Effects of Environmental Parameters on the Dual-Species Biofilms Formed by *Escherichia coli* O157:H7 and *Ralstonia insidiosa*, a Strong Biofilm Producer Isolated from a Fresh-Cut Produce Processing Plant

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ABSTRACT

Biofilm-forming bacteria resident to food processing facilities are a food safety concern due to the potential of biofilms to harbor foodborne bacterial pathogens. When cultured together, *Ralstonia insidiosa*, a strong biofilm former frequently isolated from produce processing environments, has been shown to promote the incorporation of *Escherichia coli* O157:H7 into dual-species biofilms. In this study, interactions between *E. coli* O157:H7 and *R. insidiosa* were examined under different incubating conditions. Under static culture conditions, the incorporation of *E. coli* O157:H7 into biofilms with *R. insidiosa* was not significantly affected by either low incubating temperature (10°C) or by limited nutrient availability. Greater enhancement of *E. coli* O157:H7 incorporation in dual-species biofilms was observed by using a continuous culture system with limited nutrient availability. Under the continuous culture conditions used in this study, *E. coli* O157:H7 cells showed a strong tendency of colocalizing with *R. insidiosa* on a glass surface at the early stage of biofilm formation. As the biofilms matured, *E. coli* O157:H7 cells were mostly found at the bottom layer of the dual-species biofilms, suggesting an effective protection by *R. insidiosa* in the mature biofilms.

Biofilm formation by various bacterial species in food processing plants can be a vector for food spoilage and also a food safety concern (22), especially given the ability of biofilms to provide protection to pathogenic bacteria against environmental stresses, such as disinfectant treatments (1). Biofilms formed by environmental bacteria isolated from meat and dairy processing plants could harbor pathogenic bacterial strains (6). Some *Escherichia coli* O157:H7 strains were shown to require a colonizing partner to form biofilms on glass slides (14). In our recent study, *Ralstonia insidiosa*, an environmental bacterium isolated from a fresh-cut produce processing plant with a strong biofilm-forming tendency, was shown to significantly promote the incorporation of *E. coli* O157:H7 in biofilms (17). Dual-species biofilms formed by *E. coli* O157:H7 and *R. insidiosa* showed significant increases in biomass and thickness in comparison to their respective monoculture biofilms.

Environmental factors, such as low temperature, continuous fluid flow, and limited nutrient availability, are conducive to the transition of environmental bacteria from planktonic growth to an attached form (6). Although typical refrigerating temperature (4°C) strongly inhibits bacterial proliferation on fresh-cut produce, substantial microbial growth occurs under conditions of suboptimal refrigeration (31). Low temperature is an important cue for the development of biofilms, as evidenced by the prominent expression of biofilm-related genes in *E. coli* (30). It has been reported that low temperature (12°C) enhanced the exopolysaccharide production in *E. coli* O157:H7 biofilms compared with those grown at 22°C (24). In addition, extensive application of wash water inevitably results in secondary water flow on various types of surfaces in fresh produce processing facilities, which are conducive for biofilm formation (9). The exudates of fresh-cut produce can also provide limited nutrients for microbial growth in wash water and on contact surfaces (2, 3). Bacterial medium made of lettuce juice was reported to result in higher exopolysaccharide production in aerobically cultured *E. coli* O157:H7 biofilms than either minimal salt media or common rich media (24). However, the effects of environmental factors on dual-species biofilms formed by environmental bacteria and human pathogens remain largely unknown. In the present study, marginal temperature abuse (10°C), three types of culture media with varying physio-
chemical characteristics, and a dynamic culture system were evaluated for impact on the behavior of \textit{R. insidiosa} and \textit{E. coli} O157:H7 in dual-species biofilms.

**MATERIALS AND METHODS**

**Bacterial strains and media.** \textit{E. coli} O157:H7 strain FS4052, a weak biofilm former (16), was derived from the nonpathogenic isolate from bovine feces, ATCC 43888. It harbors a stably maintained plasmid encoding for green fluorescence protein (10). \textit{R. insidiosa} strain FC1138, a strong biofilm producer, was isolated from a local fresh produce processing plant. A previous study demonstrated that these two strains interacted synergistically in forming dual-species biofilms (17).

