



Persistence of *Escherichia coli* O157:H12 and *Escherichia coli* K12 as Non-pathogenic Surrogates for O157:H7 on Lettuce Cultivars Irrigated With Secondary-Treated Wastewater and Roof-Collected Rain Water in the Field

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Treated wastewater (TW) and roof-collected rain water (RW) that meet the required microbial quality as per Food Safety Modernization Act (FSMA) regulation may serve as alternative irrigation water sources to decrease the pressure on the current water scarcity. Alternative water sources may have different water characteristics that influence the survival and transfer of microorganisms to the irrigated produce. Further, these water sources may contain pathogenic bacteria such as Shiga-toxicogenic *Escherichia coli*. To evaluate the risk associated with TW and RW irrigation on the fresh produce safety, the effect of TW and RW irrigation on the transfer of two non-pathogenic *E. coli* strains as surrogates for *E. coli* O157:H7 to different lettuce cultivars grown in the field was investigated. Lettuce cultivars “Annapolis,” “Celinet,” and “Coastline” were grown in the field at the Fulton farm (Chambersburg, PA). Approximately 10 days before harvest, lettuce plants were spray-irrigated with groundwater (GW), TW, or RW containing 6 log CFU ml⁻¹ of a mixture of nalidixic acid-resistant *E. coli* O157:H12 and chloramphenicol-resistant *E. coli* K12 in fecal slurry as non-pathogenic surrogates for *E. coli* O157:H7. On 0, 1, 3, 7, and 10 days post-irrigation, four replicate lettuce leaf samples (30g per sample) from each group were collected and pummeled in 120ml of buffered peptone water for 2 min, followed by spiral plating on MacConkey agars with antibiotics. Results showed that the recovery of *E. coli* O157:H12 was significantly greater than the populations of *E. coli* K12 recovered from the irrigated lettuce regardless of the water sources and the lettuce cultivars. The TW irrigation resulted in the lowest recovery of the *E. coli* surrogates on the lettuce compared to the populations of these bacteria recovered from the lettuce with RW and GW irrigation on day 0. The difference in leaf characteristics of lettuce cultivars significantly influenced the recovery of these surrogates on lettuce leaves. Populations of *E. coli* O157:H12 recovered from the RW-irrigated “Annapolis”

lettuce were significantly lower than the recovery of this bacterium from the “Celinet” and “Coastline” lettuce ($P < 0.05$). Overall, the recovery of specific *E. coli* surrogates from the RW and TW irrigated lettuce was comparable to the lettuce with the GW irrigation, where GW served as a baseline water source. *E. coli* O157:H12 could be a more suitable surrogate compared to *E. coli* K12 because it is an environmental watershed isolate. The findings of this study provide critical information in risk assessment evaluation of RW and TW irrigation on lettuce in Mid-Atlantic area.

Keywords: cultivar, irrigation water, food safety, *E. coli*, pre-harvest, fresh produce

INTRODUCTION

The World Health Organization encourages people to consume at least 400 g of fresh produce such as fruits and vegetables per day to prevent chronic disease including heart disease, diabetes, and obesity (World Health Organization, 2018). Lettuce is the most consumed leafy green vegetables in the United States with an annual consumption of 5.9 kg per person [U.S. Department of Agriculture (USDA), 2016]. As the consumption of fresh produce increased during the past decades, incidences for foodborne outbreaks linked to fresh produce have also increased [Sivapalasingam et al., 2004; European Food Safety Authority (EFSA), 2012; Painter et al., 2013]. In European Union, fresh produce-associated outbreaks accounted for 8.7% of all reported outbreaks in 2010 compared to 2.1% in 2009 [European Food Safety Authority (EFSA), 2011, 2012]. A total of 110 outbreaks in the U.S. between 2010 and 2017 were associated with the consumption of the contaminated lettuce; pathogenic *Escherichia coli* was responsible for 24 of these outbreaks that resulted in 506 illnesses, 159 hospitalizations, and one death [National outbreak reporting system (NORS), 2019].

Contamination of produce may occur at any point during the production of fresh produce through soil, animal manures, farming equipment, and post-harvest processes (Holvoet et al., 2015; Weller et al., 2017); however, agricultural water has been identified as a major risk factor in the contamination of leafy greens [Steele and Odumeru, 2004; Food and Drug Administration (FDA), 2008; Gerba, 2009; Jung et al., 2014; Araújo et al., 2017]. Cross-contamination of field-grown vegetables by the foodborne pathogens through the contaminated irrigation water with fecal waste or improperly composted manure is suggested to be a possible source of pathogen at the pre-harvest level (Oliveira et al., 2012). Attachment of human bacterial pathogens on fresh produce during spray irrigation can be affected by the bacterial strains, bacterial population, stress tolerance, and their biofilm formation ability (Carey et al., 2009; Ge et al., 2012; Yaron and Römling, 2014; Kljujev et al., 2018). Other factors such as produce growing conditions, plant development stage, and the leaf characteristics of cultivars also affect bacterial attachment on the fresh produce leaf (Barak et al., 2011; Ge et al., 2012; Hirneisen et al., 2012; Quilliam et al., 2012).

