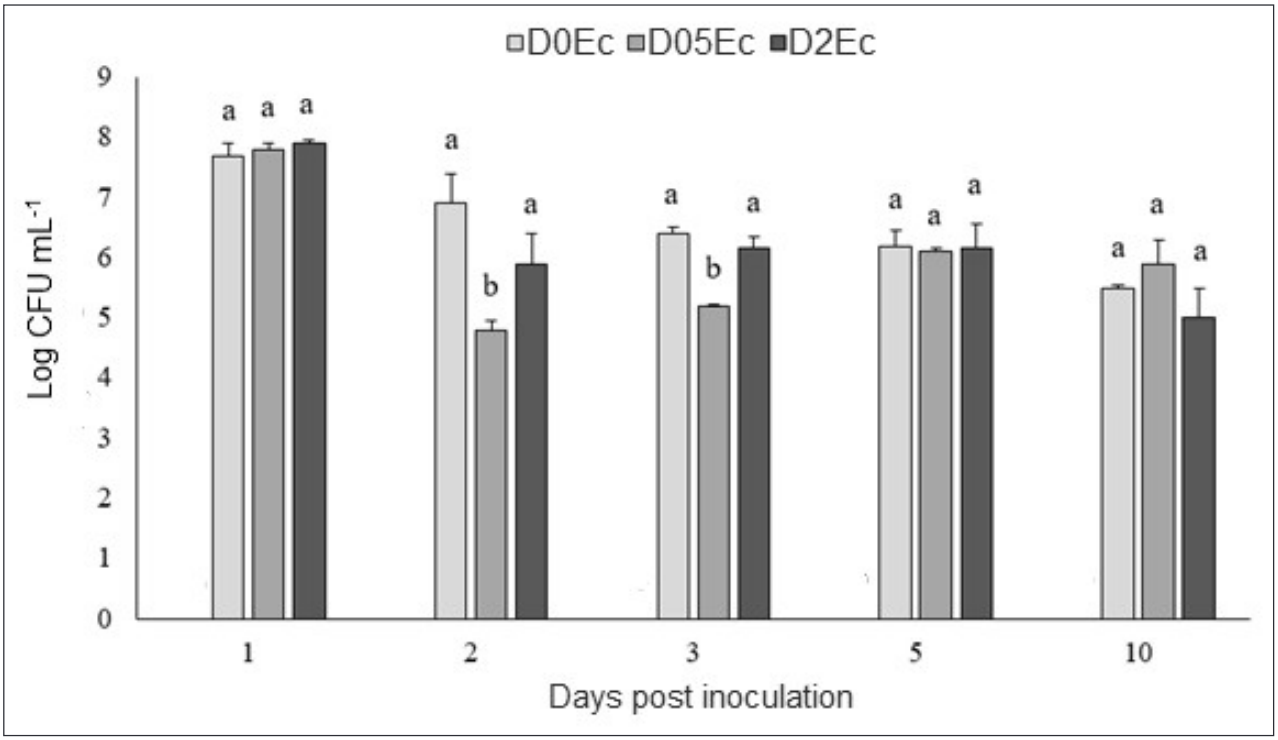

Figure 2. *E. coli* counts in the nutrient solution inoculated with 8 log CFU mL^{-1} of *E. coli* ATCC 35218 and subjected to three doses of O_3 (0, 0.5, and 2 mg L^{-1}) for 3 minutes, applied on day 2 post-inoculation. D0 maintained non-detectable levels of *E. coli* (<1 log CFU mL^{-1}) in all evaluation days. Different letters on the bars indicate significant differences between O_3 doses, according to Tukey's test ($p \leq 0.05$).