Tryptic soy agar (TSA; BD Biosciences, San Jose, CA) and tryptic soy broth (TSB; BD Biosciences) were used for routine maintenance and propagation of all bacterial strains. TSA and sorbitol MacConkey agar (BD Biosciences) containing 100 mg/liter ampicillin were used for enumeration of total aerobic counts and \textit{E. coli} O157:H7, respectively. Diluted (10%) TSB, M9 minimal salts (BD Biosciences), supplemented with 0.4% of glucose, and diluted cantaloupe juice (1.25%) were used to support biofilm formation using static culture. Diluted TSB (1%) was used for biofilm development in the drip flow continuous culture system. Cantaloupe juice was prepared from fresh cantaloupes and filtered using 330-µm perforated polyethylene Whirl-Pak filter bags (Nasco, Fort Atkinson, WI) before subjecting to 10-kGy electron beam irradiation (U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA). Electron beam–pasteurized cantaloupe juice was stored at −20°C and centrifuged at 4,000 × g for 5 min to remove precipitation prior to use.

Glucose-supplemented M9 salts, TSB, and cantaloupe juice were freeze-dried, and carbon and nitrogen contents were determined by using the Macro Elemental Analyzer Vario MAX CN (Elementar Analysensysteme GmbH, Hanau, Germany), and those in the diluted media were calculated accordingly. The glucose contents of each medium were determined by using the Glucose (HK) Assay Kit (Sigma-Aldrich, St. Louis, MO). Osmolarity of each medium was measured using a Vaporo Pressure Osmometer (Wescor, Logan, UT), following manufacturer’s instructions.

**Biofilm formation in static culture.** Monoculture and dual-species biofilms were formed on polystyrene 12-well tissue culture plates, as described previously (17). Fresh medium was inoculated with overnight cultures of \textit{E. coli} O157:H7 and \textit{R. insidiosa}, separately or in combination, to achieve a cell density of approximately 10^7 CFU/ml. To test the effect of temperature on the interaction between \textit{E. coli} O157:H7 and \textit{R. insidiosa} in dual-species biofilm formation, 3 ml of prepared cell suspension (in 10% TSB) was added in each well and incubated at 30 or 10°C, with moderate orbital shaking (80 rpm) for up to 24 h or 20 days, respectively, to allow biofilm formation in the microplate wells. To examine the effect of nutrient availability on this interaction, monoculture and dual-species biofilms were formed by culturing in 10% TSB, glucose-supplemented M9 salts, or 1.25% cantaloupe juice at 30°C, with moderate orbital shaking for 24 h. Samples were taken at the indicated time to assess the progression of biofilm formation. Total biomass production and cell counts for \textit{E. coli} O157:H7 and \textit{R. insidiosa} in the biofilm samples were determined, as previously described (17).

**Biofilm formation in continuous culture system.** To assess interactions between \textit{E. coli} O157:H7 and \textit{R. insidiosa} in forming biofilms under conditions simulating secondary water flow, a drip flow biofilm reactor (BioSurface Technologies, Bozeman, MT) was assembled, following the manufacturer’s instructions, and used to grow biofilms. Two types of surfaces, standard glass coverslip supported by a glass slide and glass fiber filter paper (Whatman Grade GF/F, Sigma-Aldrich) supported by a glass slide, were used as solid substrata for biofilm formation. Individual pieces of solid substratum were placed in the reactor holding chambers and submerged in 15 ml of fresh 1 × TSB inoculated with overnight culture of \textit{E. coli} O157:H7 or \textit{R. insidiosa}, or 1:1 mixture of both, to approximately 10^4 CFU/ml for each strain. After a 4-h incubation at room temperature (22 ± 2°C), the inoculated medium was discharged from the chambers, and fresh media (1% TSB) was continuously delivered into each channel at a rate of 2 ml/min using a Masterflex peristaltic pump (Cole Parmer, Vernon Hills, IL). The chambers hosting the solid substrata were tilted to 10° to facilitate steady flow of incoming fresh medium across the substratum surfaces and to the draining outlet.

Biofilms grown on glass coverslips were observed using a confocal laser scanning microscope (CLSM) at indicated times or subjected to cell enumeration. Biofilms were grown on glass fiber filter paper for transmission electron microscopic (TEM) examination. At each sampling point, biofilms on the substratum were gently washed with 30 ml of sterile phosphate-buffered saline (PBS) before further processing for respective analyses. For cell enumeration, biofilms were sampled by Z-pattern rubbing by using a sterile cotton-tipped swab, followed by the procedure described previously for biofilms formed under static culture conditions (17).