Traditionally, groundwater and surface water are commonly used for irrigation in the United States, where groundwater withdrawals at 57.2 billion gallons per day account for 48%

of total water usage for irrigation (Dieter et al., 2018). In Pennsylvania, nearly 58 million gallons per day of water were used for irrigation and livestock purposes, which constituted more than 10% of the total water consumption in the state [Penn State Extension (PSE), 2007]. The water scarcity has become an issue due to the unpredictable climate and the increased food demands for the growing world populations (Cabera, 2017), requiring approaches to mitigate the scarcity of irrigation waters or supplement more common water sources. Several water management strategies have been employed to overcome the water scarcity issue, including the selection of water-use efficient crops and the use of water-conservation practices such as mulching and sensible irrigation technologies (Pereira et al., 2012). Additionally, research studies have been conducted on the use of alternative water sources such as treated domestic wastewater and roof-collected rain water for fresh produce irrigation (Ahmed et al., 2011; Mizyed, 2013; Yin et al., 2018, 2019).

Domestic wastewater that contains household sewage and industrial wastewater could be a reliable alternative water source for agriculture once treated at the wastewater treatment plant [Xie, 2009; Pedrero et al., 2010; European Commission (EC), 2016]. Reuse of domestic wastewater reduces the release of nutrient-rich wastewater from the wastewater treatment plants into surface water sources such as stream and river (Scott et al., 2004). In Pennsylvania, treated domestic wastewater can be reused for the irrigation of crops intended for human consumption that would be peeled, skinned, cooked, or thermally processed before consumption or commercially processed foods [Department of environmental protection (DEP), 2012]. However, such wastewater used as irrigation water for edible crops should undergo at least a secondary treatment with filtration and disinfection steps at the wastewater treatment plants and should meet the requirements for Class B or better. For spray irrigation of edible crops, Class B wastewater should contain fecal coliforms $<2.2 \log$ CFU per 100 ml during the biweekly monitoring process and apply for a period of 15 days prior to harvest as per the wastewater reuse guidance in Pennsylvania [Department of environmental protection (DEP), 2012]. Rain water harvested from the rooftop is another strategy that may serve to cope with current water shortages; however, such water could be contaminated by the bird and rodent droppings on the roof (Zhu et al., 2004; Fletcher et al., 2008; Yin et al., 2019). Although the use of treated wastewater and roof-collected rain water has been increasingly seen as alternative

agricultural water sources to overcome water scarcity, these water sources may contain bacterial pathogens including *E. coli* O157:H7 and *Salmonella* spp. (Steele and Odumeru, 2004; Yin and Patel, 2018). Potential transfer of pathogens from contaminated irrigation waters to fresh produce could pose significant risk as fresh produce is often consumed raw (Beuchat, 2002).

Previous studies have confirmed the transfer of fecal microorganisms from the irrigation water to the irrigated vegetables (De Roever, 1998; Yin et al., 2018, 2019). Water characteristics such as total organic carbon, hardness, ion composition, pH, and indigenous microflora influenced the survival of *E. coli* O157:H7 in different water sources (Williams et al., 2007; Avery et al., 2008). Spray irrigation of spinach with wastewater and roof-collected rain water significantly altered its microbiome compositions (Gu et al., 2019). Effects of alternative irrigation waters on the transference and persistence of non-pathogenic *E. coli* surrogates from the water to the lettuce cultivars at farm levels need further investigation. This study investigated the impact of secondary-treated wastewater and roof-collected rain water on the transfer and persistence of *E. coli* O157:H12 and *E. coli* K12 as non-pathogenic surrogates for *E. coli* O157:H7 on three lettuce cultivars in the field. Lettuce cultivars “Annapolis,” “Celinet,” and “Coastline,” with different leaf structures and ~35 days of maturity time were selected. “Annapolis” cultivar has wavy red leaves, and “Celinet” variety has medium green leaves and thin petioles (Skrzyniarz, 2016; U.S. Patent No. 9,392,765). “Coastline” lettuce is a type of Batavian lettuce with medium green leaves.

