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Escherichia coli O157:H7 and *Salmonella* Typhimurium adhesion to spinach leaf surfaces: Sensitivity to water chemistry and nutrient availability

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ABSTRACT

This study investigated the effects of solution chemistry and growth conditions on bacterial deposition on spinach leaf surfaces using a parallel plate flow cell. Two food safety pathogens of concern and two non-pathogen bacterial surrogates (environmental *E. coli* isolates) were grown in ideal (LB media) and nutrient-restricted (M9 media) conditions. Bacterial attachment was quantified as mass transfer rate coefficients for cells suspended in 10 mM KCl, $CaCl_2$ and artificial groundwater, and cell and leaf surfaces were extensively characterized (zeta potential, hydrophobicity, extracellular polymer (EPS) composition). Between the pathogens, *E. coli* O157:H7 attachment was greater than that of *Salmonella* Typhimurium, attributed to measurable variability in cell surface charge and hydrophobicity. When grown in M9 media, both pathogens were significantly more adhesive to spinach surfaces ($p < 0.01$) than when grown in LB media. Surrogates did not follow this trend and showed minimal changes in adhesion kinetics and surface properties between growth conditions. EPS sugar/protein ratios were reduced in some of the highest attachment scenarios, suggesting that changes in EPS composition in favor of proteins may play a role. These results show the importance of growth conditions and solution complexities in understanding mechanisms of aqueous bacterial adhesion to food surfaces.

1. Introduction

The World Health Organization estimates that 600 million people fall ill after consuming unsafe food ever year, leading to over 400,000 deaths worldwide (World Health Organization (WHO), 2015). While consumption of fruits and vegetables is essential to improving diets and nutrition, prevalence of foodborne illness linked to microbial contamination of fresh produce remains high in both the United States and Europe (Callejón et al., 2015). Contamination has been shown to come from water sources before, during, and after harvest (Olaimat and Holley, 2012), and can be magnified by increasing pressures on water resources and occurrences of extreme weather (Bradford and Harvey, 2016; Shah et al., 2016). The potential for cross-contamination during value-added processes like cooling, washing, and packaging have been shown to further magnify the risks of foodborne illness outbreaks associated with several types of fresh produce (Kuan et al., 1016; Kusumaningrum, 2003). Leafy greens are considered especially vulnerable to microbial risks due to the common usage of sprinkler irrigation, "triple wash" and other pre-packaging methods, and high likelihood of consumption without a heating or cooking step to kill pathogens (Food and Drug Administration (FDA), 2008). In fact, over 600 foodborne illness outbreaks and 20,000 unique illnesses in the U.S. were attributed to leafy greens from 1973 to 2012 (Herman et al., 2015). Even with industrializing agricultural systems that regulate and limit direct human handling, there is a significant need for scientific research to improve our understanding of the interactions between microbes and produce. This is due to washing and handling processes that create complex and dynamic environments in which bacteria can attach and proliferate on produce surfaces.

The complexities of water chemistry in agricultural systems can vary significantly based on the climate, water source, and crop, while the approach to food safety largely remains the same. For example, nearly all farms in Salinas, California use unregulated groundwater for irrigation, while those in Yuma, Arizona use highly saline Colorado River water (Benjamin et al., 2013; Fonseca et al., 2011). Together, these locations represent 90% of U.S. leafy greens production and operate under one set of food safety standards (Leafy Greens Marketing Agreement (LGMA), 2016). Previous work has demonstrated that aqueous bacterial deposition on surfaces is sensitive to a wide range of parameters, including ionic strength, pH, and valence (Chen and Walker, 2007; Chowdhury et al., 2014). However, these trends have not been tested systematically in the context of potential microbial cross contamination and food safety of leafy greens. Similarly, the critical influence of fluid dynamics on cell-surface interactions has been

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demonstrated in a number of studies and environments, but most research on microbial adhesion and detachment from produce surfaces has been conducted under static conditions that generally involve dipping and rinsing food substrates in inoculated solutions (Kuan et al., 2017; Macarisin et al., 2012; Reina et al., 2002), rather than in situ observation methods. This work utilizes a microfluidic flow cell to assess bacterial deposition and rinsing on model spinach leaf surfaces in real time, under well-defined hydrodynamic flow conditions in simple and complex solution chemistries.

Two foodborne pathogens, *Escherichia coli* (*E. coli*) serotype O157:H7 and *Salmonella enterica* serotype Typhimurium *(Salmonella),* have been implicated in significant foodborne illness outbreaks in recent years (Cooley et al., 2007; Ao et al., 2015). Together, they account for nearly one-third of all leafy-vegetable associated outbreaks over the past 30 years (Herman et al., 2015). Industry, regulatory groups, and researchers often use nonpathogenic bacteria, like other strains of *E. coli,* as surrogates of human pathogens to study risk and improve management techniques for food safety (Harwood et al., 2005). However, the validity and usefulness of generic *E. coli* strains in mimicking pathogen fate and transport in diverse, realistic environments outside of the laboratory has been questioned (Deng et al., 2014; Sinclair et al., 2012). Additionally, few studies have considered changes in pathogen and surrogate attachment after growth in nutrient-restricted conditions, which can have a significant influence on cell surface properties (Hassan and Frank, 2004) and ultimately biofilm formation (Teh et al., 2016). This study investigates the adhesion behavior of two environmental *E. coli* strains that were previously identified as promising nonpathogenic surrogates (Cook et al., 2017), as well as that of reference strains the two known pathogens, all grown in nutrient-rich and nutrient-restricted conditions. Characterization of the deposition trends and cell surfaces for *E. coli* O157:H7 and *Salmonella* was conducted in a suite of water quality scenarios, ranging from simple to complex solution chemistries. Following pathogen experiments, adhesion of the two potential surrogates was quantified in one representative complex solution chemistry in order to provide a comparison in the most realistic conditions.

