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# *Ralstonia insidiosa* serves as bridges in biofilm formation by foodborne pathogens *Listeria monocytogenes, Salmonella enterica,* and Enterohemorrhagic *Escherichia coli*

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ABSTRACT

Biofilm formation on abiotic surfaces in fresh produce processing facilities may play a role in foodborne outbreaks by providing protective microniches for pathogenic bacteria. Our previous study showed that a strain of *Ralstonia insidiosa* isolated from a fresh produce processing plant could enhance the incorporation of *Escherichia coli* O15:H7 in biofilms under various environmental conditions. These results raised the concern that *R. insidiosa* might have the ability to incorporate other foodborne pathogens and promote their survival and growth in biofilms. To test this hypothesis, 6 strains of Shiga toxin producing *E. coli*, 2 strains of *Salmonella*, and 6 strains of *Listeria monocytogenes* were examined for dual-species biofilm formation with *R. insidiosa*. A significant increase in biomass formation was observed in 7 of the 14 *R. insidiosa*-pathogen combinations, while significantly enhanced incorporation of pathogenic cells into biofilms was seen in 12 of the 14 *R. insidiosa*-pathogen combinations. The synergistic interactions between *R. insidiosa* and the tested foodborne pathogens seemed dependent on intimate cellular contact between the two strains. Overall, this study showed that *R. insidiosa* could enhance the incorporation of biofilms of different types of foodborne pathogenic bacteria and should be considered a bridging bacterium for biofilm formation in various food processing environments.

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## 1. Introduction

Fresh-cut produce is widely recognized as a potential vehicle for foodborne outbreaks of bacterial infections (Harris et al., 2003; Lynch, Tauxe, & Hedberg, 2009). Various serotypes of *Listeria monocytogenes, Salmonella enterica*, and Shiga toxin producing *Escherichia coli* have been implicated as causal agents for fresh produce associated outbreaks (Bowen, Fry, Richards, & Beuchat, 2006; Warriner & Namvar, 2010). One particular concern related to these outbreaks is the formation of multispecies biofilms in produce production and processing environments (Carpentier & Chassaing, 2004; Silagyi, Kim, Lo, & Wei, 2009).

It has been previously shown that native microflora in dairy and meat processing facilities provided potential transference routes for foodborne pathogens (Carpentier & Chassaing, 2004; Lynch et al., 2009; Penteado, Eblen, & Miller, 2004). Particularly, certain environmental bacteria could play a key role in multispecies biofilms formation, thereby providing protective niches to planktonic bacteria for gaining enhanced resistance to daily cleaning and disinfections (Jeong & Frank, 1994; Rickard, Gilbert, High, Kolenbrander, & Handley, 2003; Van der Veen & Abee, 2011). *Acinetobacter calcoaceticus*, a strong biofilm former frequently isolated from meat processing facilities, enhanced the survival of *E. coli* O157:H7 in a dynamic culture system (Habimana, Heir, Langsrud, Asli, & Moretro, 2010). *A. calcoaceticus* was also found to serve as a "bridge" for other bacteria isolated from drinking water in forming multispecies biofilms. The absence of *A. calcoaceticus* resulted in a 75% reduction in biomass of multispecies biofilms

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(Simoes, Simoes, & Vieira, 2008). Therefore, these type of resident bacteria, known as bridging bacteria, may not only facilitate incorporation of pathogenic bacteria species into biofilms but also enhance the biomass production of biofilms, increasing the likelihood of pathogen survival (Uhlich, Rogers, & Mosier, 2010; Van der Veen & Abee, 2011).

Our previous study (Liu, Nou, Lefcourt, Shelton, & Lo, 2014) demonstrated that *Ralstonia insidiosa* isolated from a fresh-cut processing environment could enhance the incorporation of *E. coli* O157:H7 into dual-species biofilms. Other strong biofilm producers isolated from fresh-cut processing plants, including *Klebsiella pneumoniae*, *Stenotrophomonas rhizophila*, were shown to be ineffective in increasing the incorporation of *E. coli* O157:H7 in biofilms, suggesting that the enhanced incorporation of *E. coli* O157:H7 in biofilms was species or strain dependent. In another study, we observed that *R. insidiosa* increased the incorporation of multiple *E. coli* O157:H7 strains into dual-species biofilms under various temperature and nutrient availability conditions (Liu et al., 2015), indicating that *R. insidiosa* was able to adapt to diverse microenvironments in fresh produce processing plants. Based on these observations, we hypothesized that *R. insidiosa* may serve as a universal bridging species for multispecies biofilm formation allowing for enhanced incorporation of other types of pathogenic microorganisms. Thus, the goal of this research was to evaluate the effects of *R. insidiosa* on biofilm formation by foodborne pathogenic bacteria most commonly associated with outbreaks involving fresh produce, including *L. monocytogenes, S. enterica*, and Shiga toxin producing *E. coli* (STEC). In addition, selected key factors that might affect incorporation of pathogenic bacteria in biofilms, such as biofilm-cell attraction, cell-cell adherence and metabolite production, were tested using *E. coli* O157:H7-*R. insidiosa* combination as a model.