No bacterial presence was observed in the control treatment without inoculation (Figure 4A). Direct bacterial inoculation of leaves allowed the observation of *E. coli* cells within a stoma on the adaxial surface of the leaf and on the leaf surface itself (Figure 4B). Based on the topography of the chard leaf surface, it could be observed that the bacteria inhabited leaf depressions. Stomatic images corresponded to the third leaf of chard and showed high efficiency of pathogen internalization through stomata and adherence to leaf surface tissue. Most of the stomata of the examined leaves remained partially open after inoculation (Figure 4B). On the other hand, microscopic observation of the tissues after inocula-

Table 2. *E. coli* counts (log CFU mL^{-1}) in chard (cv. SCR 107) roots and leaves grown in nutrient solution inoculated with the bacteria and subjected to O_3 application.

	Treatments	Days after inoculation				
		1	2 *	3	5	10
		log CFU mL^{-1}				
Roots	D0 (control)	<1^{b} **	<1^{c}	<1^{c}	<1^{c}	<1^{c}
	D0Ec	5.28^{a}	5.65^{a}	5.88^{a}	4.86^{a}	4.87^{ab}
	D05Ec	5.44^{a}	4.35^{b}	4.13^{b}	4.27^{b}	4.41^{b}
	D2Ec	5.79^{a}	4.39^{b}	4.66^{ab}	4.41^{b}	5.21^{a}
Leaves	D0 (control)	<1^{b}	<1^{c}	<1^{c}	<1^{c}	<1^{c}
	D0Ec	3.72^{a}	3.78^{a}	4.02^{a}	4.28^{a}	4.81^{a}
	D05Ec	3.63^{a}	3.45^{b}	3.70^{b}	3.76^{b}	4.32^{b}
	D2Ec	3.21^{a}	3.67^{b}	3.81^{b}	3.89^{b}	4.34^{b}

* On day 2, the O_3 application was performed. The count was conducted after 2 h following ozonation.
** Values represent the means ($n=3$).
Different letters in the same column indicate significant differences between treatments according to Tukey's test ($p \leq 0.05$).

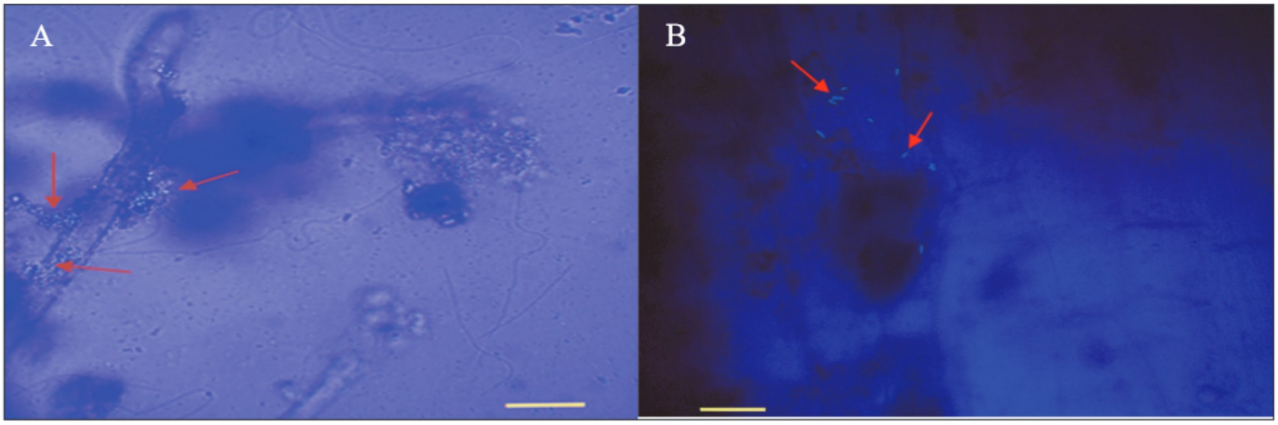

Figure 3. Chard root hair in inoculated nutrient solution with 6 to 8 log CFU mL^{-1} of *E. coli* ATCC 35218 (A). Chard petiole in inoculated nutrient with 8 log CFU mL^{-1} of *E. coli* ATCC 35218 (B). Image taken 2 days after inoculation using a Motic BA310 microscope, 40× with DAPI epifluorescence filter. Yellow line represents 100 and 10 µm in A and B, respectively. Red arrows indicate groups of *E. coli* bacteria adhered and internalized in a root hair (A) and presence within the cells of the spongy parenchyma of the petiole (B).