2. Materials and methods

2.1. Bacterial growth and preparation

Escherichia coli O157:H7 (ATCC 43888), *Salmonella* Typhimurium (ATCC 13311), and two non-pathogen environmental *E. coli*, referred to as *E. coli* B01 and B05, were acquired from the USDA (Kimberly Cook, USDA-ARS-FAESR, Bowling Green, KY). *E. coli* O157:H7 and *Salmonella* were chosen as model pathogens for this work because they represent water- and foodborne bacteria that are both rod-shaped and Gram-negative, while they have been shown to have notably different characteristics in hydrophobicity, zeta potential, and attachment (Haznedaroglu et al., 2009). *E. coli* B01 and *E. coli* B05 were chosen based on previous work that identified their potential ability to sufficiently represent these two pathogens in complex systems (Cook et al., 2017).

In brief, all bacteria were cultured in Luria-Bertani (LB) media at 37 °C overnight, before inoculation in either LB or minimal salts (M9) media for 3.5 h or 6 h, respectively, at 37 °C and harvested at mid-exponential cell growth phase (see growth curves in Supplementary Information). LB medium (Fisher Scientific, Fair Lawn, NJ) consists of 0.5% (5 mg/mL) yeast extract, 1% (10 mg/mL) tryptone, and 0.5% (10 mg/mL) NaCl prepared in deionized water. M9 medium was created using 6 mg/mL Na_2HPO_4, 3 mg/mL, KH_2PO_4, 0.5 mg/mL NaCl, and 1 mg/mL NH_4Cl, supplemented with 1% glucose, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$ in deionized water (M9 minimal medium (standard), 2010). Bacterial cell suspensions were adjusted to a concentration of 5.0×10^7 cells/mL using a counting chamber (Bürker-Türk chamber, Marienfield Laboratory Glassware, Lauda-Konigshofen, Germany) for deposition experiments or $OD_{560} = 0.2$ (approximately 10^8 cells/mL) for bacterial cell characterization.

2.2. Quantifying bacterial attachment

A parallel plate (PP) flow chamber (GlycoTech, Rockville, MA) on an inverted fluorescent microscope (BX-52, Olympus) was used to conduct bacterial adhesion and rinsing experiments. The microfluidic chamber dimensions are 6 cm × 1 cm × 0.0762 cm and is made up of a Plexiglas® block mounted by a flexible silicone elastomer gasket and a polycarbonate microscope slide (supporting isolated spinach epicuticle layer) sealed by vacuum grease. Influent bacteria suspended in one of the solutions (10 mM KCl, $CaCl_2$, or artificial groundwater (AGW)) enters the chamber from a capillary tube connected to a syringe on a syringe pump set to an average flow rate of 0.1 mL/min, which corresponds with a flow velocity of 0.8 m/h and a Péclet number of 6.47×10^{-4} (McClaine and Ford, 2002). These non-turbulent flow conditions induce a shear rate of 1.7 s^{-1} on the spinach surface and simulate expected conditions in a gentle produce washing process (Huang et al., 2017). Bacteria are imaged on the surface under 400x total magnification by using a 40 × long working distance objective (UPlanFI, Olympus).

After rinsing the epicuticle surface with DI water within the parallel plate flow cell, bacterial attachment was observed over a 30 min. period at ambient temperatures (20–25 °C), followed by a 30 min. rinse with an uninoculated (cell-free) solution of the same background solution. Images were recorded with a digital camera (Demo Retiga EXI Monochrome, QImaging) every 30 s in order to determine the kinetics of cell attachment to the surface by comparison of successive images. Flow cell experiments were conducted in 10 mM KCl, 10 mM $CaCl_2$, and 10 mM artificial groundwater (AGW) to investigate the impact of solution chemistry on deposition on the leaf surface. AGW was composed of 0.014 g KNO_3, 0.321 g $MgSO_4{\cdot}7H_2O$, 0.112 g $CaSO_4{\cdot}2H_2O$, 0.044 g NaCl, and 0.109 g $NaHCO_3$ per 1 L of deionized water (Bolster et al., 1999). This ionic strength is relatively high compared to that of most surface water, but was chosen as a baseline within the realm of possibility for surface and groundwater to maximize observable attachment, as shown by previously reported trends in microbial adhesion to the epicuticle and other solid surfaces (Rapicavoli et al., 2015).

Images from flow cell experiments were analyzed using the supplied digital camera software, SimplePCI, and Mathworks MATLAB (R2015a) in order to record and enumerate the kinetics of cell attachment. The rate of attachment during the final 10 min is used to calculate the mass transfer rate coefficients in order to minimize variability in attachment and to maximize the number of cells that can be observed. The number of bacterial cells deposited can be plotted as a function of time, and bacterial flux, J (cells per s × m^2), can be calculated according to:

$$J = \frac{m}{a}$$

where *m* is the linear slope of adhered cells versus time and *A* is microscope viewing area (153 μm × 113 μm). The corresponding bacterial transfer rate coefficient for the bacteria, *k* (m × s^{-1}), can then be calculated:

$$k = \frac{J}{C}$$

where *C* is the bulk cell concentration (cells per mL).

2.3. Surface preparation and characterization

Baby spinach leaves used in this study were pre-washed and bagged from the same brand and same grocery store, and were used for creating isolated epicuticle layers on the same day as purchase. Otherwise, leaves were stored for a maximum of 3 days at 4 °C. Fully green, healthy-looking, non-damaged leaves were selected for experiments (no visible yellowing, browning, rips, or tears) and were handled aseptically throughout their preparation. A freeze imbedding technique was used to isolate epicuticle layers from the spinach leaf surface, which was developed by Ensikat et al. (2000). The transparent wax epicuticle layer was immobilized onto a polycarbonate microscope slide in order to view bacterial deposition from below using the inverted microscope. The method, in brief, involves placing a dime-sized drop of Triethylene glycol (TEG) on a flat, rigid stainless steel surface. A pre-cut spinach leaf is then placed on top of the drop, with the top side of the leaf in contact with the TEG. The TEG and surfaces are then submerged in liquid nitrogen for approximately 10 s. Immediately after removal from liquid nitrogen, tweezers are used to remove the leaf sample, leaving the epicuticle layer imbedded in the frozen TEG droplet, and the droplet is allowed to thaw directly on the polycarbonate slide. This allows the epicuticle wax layer to transfer directly to the slide and remnant TEG can be rinsed away with DI water. Immobilized wax layers were stored at 4 °C following isolation for up to one week.