## 2. Materials and methods

### 2.1. Bacterial strains and media

The bacterial strains used in this study are listed in Table 1. *R. insidosa* strain FC1138 was isolated from food contact surfaces in a fresh-cut processing plant (Liu et al., 2013). *E. coli* O157:H7 strain FS4052 is a derivative from the non-virulent strain CDC B6-914 that carries a stable plasmid expressing green fluorescent protein (pGFP) (Fratamico, Deng, Strobaugh, & Palumbo, 1997). *E. coli* O104:H4 strain TW16133, which was associated with the 2011 German sprout outbreak (CDC, 2013), was obtained from Michigan State University EHEC Stock Center (East Lansing, MI.). *L. monocytogenes* strains NRRL B-57616, 57617, 57618 and 57622, associated with 2011 Colorado cantaloupe outbreaks, were obtained from ARS Culture Collections (Peoria, Il.). All other strains were obtained from the Environmental Microbial and Food Safety Laboratory (EMFSL) (USDA-ARS, Beltsville, MD) laboratory stock culture collections. Tryptic soy broth (TSB) and tryptic soy agar (TSA) (BD Biosciences, San Jose, CA.) were used for routine bacterial growth. Diluted (10%) TSB was used for culturing bacteria to form biofilms in static culture, while 1% TSB was used for forming biofilms in the drip flow biofilm reactor. TSA was used for enumeration of total aerobic bacteria. All strains used in this study grew well on TSA, with *R. insidiosa* forming characteristic small round colonies after 24 h incubation, allowing easy distinction of *R. insidiosa* from other strains in mixed cultures. In addition, modified Oxford *Listeria* agar (MOC, BD Biosciences), Xylose lysine tergitol 4 agar (XLT-4, Neogen, Lansing, MI), and sorbitol MacConkey agar (sMAC, BD Biosciences) were used for enumeration of *L. monocytogenes*, *S. enterica*, and Shiga toxin-producing *E. coli*, respectively.

**Table 1**
List of environmental isolate and foodborne human pathogens.

| Strain | Serotype | Source of isolation | Source |
|---|---|---|---|
| *Ralstonia insidiosa* | | | |
| FC1138 | *NA* | Produce processing plant | EMFSL |
| *Listeria monocytogenes* | | | |
| FS2005 | 1/2a | Milk | EMFSL |
| FS2008 | 4b | Milk | EMFSL |
| B-57616 | 1/2b | Clinical isolate | NRRL |
| B-57617 | 1/2a | Clinical isolate | NRRL |
| B-57618 | 1/2a | Clinical isolate | NRRL |
| B-57622 | 1/2a | Clinical isolate | NRRL |
| *Salmonella enterica* | | | |
| FS3022 | Newport | Mango | EMFSL |
| FS3060 | Poona | Cantaloupe | EMFSL |
| *Escherichia coli* | | | |
| FS4052 | O157:H7 | Human feces | EMFSL |
| FS4137 | O111:NM | Clinical isolate | EMFSL |
| FS4140 | O45:H2 | Clinical isolate | EMFSL |
| FS4143 | O26:H11 | Clinical isolate | EMFSL |
| FS4146 | O103:H2 | Clinical isolate | EMFSL |
| TW16133 | O104:H4 | Clinical isolate | MSU |

### 2.2. Biofilm formation by pathogenic bacteria and R. insidiosa

Monoculture or dual-species biofilms were produced in polystyrene 12-well tissue culture plates (BD Biosciences) following the procedure described previously (Liu et al., 2014), with slight modification. Briefly, overnight cultures of individual strains were washed in phosphate buffered saline (PBS) and cell numbers adjusted to approximately $10^9$ CFU/ml. Three ml of 10% TSB was pipette into 12-well tissue culture plates along with 30 μl aliquots of bacteria suspensions, resulting in approximately $10^7$ CFU/ml. For dual-species biofilms formation, equal amounts of each bacterial strain (30 μl) were co-inoculated into the same well. Inoculated culture plates were then incubated at 30 °C for 24 h with moderate orbital shaking (80 RPM).

### 2.3. Biofilm formation with physical separation of tested strains

*E. coli* O157:H7 strain FS4052 and *R. insidiosa* were used to test the key factors that might affect incorporation into dual-species biofilms. Special cell culture plates (12-well) with base and matching insert compartments with 0.4 μm polyethylene terephthalate membranes (Corning, Tewksbury, MA) were used to physically separate *R. insidiosa* and *E. coli* O157:H7 strains during biofilm formation. *E. coli* O157:H7 was inoculated into the base compartments of the tissue culture plates, while an equal amount of *R. insidiosa* was added into the insert compartments on top of the wells, thereby allowing for free exchange of growth medium and metabolites between the well and the insert compartments. After incubation at 30 °C for 24 h, the inserts were removed; biofilms in the base compartments were washed, and cell counts of *E. coli* O157:H7 in the biofilm were determined.