MATERIALS AND METHODS

Preparation of Bacteria Inoculum

Non-pathogenic *E. coli* strains *E. coli* O157:H12 and *E. coli* K12 (ER2420/pBeloBAC11) were used as surrogates for *E. coli* O157:H7 in water for lettuce irrigation (Fonseca et al., 2011; Ingram et al., 2011). *E. coli* O157:H12 isolated from the environment was spontaneously resistant to nalidixic acid (NA; Sigma-Aldrich, St. Louis, MO, USA) as previously described by Ingram et al. (2011). *E. coli* K12 (New England Biolabs, Ipswich, MA) contained the plasmid pBeloBAC11, which carried the chloramphenicol-resistant gene (CP; Sigma-Aldrich). The antibiotic-resistant properties enabled the differentiation of these *E. coli* surrogates from the generic *E. coli* present in the water and lettuce plants (Ingram et al., 2011).

E. coli O157:H12 and *E. coli* K12 were individually grown in 10 ml of tryptic soy broth (TSB; Fisher Scientific, Waltham, MA) that contained NA at 50 ng ml⁻¹ or CP at 200 ng ml⁻¹ at 37°C for 24 h. After incubation, overnight cultures were centrifuged at 5,000 × *g* for 15 min, followed by re-suspending bacterial pellets with 10 ml of phosphate-buffered saline (PBS, Fisher Scientific). Two milliliter portions of each bacterial suspension were transferred to 100 ml of fecal slurry and incubated at 37°C for 48 h. Bovine fecal slurry was prepared as described by Patel et al. (2010), in which dairy manure solids were obtained from the USDA-ARS Holstein dairy herd (Beltsville, MD). After 48 h of incubation, equal amount of *E. coli* O157:H12 and *E. coli* K12

cultures was mixed (8 log CFU ml⁻¹) and supplied as 1:100 ratio to each type of irrigation waters to obtain final bacterial populations at 6 log CFU ml⁻¹ in irrigation water samples.

Irrigation Water on Lettuce Grown in Field

Field experiment was conducted at the Fulton farm in Chambersburg, PA, in October 2018. Seeds of lettuce cultivars “Annapolis,” “Celinet,” and “Coastline” were planted into flats with 128 cells and then manually transplanted into the field after the growth of true leaves was observed. Approximately 150 plants of each lettuce cultivar were planted in each 4 × 2-m plot.

Three irrigation waters were used in this experiment including groundwater (GW), secondary-treated wastewater (TW), and roof-collected rain water (RW). Prior to the experimental irrigation treatments, lettuce plants were drip irrigated weekly with GW without the supplementation of *E. coli* surrogates. The GW collected from the Fulton farm (Chambersburg, PA) was chosen as the baseline water source in this study because it is the main water source used for farm irrigation, and it is also commonly used in the Mid-Atlantic area. The TW was obtained from the stage of secondary treatment that removed ~80% of organic matter in the sewage with denitrification by microbial process, followed by the filtration and ultraviolet disinfection at Chambersburg Wastewater Treatment Plant (Chambersburg, PA). RW was collected from the rain barrels located at the Fulton farm. Microbiological properties of these irrigation waters (GW, TW, and RW) were determined by using the membrane filtration method as previously described by Yin and Patel (2018) for analyzing the populations of indicator bacteria including total coliforms, fecal coliforms, and generic *E. coli*. The pH and electrical conductivity of these irrigation waters were measured with a pH meter (Orion, St. Louis, MO, USA) and a conductivity meter (HM Digital, Culver City, CA, USA) as described by Castro et al. (2009).

The irrigation treatments were initiated at 25 days of lettuce transplant in the field soil. Freshly collected irrigation treatment waters including GW, RW, and TW were inoculated with *E. coli* O157:H12 and *E. coli* K12 as previously described and used for irrigation. A total of nine lettuce plots (three plots per cultivar) were used in this study, and each cultivar plot was separately irrigated with each type of irrigation treatment water. Each lettuce plot (4 × 2 m) was spray irrigated with ~5 L of treatment water by using handheld sprayers (Forestry suppliers, Jackson, MS, USA) and lettuce plants were allowed to dry in the field for 2 h. After irrigation, four lettuce leaf samples (~30 g per sample) from each plot were randomly collected on days 0, 1, 3, 7, 10 post-irrigation. Lettuce leaf samples were aseptically harvested by cutting ~2 cm above the soil with sterile scissors and forceps and transferred to sterile Whirl-Pak bags. Lettuce samples were stored on ice and transported to the USDA laboratory for microbiological analysis.

