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Research Note

Survival of Inoculated *Campylobacter jejuni* and *Escherichia coli O157:H7* on Kale During Refrigerated Storage

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ABSTRACT

Campylobacter and pathogenic *Escherichia coli*illnesses have been attributed to the consumption of fresh produce. The leafy green, kale, is increasingly consumed raw. In comparison to other leafy greens, kale has a longer shelf-life. Due to the extended shelf-life of kale, it is warranted to examine the survival of pathogenic *Campylobacter jejuni* and *E. coli* O157:H7 inoculated on the surface of kale stored in a controlled environment at 4 ± 1.4°C, and average humidity of 95 ± 1.9% over a 23-day period. At predetermined time points (days 0, 1, 2, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21), inoculated kale was destructively sampled and the surviving bacteria determined by serial dilution and plating onto Tryptic soy agar, Charcoal cefoperozone deoxycholate agar, and Eosin methylene blue for total aerobic bacteria, *C. jejuni,* and *E. coli* O157:H7*,* respectively. Enrichment and PCR were used for detection when pathogens were not detected using serial dilution and plating. Aerobic heterotrophic bacteria increased over the 23-day period, in contrast, significant declines in the inoculated pathogens were observed. Inoculated *E. coli* O157:H7 survived longer on kale (up to 19 d); in comparison, *C. jejuni* was undetectable by day 13 using enrichment and PCR or plating. In conclusion, *C. jejuni* and *E. coli* O157:H7 declined on fresh kale over time when held at refrigerated temperatures but were still detected during the majority of the time when the kale would likely still be considered edible by consumers.

Raw kale is becoming increasingly popular among consumers due to its nutritional benefits and is commonly eaten in salads or included in beverages (Kang & Song, 2017). Studies have shown that raw kale contains more nutritional content than cooked or heavily processed (Sikora & Bodziarczyk, 2012). However, without a cooking step, pathogenic bacteria have a greater chance of persisting on fresh vegetables for consumption (Luna-Guevara et al., 2019).

Leafy greens are associated with the most produce-associated illnesses in the United States: from 2018 to 2021, there were eleven multistate outbreaks investigated by the US Centers for Disease Control and Prevention that were linked to leafy greens (Centers for Disease Control and Prevention, 2022; The Interagency Food Safety Analytics Collaboration, 2021). Serotypes of pathogenic *Escherichia coli* are associated with the majority of leafy green-borne outbreaks in the United States and Canada: from 2009 to 2018, 40 outbreaks were identified with a resulting 1,212 illnesses (Marshall et al., 2020). *Campylobacter* spp. are an emerging concern on fresh produce. From 2004 to 2012, there have been seven outbreaks of *Campylobacter* spp. from fresh vegetable consumption, and those are only the ones that have been reported/detected (Mohammadpour et al., 2018). There are several places along the supply chain that leafy greens, such as kale, can become contaminated with *E. coli* and *Campylobacter* spp. This contamination may occur where the vegetables are grown, during harvest, and after harvest (Abay et al., 2022). Pathogens are not easily removed by washing produce due to their adhesion capabilities (Berger et al., 2010). *E. coli* O157: H7 can survive for extended periods at 4°C storage; for example, 12 d on iceberg lettuce ((Delaquis, Bach, & Dinu, 2007)) and 15 d on baby spinach (Lopez-Velasco et al., 2010).

Among leafy greens, kale has a longer shelf-life, often exceeding 15 d, when stored at temperatures below 5°C and high relative humidity (Albornoz & Cantwell, 2016). Wilting and loss of chlorophyll (discoloration) are associated with major losses to kale product quality, but high numbers of naturally occurring microorganisms (aerobic bacteria, *Enterobacteriaceae*, *Pseudomonas* spp., and yeast and mold) may lead to flavor, appearance, and structure changes during storage (Ragaert et al., 2007). There are few studies that have characterized the changes to kale microbial communities during extended storage (Mansur & Oh, 2016), especially in the presence of human pathogens

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(Mansur & Oh, 2015). Some background microorganisms are reported to antagonize the growth of *E. coli* O157:H7 at permissive temperatures on produce (Lopez-Velasco et al., 2012), but may also alter the survival at refrigerated temperatures. The purpose of this study was to evaluate the survival of *C. jejuni* and *E. coli* O157:H7 inoculated separately onto freshly harvested kale, bagged, and stored under refrigeration until the kale appearance indicated it was beyond acceptable shelf-life, as indicated by exudate, yellowing or wilting. In addition, total aerobic plate counts were performed along the time course, which may aid in the prediction of product shelf-life.