The whole spinach leaves were characterized using a streaming potential analyzer (SurPASS, Anton Paar, Graz, Austria) and goniometer to assess surface zeta potential and surface contact angle (SCA), respectively. Whole leaves and isolated epicuticle layers were also visualized using light microscopy and environmental scanning electron microscopy (ESEM) (FEI Quanta 200). Spinach leaves were imaged in the ESEM in low-vacuum mode at 20 kV, without pre-treatment of samples.

2.4. Bacterial cell characterization

Bacteria were characterized following protocols previously developed for relative hydrophobicity, electrophoretic mobility (a surrogate for surface charge), size, and EPS composition. Relative hydrophobicity (%) was measured using a UV–Vis spectrophotometer (BioSpec-mini, Shimadzu Corp., Kyoto, Japan) and the microbial adhesion to hydrocarbons (MATH) test (Pembrey et al., 1999). A ZetaPALS analyzer (Brookhaven Instruments Corporation, Holtsville, NY, U.S.A.) was used to measure electrophoretic mobility, which is converted to zeta potential (mV). Extracellular polymeric substances (EPS) on the cell surface were extracted through water bath sonication (at 3.5 Hz for 20 s total) and characterized for composition of proteins and polysaccharides using the Lowry and phenol sulfuric acid methods, respectively, as previously described (Gong et al., 2009).

2.5. Statistical analysis

At least three independent repetitions were performed for characterization and flow cell experiments, including a fresh cell culture for each trial. To test for differences between water chemistry, growth conditions, and bacteria strains in all experiments listed above, a statistical single-factor ANOVA test was conducted for confidence intervals of 95% and 99% ($p < 0.05$ and $p < 0.01$, respectively). For pairs of water chemistry conditions, a *t*-test was conducted to determine statistically significant differences for confidence intervals of 95% and 99% ($p < 0.05$ and $p < 0.01$, respectively).

3. Results and discussion

3.1. Adhesion kinetics of E. coli O157:H7 and Salmonella Typhimurium

Systematic flow cell experiments were conducted to evaluate kinetics of cell attachment to spinach surfaces under a range of relevant water chemistries for the two pathogens, including 10 mM monovalent (KCl), divalent ($CaCl_2$), and multivalent (artificial groundwater (AGW)) salt solutions. The mass transfer rate coefficient (k, m/s) for bacteria was calculated using observed bacterial flux (number of cells per area per time) and bulk cell concentration (number of cells per mL), from which adhesion trends can be evaluated. Deposition behavior of *E. coli* O157:H7 and *Salmonella* Typhimurium on spinach epicuticle wax as a function of nutritional conditions during growth and water chemistry is displayed in Fig. 1. After growth in nutrient-rich conditions (LB), the average mass transfer rate coefficient, *k,* for cells in the divalent salt solution ($CaCl_2$) ($1.71 \pm 0.64 \times 10^{-7}$ m/s) was more than double that of the monovalent salt (KCl) ($8.14 \pm 1.28 \times 10^{-8}$ m/s) for *E. coli* O157:H7, which was similar to *k* in AGW ($8.25 \pm 0.70 \times 10^{-8}$ m/s). *Salmonella* Typhimurium followed the same trend when grown in LB, with *k* values of 6.57 ± 0.34, 7.32 ± 3.39, and $11.7 \pm 2.24 \times 10^{-8}$ m/s in KCl, AGW, and $CaCl_2$, respectively. The differences in average *k* between mono-, di-, and multi-valent solution chemistries were not highly significantly when grown in LB, with the exception of *Salmonella* in KCl and $CaCl_2$ ($p < 0.01$).