Microbiological Analysis

The lettuce leaf sample (~30 g) collected in sterile Whirl-Pak bag was homogenized with 120 ml of buffered peptone water (BPW) by pummeling for 2 min in a stomacher (Interscience Lab Inc., Woburn, MA, USA). Serial 10-fold dilutions of the

homogenate were made with PBS and the homogenate or its appropriate dilution was spiral plated onto the MacConkey agar (MAC; Neogen) with NA and CP for the enumeration of *E. coli* O157:H12 and *E. coli* K12, respectively. Agar plates were then incubated at 37°C for 24 h, and the bacterial populations were determined and expressed as log CFU g⁻¹. Prior to the experiment, irrigation water samples and lettuce leaves were analyzed by spiral plating onto MAC agar supplemented with NA or CP to ensure that the natural bacteria in irrigation waters and lettuce leaves were unable to grow on these agar media. The lettuce-BPW homogenate samples were incubated at 37°C for 24 h, followed by streaking a loopful of the enrichment onto MAC with NA and CP to determine the presence of the applied *E. coli* surrogates. Lettuce samples, in which *E. coli* surrogates were undetectable by the direct plating method but positive by the enrichment procedure, were reported as 1 log CFU g⁻¹ in this study.

To detect the presence of the pathogenic bacteria including *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7, the homogenates of lettuce samples with BPW were incubated at 37°C for 24 h as primary enrichment [American Public Health Association (APHA), 2005]. A portion of primary enriched sample (5 ml) was transferred to 45 ml of tetrathionate broth (Neogen), Fraser broth (Neogen), and mEHEC broth (Biocontrol, Bellevue, WA, USA) and incubated at 37°C for 24 h, followed by streaking a loopful of these enrichment broths on xylose lysine tergitol-4 agar (XLT4; Neogen), rapid *Lmono* agar (BioRad), and sorbitol MacConkey agar with CT supplement (CT-SMAC; Neogen), for the detection of *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7, respectively. Presumptive isolates of the target pathogens on selective media (XLT4, rapid *Lmono*, or CT-SMAC) were further confirmed by real-time quantitative PCR (RT-qPCR) as previously described (Yin and Patel, 2018).

Statistical Analysis

Completely randomized design was used to study the effect of irrigation treatment waters (GW, RW, and TW) on the persistence of *E. coli* surrogates on different lettuce cultivars. A three-way factorial repeated measurement ANOVA model with PROC MIXED procedure of Statistical Analysis Software (SAS 9.4, Cary, NC) was used for each of the two *E. coli* surrogates (*E. coli* O157:H12 and *E. coli* K12). Four individual samples of lettuce collected from each plot were analyzed at each sampling time point. The factors included water (GW, RW, and TW), lettuce cultivar (“Annapolis,” “Celinet,” and “Coastline”), and time (0, 1, 3, 7, and 10 days after irrigation). The standardized skewness was verified to assure normal distribution of the data. Differences among the means were analyzed at $P < 0.05$ using Fisher’s least significance difference test.