Materials and methods

Bacterial cultivation and inoculum preparation

All cultures were maintained at −80°C in 20% glycerol stock. *C. jejuni* ATCC 33291 was inoculated into Bolton Broth (ThermoFisher Scientific) with supplement (SR0155E) without blood and incubated at 37°C for 48 h at 10% CO_2. *E. coli* O157:H7 strains, ATCC 43894 and ATCC 43895, were streaked for isolation from freezer stocks onto Levine EMB Agar (ThermoFisher Scientific) and incubated for 24 h at 37°C. Individual colonies were then taken from the plate and transferred to Tryptic Soy Broth (Becton, Dickinson and Company, Sparks, Maryland) and incubated at 37°C for 24 h. The cultures were centrifuged at 2817*g* for 10 min (Eppendorf); the cell pellet was washed by decanting the supernatant followed by suspension in 0.1% peptone water (BD, Thermo Fisher Scientific). The culture was washed twice in this manner and finally resuspended in 5 mL 0.1% peptone water. After preparation of the inoculum cocktail, serial dilution and plating onto appropriate selective media (CCDA (BD, Thermo Fisher Scientific) *C. jejuni*), EMB for *E. coli* was used to determine the starting concentration of the inocula. Three individual colonies were used to create separate inoculum allowing for three biological replicates to be compared during the shelf-life trial.

Inoculation of kale

Kale was collected from a local producer (Charlotte, Virginia), and the experiment commenced within 24 h of harvest. Kale was stored in plastic bags and placed on ice during transport. The kale was sorted to remove any broken or damaged leaves, and then weighed out into 25-g allotments. For the inoculation of the kale, the 25-g allotments of kale leaves were placed in a weigh boat and a spot inoculation protocol was used to deposit 100 ul of the *Campylobacter* or *E. coli* O157:H7 culture across the kale leaf surface in 10 ul drops. Kale leaves were not coinoculated with both *C. jejuni* and *E. coli* O157:H7. The inoculated kale leaves were placed within a biosafety cabinet for ca. 1 h until the droplets dried. All kale samples were then placed into sterile whirl pack bags (Nasco). A total of 15 samples were inoculated with *Campylobacter* per replicate (45 total), 15 were inoculated with the *E. coli* cocktail per replicate, and 15 were left uninoculated per replicate. All bags were held at 4°C, ±2°C once stabilized, in plastic-lidded containers that also contained an open bottle of water to control humidity to an average of 95% humidity (ranged from 86.5% to 100%). A temperature and humidity tracker (Vertiv) was also placed with the uninoculated kale samples. The inoculated kale leaves were destructively sampled at day 0 (after drying), 1, 2, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23 (14 time points).

Microbiological sampling and enumeration

For the sampling process, one bag of kale inoculated with the *E. coli* and *C. jejuni* from each of the replicates ($n = 3$ for each microbe per time point) and one bag of noninoculated kale were removed from 4°C storage and 225 mL of 0.1% peptone water was added to each bag. The bags were placed in a lab blender (Bagmixer 400, Interscience) for 1 min at 230 RPM. After one minute, the bag was hand massaged for 30 s and blended for an additional minute. Samples were serially diluted tenfold in 0.1% sterile peptone and plated onto Tryptic Soy Agar (BD DIFCO, Franklin Lakes, NJ) and the appropriate selective media (CCDA for *C. jejuni* and EMB Agar for *E. coli)*. The noninoculated sample was serially diluted in 0.1% sterile peptone and plated on all three media types. An additional 1 ml of the 10^{-1} dilution was taken and enriched in 9 mL Bolton Broth without blood or Brilliant Green Bile Broth 2% (Himedia) for *C. jejuni* and *E. coli*, respectively. The enrichments were held at the same temperature as the plates and after 24 h, a loopful was streaked onto either CCDA incubated at 37°C in 10% CO_2 for 48 h or EMB at 37°C for 24 h. On the CCDA, only small, flat, and gray colonies were counted as *C. jejuni.* On the EMB, only colonies with a green sheen were counted as *E. coli.* All colony types were counted on the TSA. The noninoculated control plates did not contain colonies with characteristic *E. coli* or *C. jejuni* appearance when plated on EMB or CCDA, respectively. The limit of detection for serial dilution and plating is 1 log CFU/g.

PCR screening of enrichments

Additionally, at day 5 and each time point, 1.5 ml of the enrichment from the *C. jejuni* and *E. coli* samples were extracted with the Biobasic EZ-10 spin column soil DNA Minipreps kit (Biobasic) and screened with the corresponding PCR protocol to further enhance detection when cell numbers were below the 1 log CFU/g limit of detection by serial dilution and plating. The limit of detection for enrichment and PCR is ~0.27–0.67 log CFU/g.