### 2.4. E. coli biofilm formation on primed surface with R. insidiosa biofilm

The ability of *E. coli* O157:H7 to become incorporated into existing viable *R. insidiosa* biofilms was determined by inoculating *R. insidiosa* into 12 well tissue culture plates and allowing for biofilm formation for 24 h at 30 °C. After removing the overnight growth culture, the resultant monoculture biofilms were rinsed with PBS 3 times followed by inoculation with *E. coli* O157:H7 onto the culture plates with pre-formed *R. insidiosa* biofilms in fresh medium. The plates were incubated at 30 °C for another 24 h.

The ability of *E. coli* O157:H7 to become incorporated into inactivated *R. insidiosa* biofilms was determined by priming glass slide surfaces with *R. insidiosa* biofilm formation for 24 h in a drip flow biofilm reactor as described previously (Liu et al., 2015). *R. insidiosa* biofilms on glass slides were inactivated by submerging the slides in water at 80 °C for 30 min (Chmielewski & Frank, 2003; Scher, Romling, & Yaron, 2005). The glass slides with inactivated *R. insidiosa* biofilms was used as the substrata for *E. coli* O157:H7 biofilm formation in the drip flow system for 3 days at room temperature.

### 2.5. Low-temperature scanning electron microscopy (SEM)

Low-temperature SEM observations were performed using an S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Dallas, TX) equipped with a Quorum PP2000 (Quorum Technologies, East Sussex, UK) cryotransfer system. Three-day old biofilms grown on glass fiber filer paper in the drip flow biofilm reactor were excised and mounted on flat copper plate specimen holders. The samples were frozen conductively in liquid nitrogen and freeze etched inside the cryotransfer system to remove any surface contamination (condensed water vapor) by raising the temperature of the stage to −90 °C for 10–15 min. Following freeze etching, the temperature inside the chamber was lowered to −130 °C, and the specimens were coated with a 10 nm layer of platinum using a magnetron sputter head equipped with a platinum target. The specimens were transferred to a pre-cooled (−130 °C) cryostage in the SEM for observation. An accelerating voltage of 5 kV was used to view the specimens. Images were captured using a 4pi Analysis System (Durham, NC). Images were sized and placed together to produce a single figure using Adobe® Photoshop CS 5.0.

### 2.6. Biomass quantification and bacteria cells enumeration

The total biomass of biofilms grown in 12-well tissue culture plates was quantified by the crystal violet binding assay (Liu et al., 2014). To enumerate biofilms formed on tissue culture plates or drip flow biofilm reactor, cell sampling and plating were same as describe previously (Liu et al., 2014).

### 2.7. Statistics

Statistical analyses were performed using Student's T-test, one-way or two-way ANOVA, and Tukey's multiple comparison test to elucidate the effects of tested parameters on pathogenic bacteria cell counts or the biomass production, as indicated in the results. Significant differences were determined when the p value is less than 0.05. All data was analyzed using Prism 5 (GraphPad, La Jolla, CA).

## 3. Results

### 3.1. Dual-species biofilms formed by R. insidiosa and individual pathogenic bacteria

We demonstrated previously that *R. insidiosa* significantly enhanced the incorporation of *E. coli* O157:H7 strains in dual-species biofilms under a variety of growth conditions (Liu et al., 2015). In this study, besides *E. coli* O157:H7 strain FS4052, 5 strains of non-O157 EHEC, 2 strains of *S. enterica*, and 6 strains of *L. monocytogenes* were tested for potentials of biofilm formation, either as monoculture, or in combination with *R. insidiosa* strain FC1138 in 12-well tissue culture plates. In addition, a separate *R. insidiosa* monoculture biofilm was concurrently grown with each of the tested pathogenic strains to serve as control. Total biomass of the monoculture and dual-species biofilms was determined after 24 h incubation. Comparison of biofilm formation by individual pathogenic strains was facilitated by setting an arbitrarily value of 100 for the biomass value of *R. insidiosa* monocultures (a strong biofilm former) in each pathogen-*R. insidiosa* combination, such that the relative biomass values for the monoculture and dual-species biofilms could be normalized and classified based on the $OD_{590}$ values (Fig. 1). *E. coli* strain FS4140 produced monoculture biofilms comparable to that of *R. insidiosa* in biomass, and was classified as a strong biofilm former. In contrast, one *L. monocytogenes* strains (B57616), two *Salmonella* strains (FS3022, FS3060), and two *E. coli* strains (FS4143, TW16133) produced biomass approximately equivalent to 25–55% of the *R. insidiosa* biofilm biomass, and were considered moderate biofilm formers; while the remaining pathogenic strains (*L. monocytogenes* FS2005, FS2008, B57617, B57618, B57622, *E. coli* FS4052, FS4137, FS4146) were considered weak biofilm formers with less than 20% of the *R. insidiosa* biofilm biomass.