**CLSM of biofilms.** Glass coverslips supported by glass slides were used as biofilm substrata in a drip flow continuous culture biofilm reactor. After incubation for 4, 40, and 72 h, dual-species biofilms formed by \textit{E. coli} O157:H7 and \textit{R. insidiosa} were washed with PBS and stained with red fluorescent dye SYTO 61 (Life Technology Co., Grand Island, NY) for 45 min, followed by washing with 30 ml of water. Stained biofilms were subsequently examined with CLSM system using a Zeiss Axio Observer inverted microscope with 40 × 1.3 NA and 63 × 1.4 NA Plan-Apochromat objectives (Carl ZeissMicroscopy, LLC, Thornwood, NY). A 488-nm argon laser and a 561-nm diode-pumped solid-state laser with a pinhole of 24 µm passing through a main beam splitter (MBS) 488/561 filter were simultaneously used, with limits set at 495 to 530 nm for detection of \textit{E. coli} O157:H7 (green) and at 600 to 760 nm for detection of \textit{R. insidiosa} (red). Z-stack scanning was conducted to reveal the spatial composition of the dual-species biofilms, and three-dimensional imaging was generated using Zeiss Zen 2009 and Axiovision software.

**TEM of biofilms.** Glass fiber filter paper was used as a solid substance for biofilm formation. After incubation at room temperature for 72 h, bacterial cells and the glass fiber substratum were fixed in 2.5% glutaraldehyde buffered with 50 mM sodium cacodylate for 2 h and postfixed in 1% osmium tetroxide buffered with 50 mM sodium cacodylate for 2 h. Samples were dehydrated using graded ethanol series (20 to 100%), infiltrated with Spurr low-viscosity embedding resin (26) and ethanol, and polymerized at 60°C for 48 h. Sections of 90 nm in thickness were cut on an American Optical (AO) Reichert Ultracut microtome with a diamond knife (Diatome) and mounted on 400-mesh Ni grids, followed by staining with 4% aqueous uranyl acetate followed by 3% aqueous lead citrate. The cross sections of the biofilms were examined with a Hitachi HT 7700 TEM (Hitachi High Technologies America, Inc., Schaumburg, IL).
Each data point had three replicates throughout the O157:H7 and E. coli 123, u E. R. u E. coli E. coli, CFU/ml for O157:H7 in monoculture O157:H7 in dual-R. insidiosa O157:H7. Nutrient availability can significantly ¡ ¡ Static culture condition refers to biofilm formation at 30 E. coli 0.05). A slight increase was observed after O157:H7 in dual-R. insidiosa. ¡ ¡ E. coli E. coli 0.09 7.11 ¡ ¡ test was performed to compare the ¡ ¡ TSB was 6.4 strain ¡ ¡ E. coli ¡ ¡ 0.05). Therefore, it seemed ¡ ¡ 0.05). The Bonferroni test was used for post hoc determination of significant differences. Asterisk indicates significant difference between the cell counts of E. coli O157:H7 in monoculture and dual-species biofilms (P < 0.05).

**RESULTS**

Incorporation of E. coli O157:H7 in dual-species biofilms at 30 and 10° C. E. coli O157:H7 and R. insidiosa cell counts in dual-species biofilms formed in 10% TSB over a period of 24 h at 30° C (Fig. 1A) and of 20 days at 10°C (Fig. 1B) were compared with that in respective monoculture biofilms. At 30°C, the cell count of E. coli O157:H7 in dual-species biofilms was significantly greater than in E. coli O157:H7 monoculture biofilms after 8-h incubation (P < 0.05). A slight increase was observed after 24-h incubation, while the E. coli O157:H7 counts in the monoculture biofilms remained unchanged. There was no significant difference on the cell counts of R. insidiosa between monoculture and dual-species biofilms (P > 0.05). E. coli O157:H7 incorporation into dual-species biofilms followed similar patterns when incubated at 10°C (Fig. 1B). After 1 day, the E. coli O157:H7 cell count in dual-species biofilms was comparable to monoculture biofilms. Significantly increased incorporation of E. coli O157:H7 in dual-species biofilms was observed by day 5 and was maintained throughout the period of incubation of 20 days at 10°C. The comparable increase of E. coli O157:H7 cell counts in dual-species biofilms formed at either 30 or 10°C suggested that temperature was not a major factor affecting the interactions between E. coli O157:H7 and R. insidiosa in forming dual-species biofilms.

