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Differences in biofilm formation of produce and poultry *Salmonella enterica* isolates and their persistence on spinach plants

Jitendra Patel [a,*], Manpreet Singh [b], Dumitru Macarisin [a], Manan Sharma [a], Daniel Shelton [a]

[a] U.S. Department of Agriculture, Agricultural Research Service, 10300 Baltimore Ave, Bldg. 201, Beltsville, MD 20707, USA
[b] Department of Poultry Science, Auburn University, 201 Poultry Science Bldg., Auburn, AL 36849, USA

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A B S T R A C T

Spinach plants were irrigated biweekly with water containing 2.1 log CFU *Salmonella*/100 ml water (the maximum *Escherichia coli* MPN recommended by the Leafy Greens Marketing Agreement; LGMA), or 4.1 CFU *Salmonella*/100 ml water to determine *Salmonella* persistence on spinach leaves. Green Fluorescent protein expressing *Salmonella* were undetectable by most-probable number (MPN) at 24 h and 7 days following each irrigation event. This study indicates that *Salmonella* are unlikely to persist on spinach leaves when irrigation water is contaminated at a level below the LGMA standards. In a parallel study, persistence of *Salmonella* isolated from poultry or produce was compared following biweekly irrigation of spinach plants with water containing 6 log CFU *Salmonella*/100 ml. Produce *Salmonella* isolates formed greater biofilms on polystyrene, polycarbonate and stainless steel surfaces and persisted at significantly higher numbers on spinach leaves than those *Salmonella* from poultry origin during 35 days study. Poultry *Salmonella* isolates were undetectable (<1 log CFU/g) on spinach plants 7 days following each irrigation event when assayed by direct plating. This study indicates that *Salmonella* persistence on spinach leaves is affected by the source of contamination and the biofilm forming ability of the strain.

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1. Introduction

According to the latest estimates by the Centers for Disease Control and Prevention (CDC), 48 million cases of food-borne illness are reported each year in the United States causing over 128,000 people to become hospitalized and approximately 3000 deaths (CDC, 2010). This report by the CDC also noted that *Salmonella* is the leading cause of an estimated 35% of hospitalizations and 28% of deaths.

Salmonella spp. has been responsible for outbreaks linked to lettuce, basil, and bagged vegetables in Europe (NygaÌŠrd et al., 2008; Pakalniskiene et al., 2009; Sagoo et al., 2003; Crook et al., 2003). In the U.S., outbreak of *Salmonella* Saintpaul linked to consumption of imported Serrano peppers resulted in 1442 cases of illnesses (CDC, 2010).

Fresh produce can be contaminated by enteric pathogens throughout the food-chain. The sources of *Salmonella* contamination at farm level may include contaminated or inadequately composted manure, wild or domestic animals, irrigation or wash water. Leafy greens were contaminated before harvest in outbreaks linked to *Escherichia coli* O157:H7 where produce was grown in Salinas Valley, CA (Mandrell, 2010). Epidemiological investigations of other outbreaks linked to cantaloupe and tomatoes have supported pre-harvest contamination of suspect produce (Calfert, 2008; Gupta et al., 2007). *Salmonella* can be transferred from soil to lettuce leaves when soil is contaminated with *Salmonella* (Franz et al., 2007). Lapidot and Yaron (2009) reported transfer of *S.* Typhimurium from contaminated irrigation water to mature parsley plants and persistence in produce for up to three weeks after harvest. They suggested the role of curli and cellulose in the transfer or survival of this enteric pathogen in the plant. Stine et al. (2011) showed that water used to prepare pesticides in spray applications can be a source contamination of leafy greens with *Salmonella*, especially if the produce is harvested shortly after the chemical has been applied. *Salmonella* were found in 43% of the irrigation water samples used for growing chili peppers in Mexico (Gallegos-Robles et al., 2008). *Salmonella* were detected in 7% of iceberg lettuce irrigated with water contaminated with poultry manure (Ndiaye et al., 2011). Arthurson et al. (2011) reported *Salmonella* persistence on spinach leaves, roots, and in soil during 4-week evaluation period when soil was treated with cattle manure containing *Salmonella*.

* Corresponding author. Tel.: +1 301 504 7003; fax: +1 301 504 8438.
E-mail address: jitu.patel@ars.usda.gov (J. Patel).

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Animal-raising industries such as poultry farms in rural areas might be among the more important contributors to the *Salmonella* burden of waters receiving runoff from feed lots (Miner et al., 1967) and manured fields. The introduction of *Salmonella* from produce growing environments from poultry rearing and processing facilities can occur. However, the persistence of *Salmonella* isolates from poultry on leafy green commodities has not been studied. We evaluated the persistence of *Salmonella* isolated from poultry and produce on spinach leaves under preharvest conditions. The relation between their ability to form biofilms and persistence on foliar tissues was compared.

2. Material and methods

2.1. Spinach plants

The baby spinach cultivar 'Whale' (Meyer Seed Co., Baltimore, MD) was selected because of its heat tolerance and preference to the Mid Atlantic region of the U.S. Spinach plants were grown in organic sandy soil using Cone-tainers as described by Patel et al. (2010). Plants were maintained in a BSL-2 growth chamber at 20 °C and 18 °C, for day (14 h) and night (10 h), respectively, at 60% RH and 240 $\mu mol/m^2/s$ light intensity. Plants were irrigated carefully to avoid splashing the deionized water and container soil onto leaves; water was supplied as needed to maintain soil moisture at field capacity and plant turgor.