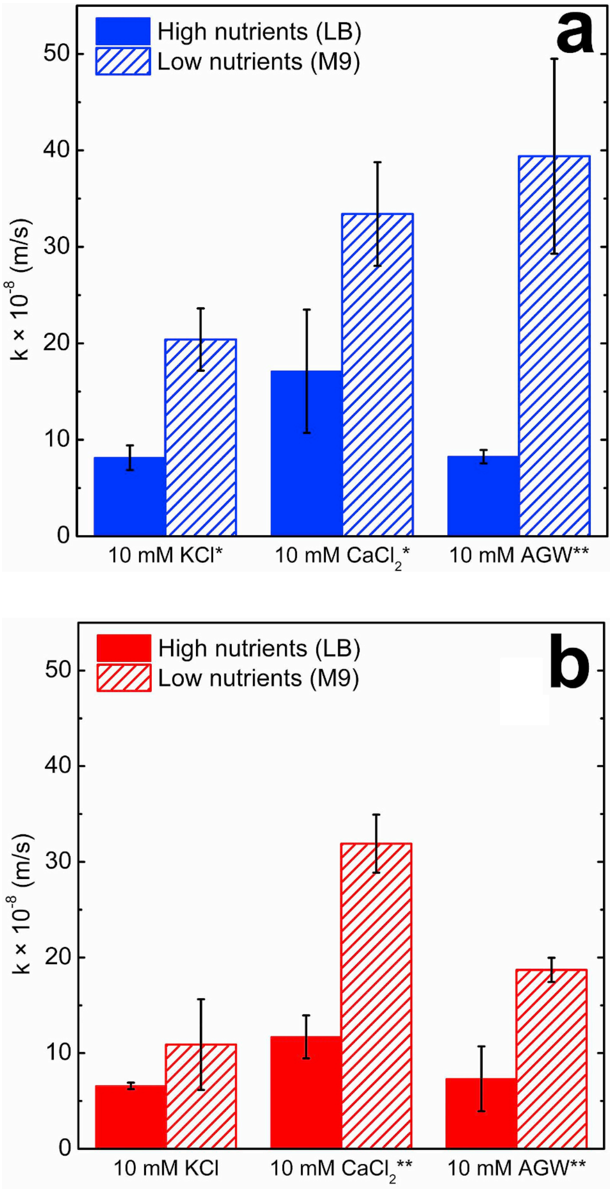

Fig. 1. Attachment of *E. coli* O157:H7 (a) and *Salmonella* Typhimurium (b) quantified by mass transfer rate coefficients in monovalent salt solution (10 mM KCl), divalent salt solution (10 mM $CaCl_2$), and a mixture of salts (10 mM AGW) over a 30 min duration. All experiments were performed in triplicate and error bars indicate one standard deviation. Significant differences between the two growth condition are denoted by *($p < 0.05$) and **($p < 0.01$).

Experiments with pathogens grown in minimal media (M9) indicate notably different deposition behavior for the cells. *Salmonella* (Fig. 1b) grown in M9 media was significantly more adhesive in $CaCl_2$

($3.19 \pm 0.30 \times 10^{-7}$ m/s) and AGW ($1.87 \pm 0.13 \times 10^{-7}$ m/s) than cells grown in LB media. *E. coli* O157:H7 was also significantly more adhesive to spinach surfaces in every solution chemistry after growth in minimal media and deviated from the trends expected based on classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of particle stability. DLVO theory can be used to characterize the forces between charged microbial cells and other surfaces interacting in aqueous environments and can therefore provide insight into the favorability of attachment under different conditions (Elimelech et al., 1995; Rapicavoli et al., 2015). For example, the presence of divalent cations in $CaCl_2$ result in charge screening and compression of the electrical double layer between bacteria and the leaf surface, which has been demonstrated to result in more favorable conditions for attachment (Hermansson, 1999). A more detailed explanation of DLVO theory is available in the Supporting Information. DLVO calculations revealed that bacteria cells and the spinach leaf surface were only modestly repulsive in all conditions (data not shown). In the absence of significant electrostatic and van der Waals interactions, hydrodynamic forces within the flow cell are expected to result in cell deposition that generally increases with solution valence (KCl, AGW, and $CaCl_2$, respectively). The attachment mass transfer rate coefficient for undernourished *E. coli* O157:H7 instead reached a maximum of $3.94 \pm 1.01 \times 10^{-7}$ m/s in the mixed valence solution chemistry (AGW).

When grown in LB, no significant differences were observed between attachment rate coefficients in the three different solution chemistries for either bacteria. In contrast, solution chemistry made a significant difference in attachment ($p < 0.05$) after cells were grown in M9 minimal media, with the exception of comparing *E. coli* in $CaCl_2$ and AGW. It is hypothesized that extracellular polymers and other structures on the bacteria surface play a role in magnifying the impacts of undernourishment on bacterial adhesion to the spinach surface in the different solution chemistries. The role of surface macromolecules in pathogen adhesion after growth in minimal media is further explored in the discussion of cell characterization results below.

In comparison to the baseline experiments in KCl and $CaCl_2$, the complex water chemistry of AGW provides more representative conditions in which mechanisms of bacterial attachment in agricultural environments can be considered. Marshall et al. (1971) demonstrated the presence of two distinct stages of aqueous bacterial attachment to surfaces: an instantaneous reversible phase and a time-dependent irreversible phase. Therefore, deposition was observed over 30 min. experiments, which would imply that adhesion is occurring within these two early stages of attachment and pre-biofilm development (Goulter et al., 2009; Van Houdt and Michiels, 2005). In Fig. 2a, the average number of *E. coli* O157:H7 and *Salmonella* cells adhered to the spinach surface during experiments in AGW is shown as a function of time, separated by M9 and LB growth conditions. For the first 5–10 min, total number of cells remains only slightly above zero due to cells reversibly attaching and then detaching from the surface. Both pathogens grown in each of the nutrient conditions show similar rates of deposition up to this point. The increasing rate of cell attachment is noticeable after the first 10 min under all conditions. The rate of deposition during the final 10 min (Fig. 2b) is where the nuances of cell type and growth condition are most distinct, therefore it is here that the mass transfer rate coefficients are calculated. Cells have likely reached an irreversible attachment phase at this point, as negligible detachment was observed over a 30 min. rinse using the same uninoculated solution following each experiment (data not shown).

When grown in LB media, *E. coli* O157:H7 and *Salmonella* adhere at similar rates in AGW, resulting in mass transfer rate coefficients of $8.25 \pm 0.70 \times 10^{-8}$ and $7.32 \pm 0.34 \times 10^{-8}$ m/s, respectively. This is in contrast with previous work that has shown that these 2 gram negative pathogens adhere differently to leafy greens when suspended in simple deionized water and rinsed over the produce surface (Takeuchi et al., 2000; Barak et al., 2002). When grown in M9 minimal media, *Salmonella* cells attached to the spinach epicuticle surface at a significantly higher rate ($1.87 \pm 0.13 \times 10^{-7}$) than in LB media ($p < 0.01$). As noted above, the maximum mass transfer rate coefficient for *E. coli* O157:H7 is observed in AGW after nutrient-restricted growth, which is also significantly higher than that of *Salmonella.* These results are in agreement with previous studies that have shown that bacteria cells under stress conditions are more likely to adhere to surfaces in order to leave planktonic conditions and begin the process of protective biofilm formation (Hassan and Frank, 2004; Nagar et al., 2016).