A synergistic interaction between *R. insidiosa* and pathogenic strains, defined in this study as a dual-species biofilm biomass greater than the sum of the monoculture biofilms ($p < 0.05$), was observed in dual-species biofilms formed by *R. insidiosa* and 7 of the pathogenic strains (*L. monocytogenes* FS2005, FS2008, B57616, B57618, B57622, and *E. coli* FS4052, TW16133). For *L. monocytogenes* strain B57617, *Salmonella* strains FS3022 and FS3060, and *E. coli* strains FS4137 and FS4146, increased biomass accumulation was observed in the respective dual-species biofilms with *R. insidiosa*, but the increases were not significantly different ($p > 0.05$) from the sum of monoculture biofilms. For *E. coli* strains FS4140 and FS4143, biomass in respective dual-species biofilms was less than the sum of the monoculture biofilms, but the differences were less than statistically significant ($p > 0.05$).

The total cell counts of each pathogenic strain in its monoculture

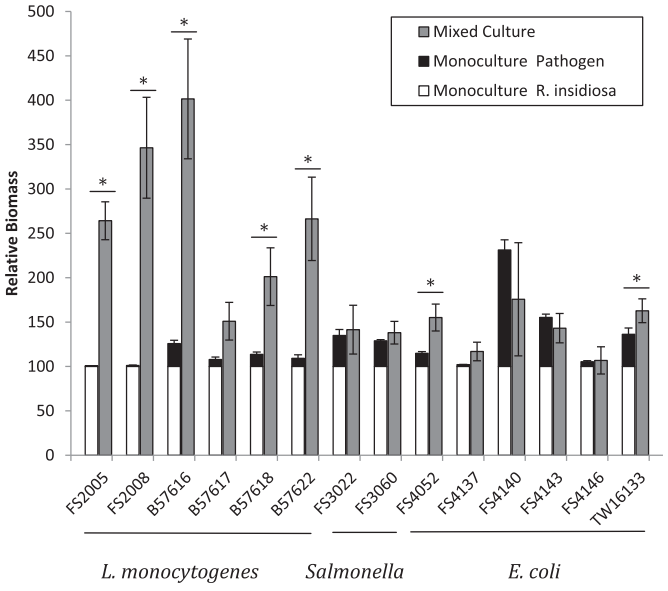


**Fig. 1.** Relative biomass accumulation in monoculture biofilms formed by foodborne pathogens (*L. monocytogenes, Salmonella* and shiga toxin-producing *E. coli*) and in dual species biofilms formed with *R. insidiosa*. Relative biomass in monoculture is shown as the sum of *R. insidiosa* and individual pathogen strains monocultures. Data was normalized by setting $OD_{590}$ reading for each *R. insidiosa* monoculture biofilms (Control) as 100. Student's T-test was used to compare the relative biomass of each tested pathogen in monoculture biofilms and in dual-species biofilms. * Indicates significant difference in pair-wise comparison at $p < 0.05$.

and dual-species biofilms are shown in Fig. 2. In monoculture biofilms, the cell counts for pathogenic stains ranged from 4.54 to 7.67 log CFU/cm$^2$. Most (12 out of 14) of the pathogenic strains showed significant increases ($p < 0.05$) in cell counts in dual-species biofilms as compared to their respective monoculture biofilms. The increases ranged from 0.36 log (*E. coli* strain TW16133) to 1.84 logs (*Salmonella* strain FS3022). On the other hand, cell counts of *E. coli* FS4143 ($p > 0.05$) and FS4140 ($p < 0.05$) decreased in dual-species biofilms as compared to the monoculture biofilms.

### 3.2. Biofilm formation by E. coli O157:H7 and R. insidiosa in monocultures separated by permeable membrane

Since secreted signal molecules are a primary means of interspecies communications during biofilm formation (Karatan & Watnick, 2009), we examined the possibility that the incorporation of pathogenic bacteria cells in the dual-species biofilms was affected by a mechanism akin to quorum sensing (Simoes, Simoes, & Vieira, 2007). *R. insidiosa* and *E. coli* O157:H7 cells were inoculated in two separate compartments separated by 0.4 μm polyethylene terephthalate filter membrane that supported free exchange of culture medium and metabolites. Then *E. coli* O157:H7 cell counts in the monoculture biofilms formed on the base compartment surface were determined after incubation at 30 °C for 24 h. No significant difference ($p > 0.05$) was observed in cell counts of *E. coli* O157:H7 in the monoculture biofilms formed with or without the presence of *R. insidiosa* in the permeable membrane-lined insert compartment (Fig. 3). This observation does not support the hypothsis that *R. insidiosa* metabolites or secreted signal molecules play a significant role in promoting the incorporation of other pathogenic bacteria cells into dual-species biofilms.