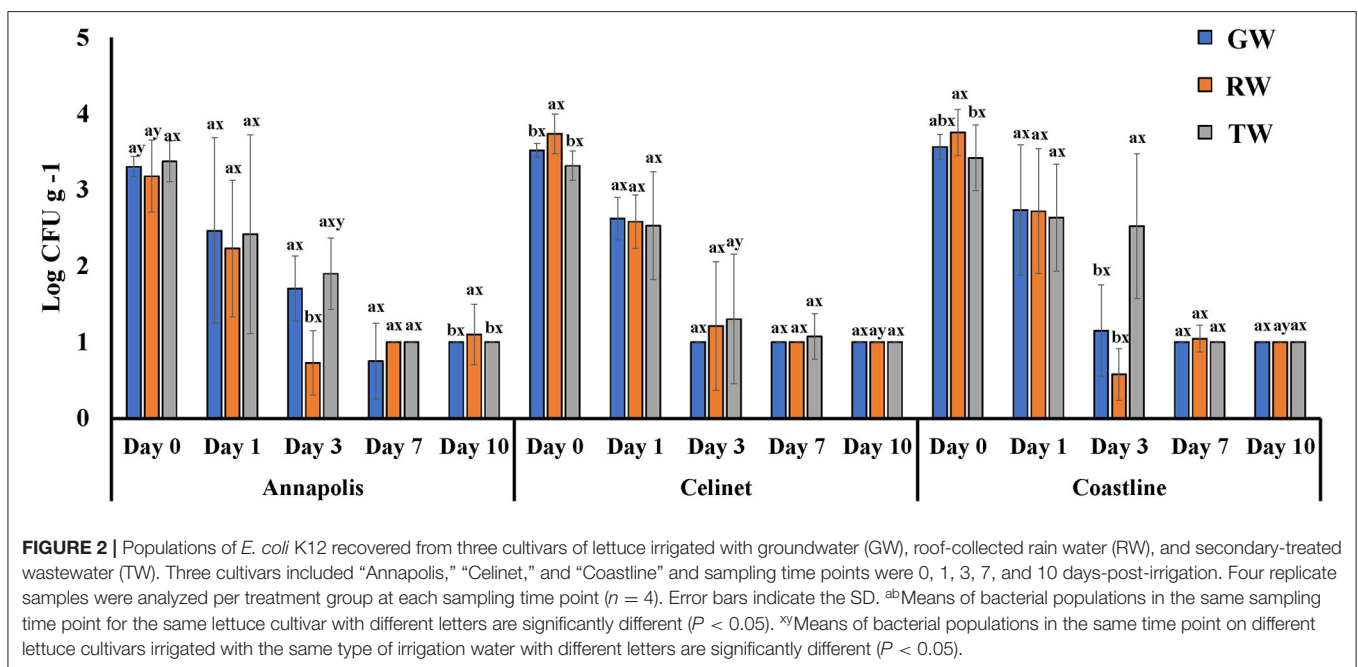
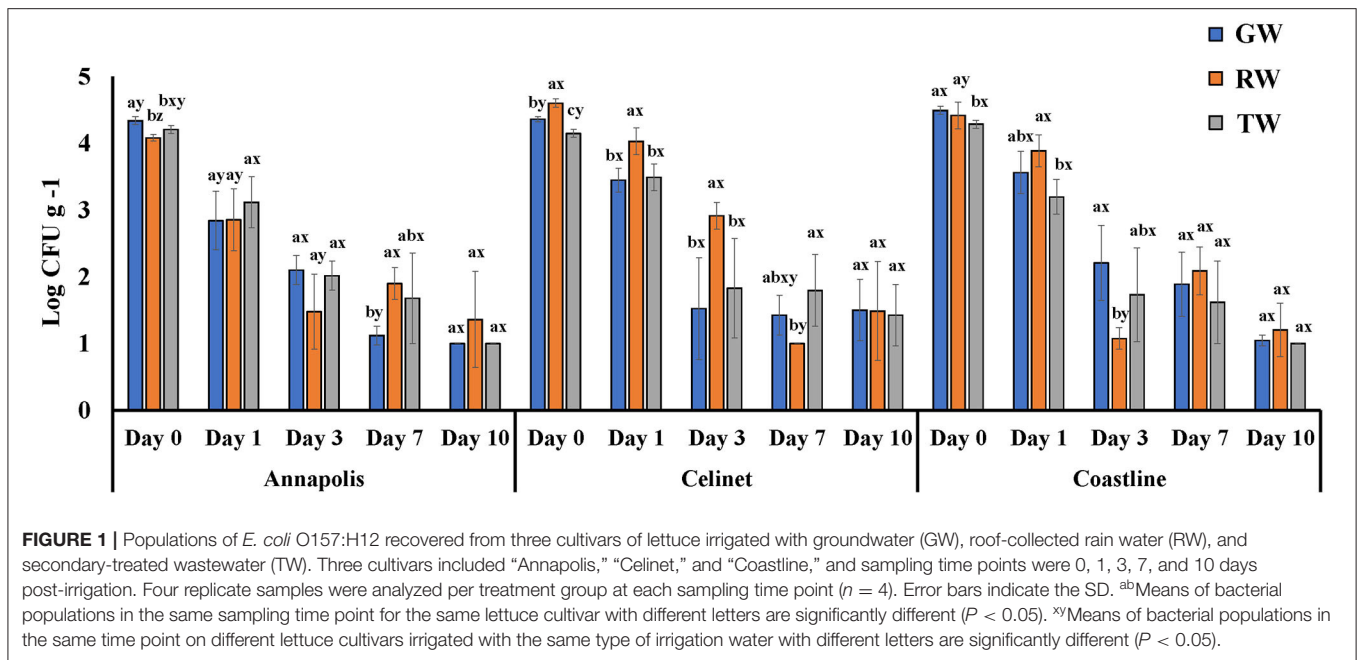
RESULTS

Prior to the supplementation of *E. coli* surrogates and fecal slurry, microbiological analysis of the irrigation waters revealed that GW sample contained 1.8 log, 1.7 log, and 1.0 log CFU per 100 ml of

total coliforms, fecal coliforms, and generic *E. coli*, respectively. Further, 0.3 log CFU per 100 ml of total coliforms was recovered from the TW and RW samples, whereas fecal coliforms and generic *E. coli* were not detected. Bacterial pathogens including *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7 were not detected from lettuce and water samples throughout the entire experiment. The values of pH and electrical conductivity of all three irrigation waters met irrigation water quality standards according to FAO’s guideline. The pH values of GW, TW, and RW were at 7.08, 7.77, and 8.21, respectively. The GW contained the highest electrical conductivity at 371 $\mu\text{s}/\text{cm}$, followed by TW (60 $\mu\text{s}/\text{cm}$) and RW (4 $\mu\text{s}/\text{cm}$).