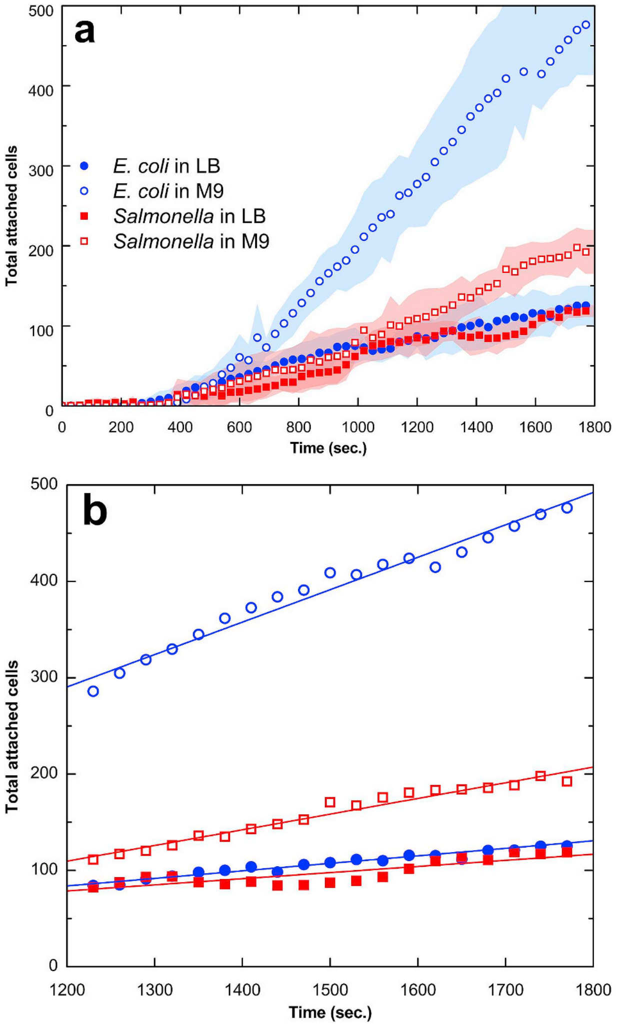

Fig. 2. Total *E. coli* O157:H7 and *Salmonella* Typhimurium cells adhered to epicuticle surface in 10 mM AGW over 30 min experiments (a) and final 10 min (b). Data points are an average of triplicate trials and standard deviation is represented by the shaded regions surrounding each curve in (a). Adhesion was observed within a 153 μm × 113 μm microscope viewing area.

3.2. Epicuticle and cell surface characterization

To further investigate the physiochemical properties of bacteria and the spinach epicuticle layer that contribute to adhesion, both whole leaves and the immobilized wax layer were characterized by streaming potential, static contact angle, and overall roughness. In addition to these surface properties, Scanning Electron Microscopy (SEM) was utilized to confirm the usefulness of the isolated epicuticle wax as representative of the whole leaf surface for relative comparison of bacterial deposition trends. The leaf surface has a slightly negative surface charge in all three solutions (-12.00 ± 0.43 mV in KCl,

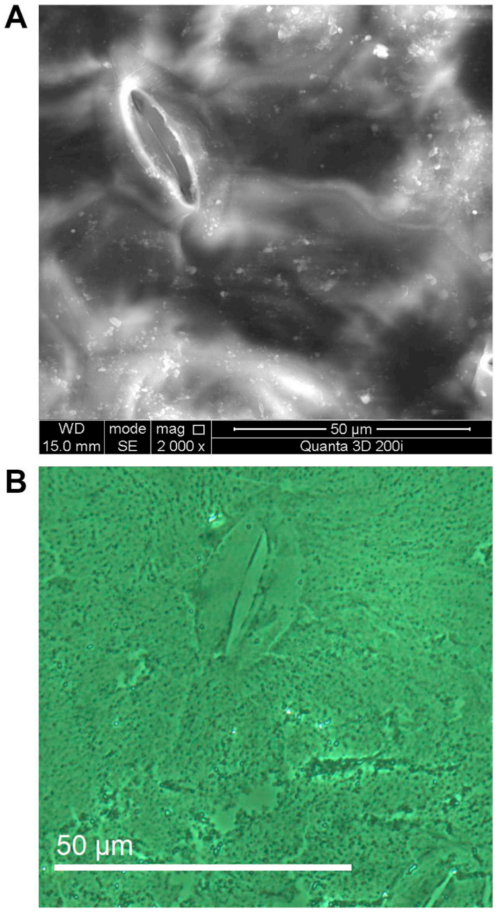

Fig. 3. Images of spinach leaf surface (top, SEM image) and isolated epicuticle wax layer on polycarbonate (bottom, light microscopy image) showing preservation of physical stomata surface structures after physical removal.

-6.81 ± 1.33 mV in CaCl$_2$, and -4.64 ± 1.44 mV in AGW). Static contact angle revealed that the immobilized wax layer was more hydrophobic than the whole leaf, but exhibited less measurement variability, making it more useful for consistent deposition experiments ($79.1 \pm 2.1^\circ$ and $69.3 \pm 8.5^\circ$, respectively). Previous work has also shown that the immobilized epicuticle wax preserves physical morphologies of the leaf surface and minimizes surface roughness (Kinsinger et al., 2016). This was further confirmed by the observation of preserved leaf surface structures, such as the stomata, within the immobilized epicuticle wax (Fig. 3).