**Dual-species biofilms formation in different nutrient-limited media.** Nutrient availability can significantly affect both growth and biofilm formation of bacteria. In this experiment, three types of bacterial growth media, glucose-supplemented M9 salts, 10% TSB, and 1.25% cantaloupe juice, were compared for supporting biofilm formation by E. coli O157:H7 and R. insidiosa. The E. coli O157:H7 strain formed biofilms poorly, while the R. insidiosa strain FC1138 showed strong biofilm formation (data not shown) on each tested medium. In comparison to monoculture biofilms, the presence of E. coli O157:H7 in dual-species biofilms increased by 1.3, 1.6, and 2.4 log, respectively, when grown in glucose-supplemented M9 salts, 10% TSB, or 1.25% cantaloupe juice (Table 1). Therefore, it seemed that enhanced incorporation of E. coli O157:H7 in dual-species biofilms with R. insidiosa consistently occurred irrespective of the nutrient profiles of growth media.

**TABLE 1. Incorporation of E. coli O157:H7 in monoculture biofilms and in dual-species biofilms formed with R. insidiosa**

<table>
<thead>
<tr>
<th>Media type</th>
<th>Culture condition</th>
<th>Monoculture</th>
<th>Dual species</th>
</tr>
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<tbody>
<tr>
<td>M9 + glucose</td>
<td>Static</td>
<td>5.81 ± 0.09</td>
<td>7.11 ± 0.27*</td>
</tr>
<tr>
<td>10% TSB</td>
<td>Static</td>
<td>5.29 ± 0.50</td>
<td>6.94 ± 0.08*</td>
</tr>
<tr>
<td>1.25% cantaloupe juice</td>
<td>Static</td>
<td>4.91 ± 0.07</td>
<td>7.38 ± 0.15*</td>
</tr>
<tr>
<td>1% TSB</td>
<td>Drip flow</td>
<td>4.98 ± 0.25</td>
<td>7.72 ± 0.16*</td>
</tr>
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</table>

* Static culture condition refers to biofilm formation at 30°C for 24 h using a 12-well microplate format. Drip flow refers to ambient temperature (22 ± 2°C) for 72 h with a drip flow biofilm reactor.

* Student’s t test compared plate counts of E. coli O157:H7 between monoculture and dual-species biofilms for each culture condition. Asterisk indicates significant difference between the cell counts of E. coli O157:H7 in monoculture and dual-species biofilms (P < 0.05).
although a larger increase was observed with diluted cantaloupe juice, which had the lowest nutrient content.

**Dual-species biofilms formation in continuous culture system.** The interaction of *E. coli* O157:H7 and *R. insidiosa* in forming dual-species biofilms was also examined using a drip flow continuous culture system. Table 1 shows cell counts of *E. coli* O157:H7 in monoculture and dual-species biofilms after a 3-day incubation. Cell counts of *R. insidiosa* in both monoculture and dual-species biofilms were not significantly different after 72-h incubation at room temperature, as previously observed with static cultures (data not shown). In contrast, cell counts of *E. coli* O157:H7 in the dual-species biofilms reached 7.72 log CFU/cm², in comparison to 4.98 CFU/cm² for the monoculture biofilms, an increase of 2.8 log.