Figure 1 presents the recovery of *E. coli* O157:H12 on the three lettuce cultivars “Annapolis,” “Celinet,” and “Coastline” with three types of irrigation waters including GW, RW, and TW. After irrigation, *E. coli* O157:H12 populations recovered from the irrigated lettuce leaves were in the range of 4.1 ± 0.1 and 4.6 ± 0.0 log CFU g⁻¹ on day 0. Results of the ANOVA table showed that lettuce cultivars significantly affected the recovery of *E. coli* O157:H12 on the irrigated lettuce leaves (Cultivar $P = 0.0013$; **Table 1**). On 0 and 1 day after irrigation with GW, RW, and TW, populations of *E. coli* O157:H12 recovered from the “Annapolis” lettuce samples were lower than the “Celinet” and “Coastline” lettuce samples that received the same type of irrigation water. Precisely, when three lettuce cultivars were irrigated with RW, *E. coli* O157:H12 populations recovered from the “Annapolis” lettuce (2.9 ± 0.5 log CFU g⁻¹) on day 1 were significantly lower than the bacterial populations recovered from “Celinet” and “Coastline” lettuce samples (4.0 ± 0.2 log and 3.9 ± 0.2 log CFU g⁻¹). Likewise, significantly lower *E. coli* O157:H12 populations were recovered from the GW-irrigated “Annapolis” lettuce on day 1 (2.8 ± 0.4 log CFU g⁻¹) and day 7 (1.1 ± 0.1 log CFU g⁻¹) compared to GW-irrigated “Coastline” lettuce samples on day 1 (3.6 ± 0.3 log CFU g⁻¹) and day 7 (1.9 ± 0.2 log CFU g⁻¹), respectively. Additionally, recovery of *E. coli* O157:H12 from the irrigated lettuce leaves was also significantly affected by the interaction effect of the irrigation water and the lettuce cultivar (water \times cultivar $P = 0.0218$; **Table 1**). Populations of *E. coli* O157:H12 recovered from different lettuce cultivars were dependent on the source of irrigation water. The recovery of *E. coli* O157:H12 was similar in the TW and GW-irrigated lettuce irrespective of the lettuce cultivars on day 3 ($P > 0.05$); however, irrigation with RW resulted in variable bacterial populations in different cultivars of lettuce when compared to the GW irrigation.

Recovery of *E. coli* K12 on the lettuce cultivars with alternative irrigation waters is shown in **Figure 2**. *E. coli* K12 populations were recovered at the lower levels than *E. coli* O157:H12 from the lettuce leaves on day 0 after irrigation ($P < 0.05$); the populations of this bacterium were in the range of 3.2 ± 0.2 and 3.8 ± 0.2 log CFU g⁻¹. Populations of *E. coli* K12 on the lettuce leaves were significantly affected by the source of irrigation waters (water effect $P = 0.0264$; **Table 1**). Higher recovery of *E. coli* K12 from RW to the leaves of “Celinet” and “Coastline” lettuce was observed (3.7 ± 0.1 and 3.8 ± 0.1 log CFU g⁻¹) compared to the lettuce samples irrigated with TW (3.3 ± 0.0 and 3.4 ± 0.1 log CFU g⁻¹) on day 0 ($P < 0.05$).



Populations of *E. coli* K12 recovered from the lettuce samples irrigated with the TW and GW were similar throughout the 10-day experimental period ($P > 0.05$). Similar results were observed with the recovery of *E. coli* O157:H12 in TW- and GW-irrigated lettuce samples. Lettuce irrigation with alternative TW irrigation did not significantly increase the transfer of *E. coli* O157:H12 compared to the control GW irrigation, irrespective of lettuce cultivar. For example, irrigation of TW resulted in the transfer of *E. coli* O157:H12 at ~ 4.2 log CFU g^{-1} on the lettuce compared to ~ 4.4 log CFU g^{-1} of *E. coli*

O157:H12 from the GW irrigated lettuce leaves immediately after irrigation ($P > 0.05$).

Regardless of the type of the irrigation waters and the lettuce cultivars, populations of these *E. coli* surrogates on the irrigated lettuce leaves gradually decreased during the 10-day experimental period. The recovery of *E. coli* O157:H12 and *E. coli* K12 from the irrigated lettuce was not significantly different 10 days after irrigation as populations of both *E. coli* strains on the lettuce leaves had become non-culturable by direct plating method (detection limit 1.0 log CFU g^{-1}), except for RW irrigation,

TABLE 1 | Analysis of variance (ANOVA) table for identifying the significant effects and their interactions with lettuce data on *E. coli* O157:H12 and *Escherichia* K12.