Bacterial surface properties play a significant role in mechanisms of cell adhesion to solid surfaces. Cells are able to modify the composition and features of their cell wall in response to specific surrounding conditions (Ploux et al., 2010) and surface characteristics have been shown to change in as little as 30 min of exposure in suspension (Chen and Walker, 2007; Kinsinger et al., 2016). Electrophoretic mobility, relative hydrophobicity, size, and extracellular polymeric substance (EPS) composition were measured at room temperature and unadjusted pH in order to assess the impact of nutrient availability and solution valence on cell surface properties. *E. coli* O157:H7 (ATCC 43888) exhibited a slightly negative charge in all conditions, ranging from -0.63 to -4.53 mV (Table 1). *E. coli* cells were also generally hydrophilic, ranging from 12.1% to 41.0% adhesion to dodecane within both growth conditions. *Salmonella* Typhimurium (ATCC 13311) cells had a wider range of surface charge that ranged from 3.75 to -20.65 mV, and were more hydrophobic (40.4%–83.7% relative hydrophobicity). Zeta potential of cell surfaces did not significantly differ between growth conditions ($p > 0.01$), which is in agreement with previous work that observed differences in transport of *E. coli* in a quartz sand column in high- and low-nutrient scenarios without corresponding changes in cell surface charge (Han et al., 2013). However, undernourished *Salmonella* cells are more hydrophilic compared to other conditions, decreasing to a range of 13.0%–48.3% relative hydrophobicity, with the largest reduction (-50% relative hydrophobicity) observed in AGW. This is similar to previous work with various bacteria that have observed lower cell surface hydrophobicity after suppressed growth rates (van Loosdrecht et al., 1987) and starvation conditions (Lagha et al., 2015). Alternatively, *E. coli* O157:H7 cells were more hydrophobic after growth in nutrient-restricted conditions and suspended in KCl and CaCl$_2$, but no significant difference was observed when suspended in AGW.

Although both the epicuticle surface and bacteria exhibited a range of negative zeta potentials (Table 1), they are relatively small in magnitude and result in DLVO calculations that predict insignificant differences in interactions between cells and epicuticle surfaces for all three combinations of solution valence. Despite similarly repulsive conditions between growth conditions, as suggested by like charges, significant differences in attachment do occur when cells are grown in M9 media, with the most significant differences in AGW. These results indicate that relatively weak electrostatic and van der Waals forces, as indicated by DLVO simulations, are not the dominant mechanism involved in cell interactions with the spinach surface, although they have been shown to be useful in predicting bacterial adhesion trends (van Loosdrecht et al., 1987).

3.3. *Influence of extracellular substances*

Bacterial attachment rate coefficients in the three water chemistries are only significantly different after growth in M9 media, which suggests that environmentally relevant nutrient conditions may magnify changes in cellular surface properties that impact adhesion to produce surfaces. The extracellular matrix has long been reported as a potential means of promoting microbial attachment, but the role of total EPS in attachment to solid surfaces remains uncertain. For example, Oh et al. (2007) found that when *E. coli* O157:H7 was grown in M9 minimal media, cells accumulated more EPS and were more adhesive to glass surfaces than when grown in ideal nutrient conditions. In contrast, Ryu et al. (2004) showed that a high EPS-producing mutant of *E. coli* O157:H7 adhered less to a stainless steel surface than the normal strain. As shown in Table 2, this study found that the influence of growth in M9 media on total EPS varies by water chemistry and between the strains of these two pathogens. *E. coli* O157:H7 generally produced more EPS (sum of sugar and protein content in mg per cell) after growth in LB media and when suspended in AGW (35.1 ± 4.4 and $11.17 \pm 2.3 \times 10^{-18}$ mg/cell after growth in LB and M9 media, respectively). *Salmonella* produced the most EPS in CaCl$_2$ (24.05 ± 1.6 and $38.13 \pm 4.4 \times 10^{-18}$ mg/cell for after grown in LB and M9 media, respectively) and did not significantly differ between growth conditions.

Cellular EPS was also characterized by its two major components: carbohydrates and proteins. Carbohydrate content increased from monovalent, to divalent, to multivalent AGW (with the exception of *Salmonella* in M9 and AGW), while trends in protein content are generally inconsistent in relation to water chemistries and growth conditions. Interestingly, the sugar/protein ratio in EPS was significantly higher in cells suspended in complex AGW than those in KCl or CaCl$_2$ (Table 2). After growth in LB and M9 media, *E. coli* O157:H7 EPS from AGW treated cultures contained 11.93 ± 1.6 and 8.28 ± 1.6 ratios of sugar to protein, respectively. *Salmonella* cells in AGW likewise decreased from 16.49 ± 4.1 and 7.82 ± 0.8 ratios of sugar to protein in their EPS after growth in LB and M9 media, respectively. For *E. coli,* this change can be attributed to a nearly 70% reduction in carbohydrate content, while protein remains relatively constant. *Salmonella* cells in AGW, on the other hand, show no change in carbohydrate content for

Table 1
Bacteria cell surface characteristics and mass transfer rate coefficients (*k*).

Solution[a]	Growth Media	Cell Radius (μm)[b]	Zeta Potential (mV)[c]	Relative Hydrophobicity (%)[d]	$k \times 10^{-8}$ (m/s)
			***E. coli* O157:H7**		
KCl	LB	0.60 ± 0.006	−4.16 ± 1.38	12.1 ± 4.0	8.14 ± 1.28
	M9	0.54 ± 0.016	−4.53 ± 1.09	25.1 ± 6.4	20.4 ± 3.23
$CaCl_2$	LB	0.58 ± 0.012	−2.22 ± 2.06	30.2 ± 12.0	17.1 ± 6.39
	M9	0.52 ± 0.010	−0.63 ± 2.83	41.0 ± 3.2	33.4 ± 5.38
AGW[e]	LB	0.58 ± 0.011	−3.65 ± 2.12	22.7 ± 2.9	8.25 ± 0.70
	M9	0.54 ± 0.021	−2.47 ± 1.62	19.0 ± 6.6	39.4 ± 10.1
			***Salmonella* Typhimurium**		
KCl	LB	0.69 ± 0.015	−20.65 ± 2.57	40.4 ± 9.8	6.57 ± 0.34
	M9	0.49 ± 0.010	−14.77 ± 2.08	13.0 ± 11.2	10.9 ± 4.74
$CaCl_2$	LB	0.56 ± 0.003	3.75 ± 1.07	72.4 ± 6.7	11.7 ± 2.24
	M9	0.53 ± 0.019	4.61 ± 1.38	48.3 ± 30.8	31.9 ± 3.04
AGW	LB	0.58 ± 0.093	−4.32 ± 1.7	83.7 ± 6.6	7.32 ± 3.39
	M9	0.52 ± 0.003	−3.99 ± 1.28	30.8 ± 3.5	18.7 ± 1.27

[a] Ionic strength of all electrolyte solutions was 10 mM.