### 3.3. Incorporation of E. coli O157:H7 in existing R. insidiosa biofilm

Since bacterial interspecies interactions can also occur by direct cell-cell contact, we examined the incorporation of *E. coli* O157:H7 cells into established *R. insidiosa* biofilms. Compared to *E. coli* O157:H7 monoculture biofilms, a significant increase ($p < 0.05$) was

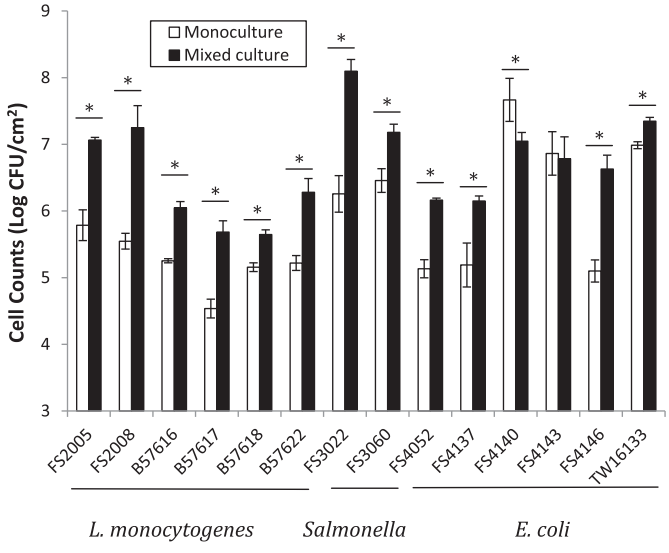


**Fig. 2.** Cell counts of tested foodborne pathogens (*L. monocytogenes, Salmonella* and shiga toxin-producing *E. coli*) in monoculture biofilms and in dual species biofilms formed with *R. insidiosa*. Student's T-test was used to compare cell counts of each tested pathogen in monoculture biofilms and in dual-species biofilms. *Indicates significant difference in pair-wise comparison at $p < 0.05$.

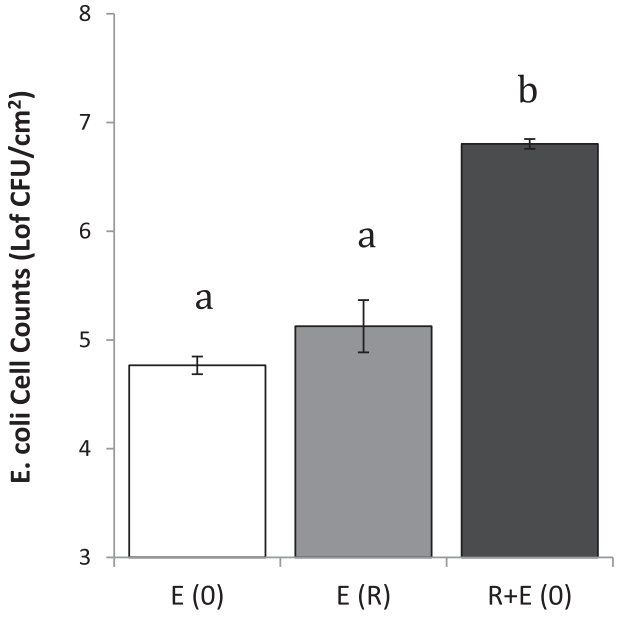


**Fig. 3.** *E. coli* O157:H7 cell counts in biofilms formed in absence and presence of *R. insidiosa*. Capital letters under the horizontal axis represent *E. coli* (E), *R. insidiosa* (R), or no bacterium (0) in the culture, letters outside the parentheses indicate bacteria grown in the base compartments, and that inside the parentheses bacterium grown in the insert. Tukey's multiple-comparison test was used to compare each set of data. Different lower case letters above the bars indicate significant difference at $p < 0.05$.

observed in *E. coli* O157:H7 cells in dual-species biofilms formed on pre-existing *R. insidiosa* biofilms after 24 h (1.93 log increase) (Fig. 4A). These increases were also significantly higher ($p < 0.05$) than in the dual-species biofilms formed by simultaneous culturing of *R. insidiosa* and *E. coli* O157:H7 (0.90 log increase). Thus, this observation indicated that the priming with *R. insidiosa* effectively further enhanced the incorporation of *E. coli* O157:H7 in the biofilms than in mixed cultures without priming.