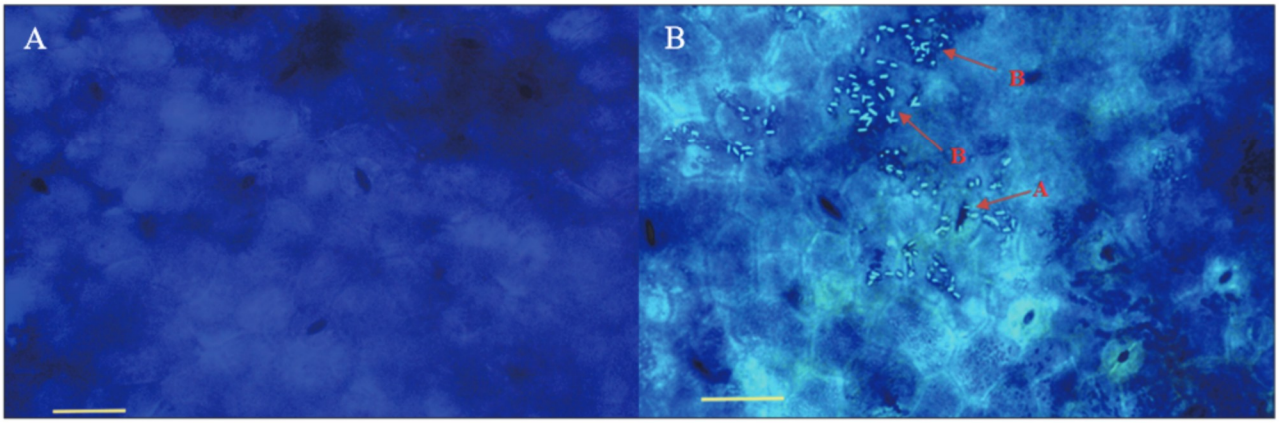

Figure 4. Chard leaf without inoculation (A) or inoculated with 6 log CFU mL^{-1} of *E. coli* ATCC 35218 (B). Images taken 2 days after inoculation using a Motic BA310 microscope, 400× with DAPI epifluorescence filter. Yellow line represents 50 µm. Red arrows indicate the presence of *E. coli* within the stomata on the adaxial surfaces of the leaves.

Table 3. Microscopic detection of *E. coli* in roots, petioles, and leaves from inoculated nutrient solution and leaves.

Inoculum localization *	Part of the examined plant	Number of positive samples for *E. coli* / Number of cuts made
Nutrient solution	Root	8/8
Leaves		0/8
Nutrient solution	Petiole	6/9
Leaves		0/9
Nutrient solution	Leaves	0/6
Leaves		6/6

* *E. coli* was introduced into the nutrient solution and leaves separately.

ting the nutrient solution with *E. coli* showed that internalization was evident in roots, petioles, and leaves. However, no pathogen was present when inoculation was performed only on leaves, roots, and petioles (Table 3).

Discussion

Experiment 1. Survival and internalization of *E. coli* from nutrient solution and O_3 application

The presence of human pathogens in vegetables is a growing concern. Cross-contamination of leafy vegetables with fecal residues from soil, through irrigation with contaminated water, or due to poor nutrient solution management suggests the possibility of internalizing human pathogens in the leaves (Koseki et al., 2011; Sun et al., 2019). Among human pathogens, *E. coli* is gram-negative, facultatively anaerobic, rod-shaped bacteria, typically 1.1 to 1.5 µm wide, 2 to 6 µm long, and occurs as single straight rods (Desmarchelier and Fegan, 2016). The World Health Organization (WHO, 2015) report titled 'Estimates of the Global Burden of Foodborne Diseases' (2015) stated that *E. coli* infections caused approximately 322 million illnesses and about 200 thousand deaths worldwide. Illnesses caused by *E. coli* are mainly due to the consumption of undercooked food or contaminated raw vegetables, so disinfection is important (Engels et al., 2012).