Source ^a	<i>E. coli</i> O157:H12			<i>E. coli</i> K12		
	DF ^b	F-value	Pr > F	DF	F-value	Pr > F
Water	2	1.73	0.1821	2	4.00	0.0264
Cultivar	2	7.07	0.0013	2	2.26	0.1179
Day	4	925.55	<0.0001	4	2,965.17	<0.0001
Water × Cultivar	4	3.01	0.0218	4	2.05	0.1063
Cultivar × Day	8	4.41	0.0002	8	6.23	<0.0001
Water × Day	8	1.58	0.1486	8	3.02	0.0077
Water × Cultivar × Day	16	3.27	0.0002	16	3.12	0.0007

^aThe main effect “water” includes groundwater, roof-collected rain water, and secondary-treated wastewater; the main effect “cultivar” includes “Annapolis,” “Celinet,” and “Coastline”; the main effect “day” includes 0, 1, 3, 7, and 10 days-post-irrigation.

^bDF, degrees of freedom.

which resulted in higher *E. coli* K12 populations on “Annapolis” lettuce in comparison to other two types of irrigation.

DISCUSSION

Our previous reports investigated the impact of RW and TW irrigation on the microbiological quality of spinach and lettuce by monitoring the transfer of indicator bacteria including total coliforms, fecal coliforms, and generic *E. coli* from the irrigation water to the produce under different growing conditions (Yin et al., 2018, 2019, 2020), and the results of these studies support the use of alternative waters for fresh produce irrigation in the Mid-Atlantic area provided that these waters contained acceptable levels of bacterial indicator populations. In the current study, the potential usage of RW and TW as irrigation water sources for fresh produce was further investigated by inoculating known concentrations of *E. coli* surrogates to the irrigation water and then determining the transfer and persistence of these surrogates on the irrigated lettuce.

The use of water contaminated with fecal materials for irrigation could be a direct route of produce contamination (Wood et al., 2010; Gorman, 2014). The TW and RW could contain fecal materials as human waste could present in TW and roof could be contaminated by bird and rodent droppings (Ahmed et al., 2011; Madoux-Humery et al., 2013). Further, as manure application is commonly used to fresh produce fields, it has been noted that leafy greens could be contaminated by fecal materials owing to fecal splash subsequent to spray irrigation. Atwill et al. (2015) reported that *E. coli* O157:H7 in simulated wildlife feces could be transferred to field-grown lettuce via splash during irrigation. Fecal slurry has been used to mimic the potential fecal splash by wild and farm animals during foliar irrigation (Chase et al., 2019). Weller et al. (2017) used rabbit fecal slurry to examine the transfer of *E. coli* on lettuce once splash contaminated by wild animal waste, and the populations of *E. coli* decreased from 8.9 log to 3.6 log MPN per lettuce head during the 10-day study between the inoculation and the harvest. It has been previously reported that *E. coli* could adapt better on the fresh

produce and spinach when applied with fecal slurry (Patel et al., 2010; Chase et al., 2017).

Several factors affect the likelihood of fresh produce becoming contaminated during irrigation including the frequency of irrigation, the microbial quality of irrigation water, the characteristic of the leaf surface, and the type of irrigation method (Gerba, 2009; Uyttendaele et al., 2015). The U.S. Food and Drug Administration (FDA) (2009) reported that the irrigation of edible crops by flooding and spraying method represents the highest risk as any bacteria in the water can be transferred to the leaf of these crops during the contact with water. Persistence of *E. coli* on irrigated produce increased by ~50% through spray (overhead) irrigation system compared to a drip irrigation system (Allende et al., 2017). In the current study, a direct contact of irrigation water with lettuce via spray irrigation method was employed to maximize the potential for microbial contamination (Solomon et al., 2002), and yet irrigation of TW did not significantly affect the recovery of *E. coli* surrogates from the irrigated leaves compared to the GW irrigation.

In this study, lettuce cultivars influenced the recovery of *E. coli* surrogates on the lettuce leaves; lower populations of *E. coli* O157:H12 were recovered from the “Annapolis” lettuce than from “Celinet” and “Coastline” lettuce. Plant variety may have different leaf surface properties including morphology, chemical constituent, and metabolic activity that could affect the bacterial colonization on phyllosphere (Heaton and Jones, 2008; Leveau, 2009; Quilliam et al., 2012). In addition, leaf surfaces harbored microbiota communities that were well-adapted to the nutrient-scarce environment, and the competition of microbiota to the invading human pathogens on the leaves could affect the persistence of these pathogens (Monier and Lindow, 2004; Pozo-Carrion et al., 2013). Leaf structural morphology of different cultivars of fresh produce have played an important role in the fate of enteric pathogens residing in lettuce foliage (Lopez-Velasco et al., 2015; Van der Linden et al., 2016; Erickson et al., 2019).