This observation, while supporting the need for direct cell-cell contact, could also suggest that *R. insidiosa* biofilms facilitate the incorporation of *E. coli* O157:H7 by passively trapping planktonic bacterial cells. To further investigate this possibility, 24 h *R. insidiosa* biofilms formed on a glass surface were inactivated by submerging in hot water (80 °C) and then used as substrata for biofilm formation by *E. coli* O157:H7. Crystal violet staining and visual inspection of the thermo-inactivated biofilms did not reveal a loss of structural integrity. The incorporation of *E. coli* O157:H7 in biofilms grown on heat inactivated *R. insidiosa* biofilms was not significantly different from that of *E. coli* O157:H7 monoculture biofilms (Fig. 4B), indicating that enhanced incorporation of *E. coli* O157:H7 into the dual species biofilms required active interactions between *R. insidiosa* and *E. coli* cells.

### 3.4. Biofilm structure under SEM

Low temperature scanning electron microscopy was used to examine the monoculture and dual-species biofilms produced by *E. coli* O157:H7 and *R. insidiosa* (Fig. 5). The monoculture and dual-species biofilms were grown on glass fiber filter paper for 3 days in the drip flow biofilm reactor with washing. The samples were inactivated by formaldehyde fumigation prior to freezing for observation as previously described (Liu et al., 2015).

An overview of the bacterial landscape of the *E. coli* O157:H7 monoculture biofilms were observed on the surface of glass fiber filter paper as uniform sheets without discernible distinct

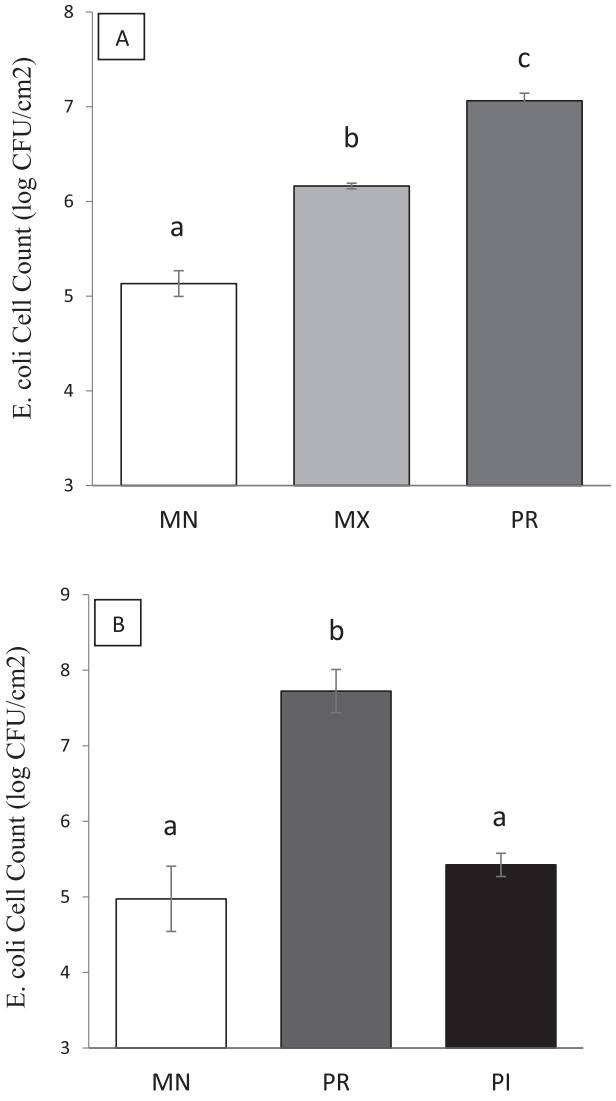


**Fig. 4.** Effect of *R. insidiosa* priming on cell counts of *E. coli* O157:H7 in biofilms. (A) *E. coli* cell counts in monoculture (MN), mixed culture (MX), and *R. insidiosa* primed (PR) biofilms grow for 24 h in microplate. (B) *E. coli* cell counts in monoculture (MN), untreated *R. insidiosa* primed (PR), and heat inactivated *R. insidiosa* primed biofilms grown on glass slides in a drip flow system for 72 h. Tukey's multiple-comparison test was used to compare each set of data. Different lower case letters above the bars indicate significant difference at $p < 0.05$.

structures. The porous property of the glass fiber paper surface was supportive of *E. coli* O157:H7 cellular proliferation, however, the observed uniform layer of cells did not show 3D biofilm structures (Image not shown). In contrast, *R. insidiosa* growth was observed as microcolonies with distinct domes and valleys, and the domed areas were seen connected through multiple conduit-like structures (Fig. 5A). Under higher magnification, *R. insidiosa* cells were seen as closely packed and possibly multilayered masses in the domed areas (Not shown). The domed areas were connected by extensive web-like extracellular structures, which were most evident in the valleys with low cell density (Fig. 5B). In addition, filamentous projections were seen extruding from the surface of microcolonies to the surrounding valley areas, with the web-like structures attached to these filamentous cells. There seemed to be numerous sub-cellular sized particles distributed throughout the web-like structures and especially on the surfaces of the filamentous projections. The morphology of dual-species biofilms was