**Microscopic examination of dual-species biofilms.** *E. coli* O157:H7 and *R. insidiosa* dual-species biofilms formed in a drip flow biofilm reactor were examined using CLSM at three time points during biofilm maturation (Fig. 2). Four hours after inoculation, aggregates of *R. insidiosa* cells were seen sporadically attached to glass surface and formed microcolony-like structures of less than 5 µm in thickness. A very small number of *E. coli* O157:H7 cells were occasionally observed in these structures (Fig. 2A). After culturing for 40 h, *R. insidiosa* and *E. coli* O157:H7 cells formed discrete mushroom-like domed structures that elevated to approximately 20 µm (Fig. 2B). *E. coli* O157:H7 cells exhibited a strong tendency of colocalizing with *R. insidiosa* cells in these structures. By 72 h of incubation, the dual-species biofilms matured into a more uniform lawn with a generally flat surface. The thickness of the biofilm reached approximately 50 µm. Under the apparent uniform surface, there were interconnected domed hollow spaces that were not stained by the STY61 fluorescent dye. *E. coli* O157:H7 cells were seen predominately located at the bottom of the biofilms but were also found interspersed among *R. insidiosa* cells (Fig. 2C). The top layer was dominated by *R. insidiosa* cells.

Vertical sections of 72-h dual-species biofilms formed on glass fiber filter paper were examined with TEM for insight into the spatial distribution of the two bacteria (Fig. 3). Using TEM examination, *E. coli* O157:H7 cells were readily distinguishable from *R. insidiosa* based on cell size and the absence of lipid exclusion bodies (Fig. 3A and 3B). TEM observations supported those from CLSM. *E. coli* O157:H7 and *R. insidiosa* were seen to form mutually exclusive layers at the areas of domed elevation in the dual-species biofilms (Fig. 3C), while cells of the two bacteria...
FIGURE 3. TEM images of monoculture and dual-species biofilms formed by E. coli O157:H7 and R. insidiosa. The images are oriented such that the supporting substrate is toward the bottom. (A) E. coli O157:H7 in monoculture biofilm. (B) R. insidiosa in monoculture biofilm. (C) An area of dome-shaped elevation showing spatial segregation of the two strains. (D) A valley area depicting comingle of the two strains. Scale bar is 1 μm.

were generally interspersed at the valleys among the elevated domed structures (Fig. 3D).

DISCUSSION

Current federal regulations require that the temperature in fresh-cut produce processing plants be maintained at 4 °C or lower (28). However, depending on the designs of the facilities, localized zones of relatively elevated temperatures can exist, which can be conducive to bacterial proliferation. In our previous study (16), resident bacteria with strong biofilm formation abilities were frequently isolated from food contact and nonfood contact surfaces in fresh-cut produce processing facilities after routine sanitization treatments, indicating that current practices need further improvement to address the food safety risks of bacterial biofilm formation. Recently, we demonstrated that R. insidiosa, a strong biofilm former isolated from a fresh-cut produce processing facility, promoted the incorporation of E. coli O157:H7 into dual-species biofilms (17). This process seemed species specific, as some other strong biofilm producers that were tested failed to promote the incorporation of E. coli O157:H7 into dual-species biofilms.

Heat-inactivated preformed R. insidiosa biofilms also failed to enhance the presence of E. coli O157:H7 in biofilms (unpublished data), arguing against a simple scaffolding role of R. insidiosa in promoting the incorporation of E. coli into biofilms. In the current study, we observed that the enhanced presence of E. coli O157:H7 in dual-species biofilms with R. insidiosa occurred under different physicochemical conditions, including low temperature and varying nutrient supplies.

Most studies concerning human pathogens in biofilms have been conducted at room temperature or higher (4, 12–14). In this study, we observed similar enhanced presence of E. coli O157:H7 in dual-species biofilms formed at either 10 or 30 °C, but not at 4 °C (no cell growth, data not shown). This observation suggested that the interaction between E. coli O157:H7 and R. insidiosa was not temperature dependent; nevertheless, permissible temperature for cellular metabolism was essential for this process. This notion is consistent with a previous study using a temperature simulating a beef slaughtering facility (15 °C), which reported that all but one environmental isolate could enhance the presence of E. coli O157:H7 in dual-species biofilms (18).