Among the sanitizer tools, ozone has attracted attention due to its high antioxidant power and, therefore, its powerful disinfectant action (Han et al., 2020; Risoli and Lauria, 2022). This property because injected ozone is decomposed into strong oxidizing agents such as hydrogen peroxide and hydroxyl anion (Martínez-Sánchez and Aguayo, 2019). In addition, ozone can be supplied in low doses by foliar spraying, soil drenching, seed preconditioning, or nutrient solutions in hydroponic systems without leaving chemical residues on crops (Risoli and Lauria, 2022). According to Martinez-Sanchez and Aguayo (2019), ozonation decreased the microbial load of water, but human pathogenic bacteria found in vegetables showed variable responses to this gas. Kobayashi et al. (2011) mentioned that to decrease the counts of *Pectobacterium carotovorum* subsp. *carotovorum*, doses of 0.1 to 1 mg L^{-1} for 1 min were required. Some studies indicated that the minimal effective doses for managing bacteria, fungi, oomycetes, and viruses were 0.5, 0.7, 0.8, and 7.9 mg L^{-1} of O_3 for 1, 16, 8, and 75 min, respectively (Kobayashi et al., 2011; Runia, 1994). Igura et al. (2004) underscored that treating with 1.0 mg L^{-1} of ozonized water for 10 min was required to reduce *Fusarium oxysporium* by 4 logarithmic cycles. For *E. coli*, Karaca and Velioglu (2014) noted that ozonated water washes (12 mg L^{-1} ozone) resulted in mean log reductions of 2.0 ± 0.3 log CFU in lettuce, spinach, and parsley. Our results indicated that after 3 min of exposure to O_3, *E. coli* counts showed a significant reduction in the nutrient solution with O_3 doses of 0.5 and 2 mg L^{-1}. However, the lowest dose was the most effective (Figure 2). This is because O_3 interacts with various compounds in aqueous solutions, such as nitrates, phosphates, and sulfates. Nitrates can generate free radicals in the presence of ozone, accelerating the decomposition of this gas. In contrast, phosphates are less reactive with ozone but may act as catalysts, promoting the formation of free radicals that expedite ozone breakdown, especially at high concentrations. Reactive sulfate radicals can be generated through the interaction between ozone and sulfates, decreasing the ozone concentration (Lim et al., 2022).

Anions and cations in the nutrient solution could provoke rapid ozone degradation, reducing its effectiveness as a sanitizer, which is crucial for systems requiring precise ozone control to eliminate pathogens. In the roots, there was a reduction in *E. coli* counts on days 2 and 5 post-inoculation. However, on days 3 and 10, there were no significant differences in *E. coli* counts between D2Ec and D0Ec treatments.

Regarding roots and leaves, a decrease in *E. coli* counts post-inoculation was also observed for both applied O_3 doses, although 0.5 mg L^{-1} was more efficient in these plant organs (Table 2). Initially, a reduction in *E. coli* populations in the roots was observed upon applying ozone treatments. However, the effect of ozone does not last long and decreases over time. When used at higher doses, ozone can reduce the total bacterial load by affecting different microbial populations in addition to *E. coli*, decreasing competition and favoring the growth of this pathogen. In addition, a high dose of ozone would cause surface damage to the roots, increasing the availability of resources for *E. coli* growth.

According to Khadre and Youssef (2001), the O_3 mechanism action on microorganisms is a complex process because O_3 attacks numerous cellular constituents, including proteins, unsaturated lipids, and respiratory enzymes in cell membranes, peptidoglycans in cell envelopes, enzymes and nucleic acids in the cytoplasm, and proteins and peptidoglycans in spore coats and virus capsids. Conversely, Rangel et al. (2022) point out that low O_3 doses interfere with cell viability, likely due to increased reactive ROS levels, which are responsible for causing a detrimental effect on lipid, protein, and DNA metabolism. In addition, O_3 promoted alterations in the *E. coli* membrane due to provoking wrinkled and deformed cells, with membrane damage and the presence of invaginations (Thanomsub et al., 2002; Rangel et al., 2022).