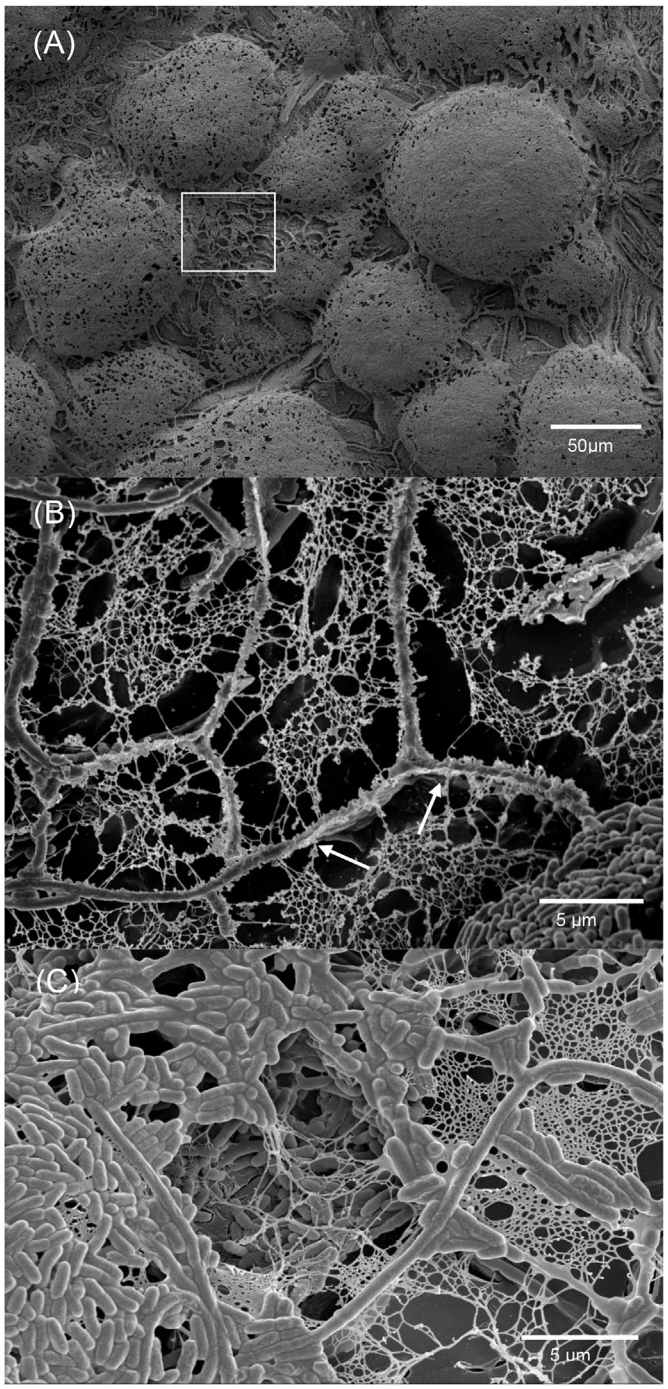


**Fig. 5.** Scanning Electron Microscopy (SEM) of Biofilms. (A). Landscape view of *R. insidiosa* monoculture biofilm showing the microcolonies and valleys. Box highlights the valleys; (B). Valley area of *R. insidiosa* biofilm. Lower-right corner is the edge of a microcolony. Arrows point to the joints of web-like structures and filamentous projections; and (C) Valley area of *E. coli* O157:H7 - *R. insidiosa* dual-species biofilm. Left is a partial microcolony.

very similar to that of the *R. insidiosa* monoculture biofilms (Fig. 5C). Currently we are unable to confidently distinguish *E. coli* cells from *R. insidiosa* using SEM. However, when the dual species-biofilms were examined using transmission electron microscope (TEM), *R. insidiosa* and *E. coli* O157:H7 cells exhibited segregated spatial distribution, with *E. coli* O157:H7 microcolonies primarily found proximal to the matrix surface and *R. insidiosa* cells

overlaying on top and in the domed areas (Liu et al., 2014). Therefore, it is likely that the observation of the dual-species biofilms using SEM is indeed simply a reflection of the characteristics of *R. insidiosa* biofilms.