In fresh-cut produce processing facilities, nutrient availability varies greatly in different microniches, including places deemed hard to clean (21). Limited nutrient content can promote biofilm formation among a number of bacterial species (8, 13, 19, 29). The growth media used in this study varied greatly in both nutritional value and physical characteristics (Table 2). Glucose-supplemented M9 salts had high nitrogen and carbon contents in the form of readily available glucose and ammonium and osmotic pressure closest to that of bacterial cells. In 10% TSB, limited glucose (170 mg/liter), high nitrogen (247 mg/liter), and trace amount of vitamins and other inorganic molecules that might facilitate the growth of bacterial species were
TABLE 2. Physiochemical characteristics of growth media used in this study

<table>
<thead>
<tr>
<th>Media type</th>
<th>Carbon (mg/liter)*</th>
<th>Nitrogen (mg/liter)*</th>
<th>Glucose (mg/liter)</th>
<th>Osmolarity (mmol/kg)</th>
</tr>
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<tbody>
<tr>
<td>dH₂O</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>29.7 ± 7.4</td>
</tr>
<tr>
<td>M9 + glucose</td>
<td>1.714 ± 65.3</td>
<td>280.6 ± 25.7</td>
<td>2.234 ± 127</td>
<td>260.0 ± 7.2</td>
</tr>
<tr>
<td>10% TSB</td>
<td>940.5 ± 24.2</td>
<td>246.7 ± 6.6</td>
<td>170 ± 9</td>
<td>56.7 ± 4.2</td>
</tr>
<tr>
<td>1.25% cantaloupe juice</td>
<td>330.0 ± 0.3</td>
<td>8.4 ± 0.1</td>
<td>211 ± 5</td>
<td>38.7 ± 3.2</td>
</tr>
<tr>
<td>1% TSB</td>
<td>94.0 ± 2.4</td>
<td>24.7 ± 0.7</td>
<td>17 ± 1</td>
<td>41.7 ± 0.6</td>
</tr>
</tbody>
</table>

* Carbon (C) and nitrogen (N) contents were determined by using glucose-supplemented M9 salts (1 ×), TSB (1 ×), and cantaloupe juice (100%). For the diluted TSB and cantaloupe juice, values shown in the table were based on calculation.

provided (5). The diluted cantaloupe juice (1.25%) contained significantly lower glucose (211 mg/liter) and nitrogen (8.4 mg/liter), which could have induced bacterial starvation (23), and had osmotic pressure closer to that of water. These differences suggest that diluted cantaloupe juice represented a stronger stress for the growth of bacteria. Interestingly, the increased incorporation of E. coli O157:H7 in dual-species biofilms occurred in all tested media regardless of the nutrient limitation or composition. However, the increase was most noticeable in biofilms formed in diluted cantaloupe juice (Table 1), consistent with a notion that starvation stress promoted the incorporation of E. coli O157:H7 in dual-species biofilms.

In food processing facilities with extensive water usage, secondary water flow over equipment surfaces, such as conveyor belts and interfaces of flumes, commonly demonstrates a low shear force (15). The drip flow biofilm reactor is designed to simulate secondary water flow on surfaces (11, 25). Compared with a microtiter plate–based static system, greater enhancement of E. coli O157:H7 incorporation into dual-species biofilms was observed with the drip flow system (Table 1; increase of 2.74 versus 1.65 log in 10% TSB under static conditions). However, it cannot be excluded that the observed difference could be due to factors not controlled in this study, such as the difference in incubation time, substratum surface characteristics, and effective nutrient availability. Under the conditions used in this study, E. coli O157:H7 seemed incapable of attaching to the glass surface independent of R. insidiosa cells at the initial stage (4-h postinoculation) of biofilm formation. Other researchers also observed inability of E. coli O157:H7 to form biofilms in the presence of low shear force, even though biofilms were formed in static culture (8, 14). E. coli O157:H7 showed strong colocalization with R. insidiosa in attachment to the glass surface at the earlier stage (40-h postinoculation) of biofilm formation. However, E. coli O157:H7 cells were seen predominantly located closer to the bottom of the mature biofilms (72-h postinoculation).

Differences in microbial metabolic capabilities can result in the presence of chemical gradients and intermingled spatial arrangement of bacteria species in multispecies biofilms (20, 27). Because R. insidiosa growth requires oxygen (7) and E. coli O157:H7 is a facultative anaerobe, the layered spatial segregation of R. insidiosa and E. coli O157:H7 cells observed in this study could be due to different respiration properties of the two species. In addition, the different catabolite metabolisms of the two species might play a role in the spatial arrangement. Regardless of the mechanisms, such layered spatial distribution could confer better protection for E. coli O157:H7 cells to various environmental stressors, including sanitization treatments. Therefore, the risks of biofilm formation by resident bacteria, such as R. insidiosa, in fresh produce processing environments should be further evaluated.

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