Primers C412F and C1228 R were used to detect the inoculated *Campylobacter* from the enrichments (Linton et al., 1996). DNA (2 μL) extracted from the Bolton broth enrichments were amplified in 25 μL reactions containing 12.5 μL Gotaq Green Master Mix 2X (Promega), 1 μL magnesium chloride 25 μM (Qiagen), 7.5 μL nuclease-free water (Qiagen), and 0.2 μM of each primer (Integrated DNA Technologies). Nuclease-free water was used for the negative control. DNA from *C. jejuni* ATCC 33291 was used for a positive control. PCR conditions were 95°C for 2 min. followed by 35 cycles of 95°C for 2 min, 57°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 2% agarose gel using 1× Tris-acetate-EDTA buffer stained with ethidium bromide. The gel was visualized under a UV transilluminator (Bio Rad).

A multiplex PCR was used for the detection of *E. coli* using the *phoA* gene designed to detect members of the *E. coli* species (Kong et al., 1999) and *eae* designed to detect Enterohemorrhagic pathotypes (Vidal et al., 2005). The sizes of the expected mPCR products and concentrations of the primer pairs are listed in Table 1. The mPCR amplification was conducted in a total reaction volume of 25 μL containing 12.5 μL Gotaq Green Master Mix 2X, 1 μL magnesium chloride 25 μM, 7.5 μL nuclease-free Water, 2 μL of DNA template, and the corresponding primers. Nuclease-free water was used for the negative control. DNA from Enterohemorrhagic *E. coli* O157:H7 ATCC 43895 was used for positive controls. The mPCR conditions were 95°C for 5 min. followed by 28 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 2% agarose gel using 1× Tris-acetate-EDTA buffer stained with ethidium bromide. The gel was visualized under a UV transilluminator. Bands of corresponding size were excised and prepared for sequencing using a gel extraction kit before DNA sequencing.

Experimental design and statistical analysis

The experiment was conducted in triplicate using three independent biological replicates. All statistics were performed in R: A Language and Environment for Statistical Computing (V4.1.2; R Core Team). The following packages were utilized: “tidyverse” and “readxl”

Table 1
A list of the *Escherichia coli* primers used and their associated properties

Gene	Sequence (5′ to 3′)	Size (bp)	Target bacteria	Concentration	Reference
eae - F	TCAATGCAGTTCCGTTATCAGTT	482	*EHEC*	0.4 μM	Vidal et al. (2005)
eae - R	GTAAAGTCCGTTACCCCAACCTG			0.4 μM	
phoA - F	GTGACAAAAGCCCGGACACCA TAAATGCCT	903	*E. coli control*	0.24 μM	Kong et al. (1999)
phoA - R	TACACTGTCATTACGTTGCGGATTTGGCGT		*E. coli control*	0.24 μM	

to upload the data, "knitr" to view the data, and "ggplot2" to construct the graph. A linear regression was used to look at the relationship between the day (time point) and log CFU. Relationships were considered significant at $P \leq 0.05$.

Results

The initial levels of *E. coli* on the inoculated kale were 4.8 log CFU/g and after 13 days could no longer be detected by serial dilution and plating on EMB (Fig. 1a). No colonies with characteristic metallic green sheen on EMB were observed on the noninoculated control leaves throughout the experiment (23 d). *E. coli* O157:H7 were intermittently detected by enrichment and PCR until day 19 but overall were not detected at the end of the study. *C. jejuni* recovered from day 0 inoculated kale was 1.8 Log CFU and was detected by serial dilution and plating on CCDA through day 7 (Fig. 1b). *Campylobacter* was undetectable through PCR and enrichment from day 13 onward. *E. coli* or *Campylobacter* were not detected by serial dilution or plating methods or by PCR on noninoculated kale. The dataset is available (Bywater & Ponder, 2022).

Aerobic bacteria (heterotrophic plate counts, HPCs) associated with the freshly harvested kale increased gradually during storage (Fig. 1c). No significant relationship was observed between HPC log CFU and day ($P < 0.9$). At d 17, some wilting and color changes were observed at the edges of the leaves. By d 23, all leaves were visually judged by researchers MP and AB, no longer acceptable due to their yellow color and wilted appearance. Despite the appearance change and increase in HPC, there was no mold, exudate, or blackening observed.