## 4. Discussion

### 4.1. R. insidiosa behaves like a bridging bacterium

In a polymicrobial community, certain species or strains interact synergistically with a multitude of other species in forming biofilms and are referred to as a bridge bacterium (Rickard et al., 2003). For example, *Fusobacterium nucleatum*, a prevalent bacterium in human plaque, positively interacts with bacteria from 7 different genera, thereby connecting the planktonic bacteria to the biofilms and enhancing biofilm production by mediating their adhesions to surfaces (Shaniztki, Ganeshkumar, & Weiss, 1998). *R. insidiosa* is widely present in diverse environmental niches, including water treatment and supply systems (Coenye, Goris, De Vos, Vandamme, & LiPuma, 2003; Ryan, Pembroke, & Adley, 2011), making it one of the most common contaminants of food and food processing environments. Our preliminary data (unpublished) suggests that *R. insidiosa* is highly efficient in nutrient utilization and proliferates well in oligonutrient environments, which could be advantageous for bacteria serving as a bridging or pioneering species.

We have previously demonstrated that the species-specific interaction between *R. insidiosa* and *E. coli* O157:H7 results in increased populations of *E. coli* O157:H7 in dual-species biofilms (Liu et al., 2014). In this study, we show that this enhanced incorporation can occur with other foodborne pathogenic bacteria, including 6 *L. monocytogenes* strains (representing 3 serotypes), 2 serovars of *S. enterica*, and 4 of 6 serotypes of Shiga toxin-producing *E. coli*; although the increased cell counts of the foodborne pathogens were not always accompanied by equally increased biomass production in dual-species biofilms. Especially notable is the significantly elevated biomass levels in dual-species biofilms for most (5 of 6) *R. insidiosa-L. monocytogenes* combinations. This suggests that large amounts of additional extracellular matrices were produced in the dual-species biofilms, which could render the biofilms more robust and more protective than either of the monoculture biofilms. Zammer and colleagues also observed increased biomass production when co-culturing *L. monocytogenes* with *Staphylococcus epidermidis* (Zameer, Kreft, & Gopal, 2010). Increased biomass production was also observed in the dual-species biofilms formed by *R. insidiosa* with environmental strains isolated from fresh-cut processing facilities (unpublished data), including *Pantoea agglomerans* and *Rahnella aquatilis*. Taken together, these observations suggest that *R. insidiosa* plays an important role as a bridging bacterium (Rickard et al., 2003) that is critical for multispecies biofilm formation, and provides a microenvironment for pathogenic bacterial accumulation and survival.

### 4.2. Role of cellular contacts in the interactions between R. insidiosa and the pathogenic strains

Interspecies communication and interactions can occur through diffusible signaling molecules or by intimate cellular contact (Flemming & Wingender, 2010; Karatan & Watnick, 2009). Data acquired in the present study suggested that cellular contact with *R. insidiosa* cells was required for increased incorporation of *E. coli* O157:H7 into dual-species biofilms, as opposed to a diffusible signal These observations are consistent with previous findings by Uhlich and colleagues (Uhlich et al., 2010), who also observed that direct cell-cell contact with companion strains was required for *E. coli* O157:H7 biofilm formation.

There could be at least two mechanisms for *R. insidiosa* enhancing the incorporation of other species (potentially pathogens) in heterogeneous biofilms. Bridging bacterium could serve as a primary colonizer by attaching to solid substrates and excreting extracellular polymeric substances that provides microniches for the secondary colonizers (Rickard et al., 2003). Alternatively, it could co-aggregate with other bacteria to form clusters that can deposit on surfaces to initiate colonization (Bos, van der Mei, Meinders, & Busscher, 1994; Rickard et al., 2003). Existing *R. insidiosa* biofilms strongly promoted the incorporation of *E. coli* O157:H7 cells into biofilms, suggesting that co-aggregation was not obligatory. However, heat inactivated *R. insidiosa* biofilms failed to promote the incorporation of *E. coli* O157:H7 into biofilms, indicating the necessity of active cell-cell interactions. Scanning electron microscopy showed extensive web-like structures and filamentous projections connecting *R. insidiosa* biofilm microcolonies. The role of those structures in attracting other bacterial species should be further investigated.

Although we were unable to demonstrate that the compounds or metabolites secreted by *R. insidiosa* could promote *E. coli* O157:H7 incorporation into biofilms, it cannot be ruled out that signal molecules play a role in *R. insidiosa* communicating with other bacteria species and in promoting multispecies biofilm formation. *Ralstonia solanacearum*, a closely related phytopathogen, is well known for production of acyl-homoserine lactones (AHL), the putative cell-cell signaling molecules in biofilm development (Flavier, Ganova-Raeva, Schell, & Denny, 1997). It has been documented that both *E. coli* and *Salmonella* produced receptors for AHL molecules in order to detect surrounding microbial community (Dyszel et al., 2010; Michael, Smith, Swift, Heffron, & Ahmer, 2001). Further investigation of the potential roles of signal molecules in *R. insidiosa* interacting with foodborne pathogens may shed more light on the understanding of polymicrobial biofilm formation in food processing environments.

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