2.2. Bacterial strain and inoculation

Study 1: A green fluorescent protein-labeled *Salmonella* Typhimurium obtained from Dr. Erickson, Univ. of Georgia, Griffin, GA was used in the study. The strain with GFP expressing ability would aid in its identification by confocal microscopy as required. The strain was cultured from −80 °C stocks in TSB supplemented with 10% glycerol. Frozen strain was partially thawed at room temperature (∼22 °C) for 15 min, inoculated in TSB containing 100 μg/ml ampicillin, and incubated at 37 °C for 24 h. The culture was grown in filter-sterilized water extracts of dairy manure solids for inoculation on spinach leaves. The bovine fecal solid extract was prepared as described by Patel et al. (2010). Individual 4-week old spinach plants were spray inoculated every 2 weeks with 3 ml water containing 130 (low) or 13,000 (high) *S*. Typhimurium per 100 ml. Spinach plants sprayed with water extracts of dairy manure solids served as controls.

Study 2: Five *Salmonella enterica* serovars associated with produce commodities (*S.* Thompson, *S.* Tennessee, and *S.* Negev, *S.* Braenderup, and *S.* Newport) were obtained from our Environmental Microbial and Food Safety Laboratory culture collection. Five *S. enterica* serovars isolated from poultry (*S*. Thompson, *S*. Newport, *S*. Seftenberg, *S*. Typhimurium, and *S.* Enteritidis) were obtained from Poultry Science Department (Auburn University, Auburn, AL). Individual strains were grown in bovine fecal extract as described above. Two separate cocktails of either produce or poultry *Salmonella* isolates were prepared by combining 5 ml of each produce isolate together or each poultry isolate together, respectively. The *Salmonella* populations of each cocktail was determined by spot plating (20 spots of 5 μL) of appropriately diluted cocktail suspension on XLT4 agar (Acumedia, Lansing, MI). Individual 4-week old spinach plants were spray inoculated every 2 week with 3 ml water containing low or high inoculums of produce or poultry isolate cocktail. Sterile bovine fecal solid extract was used on spinach plants as control.

2.3. Bacterial analysis

Four replicates of each plant were analyzed 24 h post irrigation and every week for up to 5 weeks for surviving populations of *Salmonella* using both spiral plating on XLT 4 and most probable number (MPN) procedure. Plant shoots (stem and leaves) were harvested aseptically by cutting the stem just above the soil surface with sterile scissors; shoots were transferred to sterile whirl-pak bags (Nasco, Ft. Atkinson, WI, USA) and kept on ice until analysis, and 25 ml buffered peptone water (BPW, Acumedia) was added to whirl-pack bag which were then sonicated for 30 s (Astrason ultrasonic cleaner, Plainview, NY, USA) followed by pummeling for 2 min (Bagmixer, Interscience, St. Norms, France). Homogenates were spiral plated on XLT4 agar and incubated at 37 °C for 24 h. *Salmonella* colonies were counted by Protocol colony counter (Symbiosis USA Inc., Frederick, MD). Further, surviving *Salmonella* populations were determined by 8-tube MPN assay. Spinach plant suspensions in BPW were incubated at 37 °C for 24 h for primary enrichment. Enriched suspensions (200 μl) were dispensed to 1.8 ml tetrathionate broth (Acumedia) and serially diluted for an 8-tube MPN assay in 96-deepwell microplates (2.2 ml/well, Fisher Scientific, Newark, DE), and incubated at 37 °C for 24 h. After incubation, each suspension was streaked on XLT4 agar and incubated at 37 °C for 24 h.

2.4. Biofilm formation

Overnight cultures of individual poultry and produce *Salmonella* isolates grown in TSB were diluted 1: 10000 in either TSB or 1:10 TSB, and 200 μl were deposited in wells of a sterile 96-well polystyrene microtiter plate (Fisher Scientific) and incubated under static conditions at 30 °C for 48 h. For each replicate experiment, eight wells were inoculated for each serovar in each growth medium. Growth medium devoid of bacterial inoculum served as a negative control. Biofilm formation in microtiter plates was determined by crystal violate assay as described by Patel and Sharma (2010).

The impact of a distributed shear on biofilm formation on stainless steel and polycarbonate surfaces was determined with the Center for Disease Control (CDC) biofilm reactor (BioSurface Technologies Corp, Bozeman, MT). Stainless steel and polycarbonate coupons (1 cm diameter, Biosurface Technologies) were inserted into the reactor coupon holders and the reactor was autoclaved (121 °C for 15 min). Reactors were allowed to cool, and 500 ml of 10% TSB was added in the reactor. The reactor was then inoculated with 1 ml of individual *Salmonella* strain containing 7 log CFU/ml. Reactor was placed on stir plate, connected to the peristaltic pump (Cole Parmer, Vernon Hills, IL), and 10% TSB was supplied to the reactor from 20 L carboy via silicon tubing (3/16″ internal diameter, Cole Parmer) at a flow rate of 0.3 mL/min. A baffle rotation was set at 125 rpm on stir plate while fluid flow was continued for 24 h. The coupon holders were removed; coupons were immersed in 10 ml PBS for 5 s to remove residual carry over. Coupon was transferred to a sterile 6-well plate (Fisher Scientific) containing 10-ml PBS and scrapped with sterile Teflon 'Policeman' scrapper (Fisher Scientific) each side of the coupon for 30 s to remove attached cells. Appropriately diluted suspensions containing scrapped *Salmonella* were spiral-plated on XLT4 agar. Following overnight incubation at 37 °C, presumptive *Salmonella* black colonies were enumerated using Protocol colony counter.

2.5. Curli expression

Overnight cultures of individual *Salmonella* serovars grown in TSB were streaked on tryptone agar supplemented with Congo Red (40 μg/ml) and Coomassie brilliant blue (20 μg/ml) (Romling et al., 2003), and incubated in plant growth chamber and at 22 and 37 °C for 48 h. Curli expression in strains is indicated by red colonies as a result of dye uptake.

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