Discussion

Campylobacter and *E. coli* counts both declined in refrigerated storage over the duration of the shelf-life study. At the end of 23 days of storage at 4°C, neither *C. jejuni* nor *E. coli* were detected through plating, enrichment, or PCR. Growth of the pathogens would not be expected since neither is psychotropic; therefore, storage conditions would prevent the growth of *E. coli* and *Campylobacter sp*. Aerobic heterotrophic plate counts started at 4.8 ± 0.53 log CFU/g and increased to 6 Log CFU which is consistent with an increase in psychrotrophic bacteria growing at 4°C (Xylia et al., 2021). In this study, the initial aerobic mesophilic bacteria load is lower compared to other reported studies (Mansur & Oh, 2015). This may be due to difference in cultivar, temperature, and environmental conditions. Previous studies have also purchased kale from supermarkets where the time from harvest was not provided. In this study, kale was obtained from the field and used within 24 h of harvest. A lower starting inoculum and fresher kale may explain why little evidence of bacterial spoilage was seen until d 19, despite other studies reporting shorter shelf lives of kale (Mansur & Oh, 2015).

Die-off behavior of the two pathogens on kale during refrigerated storage varied. *E. coli* counts were higher over the course of the study

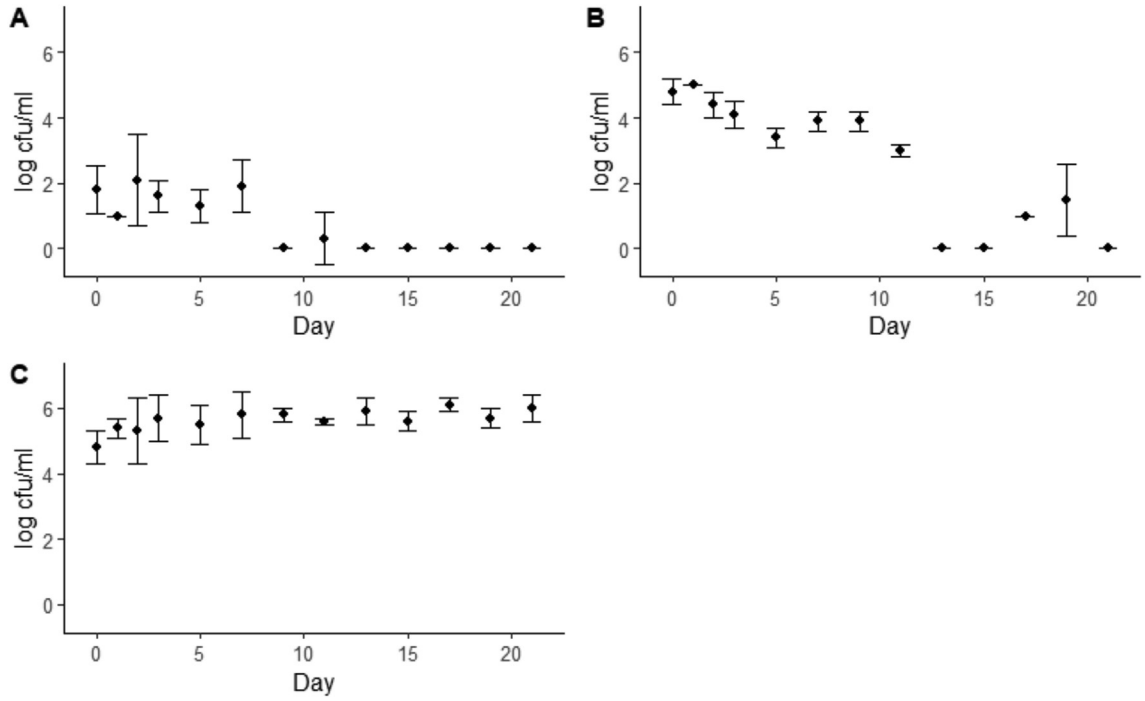

Figure 1. Effect of time on Log CFU/g of *C. jejuni* (1a), *E. coli O157:H7 (*1b), and aerobic heterotrophic bacteria (1c) from kale stored at 4°C. Values shown are the average of all three replicates. Error bars represent the standard deviation among the three replicates. HPCs are from TSA, *Campylobacter* counts are from CCDA, *E. coli* counts are from EMB.