On the other hand, after day 2 post-inoculation, the *E. coli* counts increased in nutrient solution, reaching levels higher than 5 log CFU mL^{-1} (Figure 2). This trend suggests that, despite the initial decrease in *E. coli* load, the O_3 efficacy might be limited in controlling this pathogen. In this sense, Thanomsub et al. (2002) mentioned that inoculated loads higher than 5 log CFU mL^{-1} were not affected by O_3 application despite a longer application time of 150 min. Moreover, the

O_3 application also had a limited effect on preventing *E. coli* internalization, considering the persistently detectable counts on leaves after inoculation of the nutrient solution. Therefore, it is possible that these microorganisms were internalized prior to O_3 application or that the surviving population, after O_3 treatment, managed to internalize at a later stage. The results of Koseki et al. (2011) noted the internalization of *E. coli* into the leaves when the nutrient solution was inoculated with 6 log CFU mL^{-1}. In the same study, internalization of the leaves was not observed after *E. coli* inoculation of 3 log CFU mL^{-1}. These authors established a logistic regression probabilistic model, based on the assumption of a binomial distribution of the positive or negative events, which determined initial *E. coli* of 6 log CFU mL^{-1} was 6.93 times more likely to contaminate roots than 3 log CFU mL^{-1}. The *E. coli* dose used in the present study was 6 to 8 log CFU mL^{-1}, which coincides with the dose range capable of contaminating roots and petioles of the chard plants.

Experiment 2. Internalization of inoculated *E. coli* in the nutrient solution and chard tissue

Previous studies have mentioned the presence and internalization of foodborne pathogens in leafy vegetables grown in soil and hydroponic cultivation and a large vegetable number including lettuce, spinach, tomatoes, radish sprouts, and cabbage (Xylia et al., 2022). Also, routes of entry for pathogens include plant roots, leaf stomata, lateral junctions of roots, flowers, or damaged tissue such as wounds, cut surfaces, any niches caused by mechanical damage, or even the use of contaminated seeds (Erickson, 2012; Sun et al., 2019; Grivokostopoulos et al., 2022; Xylia et al., 2022; Zhao et al., 2022). The capacity of foodborne pathogens to adhere to fresh produce surfaces is a significant component in their internalization into plant tissues. Adhesion is the initial step in the infection process (Xylia et al., 2022). In a study conducted on *E. coli* and *Salmonella typhimurium*, pathogens were able to penetrate the inoculated iceberg lettuce leaves through the stomata with 8 log CFU mL^{-1} (Kroupitski et al., 2011). *Salmonella* is known to penetrate the epidermis through open stomata and colonize within the stomata or substomatal cavities of the plant leaves (Grivokostopoulos et al., 2022). Studies related to *E. coli* O157:H7 strain mentioned 20 to 100 µm penetration below the surface of lettuce leaves (Seo and Frank, 1999). In addition, *E. coli* O157:H7 cells appear to show strong stomatal tropism for the protective cells of arugula, spinach, and lettuce (Sun et al., 2019). This phenomenon occurs due to chemotaxis processes and the bacteria's flagellar motility towards nutrients produced by photosynthetic guard cells and mesophyll cells (Kroupitski et al., 2009). According to Wright et al. (2017) and Zhao et al. (2022), *E. coli* internalization varied greatly by plant species, tissue type, and initial inoculum. In the present study, inoculating *E. coli* directly into leaves enhanced its internalization in the tissue. Therefore, internalization of *E. coli* could occur both from the inoculated leaves or nutrient solution, although it would be from the roots to the aerial part, probably due to the natural movement of water within the plant (Figure 3B; Table 3). This assumption is supported by the studies of Zhou et al. (2018), where infiltrated tomato fruits with red fluorescent microspheres and *E. coli* O157:H7 –pGFP showed by microscopy confined bacteria in xylem vessels. These authors found concentrated immediately below the stem scar, with a sharp decline in population with vertical distance from the stem scar. This evidence means that xylem vessels play a critical role in pathogen internalization. Moreover, this process is passive rather than active, as infiltration and movement of both microspheres (non-living) and *E. coli* O157:H7 cells (living) behaved similarly inside tomatoes. Other mechanisms or pathways of *E. coli* internalization have been identified. Saldaña et al. (2011) note that *E. coli* O157:H7 uses a specific T3SS effector to open the stomatal guard cells of spinach leaves, allowing its internalization into plant tissues. Conversely, pathogen internalization depends on the plant species (Golberg et al., 2011; Wright et al., 2017) and the variety or cultivar (Golberg et al., 2011). Additionally, Esmael et al. (2023) indicate that fresh produce plants' root exudate is nutritionally rich and attracts *Salmonella enterica* to the roots of lettuce plants. Though human pathogens cannot directly infiltrate via root cells, root cracks, and lateral root emergence provide sites for the internalization of *S. enterica* and *E. coli* O157:H7 into root tissues and, in some cases, between the epidermal cells. Klerks et al. (2007) reported higher *S. enterica* colonization in the shoot-root transition area; once internalized, pathogens like *Salmonella enterica* can be found in the endodermis, parenchyma, vascular system, and pericycle of lettuce roots.