The recovery of *E. coli* surrogates from the irrigated lettuce varied with *E. coli* strains. *E. coli* O157:H12 populations recovered from the irrigated lettuce leaves were ~1 log CFU g⁻¹ greater than the recovery of *E. coli* K12 on the lettuce leaves on day 0 following irrigation. Previous reports have suggested that bacterial attachment to produce surfaces varied depending on the sources of the bacteria being isolated (Patel et al., 2010; Sharma et al., 2016). It is possible that *E. coli* O157:H12 strain attached at a higher rate to lettuce leaves than *E. coli* K12 because of its environmental origin characteristic.

Plant surfaces are considered harsh environments for the enteric organisms and the pre-harvest conditions in the field such as the competition of natural flora, solar radiation, and temperature pose additional challenges for the survival of microorganisms (Aruscavage et al., 2006). The survival profile of *E. coli* surrogates on the lettuce observed in this study was similar to the results of other previous ones, where spinach and lettuce were directly spray-inoculated with *E. coli* surrogates (Moyne et al., 2011; Gutiérrez-Rodríguez et al., 2012; Lopez-Velasco et al., 2015). In the current study, ~2–3 log reductions in *E. coli* surrogate populations were observed during the 10-day

experimental period. The mean *E. coli* O157:H12 levels decreased from $\sim 4.5 \log \text{CFU g}^{-1}$ on day 0 to below detection limit ($1.0 \log \text{CFU g}^{-1}$) after 10 days of irrigation treatments. Decline in *E. coli* populations on fresh produce surface between the inoculation and the harvest was also previously reported; *E. coli* populations on “Green Tower” lettuce leaves grown in the field reduced from 8.9 log to 3.6 log MPN per sample after 10 days of inoculation (Weller et al., 2017).

The current study supports the use of alternative RW and TW as irrigation waters for fresh produce since the recovery of *E. coli* surrogates from the irrigated lettuce leaves were mostly comparable to the GW irrigation. RW is commonly collected by the rooftops of the households, temporarily stored in barrels, and used as small-scale practices in the United States (Campisano et al., 2017). Large-scale rainwater harvesting systems may be used as demonstrated by Lani et al. (2018) and Zhang et al. (2020); however, the performance of these harvesting systems may be affected by the rainfall pattern, the roof area, and the tank size (Morales-Pinzón et al., 2014). Unlike GW and RW, TW could be considered as a reliable and stable water source since it is not significantly affected by the season, climatic condition, or precipitation level (Zhang and Shen, 2019). There were no pathogenic bacteria including *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 found in water samples and the irrigated lettuce in the current study; however, microbiological quality of these alternative irrigation waters must be confirmed prior to use as irrigation water sources for fresh produce.

CONCLUSION

The current study investigated the applicability of RW and TW as alternative agricultural water sources for fresh produce irrigation. The transfer of two non-pathogenic *E. coli* surrogates from GW, RW, and TW to “Annapolis,” “Celinet,” and “Coastline” cultivars of lettuce grown in the field was examined. The recovery of

E. coli on the irrigated lettuce was affected by bacterial strains, irrigation water types, and lettuce cultivars. The recovery of *E. coli* surrogates from the RW and TW irrigated lettuce samples was comparable to the populations of these bacteria recovered from the GW-irrigated lettuce. The *E. coli* surrogates transferred via irrigation waters persisted for at least 7 days on the lettuce leaves irrespective of water sources and lettuce cultivars, which suggests that incidental contamination of lettuce with irrigation water during the later stage of harvest could pose potential health concern and may require additional mitigation strategies to remove bacteria attached to the lettuce leaves. The findings of the current study provide data on the transfer of *E. coli* surrogates from RW and TW to the lettuce leaves that can be used in quantitative risk assessments of fresh produce grown in the Mid-Atlantic area.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

H-BY and JP developed the project idea. H-BY, NG, C-HC, AB, and JP designed and performed the experiment. H-BY, NG, C-HC, and AB collected and organized the data. H-BY initiated the manuscript, and JP revised the manuscript. All authors contributed to manuscript revision, and read and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer YL declared a shared affiliation, though no other collaboration, with several of the authors H-BY, C-HC, AB, JP to the handling editor.

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