than the *C. jejuni* counts. The inoculum for both pathogens applied to the kale was 4.0–4.5 Log CFU; after drying, the recovery of *C. jejuni* was reduced by 2.2 ± 0.76 log CFU but no significant change in *E. coli* occurred after inoculation. The decrease in *C. jejuni* was likely due to exposure to atmospheric levels of oxygen during drying before packaging. Increased oxygen concentrations have been reported to reduce *Campylobacter* survival (Thomas et al., 2020). This method of inoculation has been used in other studies examining the survival of *E. coli* O157:H7 on leafy greens and has not been reported to significantly decrease *E. coli* O157:H7 immediately after drying ((Lopez-Velasco et al., 2010) Mansur & Oh, 2015; Kang & Song, 2017). *E. coli O157:H7* log CFU on unpacked Romaine lettuce has been reported to decrease by only 0.6 log over 8 days (Bhullar et al., 2021). In comparison to this study, the *E. coli* reduced 0.9 log over 7 days persisting until day 19. Interestingly, one-third of the samples here on day 19 tested positive for *E. coli* by PCR but 0/3 samples were positive on day 17 indicating that persistence is variable. Increased wilting and yellowing were visible on day 19 kale leaves, potentially providing additional nutrients for stress response and repair. Previous studies in minimally processed romaine lettuce have demonstrated that lettuce deterioration was a significant determinant in STEC *E. coli* O157 survival in cold storage (Leonard et al., 2021). It may also be possible the *E. coli* O157:H7 cells themselves are dead but the DNA has persisted on the kale.

Despite *Campylobacter's* shorter survival period during storage, it should still be considered a hazard when it comes to fresh refrigerated products, like kale. Low temperatures may aid the survival of *Campylobacter*. For example, survival of *C. jejuni* in water at 4°C improved to 29–60 days compared to 4–7 days at 25°C (González & Hänninen, 2012). *C. jejuni* reductions on refrigerated minced beef and chicken stored in plastic bags were 1 log reduction in 6 days; in contrast when stored at 22°C, only 1 day was needed for the same 1 log decrease (Barrell, 1984). In addition to prolonging survival, temperature also plays a factor in cells entering the viable but nonculturable state (Wei & Zhao, 2018).

Enrichment and PCR confirmations were run when target colonies could no longer be detected on the appropriate media. Even though the limit of detection in this study was low at 1 log CFU/g, viable but nonculturable bacteria needed to be considered. Many bacteria, including *C. jejuni* and *E. coli* O157:H7, enter a VBNC state when put under stressful conditions, like the refrigerated temperatures in this study (Zhao et al., 2017). Although undetectable through traditional culturing methods, pathogenic VBNC bacteria still have the ability to result in illness. Enrichment and PCR likely allowed us to detect fewer than 10 CFU of stressed but viable bacteria. It is possible that if the bacteria were VBNC, the limit of detection was higher because growth did not occur during the enrichment step; therefore, requiring more samples. The presence of VBNC bacteria may explain the variability among the replicates in the later days of the experiment since the small number of surviving but not growing bacteria would be difficult to detect with traditional PCR.

Environmental point source contamination and produce handling may result in different starting contamination or survival dynamics than observed in this study. Previous studies have demonstrated a 1.23 log CFU reduction in *E. coli* O157:H7 when kale was washed with water at 55°C and subsequently refrigerated (Kang & Song, 2017). This compares to the 0.6 log CFU reduction in this study, with no washing, from day 1 to day 2, suggesting that washing maybe a useful additional step to reduce initial contamination. Quantification of *E. coli* from naturally contaminated leafy greens has reported populations around 1 log CFU (Johnston et al., 2005); however, naturally occurring populations of *Campylobacter* on produce have not been reported to the best of our knowledge. It is likely that the initial inoculum chosen for this study to facilitate quantification exceeds that which would be expected on kale due to field contamination and would therefore represent a worst-case scenario.

The microbial load of *C. jejuni* and *E. coli* on fresh kale reduced over time in a temperature and humidity-controlled environment but was still present when the product would still likely be consumed. Due to this, it is still important to practice safe sanitation management when handling raw leafy greens as higher pathogenic counts have been observed, especially in summer months (Xylia et al., 2021). In this study, the kale was not washed in chlorine prior to inoculation and was refrigerated under high humidity as recommended for extended shelf-life. These results cannot be applied to situations where refrigeration is not used for storage, which may include informal markets or other locations where refrigeration is not available. *E. coli* O157:H7 has been documented to grow on kale at temperatures above 25°C (Mansur & Oh, 2016). This highlights the need for good agricultural practices and proper postharvest handling. Future work looking at the growth of *Campylobacter* spp. on vegetables at different storage conditions and different packaging configurations is needed.

CRediT authorship contribution statement

Auja Bywater: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kathleen Alexander:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Joseph Eifert:** Writing – review & editing, Conceptualization. **Laura K. Strawn:** Writing – review & editing, Software, Resources, Data curation, Conceptualization. **Monica A. Ponder:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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