The internalization of human pathogenic bacteria within leafy vegetables and herbs found that iceberg lettuce and arugula exhibited a remarkable internalization rate, with incidences of 81 and 86%, respectively (Golberg et al., 2011). In contrast, Romaine, red lettuces, and basil showed moderate internalization incidence with 16, 15, and 46%, respectively. On the other hand, parsley and tomato leaves showed a lower incidence of internalization, with 1.9 and 0.56%, respectively (Golberg et al., 2011). Wright et al. (2017) noted differences in the *E. coli* O157:H7 Sakai internalization between lettuce and spinach, indicating that tissue type and starting inoculum are factors to consider for bacteria internalization. Therefore, the process of internalization of human pathogens is not simple and involves multiple factors to consider, such as translocation from roots to leaves may occur relatively more easily in a hydroponic system because soil microbiota competes for root colonization (Warriner et al., 2003). The mechanism for the migration of human pathogens from root cortex to root vasculature through Casparian strips and endodermis, and from roots to phyllosphere through the vascular system has not been studied yet (Esmael et al., 2023).

Conclusion

The O_3 application to the nutrient solution at doses of 0.5 and 2 mg L^{-1} reduces *E. coli* counts by 2-log CFU g^{-1}, showing a limited effect in eliminating and preventing pathogen presence and internalization in the chard plant tissue. Higher ozone doses decrease the overall bacteria load, reducing competition and potentially enhancing *E. coli* growth. Furthermore, elevated ozone levels can damage root tissue, which may increase the resources available for *E. coli* proliferation. However, some factors must be considered for the real effectiveness of the O_3 application, such as the initial inoculum load and the treatment duration, among others. Besides, the results of this study show that *E. coli* can internalize from the roots to the aerial tissue like petioles. On the other hand, when the leaves are directly contaminated, the bacteria are internalized through the stomata and found in the tissue. No bacterial cells were detected in the petiole or roots of these plants, suggesting that colonization does not occur in that direction. These findings highlight the complexity of the interaction between *E. coli* and chard plants and the challenge of effectively controlling internalized pathogens in leafy vegetables. Further research is needed to better understand the mechanisms of internalization and

develop more efficient and safe methods to reduce the risk of human pathogen contamination in leafy vegetables.

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Addresses of authors:
M. Auxiliadora Faicán-Benenaula[1,2,3], Cristian Hernández-Adasme[3], Alejandra Machuca[3] and Víctor Escalona Contreras[3]
[1] Instituto de Tecnología de Alimentos, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe, Argentina
[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Santa Fe, Argentina
[3] Centro de Estudios Postcosecha (CEPOC), Facultad de Ciencias Agronómicas, Universidad de Chile, Santiago, Chile