[b] Spherical radius calculated from experimentally measured length and width of individual cells.

[c] Zeta potential calculated from electrophoretic mobility using ZetaPALS analyzer.

[d] Relative hydrophobicity as indicated by microbial adhesion to hydrocarbon (MATH) test, which measures the percent of cells partitioned in dodecane versus electrolyte.

[e] Artificial groundwater.

Table 2
Extracellular polymeric substance (EPS) composition for *E. coli* O157:H7 and *Salmonella* Typhimurium.

Solution[a]	Growth Media	Sugar × 10^{-18} (mg/cell)[b]	Protein × 10^{-18} (mg/cell)[c]	Sugar/protein ratio
		***E. coli* O157:H7**		
KCl	LB	0.65 ± 0.2	0.79 ± 0.3	0.94 ± 0.5
	M9	4.74 ± 0.3	0.98 ± 0.1	4.82 ± 0.2
$CaCl_2$	LB	15.92 ± 2.6	2.89 ± 0.3	5.57 ± 1.1
	M9	5.63 ± 1.8	2.56 ± 0.3	2.68 ± 1.7
AGW[d]	LB	32.41 ± 4.2	2.73 ± 0.3	11.93 ± 1.6
	M9	9.96 ± 2.3	1.20 ± 0.1	8.28 ± 1.6
		***Salmonella* Typhimurium**		
KCl	LB	6.51 ± 4.3	4.68 ± 1.2	1.36 ± 0.9
	M9	5.82 ± 4.2	11.23 ± 5.9	0.52 ± 0.2
$CaCl_2$	LB	7.94 ± 2.7	16.11 ± 2.9	0.52 ± 0.3
	M9	27.09 ± 3.5	11.04 ± 1.2	2.45 ± 0.2
AGW	LB	13.25 ± 0.5	0.84 ± 0.2	16.49 ± 4.1
	M9	12.89 ± 0.1	1.66 ± 0.2	7.82 ± 0.8

[a] Ionic strength of all electrolyte solutions was 10 mM.

[b] Based on the phenol−sulfuric acid method with xanthan gum as the standard at 10^8 cells/mL.

[c] Based on the Lowry method with BSA as the standard at 10^8 cells/mL.

[d] Artificial groundwater.

cells grown in nutrient-restricted conditions, while protein content doubles. The ratio of sugar/protein was generally higher for cells grown in high-nutrient LB than for those in M9, with the exception of *E. coli* O157:H7 in KCl and *Salmonella* in $CaCl_2$. This suggests that changes in the makeup of the extracellular matrix in favor of proteins may play a role in promoting bacterial attachment to produce surfaces. Haznedaroglu et al. (2008) also observed that increases in the EPS sugar/protein ratio amongst environmental *E. coli* isolates decreased adhesion to quartz sand in 10 mM KCl.

Several bacterial strains have been shown to be more adhesive and ultimately more likely to form a biofilm when grown under nutrient-restricted conditions, which is hypothesized to be a protection mechanism under stress conditions (Teh et al., 2016; Reeser et al., 2007; Sheng et al., 2008). Exposure to complex water chemistry may induce similar responses, as evidenced by the higher sugar/protein ratio in EPS of *E. coli* O157:H7 cells in AGW (Table 2). Alteration of the combination of carbohydrates and proteins on the cell surface can create a more heterogeneous distribution of surface charge that has been shown to increase bacterial adhesion (Sheng et al., 2008). Junkins and Doyle (1992) have shown that *E. coli* O157:H7 cells are more adhesive when grown under conditions that favor EPS production. This is due to the chemical and structural heterogeneity that creates multiple specific interactions with a given substrate surface (Li et al., 2016; Sharma et al., 1985). Hassan and Frank (2004) showed that this trend applies to adhesion of *E. coli* O157:H7 on produce surfaces as well.

Using the same strains of *E. coli* O157:H7 and *Salmonella* Typhimurium employed in this study, Cook et al. (2017) previously demonstrated that growth in low nutrient conditions increases biofilm formation. It has been speculated that changes in bacterial attachment and biofilm formation can be attributed to production of specific surface macromolecules. For example, curli and cellulose surface structures have been previously shown to enhance biofilm formation, but not initial attachment, of other *E. coli* O157:H7 strains on stainless steel (Ryu and Beuchat, 2005). However, curli expression has been found to be uncommon for pathogenic *E. coli* O157:H7 strain ATCC 43888 on the whole (Uhlich et al., 2001). While the literature on curli and cellulose production for *Salmonella* Typhimurium is more limited, some strains have been shown to produce curli and cellulose after incubation at 30 °C in static conditions, and ATCC 13311 was found to produce curli, but not cellulose on LB agar (Cook et al., 2017; Solomon et al., 2005). Curli fimbrae and cellulose may aid *Salmonella* in attachment, but flagella have been shown to be the most important factor in adherence to plant surfaces and flagella are still produced when grown at 37 °C (Tan et al., 2016; Wheatley and Poole, 2018). Although not included within the scope of this study, nutrient deprivation and media composition may induce stress responses in pathogen cells that have been shown to result in curli fimbrae production that can increase bacterial attachment in some cases (Olsen et al., 1993; Carter et al., 2016). However, the lack of any clear trends in protein content within cellular EPS implies that production of these surface macromolecules is not the only mechanism of increased attachment to spinach surfaces in this study. Curli are not likely to play a significant role in the attachment of *E. coli* O157:H7 cells grown in ideal nutrient conditions, and this work employs conditions that are not ideal for cellular curli production in either

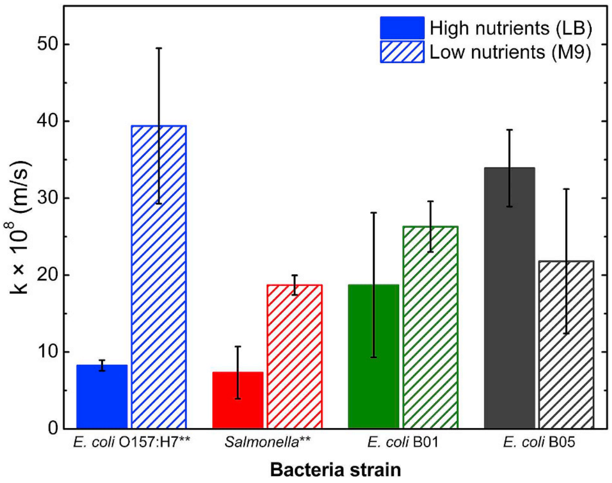

Fig. 4. Pathogen and surrogates attachment mass transfer coefficients in 10 mM AGW. Error bars indicate one standard deviation. Significant differences between the two growth condition are denoted **($p < 0.01$).

growth scenario, including growth at 37 °C and harvest at mid-exponential growth phase (Hufnagel et al., 2015). Additionally, this work has only employed reference strains for *E. coli* O157:H7, ATCC 43888, and *Salmonella* Typhimurium, ATCC 13311, and therefore does not account for the variability that has been documented within these serotypes (Ratnam et al., 1988; Beltran et al., 1991). Further research is needed to better understand surface structures of undernourished cells and should employ multiple strains of each pathogen in order to account for variation in bacterial physiology.

3.4. Adhesion kinetics of pathogens versus environmental isolates

Additional experiments were conducted in the parallel plate system to compare the two pathogens to two non-pathogenic environmental isolates in the representative AGW. *E. coli* B01 and *E. coli* B05 have been previously identified as potential non-pathogen surrogates for *E. coli* O157:H7 and *Salmonella* Typhimurium, respectively (Cook et al., 2017). Attachment trends of these four bacteria on the wax epicuticle layer from the spinach leaf were compared in complex AGW, after being grown in either LB or M9 media (Fig. 4). When grown in LB media, the environmental isolates attach similarly to their respective pathogen models, which is promising for their usefulness in predicting pathogen behavior. However, as described above, both pathogens were highly affected by nutrient conditions, becoming significantly more adhesive when grown in M9 minimal media. Environmental isolates did not follow this trend and instead showed insignificant differences in mass transfer rate coefficients between the two growth conditions ($p > 0.05$).

Results of characterization of the surrogates' cell surface properties do not different significantly from pathogenic *E. coli* O157:H7 in zeta potential and relative hydrophobicity (Table S1). None of the cell surface properties measured in this study can wholly describe the differences between the pathogens and non-pathogen isolates. However, the high variability in adhesion trends and EPS composition amongst *E. coli* B05 samples may offer some insight into the unpredictability of bacteria in the natural environment in comparison to pathogen species.

4. Conclusions

In summary, the initial phases of biofilm formation were directly observed for two well-known foodborne pathogens and two non-pathogen surrogates in this study. Previous studies on foodborne pathogen attachment to produce surfaces have largely been conducted under simple and static laboratory conditions. Through dynamic flow experiments, this work has shown that growth conditions and solution complexities have significant effects on mechanisms of bacterial adhesion at interfaces of water and spinach leaf surfaces. Specifically, these results show that deposition of these two pathogen strains on spinach epicuticle layers significantly increases when cells are grown in nutrient-restricted conditions, implying that food safety research that only includes well-nourished cells may underestimate attachment to produce surfaces. This difference in adhesion may be partially attributed to increasing cell surface charge heterogeneity, as characterized by changes in EPS composition and minimal changes in overall cell surface charge for both *E. coli* O157:H7 and *Salmonella* Typhimurium. The pathogens followed similar adhesion trends in every tested condition, with the most significant differences observed with suspension in AGW after growth in M9 media ($p < 0.01$), which illustrates the role of complex, environmentally relevant water chemistries in magnifying changes on the cell surface. This ideal water chemistry scenario offers insight into the fundamental mechanisms that result in highly variable transport behavior by *E. coli* and *Salmonella* exposed to realistic aquatic environments, such as wastewater systems (Li et al., 2015). Further, these results contribute to potential explanations for previous work that has reported increased bacterial attachment and biofilm formation on solid surfaces after exposure to stress conditions (Kinsinger et al., 2016; Oh et al., 2007; Sharma et al., 1985; Ryu and Beuchat, 2005). Additionally, we have demonstrated that two environmental isolates that are meant to mimic these pathogens are less impacted by non-ideal nutrient conditions and do not show significant changes in attachment as a result. The minimal media conditions and complex water chemistry employed in this study aim to represent more realistic scenarios in which bacteria pose risks of cross contamination in a food processing plant. The results contribute to understanding the effects of these environments on bacterial adhesion and ultimately improving removal and inactivation of foodborne pathogens, as well as presenting a potential challenge to using stress-tolerant environmental microbes as food safety surrogates. Understanding the changes in surface properties that pathogens and surrogates undergo when stressed, and the resulting influence on adhesion to organic surfaces, is important for ultimately preventing adhesion and biofilm development that can lead to foodborne illness outbreaks.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.10.002.

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